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ELECTRON CAPTURE NEGATIVE IONIZATION MASS SPECTROMETRY OF STEROIDS AND SIMILAR ELECTROPHILIC COMPOUNDS

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Major professor

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ELECTRON CAPTURE NEGATIVE IONIZATION MASS SPECTROMETRY OF STEROIDS AND SIMILAR ELECTROPHILIC COMPOUNDS

By

Helen Kathe Mayer

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ABSTRACT

ELECTRON CAPTURE NEGATIVE IONIZATION MASS SPECTROMETRY OF STEROIDS AND SIMILAR ELECTROPHILIC COMPOUNDS

By

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Electron capture negative ionization mass spectrometry (ECNI-MS) is a sensitive and selective technique for highly halogenated compounds or those containing π-conjugation. Extended conjugation is particularly important for the high ECNI responses of certain ketosteroids. Combinations of functional groups within the steroid nucleus have been correlated to ECNI response. Because steroids often have other potential electron-stabilizing substituents, monocyclic, bicyclic, and quinone compounds also have been studied to evaluate individual structure-response effects. Steroids that are chemically oxidizable to molecules with extended conjugation have been successfully used assayed by ECNI-MS.

Combinations of double bonds, carbonyl groups, halogens, cyano groups, and epoxides within the steroid nucleus have been studied. For enhanced ECNI response, the following structural features are important: a cross-conjugated system with a neighboring group, such as 1,4-dien-3,11-dione; a linearly-conjugated system, such as 4-en-3,6-dione; a fluorine atom alpha to a carbonyl group in a highly-conjugated steroid; or an epoxide alpha to a carbonyl group.

Sensitivity of the ECNI-MS technique is dependent on instrumental parameters as well as the electron-capture properties of the molecule. The choice of reagent gas, source temperature, and source pressure are important factors in ECNI response and can differ for different types of compounds. To capture an electron, a compound must have a positive electron affinity. Electron affinities for most compounds studied are not known; estimates were obtained by measuring the reduction potential.

Some steroids, usually steroidal drugs, can be chemically oxidized to effective electron-capturing species. Not only are chromatographic properties improved, but a degree of selectivity is imparted because these oxidized products have a higher ECNI response than the oxidized product of endogenous steroids. Pyridinium chlorochromate has been the oxidant of choice, but alternative methods, including electrochemical synthesis, were studied.

Several assays for steroids were developed using the chemical oxidation technique. These include the determination of dexamethasone in human plasma and the determination of prednisolone in mouse plasma. Anabolic steroids do not oxidize to highly electron-capturing species, except for fluoxymesterone; preliminary studies show that conventional ECNI derivatives like heptafluorobutyrate may be the best choice for activating this group of compounds.

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ABA	Abscisic acid
C18	Octadecahydryl
CI	Chemical ionization
cv	Coefficient of variance
CV	Cyclic voltammetry
DFTPP	Decafluorotriphenylphosphine
DIP	Direct insertion probe
DMF	Dimethylformamide
DPP	Differential pulse polarography
DST	Dexamethasone suppression test
$E_{1/2}$	Half-wave reduction potential
EA	Electron affinity
EC	Electrochemical detection
ECD	Electron capture detector
ECN	Effective carbon number
ECNI	Electron capture negative ionization
EFSIM	Electric field switching selected ion monitoring
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FAB	Fast atom bombardment
FD	Field desorption
FID	Flame ionization detector
Florox	*o*-Pentafluorobenzyloxime
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HDL	High density lipoprotein
HFB	Heptafluorobutrate
HP	Hewlett Packard

HPLC	High performance liquid chromatography
IOC	International Olympic Committee
LOD	Limit of detection
LUMO	Lowest unoccupied molecular orbital
MFSIM	Magnetic field switching selected ion monitoring
MO-TMS	Methoxyamine-trimethylsilyl
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSD	Mass selective detector
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MSU	Michigan State University
NCI	Negative chemical ionization
OFN	Octafluoronaphthalene
PAH	Polyaromatic hydrocarbon
PCC	Pyridinium chlorochromate
PCI	Positive chemical ionization
PFK	Perfluorokerosene
PFP	Pentafluoropropionyl
QRE	Quasi-reference electrode
RIA	Radioimmunoassay
S/N	Signal-to-noise ratio
SCE	Standard calomel electrode
SHE	Standard hydrogen electrode
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
TBAF	Tetrabutylammonium fluoroborate
TFA	Trifluoroacetyl
TIC	Total ion chromatogram
TLBE	Thin-layer bulk electrolysis
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMIS	Trimethylsilylimidazole
TMS	Trimethylsilyl
TPAP	Tetra-*n*-propylammonium perruthenate
UV	Ultraviolet

Chapter 1: Introduction

Detection of small amounts of analyte in complex matrices is an important problem in analytical chemistry, especially with the current emphasis on environmental and biological samples. In the biological and environmental sciences, for example, the problem becomes exceedingly difficult because of the amount and variety of interfering compounds which are often unknown to the investigator. Sensitive and selective methodology for sample analysis needs to be further developed to detect small amounts of analyte with a minimum of sample preparation. Confirmation of the analyte of interest is another problem. Proving with certainty the presence or absence of a component is critical in many analyses in both the environmental and biomedical fields. In environmental samples, the absence of a component may mean a costly and time-consuming clean-up of a ecosystem could be avoided. In the biomedical field, the absence or presence of a substance may mean the confirmation of a disease, the success of a treatment, or the detection of the use of illicit drugs.

An example of an analysis problem in the biological sciences is the detection of drugs in urine or plasma. High pressure liquid chromatography (HPLC), gas chromatography (GC), and immunoassays have been commonly used. Although they can confirm the absence or presence of a drug, specificity can suffer, especially for structurally-similar compounds. Often, all interfering compounds must be removed in time-consuming extraction steps. Also, a chromatographic peak provides only retention time data as a parameter for comparison to standards. Gas chromatography-mass spectroscopy (GC-MS) is a superior technique for trace analysis because it also provides structurally-related information along the mass axis.

Electron-impact mass spectrometry (EI-MS) is the most popular identification technique. For many classes of compounds, the molecular ion is small or absent. Major fragmentation peaks within a class of compounds are often similar in *m/z* values

and cannot be used for quantitation. Chemical ionization (CI) is often used to obtain complementary fragmentation patterns to those obtained by EI. CI spectra can provide additional structural information in the form of a molecular ion or an adduct ion due to adduct formation with the reagent gas (1).

For qualitative analysis, fragmentation patterns provide a "fingerprint" for the analyte of interest. For quantitative analysis, fragmentation is often more a burden than a blessing. The most abundant ion in the spectrum may not be the one which distinguishes the analyte from the matrix. Molecular ions of low abundance make detection of small amounts of analyte difficult.

Electron-capture negative ionization mass spectrometry (ECNI-MS) has been utilized in quantitative analysis because less fragmentation is obtained. Because negative ions usually have low internal energies, they are unable to fragment (2). The most abundant ion in the spectrum is usually the molecular anion. In the mass spectra of steroids, the molecular ion is often the base peak in the spectrum, although losses of radicals such as hydrogen and CH_3 and small neutrals like HF have been observed. In selected ion monitoring (SIM), ion currents at a few *m/z* values are transmitted to the detector. By using the SIM mode in ECNI and selecting only the molecular anion, small amounts of analyte can be detected. The sensitivity of ECNI is also impressive. In fact, the formation of a molecular anion under ECNI conditions is about 400 times more rapid than the formation of $[M+H]^+$ under positive chemical ionization (PCI) conditions or the formation of $M^{-\cdot}$ under negative chemical ionization (NCI) conditions (3). The reaction rate for gas phase PCI and NCI reaction is about 10^{-9} cm^3s^{-1}. For electron capture, the rate constant is 4×10^{-7} cm^3s^{-1}, or about 400 times greater.

Another problem plaguing the analysis of biological samples is the high background from the matrix; for example, the presence of endogenous steroids in the analysis of urine can cause interference in the detection of synthetic corticosteroids.

Corticosteroids are substituted pregnanes, some of which occur naturally in the adrenal cortex. ECNI is a selective technique that responds best to highly electrophilic species, i.e., highly halogenated or highly conjugated (α,β-unsaturation) compounds. Compounds which have negative electron affinities will not respond, eliminating much of the background and often simplifying sample clean-up.

Many electrophilic compounds have been analyzed via ECNI, especially in the biological and environmental sciences for quantitative analysis. Polychlorinated compounds and pesticides (4), aromatics (5), derivatized amino acids (6), mycotoxins in food (7), amines (8), prostaglandins (9), pharmaceuticals (10), and steroids (11) have been analyzed using this technique.

Formation of Negative Ions in Mass Spectrometry

Types of Reactions

Negative ions can be formed in a mass spectrometer ion source by the following mechanisms:

(1) $AB + e^- (\sim 0.1\ eV) \rightarrow [AB^-]^* \rightarrow AB^{\cdot -}$ Resonance capture

(2) $AB + e^- (0\text{-}15\ eV) \rightarrow [AB^-]^* \rightarrow A + B^-$ Dissociative resonance capture

(3) $AB + e^- (> 10\ eV) \rightarrow A^- + B^+ + e^-$ Ion-pair formation

(4) $AB + C^- \rightarrow AB^- + C$ Negative ion-molecule reaction

Resonance electron capture is the most useful of all these pathways because the molecular anion predominates. Resonance capture requires a thermal electron (an electron of energy 0.1 eV or less). The anion is produced in the vibrationally excited

state, $[AB^-]^*$, which is dependent upon molecular structure and electron energy. If the excess vibrational energy is not removed or delocalized, dissociation will occur. The molecular anion can be stabilized by delocalization of charge via a conjugated system, collisional stabilization, or emission of infrared photons.

Dissociative electron capture occurs when an electron of 0 to 15 eV is captured. Direct rapid cleavage or molecular rearrangement (12) can occur. This mode is not as useful in quantitative analysis as the resonance capture mode. In resonance capture, all of the ion current is concentrated in the molecular anion. The ion current in dissociative resonance capture is shared between two or more fragments, resulting in a decrease in detectability.

Ion-pair formation occurs when the electron has over 10 eV of energy. Both a positive and a negative ion are formed, with the electron carrying away the excess energy. In an ion source operated under EI conditions, the ion-pair formation mechanism predominates. The majority of negative ions formed are of low mass and are not indicative of the parent molecule (e. g., OH^-, CN^-, NO_2^-, etc.); consequently, there is little molecular ion information unless the anion is unusually long-lived. Negative ion formation under low pressure and high electron energy conditions have been reviewed by Bowie (2) and Budzikiewicz (13).

Negative ion-molecule reactions truly deserve the designation of negative chemical ionization (NCI) because a negative ion in the source (e. g., H^-, OH^-, CH_3O^-) reacts with the analyte. Many reactions can occur, including charge transfer, proton transfer, hydride transfer, oxygen exchange with either halogens or hydrogen, and anion-molecule adduct formation (14).

Of most interest to this work are those reactions which occur under ECNI conditions, namely resonance and dissociative capture.

How are Thermal Electrons Formed?

In ECNI, a high-pressure chemical ionization (CI) source is used. A nonreactive reagent gas (such as methane, ammonia, isobutane, etc.) is introduced into the source at the pressure of 0.1 to 1 torr and bombarded by a beam of electrons, generating positive ions and thermal electrons. Using methane as an example, the following reactions can occur (15):

$$CH_4 + e^-_{primary} \rightarrow CH_4^{+\cdot} + e^-_{primary} + e^-_{secondary}$$

$$CH_4^{+\cdot} + CH_4 \rightarrow CH_5^+ + CH_3^{\cdot}$$

$$e^-_{secondary} + CH_4 \rightarrow e^-_{thermal} + CH_4$$

The ionizing energy is about 200 eV to ensure good penetration of electrons into the high pressure source. Ionization of methane removes about 30 eV from the bombarding electron while more energy is removed from the electron by nonionizing collisions with methane (14). One primary electron can potentially produce about six to ten ion-electron pairs. The positive ions formed undergo other collisions, such as those to form CH_5^+. The secondary electrons collide with methane, producing the thermal electrons, which can be captured by high electron affinity analytes. The high pressure gas also provides stabilization of the molecular anion.

A low-energy electron distribution is formed by the modulating gas. This distribution varies from instrument to instrument and can cause differences in ECNI spectra. In one particular experiment, it was shown that 48% of the low-energy electrons had energies near thermal, 5% had energies less than 0.15 eV, and 12% had energies ranging from 0.15 to 0.45 eV (16).

What Happens After Electron Capture?

Processes Directly Following Electron Capture. After the capture of a thermal electron, an unstable molecular anion $[AB^-]^*$ is formed. The processes that this unstable anion can undergo are the following: stabilization by collisions with the modulating gas or radiative emission, dissociation into A^- + B, or autodetachment of the electron. After forming the negative anion, reactions which can neutralize or change the anion can occur, including associative detachment of the electron, displacement of certain functional groups on the molecule, proton transfer, charge exchange, and association (clustering) (17). In addition to these one-step reactions, Sears and co-workers (8) have proposed four mechanisms to explain the formation of unusual ions (see Figure 1.2).

Stabilization *vs.* Autodetachment *vs.* Dissociation. Stabilization of the anion, dissociation, and autodetachment of the electron are the three primary mechanisms by which the unstable intermediate $[AB^-]^*$ forms stable products.

If excess internal and translational energy are present, the capture of an electron can lead to the process depicted in Figure 1.1 (b). For dissociative capture reactions, energy maxima are determined by energy differences between unoccupied orbitals, the potential energy of the molecule, and the Franck-Condon factors describing the interaction between the molecule and the dissociating pair (18).

Electron capture can occur by two methods. If the electron affinity (EA) is negative (Figure 1.1 (c)), then the extra electron on intermediate $[AB^-]^*$ may autodetach or the intermediate may dissociate to $A^\cdot$ + B^- if the Franck-Condon transition is above the dissociation limit. The autodetachment process is quite rapid; therefore, these compounds rarely form detectable negative ions. Rapid autodetachment leaves little possibility for collisional stabilization unless the source presure is very high. Stabilization by radiative emission is also improbable.

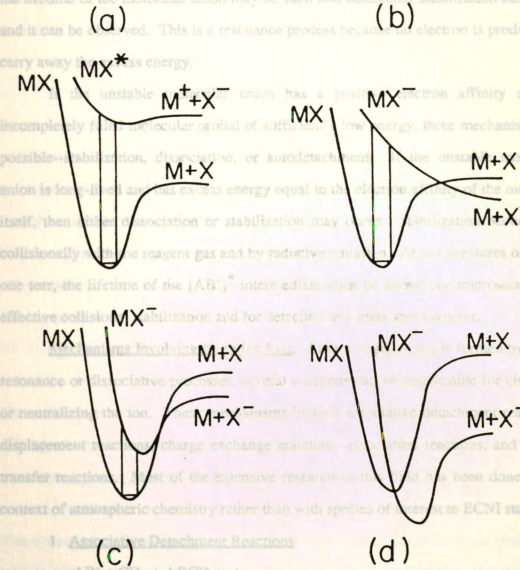


Figure 1.1: Potential energy curves for negative ion formation. (a) Ion-pair formation. (b) Dissociative electron capture. (c) Electron attachment, EA of MX < 0. (d) Electron attachment, EA of MX > 0. (from reference 17).

When the electron affinity is positive (Figure 1.1 (d)), two types of electron capture can occur. If the potential energy curve for AB^- intersects the vertical Franck-Condon region, the reactions are similar to those discussed for Figure 1.1 (c). If the potential energy curve does not intersect the Franck-Condon region and the excess energy of the unstable intermediate $[AB^-]^*$ can be distributed into vibrational modes, the lifetime of the molecular anion may be such that collisional stabilization can occur and it can be observed. This is a resonance process because no electron is produced to carry away the excess energy.

If the unstable molecular anion has a positive electron affinity and an incompletely filled molecular orbital of sufficiently low energy, three mechanisms are possible--stabilization, dissociation, or autodetachment. If the unstable molecular anion is long-lived and has excess energy equal to the electron affinity of the molecule itself, then either dissociation or stabilization may occur. Stabilization occurs both collisionally with the reagent gas and by radiative emission. At gas pressures of about one torr, the lifetime of the $[AB^-]^*$ intermediate must be above one microsecond for effective collisional stabilization and for detection in a mass spectrometer.

Mechanisms Involving Negative Ions. After a negative ion is formed by either resonance or dissociative processes, several reactions can be responsible for changing or neutralizing the ion. These mechanisms include associative detachment reactions, displacement reactions, charge exchange reactions, association reactions, and proton transfer reactions. Most of the extensive research in this field has been done in the context of atmospheric chemistry rather than with species of interest to ECNI studies.

1. Associative Detachment Reactions

$$AB^- + CD \rightarrow ABCD + e^-$$

In this case, the analyte reacts with a neutral (designated by CD) in the ion source or is lost on the ion source walls. At times, the rate of this reaction could almost equal the rate of formation of AB^-, resulting in the loss of signal (17).

Another source of negative ion and electron loss is in recombination reactions with positive ions (19). Because both negatively and positively charged species are present, the gas in the chemical ionization volume can be considered a plasma. In a plasma, ambipolar diffusion predominates, meaning that electrons and positive ions diffuse at the same rate if their populations and energies are equal. Ambipolar diffusion is responsible for much of the ion and electron loss on the walls of the source.

2. Displacement Reactions

$$AB^- + CD \rightarrow ACD + D^- \quad \text{or} \quad AB^- + CD \rightarrow AC^- + B + D$$

These reactions occur if the thermodynamics are favorable and proton transfer is unfavorable. For example, the OH^- ion will displace the Cl^- in CH_3Cl. However, in the reaction of OH^- with CH_3CN, proton abstraction is favorable and will occur rather than the displacement of CN^-. These reactions are analogous to S_N2 reactions in the solution phase, and even result in inversion of configuration (17). Reaction efficiencies can differ greatly depending on the nature of the nucleophile and the leaving group.

3. Charge Exchange Reactions

$$AB^- + CD \rightarrow AB + CD^-$$

These reactions can occur if the electron affinity of CD is greater than the electron affinity of AB. It is estimated that these reactions are 30 to 100% efficient (17).

4. Association (Clustering) Reactions

$$AB^- + R \rightarrow ABR^-$$

These reactions are analogous to the reactions with reagent gas observed in positive CI spectra. Most of the kinetics for these reactions have been studied in the context of atmospheric chemistry.

5. Proton Transfer Reactions

$A^- + YH \rightarrow AH + Y^-$

These reactions occur when using CI gases that are strong bases such as OH^-, F^-, NH_2^-, or OCH_3^-. Anion-adduct formation may occur but, more commonly, $[M-H]^-$ ions are formed. This process has been used advantageously in analytical processes; for example, the OH^- ion has been used in the negative ion detection of substituted cholestanes and cholesteryl esters (20). The OH^- ion was formed in the ion source using a 2:1 mixture of CH_4 and N_2O. In the spectra of all but two of the 35 steroids studied, $[M-H]^-$ ions were present, often as the base peak. For about one-third of the compounds, additional ions are found at $[M+43]^-$, $[M+25]^-$, and $[M+15]^-$, which are attributed to reactions of $[M-H]^-$ with the N_2O reagent gas. The $[M-3]^-$ ion was found in compounds containing hydroxy groups or double bonds. This ion is due to H_2 loss following proton abstraction. The $[M-5]^-$ ion present in some compounds probably is formed in the same way after loss of two H_2 molecules. Other, usually low abundance ions, such as $[M-19]^-$, $[M-21]^-$, and $[M-37]^-$, are attributed to proton abstraction followed by losses of water, water and H_2, and two water molecules, respectively. Not many fragment ions are noted because the fragmentation of Y^- is usually limited due to the exothermicity of this reaction appearing as vibrational energy of the AH bond (21).

Often an oxygen-containing anion is not added on purpose; however, proton abstraction can occur as a by-product of low-level contamination of oxygen-containing species such as O^-, O_2^-, or OH^- in the ion source. Some molecules undergo both electron-capture and ion-molecule reactions; for example, in the presence of oxygen, diazepam produces a $[M-H]^-$ ion as well as a $M^{-\cdot}$ ion (22). The $H^\cdot$ is lost from the carbon adjacent to the amide carbonyl after $M^{-\cdot}$ is formed, resulting in a more conjugated anion in which the charge is delocalized. It is postulated that the same bond is reduced in polarographic analysis. In a study that compared the ECNI spectra

obtained on different instruments, diazepam was used to determine the presence of oxygen in different ion sources (15).

The rate of the electron capture reaction *vs.* the rate of the deprotonation reaction will determine whether $M^{-\cdot}$ or $[M-H]^-$ is observed. For example, it has been observed that the change in selectivity of polyaromatic hydrocarbons (PAHs) with instrumental parameters is due to the competitive formation processes of $[M-H]^-$ and $M^{-\cdot}$ (23). PAHs with electron affinities greater than 0.5 eV produce $M^{-\cdot}$ while those with electron affinities less than 0.5 eV produce $[M-H]^-$ as their base ion in methane ECNI spectra. Another study with PAHs has confirmed that the $[M-H]^-$ ion is from a reaction of the PAH molecule with OH^- (24). In this study, water was added to the nitrogen reagent gas to produce OH^-, $(H_2O)OH^-$, and $(H_2O)_2OH^-$ in the ion source, and the ECNI mass spectra of fluorene, anthracene, and fluoranthrene were recorded. The production of $[M-H]^-$ from fluorene was enhanced, as was the production of $[M-H]^-$ from anthracene. Fluoranthrene, however, produced only $M^{-\cdot}$ ions because it rapidly captures an electron.

The ECNI spectra of PAHs are not the only cases in which the electron affinity of the compound contributes to the formation of $[M-H]^-$ ions. Crow and co-workers (25) studied a series of bromobenzene and chlorobenzenes with one to six substituents. Under methane ECNI conditions, only those with a significant amount of halogenation (pentachlorobenzene, hexachlorobenzene, tetrabromobenzene, and hexabromobenzene) had $M^{-\cdot}$ ions; the rest produced $[M-H]^-$ as their highest mass anion.

The parent structure may also have some role in the production of $[M-H]^-$ ions over $M^{-\cdot}$ ions. In the methane ECNI study of polychloroanisoles with three, four, or five chlorine substitutions (26), compounds with a hydrogen *ortho* to a methoxy group produced $[M-H]^-$ ions, while the others, containing *ortho* chlorines, produced molecular anions.

The mass spectra of many other compounds display $[M-H]^-$ as their highest mass ion, rather than $M^{-\cdot}$. In these studies, the cleanliness of the source with respect to oxygen content is not known. ECNI studies of brassinosteroids (27) and barbiturates (28) show that the spectra of both have $[M-H]^-$ ions. The ECNI spectra of many derivatives have $[M-H]^-$ ions, including the trimethylsilyl derivatives of cytokinins (29) and the pentafluorobenzyl derivative of 3-ketovalproic acid (but none of the other derivatized valproic acid metabolites studied) (30).

The presence of oxygen can result in unusual ions other than $[M-H]^-$. Many of these have been reviewed by Budzikiewicz (13) and Stemmler and Buchanan (24). Chlorinated compounds can give species such as $[M+O-Cl]^-$ and $[M+O-Cl-H_2]^-$. PAHs produce ions corresponding to $[M+O-H]^-$ and $[M+O_2]^{-\cdot}$ from ion-molecule reactions with $O_2^{-\cdot}$. Ions corresponding to $[M+O-2H]^{-\cdot}$ and $[M+2O-2H]^{-\cdot}$ are attributed to the wall-catalyzed reactions discussed in the next section.

The Formation of "Unusual" Ions. Much of the fragmentation of negative ions is simple; losses such as HF, CH_3, and Cl are often observed. Many other fragment and adduct ions are not so easily explained. Competitive processes in the source can play a large role, and these processes can often involve multistep mechanisms if the anion is sufficiently stable. Sears and co-workers have treated this subject in great detail (8).

The basis of the Sears, *et al.* model is that there are many competing reactions in a chemical ionization source. What is actually observed in the mass spectrum depends on the rates of these reactions as shown by the following scheme:

$$\begin{array}{ccc} M & \xrightarrow{e^-} & M^- \\ \text{process X} \downarrow & & \\ M_i & \xrightarrow{e^-} & M_i^- \end{array}$$

In this case, the analyte molecule, M, can either capture an electron to form $M^{-\cdot}$ or can undergo process X to form M_i, which potentially can capture an electron. If M_i has a similar or greater electron-capturing ability as M, M_i^- may appear in the spectrum or could even be represented by the most abundant peak. If M_i has a much greater electron-capture capability than M, process X does not have to be efficient for M_i^- to dominate the spectrum.

Four possible mechanisms have been proposed for the production of unusual negative ions (8) as illustrated in Figure 1.2.

Route A first involves electron capture to form $M^{-\cdot}$. After electron capture, neutralization by positive ions is possible. Alternately, wall reactions can occur, forming the neutral species Z, which can potentially capture an electron. $M^{-\cdot}$ must be at least as intense as Z^- in the ECNI spectrum for this pathway to occur. All pathways A through D have the potential of ending in neutralization.

Route B involves a reaction between the analyte molecule M and a positive ion to form $M^{+\cdot}$ (this ion does not necessarily have the same structure as M). After a reaction with an electron, a neutral, X, is formed which could potentially capture an electron.

Route B explains the formations of $[M\text{-}O]^{-\cdot}$ ions observed in the mass spectrum of the trifluoroacetyl derivative of aminophenanthrene (8). Prominent ions appear at $M^{-\cdot}$, $[M\text{-}HF]^{-\cdot}$, $[M\text{-}CH_3OH]^{-\cdot}$, $[M\text{-}H_2O]^{-\cdot}$, $[M\text{-}O]^{-\cdot}$, and $[M+CH_2]^{-\cdot}$. The $[M\text{-}O]^{-\cdot}$ ion is difficult to justify using conventional ideas about ECNI spectra. Route A is unlikely because there is no precursor ion of sufficient intensity. Route C is also unlikely because the $[M\text{-}O]^{-\cdot}$ ion is fairly prominent. Route D does not occur in the other fluorinated derivatives of aminophenanthrene, so it is unlikely it would occur here; therefore, route B is the only other alternative. In this molecule, oxygen is present only in a carbonyl group. It is expected that the positive ion formation of this derivatized aminophenanthrene by CH_5^+ and $C_2H_5^+$ regent ions is at the usual fast

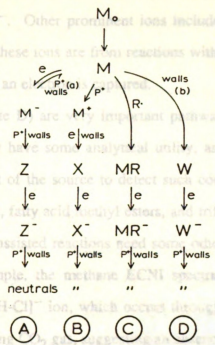


Figure 1.2: Mechanisms of formation of unusual negative ions (from reference 8).

rate of ion-molecule reactions. To test this hypothesis, reagent gas is doped with a substance which blocks the initial positive ion-molecule reaction while not disturbing the electron reactions. In this experiment, triethylamine was used as the dopant. In the presence of triethylamine, a very low abundance of $[M-O]^{-\cdot}$ was observed; therefore, the mechanism for the formation of $[M-O]^{-\cdot}$ must first involve a positive-ion reaction with the reagent gas. At this time, the identities of the species M^+ and X are not known; not much is known about the products generated by the recombination of large positive ions and electrons or by the neutralization of positive ions on a metallic source. The expected large energy release from these processes may make unconventional rearrangements possible.

Route C involves a reaction of the analyte with a radical from the modulating gas. The resulting species, MR, can capture an electron to form $MR^{-\cdot}$. Compounds

which can act as free radical traps include tetracyanoethylene (31), naphthalene (23), and PAH isomers (32). The base ion for tetracyanoethylene is at *m/z* 103 which corresponds to $[M+H\text{-}CN]^{-\cdot}$. Other prominent ions include those at $[M+CH_3\text{-}CN]^{-\cdot}$ and $[M+C_2H_5\text{-}CN]^{-\cdot}$. All these ions are from reactions with radicals formed from the methane reagent gas before an electron is captured.

Wall reactions (route D) are very important pathways that can form unusual ions. These reactions may have some analytical utility, as demonstrated by studies using chlorine pretreatment of the source to detect such compounds as trimethylsilyl derivatives of organic acids, fatty acid methyl esters, and trifluoroacetyl derivatives of alcohols (33). Many wall-assisted reactions need some other species in the source in order to occur. For example, the methane ECNI spectrum of both fluoranil and chloranil contains the $[M+H\text{-}Cl]^{-\cdot}$ ion, which occurs through a wall-assisted reaction. This ion is not observed using CO_2 gas, suggesting an absence of gas-phase or surface-bound free radicals under CO_2 conditions.

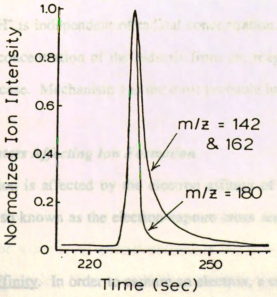


Figure 1.3: Single ion reconstructed chromatograms of the methane ECNI-MS analysis of floranil. The peak at *m/z* 180 is the molecular anion; *m/z* 162 corresponds to $[M+H\text{-}F]^{-\cdot}$; *m/z* 142 corresponds to $[M\text{-}HF]^{-\cdot}$ (from reference 8).

Ions formed from wall reactions tend to have non-Gaussian profiles. Single ion reconstructed chromatograms of the methane ECNI analysis of fluoranil are shown in Figure 1.3. In this case, the non-Gaussian profiles of peaks at *m/z* 142 (corresponding to $[M-2F]^{-\cdot}$) and 162 (correponding to $[M+H-F]^{-\cdot}$) are characteristic of wall reactions while Gaussian profile of ion current at *m/z* 180 corresponds to $M^{-\cdot}$. Neutral species that can form ions often stick to the source wall, and they move out of the ion source more slowly, causing the non-Gaussian profile.

Other researchers have used the Sears models to postulate mechanisms for unusual ions. For example, an unusual ion, MH^-, is observed in the ECNI spectrum of chloroprothixene (34). Three mechanisms are possible for the formation of this ion: (1) electron capture followed by $H^{\cdot}$ transfer from the methane gas (route A in the Sears model), (2) $H^{\cdot}$ transfer followed by electron capture (route C), or (3) reaction on the source wall (route D). To determine the mechanism, several experiments were performed. When a gold-plated source is used, the MH^- ion remains unchanged; therefore, this ion does not result from a wall reaction, disproving mechanism 3. There is also no change in the $[M-H]^-/MH^-$ ratio with a change in emission current, suggesting that MH^- is independent of radical concentration. Mechanism 2, which is dependent on the concentration of the radicals from the reagent gas, is not the likely mechanism in this case. Mechanism 1 is the most probable in this case.

Molecular Parameters Affecting Ion Formation

Ion formation is affected by the electron affinity of the analyte, the electron attachment rate (also known as the electron-capture cross section), and the lifetime of the ion.

Electron Affinity. In order to capture an electron, a compound must have low-energy vacant orbital which correlates to a positive electron affinity (EA) (14). Systems with extensive π-conjugation result in lower-energy unoccupied orbitals. For

example, the electron affinity of benzene is negative and it will not form a stable anion. On the other hand, pentacene has larger orbitals which can overlap more effectively, resulting in low energy unoccupied orbitals. The electron affinity of pentacene is positive (35).

Fragmentation also is dependent on electron affinity. To fragment AB into A^-, the A-B bond dissociation energy and the total fragment energy must be less than the EA of A. Thus, the formation of molecular anions is favored by high, positive electron affinities, as shown for PAHs (23).

According to Field (35), molecular electron affinities generally are affected by the same structural factors that increase the wavelength of light absorption. In light absorption, the electron is usually promoted from an occupied to an unoccupied orbital, the same lowest unoccupied orbital that captures an electron. Not all the absolute energies are the same, but the trends should be similar for a series of compounds.

Several methods have been used to experimentally determine electron affinity. For example, gas-phase electron affinities may be derived from gas-phase electron transfer equilibria with a pulsed electron high-pressure mass spectrometer. Using this method, the electron affinity values for 21 quinones were determined (36).

Electron affinity is difficult to measure experimentally; therefore, estimation or approximation methods have been developed. *Ab initio* calculations have been used to calculate electron affinity directly, but with great difficulty. Another method of approximating the trends in electron affinities is by calculating the lowest unoccupied molecular orbital (LUMO) energy. Laramee and co-workers (18) calculated the LUMO energies for a series of polychlorodibenzofurans and polychlorodibenzo-*p*-dioxins using the "Complete Neglect of Differential Overlap" method. No molecular anion was observed for molecules with high LUMO energies, i. e., above 1.6 eV.

Another electron affinity estimation method was proposed by Chen and Wentworth (37). The half-wave reduction potential in aprotic solvents ($E_{1/2}$) was correlated to experimental values for a series of compounds. The relationship is expressed by $EA = E_{1/2} + 2.49 \pm 0.2$ eV.

The rate of electron attachment (cross section) can vary by orders of magnitude, while the cross section for electron impact varies very little (17). Cross sections are influenced by structural differences between molecules; for example, electron attachment rate constants increase as chlorine substitution increases. The rate constant also increases as the halogen changes from F to Cl (38).

Ion Lifetimes. The lifetime of an anion before it undergoes autodissociation can also vary greatly from 10^{-15} to 10^{-4} s. In order to have significant collisional stabilization under CI conditions, an anion must have a lifetime of at least 10^{-6} s. The ion lifetime is dependent on molecular structure and electron energy. At very long ion lifetimes (>10 μs), molecular anions can be observed under low-pressure conditions. Molecular anion lifetimes are listed in Table 1.1.

Table 1.1: Lifetimes of Molecular Anions (adapted from reference 17)

M^-	t (μsec)
$C_6H_5NO_2^-$	~40
Phthalic anhydride$^-$	313
o-$ClC_6H_4NO_2^-$	17
o-$C_6H_4(NO_2)_2^-$	463
m-$NO_2C_6H_4COCH_3^-$	310
o-$CH_3OC_6H_4NO_2^-$	16
$C_6H_5F^-$	<1 x 10^{-6}
$C_6F_6^-$	12
$C_6H_6^-$	1 x 10^{-6}
$C_6H_5CN^-$	5
$C_6F_5CN^-$	17
O_2^-	2 x 10^{-6}

Long-lived ions have positive electron affinities, usually imparted by conjugation and/or electron-withdrawing substituents like halogens, CN^-, or NO_2^-. These molecules tend to be large, so that the excess energy can be stabilized through many degrees of freedom; therefore, this can be called a "resonance" process. At ion lifetimes above 10 μs, molecular anions are observed under EI conditions of low pressure and high electron energy. Internal energies of a molecule increase the fragmentation, as shown for a series of polychlorodibenzo-*p*-dioxins (18). The branching ratio, log ($[M\text{-}Cl]^-$)/(Cl^-), is linearly related to the energies of the low-lying unoccupied orbitals (LUMO). The internal energy of $[M\text{-}Cl]^-$ increases with increasing degree of chlorination.

Experimental Parameters Influencing Ion Abundances

ECNI ion abundances and overall sensitivity are affected greatly by the choice of instrumental conditions. The following instrumental parameters will be discussed in more detail: source temperature, source pressure, reagent gas, sample concentration, electron energy, emission current, and lens potential.

Source Temperature. In general, ECNI spectra are highly dependent on source temperature. As the temperature increases, dissociative electron capture increases because negative ion lifetimes and the forward rate of clustering reactions are decreased. At low source temperatures (< 100 °C), the molecular anion formation is enhanced for a variety of compounds including hexabromobiphenyl (39), diazepam (22), and PAHs (24).

Sensitivity is also affected by source temperature; however, different compounds behave differently to increases in temperature. For example, Low and co-workers (23) studied the response of three PAHs, anthracene, fluorene, and fluoranthene, at temperatures varying from 90°C to 230°C. The response of anthracene and fluorene increased slightly with increasing temperature, but the

response of fluoranthrene fell off dramatically. Thus, compounds which exhibit primarily deprotonation, such as fluorene, have best sensitivity at high temperatures (> 150°C) while those favoring resonance capture, such as fluoranthrene, prefer low temperatures (< 100°C) (23). Compounds that undergo primarily dissociative resonance capture usually have a higher response at low source temperatures (39).

Temperature also affects the formation of ions resulting from reactions other than electron capture (8). Positive ion-electron recombination reactions decrease with increasing temperature, while positive ion-negative ion recombination reactions increase with increasing temperature.

In a study of fluorinated derivatives of chlorophenols (40), the effects of source temperature were dependent on the type of derivative. Derivatives used included pentafluorobenzyl, pentafluorobenzoyl, and 3,5-bis(trifluoromethyl)benzoyl. Temperature effects are minimal for compounds undergoing dissociative electron capture, such as the 3,5-bis(trifluoromethyl)benzoyl derivative of chlorophenol, but are important for those which can undergo resonance electron capture, such as the pentafluorobenzyl and pentafluorobenzoyl derivatives.

Source Pressure. Control of the ion source pressure is thought to be the most critical parameter for the reproducibility of ion abundances (14). As with source temperature, the optimum source pressure is dependent on the compound. In a study of PAHs (23), fluorene, which exhibits mainly $[M-H]^-$ ions, has an optimum sensitivity at low source pressure (< 0.065 kPa). For fluoranthrene which produces mainly $M^{-\cdot}$, the source pressure was not as critical.

Increasing the pressure increases the number of ion-electron pairs formed until the electron beam is stopped by gas molecules. Also, an increase in pressure will increase the collisional frequency, which affects the rate of electron thermalization and collisional stabilization of the $[AB]^{-*}$ intermediate. Usually, increasing the pressure increases the response until a maximum is reached. The initial increase in response is

probably due to collisional stabilization (41). After the maximum, the loss in response is probably from ion transmission losses (15). The pressure maximum is different for different reagent gases (18).

Reagent Gas. The reagent gas can have three functions in negative chemical ionization. The first is to convert primary electrons into thermal electrons. This allows resonance and dissociative electron capture. The gas can also react with the analyte. Charge transfer can occur if electron affinity of the reactant gas is less than the electron affinity of the substance. True chemical ionization can also occur between ions from the gas and analyte molecules.

In Table 1.2, the second-order electron thermalization rate constants and the number of collisions (Z) required for vibrational quenching of photoexcited bromobenzene positive ions are listed. In general, electron thermalization increases with increasing complexity of the reagent gas, i. e., increasing molecular size, dipole moment, and π character (42).

Table 1.2: Electron Thermalization rates (K_f), number of collisions required for vibrational quenching (Z), and ionization potentials (IP) for various reagent gases (adapted from reference 42).

Gas	K_f (cm^3s^{-1})	Z	IP
He	6.4×10^{-12}	150	24.59
Ar	1.3×10^{-13}	77	15.76
O_2	1.3×10^{-10}	30	12.07
N_2	2.2×10^{-11}	70	15.58
CH_4	8.6×10^{-10}	40	12.51
CF_4	--	--	16.25
CO_2	5.8×10^{-9}	18	13.77
NH_3	5.9×10^{-9}	--	10.18
COS	--	8	11.17
H_2O	--	8	12.61

In order to form stable molecular anions, the second-order rate constant for collisional stabilization is important. This rate constant should be fast with respect to the autodetachment rate constant. The collisional stabilization rate has not been measured; however, an assessment of this rate can be determined using the *Z* number, the number of collisions necessary for vibrational quenching. The lower the *Z* value, the more efficiently excess internal energy is removed from the excited ion (43). Quenching efficiency for gases not containing unsaturation or heavy atoms can be correlated by the relation $NM_r^{1/2}$ where N is the number of atoms and M_r is the reduced mass. Gases containing heavy atoms and unsaturation have vastly improved quenching efficiencies (42).

Many studies have been done comparing the effects of the type of reagent gas on ion abundances. If the major ion is $M^{-\cdot}$, as in the case of anthraquinone and bis (*N,N*-diethyldithiocarbamato) nickel (II), only minor differences are noted in the mass spectra with various reagent gases including He, Ne, N_2, CH_4, Ar, Kr, Xe, *i*-C_4H_{10}, and CO_2 (44). If the most abundant ion is not the molecular ion, changing the reagent gas can have a more significant effect on the relative abundances of the ions. For example, polybrominated biphenyls (PBB) and bromobenzenes were studied under methane and nitrogen ECNI conditions (39). When nitrogen was used as the reagent gas, the relative abundance of Br^- was enhanced as compared to $M^{-\cdot}$. The ratio of Br^- to $M^{-\cdot}$ was small under methane ECNI conditions.

In studies with polychlorodibenzo-*p*-dioxins (18, 41), six different reagent gases were used: methane, hydrogen, helium, sulfur hexafluoride, xenon, and argon. Pressures were varied between 0.1 and 1.0 torr and the relative abundances of the major fragment ions were recorded. Only a weak signal for Cl^- was observed when using sulfur hexafluoride as the reagent gas during the analysis of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin because the reagent gas removed all of the thermal electrons. When helium is used, the relative abundances of $M^{-\cdot}$, $[M\text{-}Cl]^-$, and Cl^-

remain constant over a pressure range of 0.1 to 0.8 mmHg. This can be used advantageously in GC-MS work because helium is the carrier gas of choice. Also, competing ion-molecule reactions are not observed with helium as they are with hydrogen. Using hydrogen, $M^{-\cdot}$ is the dominant ion at low pressures, but at high gas pressures, Cl^- predominates. Argon shows similar pressure-related behavior. Xenon exhibits unique behavior with varying pressures. At low pressures, $M^{-\cdot}$ dominates. As the pressure increases, the relative abundance of $M^{-\cdot}$ decreases at pressures from 0.02 to 0.29 mmHg at which point $M^{-\cdot}$ again becomes the dominant ion.

The variation of ECNI response with type of reagent gas has also been studied. One of the primary studies was performed by Gregor and Guilhaus (44) on anthraquinone and bis (*N,N*-diethyldithiocarbamato) nickel (II) using nine reagent gases. The relative responses were in the following order: $He < Ne \sim N_2 < CH_4 \sim Ar \sim Kr < Xe < i\text{-}C_4H_{10} < CO_2$. Both analytes form mainly molecular anions. In order to prove that the responses were from just the effect of the reagent gas, it was determined that the sample did not in any way participate in the thermal electron formation or collisional stabilization processes. The gas pressure for maximum sensitivity varies with each reagent gas; in general, the gas pressure corresponding to the highest sensitivity decreases with increasing atomic or molecular weight of the gas. When monoatomic gases are used as the reagent gas, the sensitivity of the analyte increases with increasing atomic number of the gas. When polyatomic gases are used, the sensitivity of the analyte increases with the available degrees of freedom of the reagent gas, with the exception of CO_2. CO_2 is thought to be a very effective electron thermalizer because it has a large quadrupole moment, a low-lying vibrational threshold, and can thermalize sub-excitation electrons by resonance scattering processes.

Other studies have confirmed the observations made by Gregor and Guilhaus about reagent gas type. Laramee, *et al.* studied 1,2,3,4-tetrachlorodibenzo-*p*-dioxin

using argon, xenon, sulfur hexafluoride, hydrogen, and helium (41). Heavier mass gases, such as methane, argon, and xenon, have the best sensitivities at low source pressure.

Sensitivities of fluoranil as well as nitrobenzene with fluorine, bromine, chlorine, nitro, and CF_3 substitutions were determined using six different reagent gases (43). In general, the order of relative response was Xe < He < Ar ~ N_2 < CH_4 ~ CO_2. The use of CO_2 and CH_4 as reagent gases increased the sensitivities of the analytes by one to three orders of magnitude more than the other gases.

The relative response of fluorocarbons with seven different reagent gases has been studied by Huang and co-workers (42). In general, the order of relative response for hydrogen- or oxygen-containing compounds, such as perfluoro-*t*-butanol and perfluorobis(isopropyl) ketone was He < CF_4 ~ Ar < CH_4 < CO_2 ~ air ~ NH_3. For $M^{-\cdot}$ forming compounds that included perfluorohexane, perfluorobenzene, and perfluoro-*trans*-decalin, the relative response was He < CF_4 < Ar ~ air < CO_2 ~ NH_3 < CH_4. The actual order was very compound dependent. Carbon dioxide or ammonia is best for the detection of hydrogen- and oxygen-containing fluorocarbons in which dissociative resonance capture predominates. For compounds which exhibit resonance capture, methane is the best reagent gas.

Certain reagent gases can also simplify ECNI mass spectra. For example, CO_2 has been suggested as a model reagent gas for several reasons (43). The spectra are simpler because only resonance and dissociative electron capture processes are observed for compounds that often undergo source reactions involving gas-phase and surface-bond free radicals. The fluoranil and substituted nitrobenzene compounds tested still had high sensitivity, comparable to that of methane ECNI.

In a study of polychlorinated compounds, such as heptachloroepoxide, dieldrin, and hexachlorobenzene, the response and spectra of several reagent gases, including nitrogen, ethylene, and methane were compared (45). When using N_2, no rhenium

ions were observed, thus reducing the background. However, a lower ECNI response for the compounds tested was obtained using nitrogen rather than methane. C_2H_4 was a poor reagent gas because there is more background, lowered response, and a chance of ion-molecule adduct formation.

Concentration. Concentration of the analyte can affect the appearance of the spectra as well as the response. For certain compounds, such as the amino acid carboxy-*n*-butyl ester N-pentafluoropropionate, the ECNI mass spectrum is biased towards high mass fragments at high concentrations (23). This may be due to intermolecular reactions. This effect was not noted for compounds such as PAHs. Radical incorporation reactions are enhanced by low sample concentration (15). At high concentrations, the response becomes nonlinear and the chromatographic peak shape becomes non-Gaussian (5). Often, saturation of the detector with analyte results in flat-top chromatographic peaks.

Electron Energy. The electron energy will determine the penetration of electrons into the reagent gas as well as the efficiency of ion pair production. The electron energy has a very minor effect on the relative ion abundances of decafluorotriphenylphosphine (DFTPP) and chlordane (5).

Emission Current. The emission current determines the number of electrons entering the ion source. As the emission current is increased, so is sensitivity because the ion population is increased. The effects of emission current were studied using DFTPP and chlordene (5). The relative abundance of ions from DFTPP does not change with increasing emission current. Many of the chlordene ions, however, are dependent on emission current. The types of ions most likely to change with increasing ionization current result from radical reaction prior to ionization.

Lens Potentials. The lenses controlling the extraction and transmission of ions can affect the relative ion abundances. The effects of lens potential on ion abundances

is complex, often depending upon interactions between the lenses. The most complete work on this topic was done on a HP 5985 quadrupole mass spectrometer (5).

The repeller potential has a minor effect on ion abundances, but can affect the voltage profiles of the other lenses. The drawout lens accelerates ions to the ion focus lenses. Higher mass ions are slightly affected by the drawout lens. The ion focus lens collimates the ion beam and has the largest effect on relative ion abundances, which is especially pronounced for low-mass fragment ions such as Cl^- or $C_6F_5^-$. This lens has an important effect on the comparison of ECNI mass spectra measured on different instruments. To overcome the quadrupole fringing fields, the entrance lens is used whose potential affects the abundance of the high-mass ions.

Applications of ECNI-MS

Selectivity

ECNI is more selective than positive CI because not all molecules have low-lying vacant orbitals or virtual vacant orbitals. In conjugated systems, the π^* orbital is delocalized and electron-withdrawing groups (like F, Cl, CN) lower the energy of these orbitals making them more accessible. Positive ions are generated by removing valence-level electrons, as can be done for all compounds (14). In order to generate a negative ion, a compound must have a positive electron affinity. Because many organic compounds have negative electrons affinities, ECNI can be very selective for certain classes of compounds.

Ionization efficiency is determined by the electron affinity and the electron-capture cross section of the analyte, which can vary over a wide range such as a factor of 10^6 between the values for hexane and carbon tetrachloride (16). After colliding with a thermal electron, a molecule can lose this electron by collisions. For small molecules, the electron loss process can be very rapid (about 10^{-13} s), but for large

molecules, the excess energy can be redistributed and the rate constant for electron loss is about 10^{-6} s. Thus, the negative anion can be more readily observed.

Sensitivity

As with selectivity, the sensitivity of compounds to electron capture techniques varies greatly. Sensitivity is dependent on the electron-capture cross section which can vary greatly. In contrast, the cross section only varies by one or two orders of magnitude in the positive ion mode (46). Therefore, one can expect widely varying and unpredictable responses under ECNI conditions. For compounds exhibiting electron-capture properties, less than 10 ng of sample can give a discernible spectrum. For highly electrophilic compounds, saturation of the detector signal can occur with as little as 100 ng. To saturate the detector with positive ion current, micrograms of sample are needed. The reaction rate for gas phase positive and negative ion reactions (PCI and NCI) is about 10^{-9} cm^3s^{-1}. For electron capture, the rate constant is 4×10^{-7} cm^3s^{-1} or about 400 times greater (3). Thus, sensitivity should be 400 times greater by ECNI than by PCI or NCI.

For selected compounds with high electron-capture cross sections, ECNI can be 100 to 1000 times more sensitive than positive EI (3). Detection limits for highly fluorinated compounds like perfluoromethylcyclohexane and perfluoro-1,3-dimethylcyclohexane are 2 to 3 fg (47). This is a three to four times better detection limit than that obtained by GC-ECD. In theory, the detection limit of the GC-ECD is on the order of 330 attomoles (48). Because ECNI-MS measures ion abundances rather than a decrease in standing current, Hunt and Crow have estimated that ECNI could be 10 to 100 times more sensitive than ECD (49). It has been noted (47) that noise rather than signal determines the detection limit in ECNI. This noise is thought to be general chemical background.

Types of Compounds

ECNI-MS has found utility in the analysis of many electrophilic compounds, mainly highly halogenated ones (5). Highly conjugated compounds also have been effectively analyzed (10). For compounds without electrophilic groups, halogenated derivatives have been prepared prior to analysis (50). For a review of halogenated derivatives, please refer to Chapter 3.

Compounds that have been analyzed by ECNI-MS with no further derivatization include *s*-triazines (51), trichothecenes (7), aflatoxins (52), polycyclic aromatic hydrocarbons (53), dihydropyridine calcium-channel blockers (54), lorazepam (55), and steryl fatty acid acyl esters (56).

Many biological compounds can be analyzed by ECNI-MS only after derivatization. Derivatization is necessary because the molecule is thermally labile and/or has poor electron capture response. Corticosteroids have been analyzed after MO-TMS derivatization (57). Valproic acid (30) and histamine (58) have been analyzed as their pentafluorobenzyl derivatives. A heterocyclic amine thought to be a carcinogen, 2-amino-3,8-dimethylimidazo [4,5-*f*] quinoxaline, has been analyzed as its 3,5-*bis*-trifluoromethylbenzyl derivative (59).

Electron Capture Response Studies

Electron Capture Negative Ionization Mass Spectrometry Response Studies

The effect on halogen substitution on ECNI-MS response has been studied for a series of brominated dibenzodioxins and dibenzofurans (60). The presence of at least two bromines or a bromine with at least one other chlorine is required for high response. In the ECNI mass spectra of these compounds, Br^- ions usually predominate; in fact, an assay was developed for brominated compounds based on monitoring only the ion current corresponding to the Br^-.

Crow and co-workers (25) compared the methane positive CI, methane ECNI, methane/CH_2Cl_2 chloride attachment NCI, and methane/O_2 NCI source conditions using chlorobenzenes and bromobenzenes with one to six substitutions. The compounds all have fairly similar positive CI responses, except for tetrabromo- and hexabromobenzene which had low responses. In the ECNI mode, bromobenzene, chlorobenzene, and dichlorobenzene cannot be detected. The tri- and tetrachlorobenzenes have fairly low responses. In these cases, the major ion is Cl^-; minor ions include $[M-H]^-$ at about 10% relative abundance. No molecular anion is observed. The di- and tribromobenzenes have responses in ECNI similar to that obtained during positive CI. Their spectra consist of mainly Br^-, with $[M-H]^-$ as a very minor ion (< 0.5%). The only compounds that have molecular anions at significantly higher responses than under positive CI conditions were pentachlorobenzene, hexachlorobenzene, and tetrabromobenzene. Hexabromobenzene had a molecular anion and about the same response as in positive CI. Interestingly, hexabromobenzene has a lower response than tetrabromobenzene.

The methane/CH_2Cl_2 chloride attachment NCI spectra generally are not very abundant. The monochlorides and dichlorides were not detected; the trichlorides have molecular anions, but very poor sensitivity, worse than positive CI or ECNI. Only pentachlorobenzene and hexachlorobenzene have sensitivities comparable to that achieved with ECNI.

The methane/O_2 NCI technique is the best choice for many of the compounds. For all brominated benzenes except monobromobenzene, it is the most sensitive detection method. This detection method is also superior for the chlorinated benzenes containing more than three compounds.

In a study of the responses of chlorinated phenols, biphenyls, and aliphatic bicyclics, the trends in response for ECD and ECNI were compared (63). In the phenol and biphenyl systems, molar response increases with increasing chlorine

number for both ECD and ECNI. In each case, the ECNI response falls slightly for the fully chlorinated species. However, for the chlorinated bicyclics, the increase of molar response with increasing chlorine number is observed only in ECNI; the ECD response does not vary.

Monochlorinated and dichlorinated ethyl acetates have recently been studied by ECNI-MS and ECD (62). The position of the chlorine substitution is vitally important; the highest ECD response monochloride is $CH_2ClCOOCH_2CH_3$ while the highest response dichloride is $CH_2ClCOOCH_2CH_2Cl$. The presence of two chlorine substituents on the same carbon increases the response slightly, but not significantly. The standard deviations for this ECD work were quite high (ranging from 10 to 16%). The standard deviations for the ECNI work were not even reported; this is probably because typically ECNI-MS standard deviations are higher than ECD standard deviations. The trends in ECD and ECNI response are the same.

In a study of fluorinated the pentafluorobenzyl, pentafluorobenzoyl, and 3,5-*bis*(trifluoromethyl)benzoyl derivatives of phenol (40), it was noticed that the relative ECNI molar response showed the same trend regardless of source temperature. Each of these derivatives have spectra consisting of essentially one peak. The relative trends are the same when compared to the trends in the ECD response.

GC-ECD Response Studies

ECD differs from ECNI in the method of detection. In ECNI, thermal electrons formed from a reagent gas are captured by the analyte and the resulting negative ions are detected. In contrast, a standing current in ECD is formed from the ^{63}Ni foil in the ECD detector. The decrease in standing current when an analyte captures an electron is measured.

Many more response studies for steroids have been done using ECD rather than ECNI-MS. In studies with haloacyl derivatives of testosterone and diethylstilbesterol

(63), monochloroacetyl and chlorodifluoroacetyl derivatives capture electrons more efficiently than trifluoroacetyl (see Table 1.3). Pentafluoropropionyl and heptafluorobutyryl derivatives capture electrons more efficiently than trifluoroacetyl, but not quite as well as chlorodifluoroacetyl. However, the features impart by the heptafluorobutryl derivatives are considered the best compromise between sensitivity and volatility.

Unsaturated steroids have been studied using ECD. The earliest study was done by Lovelock and co-workers (64), the person who invented the ECD detector. As shown in Table 1.4, the 4-ene-3-one system is thought to be the basis for enhanced response. A steroid with three isolated ketones has the same response as a steroid with only one ketone. A 1-ene-3-one does not have an appreciable increase in response; however, a 4-ene-3-one increased the response as in 4-androsten-3,17-dione. The 17-position seems significant to the authors because 4-cholesten-3-one and testosterone (4-androsten-3-one-17-ol) have lower responses than compounds with a 17-one. The 4-cholesten-3-one response is lower than that of 4-androsten-3-one by a factor of two.

Table 1.3: Relative Response of the Electron-Capture Detector to some Haloacyl Derivatives (adapted from reference 63)

	Compound	
Derivative	**Testosterone**	**Diethylstilbesterol**
Acetyl	1.0	--
Monochloroacetyl	40	2.7
Chlorodifluoroacetyl	340	--
Dichloroacetyl	--	2.6
Trichloroacetyl	--	2.1
Trifluoroacetyl	4	1.7
Pentafluoropropionyl	50	--
Heptafluorobutyryl	190	--
Perfluorooctonyl	600	--

Since the authors admit that from a *single* test the reproducibility of 4-androsten-3,17-dione was within 17%, a difference of a factor of two may not be very significant. Comparing the response for testosterone to the response for 4-androsten-3,17-dione is unfair because testosterone has a hydroxy group which is more likely to remain on the injector or column. Then the authors stated that the location of the carbonyl group in the D-ring is not important because progesterone, 4-androsten-3,17-dione, and 4-androsten-3,16-dione have the same response. Perhaps this is true because the 17-position is not important for enhanced response.

The 1,4-diene-3-one system is interesting. 1,4-Androstadien-3,17-dione has one fifth the response of 4-androsten-3,17-dione. However, when an 11-ketone is added to the 1,4-androstadien-3,17-dione, the response increases by a factor of 100.

The other ECD response study for steroids used substituted 17α-acetoxyprogesterones (65). Methyl groups in the A or B ring have no effect, but substitution at the C-16 position are important. A methyl group at C-16 increases the response by a factor of two to three, while a methylene group has two to six times the response of a methyl group. The 1,4-diene-3-one group has a very small increase in response over that of a 4-ene-3-one group. Much more significant are the responses of the linearly conjugated 4,6-diene-3-one system. The addition of a 6-methyl group to this system increases the response by six. The most sensitive compound in this study is 4,6-pregnadien-3-one-6β-methyl-16-methylene-17α-hydroxy acetate (melengestrol acetate), which combines all the important features for enhanced response--linear conjugation, a 6-methyl group, and a 16-methylene group.

Table 1.4: Electron Absorption Coefficients of Steroids (adapted from reference 64)

Compound	Absorption Coefficient
Androstane	0.01
Cholestane	0.01
Cholesterol	0.03
Androstane-3,17-dione	0.10
Allopregnane-3,11,17-trione	0.50
4-Cholestene-3-one	11.3
Testosterone	8.4
19-Nortestosterone	9.1
3,5-Cholestadiene-7-one	96.5
17β-hydroxy-1,4,6-androstatrien-3,17-dione	85.0
1-Androsten-3,17-dione	0.21
1,4-Androstadiene-3,17-dione	4.5
4-Androsten-3,17-dione	23.5
4-Androsten-3,16-dione	19.2
Progesterone	22.2
4-Androsten-11-ol-3,17-dione	76.0
1,4,6-Androstatriene-3,17-dione	167
6-Ketoprogesterone	143
4-Androsten-3,11,17-trione	248
4-Pregnene-3,11,20-trione	250
1,4-Androstadien-3,11,17-trione	535
4,4-Dimethyl-5-cholestene-3-one	0.03
Estrone	0.03

All values are relative to the absoption coefficient of chlorobenzene, which is taken as unity, and are for electrons with thermal energy.

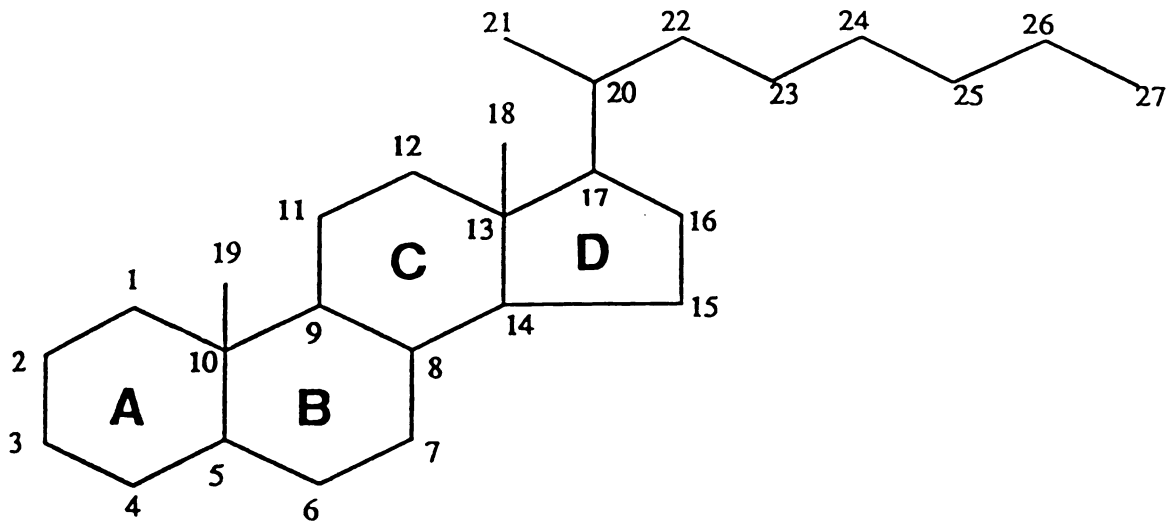
The Analysis of Steroids Using ECNI-MS

ECNI-MS has been shown to be a sensitive and selective technique for the analysis of compounds that are either highly halogenated or highly conjugated. Steroids are typically neither highly halogenated nor highly conjugated nor do they survive the GC intact; therefore, steroids usually are derivatized before ECNI analysis. Previous work in this laboratory (10, 66-70) has shown the feasibility of using chemical oxidation before ECNI analysis for the analysis of selected corticosteroids, namely dexamethasone in plasma and 6β-hydroxycortisol in urine.

A steroid is a fused four-ring system composed of three cyclohexane rings and one cyclopentane ring. In the IUPAC system, the parent name of a steroid results from the number of carbons in its skeleton; for example, a nineteen carbon steroid is an androstane. The numbering of the carbon atoms is shown in Figure 1.4.

Analytical Techniques for Steroid Analysis

Radioimmunoassay (RIA) has been widely used in the analysis of steroids. In general, radioimmunoassays provide a precise, low-cost, easy, and sensitive analysis with minimal or no sample extraction. Very small amounts of sample are needed; this makes RIA a valuable technique in cases were very little sample is available, as in the analysis of dexamethasone in newborns (71). A problem with the RIA analysis is precision; coefficients of variance are usually not better than 5%. Contributions to this error include specific activity of the label, incorrect optimization of assay conditions, non-specific binding effects, sample contamination, incomplete phase separation, and errors in counting of the radioactivity (72). The most significant disadvantage of the RIA assay is the cross-reactivity with structurally-similar steroids. Also, RIA can evaluate only one steroid at a time; another assay must be performed to quantitate another steroid.



Key for Abbreviations

A = Androstane C_{19} structure

P = Pregnane C_{21} structure

C = Cholestane C_{27} structure

α or β before a letter refers to the configuration at C-5 of steroids; e. g. αA for 5α-androstane

Superscripted number after a letter denotes a souble bond; for example, A^4 = 4-androstene

Figure 1.4: Steroid numbering system.

RIA analyses have been developed for all classes of steroids. For dexamethasone, RIA analysis (73-77) is the technique of choice for clinical analysis. RIA is fairly sensitive with a limit of detection (LOD) of 20 pg with a coefficient of variation on the order of 8%. Known interferences include cortisol (2% as active as dexamethasone), 9α-fluoroprednisolone (5%), paramethasone acetate (19%), and flumethasone (11%). For the analysis of 6β-hydroxycortisol, the cross-reactivity with cortisol is so high (17%) that an extraction step is necessary (78-79). The limit of detection is 25 pg with a coefficient of variance of 5% using 1 μL of urine or 50 μL of plasma (80).

Immunoassays based on enzymes have the same advantages of sensitivity and selectivity as their RIA counterparts, but do not have the problems associated with the handling and disposing of radioactive materials. An enzyme-linked immunosorbent assay (ELISA) for urinary 6β-hydroxycortisol (86) has been developed. Like radioimmunoassays, ELISA methods are quite sensitive, with a detection limit of 10 pg and quite good precision. The major problem with ELISA is the same as with RIA; that is, cross-reactivity with other steroids. In this case, there was a 1.2% cross-reactivity with cortisol and 6% with 6β-hydroxycortisone.

Thin-layer chromatography (TLC) is used in the quick screening of drug formulations (81). To confirm the results of the assay, CI-MS using a direct-probe inlet was used. Detection limits of this hour-long TLC assay were on the order of 25 ng. TLC is not a useful technique for determining steroids in biological fluids where the concentration is much lower. On the other hand, a complementary high performance liquid chromatography (HPLC) method had a detection limit of 2 ng.

HPLC techniques have been used in the analysis of corticosteroids (82-85), often analyzing more than one steroid at a time. The major advantage of the HPLC method is there is no need for derivatization; however, HPLC methods suffer from problems with sensitivity and selectivity. The most common detector used is

ultraviolet (UV). Almost all steroids absorb at the same UV frequencies which makes the extraction steps before analysis critical. In fact, some researchers will knowingly sacrifice analyte to be sure that the interferences are removed. The best limit of detection is 0.5 ng/mL for a HPLC method for the analysis of dexamethasone in plasma. More typical detection limits range from 4-10 ng/mL for a variety of steroids, including the simultaneous detection of prednisolone and prednisone.

HPLC-MS has been used to make liquid chromatography a more specific detector for corticosteroids (88-90). Although the HPLC inlet offers the advantage of direct analysis without derivatization, the sensitivity is not as good as comparable GC-EI-MS methods. In one study comparing LC-MS with GC-MS (90) for the analysis of serum cortisol, the LC-MS technique was half as sensitive as the GC-EI-MS technique employing methoxyamine-trimethylsilyl (MO-TMS) derivatives.

Gas chromatographic methods have long been used in steroid analysis. The hydroxy group on steroids accounts for their low volatility because of hydrogen bonding and thermal lability. In order to improve thermal stability and chromatographic properties, derivatives have been used (50). In the past, various detectors, such as flame ionization (FID) and electron capture (ECD) detectors have been used. At the present time, GC-MS is the detector of choice because of the specificity imparted by the mass axis.

Derivatization reagents should have the following desirable characteristics (87):

(1) Only one derivative must be made from the parent compound. More than one derivative complicates the GC chromatogram and also makes quantization difficult.

(2) The reaction must be complete. Often an internal standard is reaction simultaneously in order to compensate for incomplete reactions.

(3) The by-products should not interfere with the separation or mask peaks which represent the analyte. Ideally, one or two peaks are obtained, one for the reaction product and the other for the unreacted moiety. For example, a by-product such as the leftover derivatization agent should be removed.

(4) The derivative should be stable so that it does not degrade before GC analysis.

(5) The derivatives must not interfere with the separation of the parent compounds; i. e., the derivatives of two different compounds should be different.

(6) Other important advantages derivatization may have is the improvement of chromatography and the incorporation of a clean-up step.

(7) For GC-MS, the majors ions should be dependent on the parent molecule, not the derivatizing moiety.

The Analysis of Corticosteroids Using Mass Spectral Techniques

The synthetic corticosteroid dexamethasone has been successfully detected using GC-EI-MS techniques. One of these techniques, based on the trimethylsilyl-enol-trimethylsilyl-ether (tetra-TMS) derivative of dexamethasone was used in the determination of dexamethasone in human plasma (66). The detection limit was approximately 0.15 ng/mL. Replicate injections of sample were not possible; the extract was dissolved in 5 μL of hexane and 3-4 μL of this extract was injected into the GC-MS. The injection of such a large fraction of the sample resulted in the accumulation of contaminants on the GC column, requiring frequent removal of short sections of the column. The other method (91) used the tetra-TMS derivative to confirm the presence of dexamethasone in bovine tissues after analysis by normal-phase HPLC. The sample extraction was complex: after a three-phase liquid-liquid extraction, the sample was injected onto a coupled-column HPLC system. The detection limit of this method was 6 ppb. Problems with the EI analyses include

choice of ions to monitor. The molecular ion has a relative abundance of less than 10%. The ions monitored, $[M-335]^+$ and $[M-375]^+$, are not particularly diagnostic. In order to overcome this difficulty, high resolution selected ion monitoring (HR-SIM) was used.

Chemical ionization methods have found limited use in the analysis of corticosteroids. Both MO-TMS and tetra-TMS derivatives were used for a GC-CI-MS study of dexamethasone (92). The $[M+1]^+$ ion of the tetra-TMS derivative had a higher relative abundance than for MO-TMS (80% vs. 50%), so the tetra-TMS was used for the development of an assay for the determination of dexamethasone in human plasma. The detection limit of pure sample was about 100 pg; however, the limit of detection in plasma was not determined. An estimate of the detection limit can be obtained by noting that the lowest value on the calibration curve was 500 pg.

Electron Capture Negative Ionization Mass Spectrometry

ECNI-MS has been shown to be a very sensitive technique for compounds with electrophilic substitutions. Not many steroids are inherently electron capturing; therefore, a derivatization step is needed to improve sensitivity. Many conventional electron-capturing derivatives have been used for this purpose. For example, the pentafluorobenzyloxime derivative of testosterone has been analyzed by isobutane ECNI (93), but its base peak was $[C_6F_6CH_2]^-$ and another prominent ion was $[M-HF]^{-\cdot}$ (see Figure 1.5). The former ion could be found in any such derivative of a steroids and thus lacks selectivity. Selected ion monitoring (SIM) of the $[M-HF]^{-\cdot}$ ion gave a detection limit of about 20 pg.

Having a large proportion of the ion current carried by the reagent-specific ion causes many difficulties in the specificity of the analysis. In order to overcome this problem, researchers have developed MO-TMS methods for steroids with substitution in the form of double bonds, carbonyl, and hydroxyl groups (57, 88, 94).

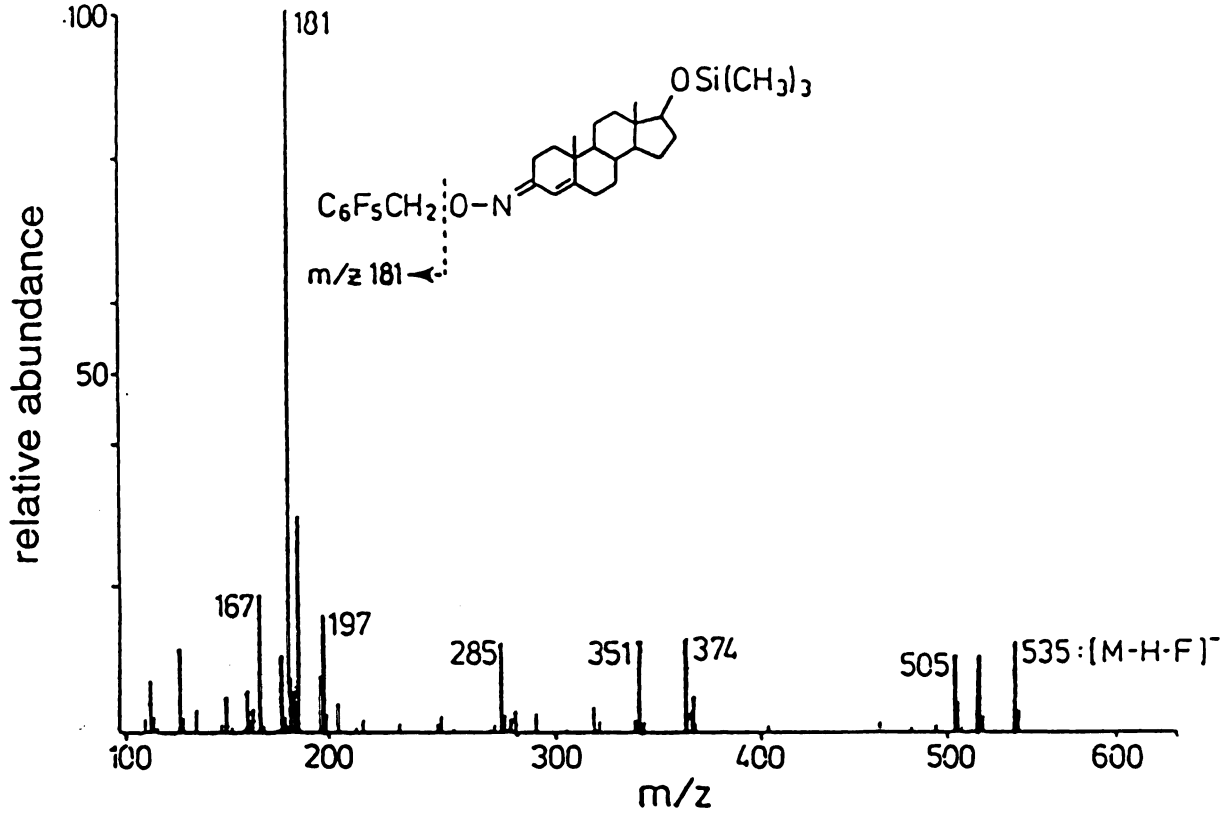


Figure 1.5: ECNI mass spectrum of pentafluorobenzyloxime, TMS-ether derivative of testosterone. The base peak at *m/z* 181 represents the pentafluorobenzyl anion (from reference 93).

Corticosteroids such as prednisone, prednisolone, dexamethasone, and betamethasone fall into this category. The MO-TMS derivative does not confer any electron-capturing properties to the steroid; however, the derivatization process enables the compound to survive the GC intact. In the case of the MO-TMS derivative of prednisolone acetate, the $M^{-\cdot}$ ion was formed and fragmentation was minimal. The limit of detection was 80 pg. The spectra are very different for prednisolone, dexamethasone, and betamethasone. $M^{-\cdot}$ or $[M-H]^-$ is very small, less than 10% abundance. The base peak often is $[M-2(NOMe)-TMS]^-$, which does correspond to the parent molecule. Fragmentation is much more prevalent. Even so, quantitation of 0.1 to 10 ng of corticosteroids in human aqueous humor are possible by this method. Along with problems associated with fragmentation, other problems with the MO-TMS derivative include the possibility of forming chromatographically-separable isomers, which makes quantitation difficult. Also, the derivatization procedure occurs in two steps; this may be less efficient than a one-step procedure.

A recent method for the analysis of dexamethasone in plasma and synovial fluid is based on the use of a tri-TMS derivative followed by GC-ECNI-MS analysis (95). The ion monitored corresponds to the $[M-TMSOH-TMS]^-$; very little other fragmentation is noted. The limit of detection is 0.1 ng/mL with a coefficient of variance of 6%.

Use of the Oxidation Methodology

When using conventional derivatization procedures to improve the vapor-phase characteristics and the electron-capture properties of the analyte for further analysis by ECNI-MS, there are two significant disadvantages. One is that any compound with the derivatizable functional group (such as hydroxy or amino group) will react with the derivatizing agent, thus forming species which can be detected by ECNI-MS and which may interfere with the detection of the analyte. Another disadvantage often

observed is that the major ions are indicative of the derivatization moiety rather than the parent molecule.

In order to overcome these disadvantages, an approach to derivatization using chemical oxidation has been used in this laboratory (10, 66-70). Using a mild oxidizing agent such as pyridinium chlorochromate (PCC), hydroxysteroids are converted to ketosteroids. Under ECNI conditions, these ketosteroids either form a molecular anion or undergo simple dissociation such as losing hydrogen, a methyl group, or HF. With little or no fragmentation, sensitivity is improved.

The real advantage of the oxidation methodology is the selectivity against background. Steroids amenable to this technique are oxidized to highly electrophilic species; other endogenous steroids form species which do not efficiently capture electrons. The decrease in background is best illustrated in Figure 1.6. In this experiment, a urine sample was spiked with 6β-hydroxycortisol. The oxidized product of 6β-hydroxycortisol, 4-androsten-3,6,11,17-tetrone, has an enhanced ECNI response due to the linearly conjugated 4-ene-3,6-dione system. The EI-MS reconstructed total ion chromatogram (Figure 1.6(a)) illustrates the response from a non-selective detector. All of the steroids in urine elicit a response. The spiked 6β-hydroxycortisol has a small response compared to the other steroids. If the selective ECNI-MS detector is used instead, the background virtually drops off (Figure 1.6(b)), and the signal due to the oxidized 6β-hydroxycortisol is significantly enhanced. Most of the trouble in developing sensitive ECNI methods is not in the response of the compound of interest but in the presence of background. The described oxidation methodology can help overcome this background problem.

Assays developed for steroids using the oxidation methodology are relatively sensitive. Steroids which can be successfully assayed include dexamethasone, betamethasone, prednisolone, prednisone, and 6β-hydroxycortisol. The limit of detection for the analysis of dexamethasone in plasma is 0.25 ng/mL (66). This is

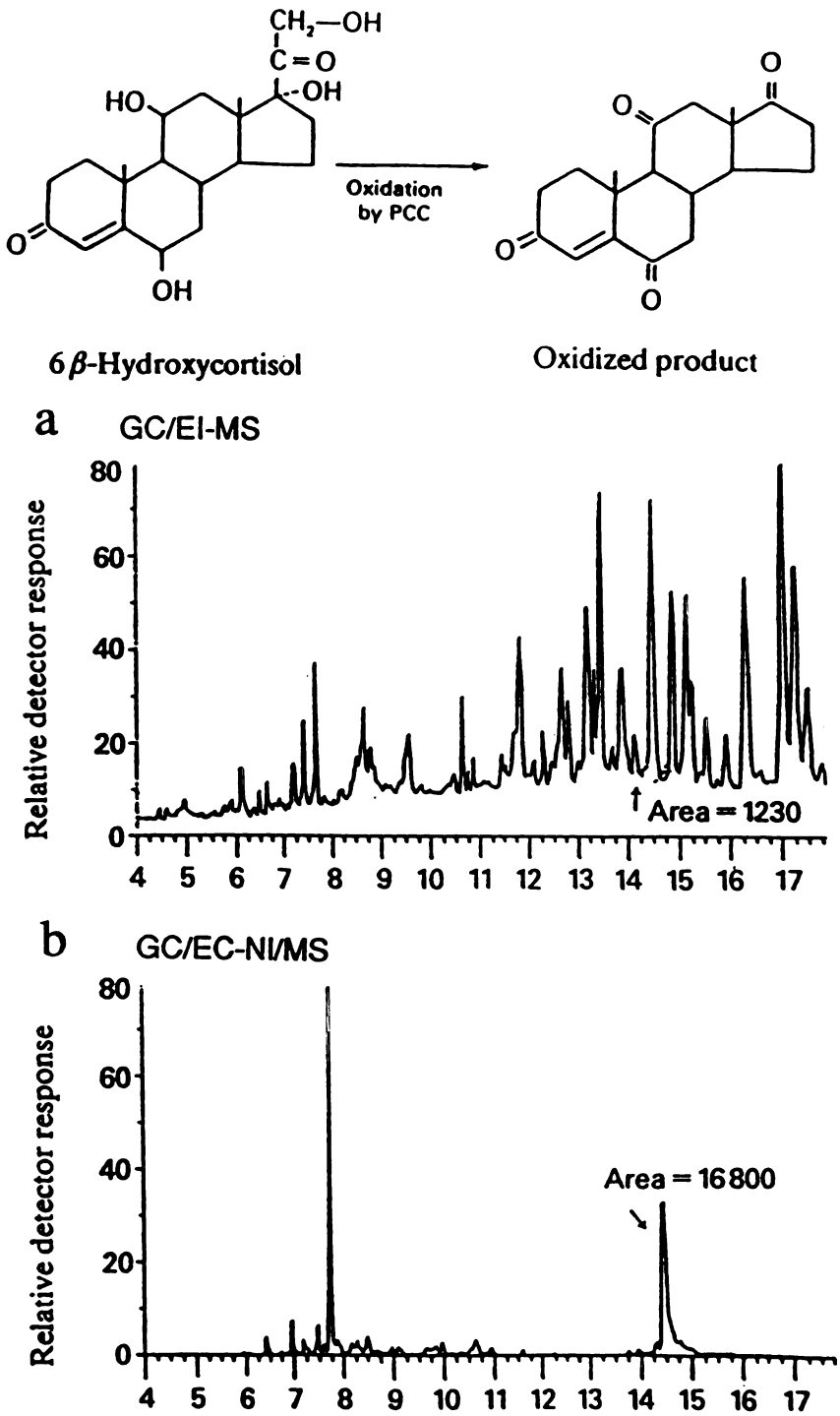


Figure 1.6: The oxidized product of 6β-hydroxycortisol is 4-androsten-3,6,11,17-tetraone. A sample of urine was spike with 6β-hydroxycortisol, extracted, and oxidized using PCC. (a) TIC from GC-EI-MS analysis (about 56 ng of analyte on-column). (b) TIC from GC-ECNI-MS analysis (about 44 ng of analyte on-column). Note the improvement in selectivity and sensitivity (adapted from reference 69).

much higher than the detection limit of the instrument which is 160 fg pure standard. One of the reasons for this disparity is the high value of the blank because the internal standard used, $^{13}C_6$,$^{2}H_3$-dexamethasone, had a significant amount of unlabeled dexamethasone present.

In comparison to the ideal derivative described previously, the oxidation methodology fares well. Only one derivative is made from each compound using the PCC reaction. Reaction efficiencies have been estimated at 50-95% (96) depending on the analyte. An isotopically-labelled or structurally-similar internal standard can compensate for the inefficiency of the reaction or losses during the clean-up procedures. By-products from the reaction itself are rare, but if the do exist, they are usually not as electron-capturing as the analyte. Because the derivatives are extremely stable, they can be stored for months in the freezer. After oxidation, chromatography of the steroids is improved over parent compounds. Interferences are minimized because the analyte oxidizes to a higher-response compound than the matrix; therefore, stringent clean-up procedures before GC-MS analysis are not needed. The major ion in the ECNI mass spectrum of oxidized steroids is always dependent on the parent molecule as either the molecular anion or an ion based on a simple loss from the parent molecule is formed.

The major problem with the oxidation methodology is that the derivatives of two different compounds are not necessarily different. For example, the steroidal drugs prednisone or prednisolone differ only by the substituent at C-11, which is either a hydroxy or carbonyl group. Upon oxidation with PCC, both prednisone and prednisolone form the identical product, 1,4-androstadien-3,11,17-trione (see Figure 1.7). One method of analyzing prednisone in the presence of prednisolone is by careful choice of the oxidizing agent. If sodium bismuthate is used, the 11-hydroxy group will not react; thus, prednisone can be analyzed without interferences.

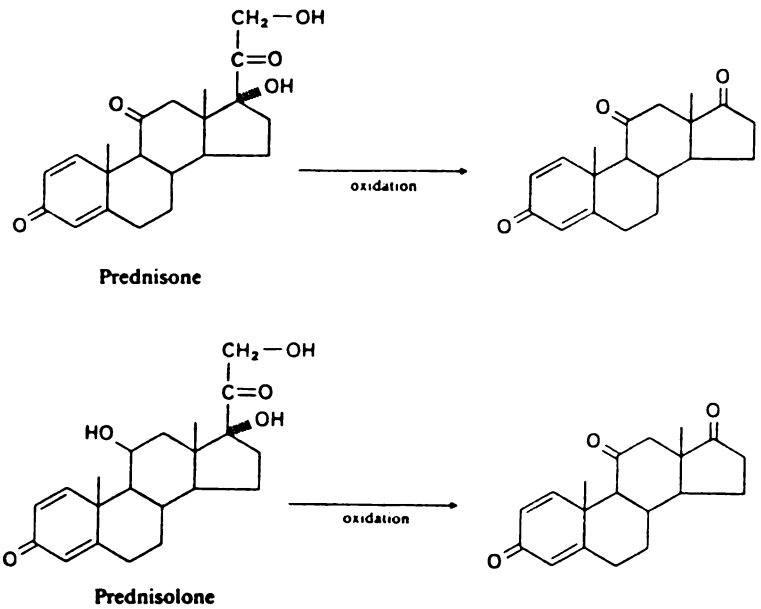


Figure 1.7: Prednisolone and prednisone are oxidized to the same product, 1,4-androstadien-3,11,17-trione.

In an attempt to improve the selectivity and sensitivity of the oxidation methodology, ECNI-MS-MS was tried (68). Oxidized dexamethasone was analyzed using selected reaction monitoring (SRM) of the ion currents corresponding to the reactions *m/z* 330 ($M^{-\cdot}$) → *m/z* 310 ($[M\text{-}HF]^{-\cdot}$). When the GC inlet was used, there was no noticeable difference between the samples analyzed by SRM and those analyzed by conventional SIM. The major advantage to SRM is that the direct inlet probe can be used, which significantly simplifies and shortens the analysis.

Objectives of This Study

The analysis of steroids in biological matrices, such as urine or plasma, is a complex problem. One possible way to solve this problem involves the use of chemical oxidation followed by GC-ECNI-MS analysis. The chemical oxidation step converts the hydroxysteroids into ketosteroids, thus improving their chromatographic properties. Also, this step imparts a degree of selectivity because the oxidized product of the steroid of interest, usually a steroidal drug or 6β-hydroxycortisol, will have a higher ECNI response than endogenous steroids.

In this study, three aspects of the chemical oxidation methodology were further developed: (1) the oxidation of steroids by alternative methods, (2) the study of the relationship of structure to ECNI response of steroids and other cyclic compounds, and (3) the development of specific assays for dexamethasone, prednisolone, and anabolic steroids.

Pyridinium chlorochromate (PCC) has been the oxidizing agent of choice for the conversion of hydroxysteroids into ketosteroids. Virtually no selectivity is imparted by this technique; all hydroxy groups within the steroid nucleus are converted into carbonyl groups. The efficiency of conversion ranged from 50 to 95% depending on the parent compound. Optimum reaction times are in the six to eight hour range. If this method is to be used in a clinical setting, the oxidation and clean-up procedure must be on the order of eight hours, a typical work day. In an attempt to improve selectivity, efficiency, and reaction time, other oxidation methods were investigated. Electrochemical oxidation was primarily studied because of its potential to solve problems inherent in the PCC methodology.

The success of the chemical oxidation methodology is dependent on the suppression of the interfering compounds. This occur because these compounds do not oxidize to highly electron-capturing species. In the investigation of the effects of structure on the ECNI response of steroids, different combinations of double bonds, carbonyl groups, halogens, and epoxides were studied. Smaller cyclic systems, such as cyclohexanes, quinones, and terpenes, were studied to compare the effect of molecule size on the ECNI response. In order to further understand the structure-response correlation, the ECNI response will be compared to the electrochemical reduction potential which can be considered the ability to capture an electron in the solution phase. The results from the structure-response study will be used as a guide for choosing steroids and other biological compounds amenable to ECNI assay development.

Finally, this project also involved the use of chemical oxidation for assay development. One particularly exciting area is in the development of assays for anabolic steroids. Because many anabolics have low responses under ECNI conditions, other electron-capturing derivatives such as trifluoroacetyl and pentafluorobenzyl, were investigated.

Two collaborative projects involving the analysis of steroids were done using the chemical oxidation methodology. The analysis of dexamethasone in human plasma was done in collaboration with Dr. Clinton Kilts of Duke University. The other, involving the determination of prednisolone in mouse plasma, was done in collaboration with Dr. Pamela Fraker of Michigan State University.

The results of these studies will further the understanding of the process behind the ECNI response of ketosteroids as well as provide further examples of the utility of the chemical oxidation methodology.

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Chapter 2: Electrochemical Studies of Steroids

The oxidation of hydroxysteroids to ketósteroids is the first step in the chemical oxidation assay for steroids in urine or plasma. In previous work in this laboratory (1), pyridinium chlorochromate (PCC) was chosen as the best reagent for the oxidation step. Several problems are encountered with the use of PCC. Depending on the compound, the yields range from 50 to 100%; therefore, complete oxidation is not available for all compounds. In order to achieve high yields, long oxidation times (5-10 hours) are necessary (1). Because this assay is designed to be done in a typical eight-hour work day in a clinical setting, a sufficient shortening of the PCC oxidation step could mean that the extraction from plasma and the oxidation steps could be accomplished in the same day rather than the two days now needed. Under PCC conditions, all hydroxysteroids are oxidized to ketosteroids; often a greater degree of selectivity is needed. For example, using PCC, both prednisone and prednisolone are oxidized to the same compound, 1,4-androstadien-3,11,17-trione. A methodology that could distinguish the two steroidal drugs would be useful.

Electrochemical oxidation has been proposed to overcome some of the problems with PCC oxidations. If the oxidation potentials of two steroids are different, electrochemistry could selectively oxidize the one with the less positive potential. Electrochemical oxidation also has the potential for more efficient conversions than by chemical means in a shorter period of time.

Steroid reduction potentials have been determined in order to compare the capture of an electron in the condensed phase to the capture of an electron in the gas phase. The electrochemical reduction potential in aprotic solution can be correlated with the electron affinity of the compound as done by Chen and Wentworth (2).

Electrochemical Synthesis of Ketosteroids

Very few examples of direct oxidative conversion of alcohols to ketones exist in the literature; this is due to the high oxidative potentials required (3). However, many examples of the use of oxidation for the detection of steroids exists, especially with HPLC-EC (electrochemical detection).

Several researchers have used electrooxidation for organic synthesis. Shono and co-workers (4) used the anodic oxidation of the dienol acetates of steroids to mimic liver microsomal γ-hydroxylation of 4-en-3-ketosteroids. A constant potential of 2.0 V (vs. SCE) with a platinum electrode was used. Electrochemical oxidation also was used to synthesize 9-substituted products of 1,3,5(10)-estratrien-3-methoxy-17-one (5). The oxidation was performed in an undivided cell with two platinum electrodes at a constant current of 800 mA for 40 min. Substitution at the 9-position depends on the solvent system used; for example, the use of methanol resulted in 9-methoxy products. When methanolic sodium cyanide is used as the solvent, three major products were formed with the cyano group at either the 2-, 3-, or 10β-positions (6).

Some electrosynthetic procedures used modified electrodes. The oxidation of cholesteryl acetate dibromide (7) using a lead dioxide electrode resulted in an 85 to 93% yield of products. After debromination, the major products were 15-hydroxycholesteryl acetate, 24-dehydrocholesteryl acetate, and 25-dehydrocholesteryl acetate. The selective oxidation of 3-hydroxysteroids has been accomplished using a nickel oxide hydroxide electrode (8). The yields for this selective oxidation are generally low, ranging from 22 to 78%.

Electrochemical detection of steroids after HPLC separation has been accomplished by oxidation. Ethynylestradiol (1,3,5(10)-estradien-17α-ethynyl-3β,17β-diol) can be oxidized with a mercury electrode (9). When the potential is held for some time at the switching potential of the cyclic voltammagram, sharp reduction

waves are noted. These data suggest that the oxidation process produces an insoluble mercury compound. Ethynylestradiol is not oxidized when either gold, platinum, or glassy carbon electrodes are used. Phenolic steroids can be easily oxidized electrochemically, but the oxidation is more difficult for saturated steroids. A HPLC method (10) with electrochemical detection was developed using acetonitrile containing sodium perchlorate as the mobile phase. The oxidation potentials (vs. SCE) ranged from 1.08 V for ergosterol to 1.78 V for campesterol. The detection limits varied between 10 and 100 ng. In some cases, this was an improvement in sensitivity over that of UV detection by a factor of ten.

Some ketosteroids are more effectively detected if they are derivatized before HPLC-EC. Because reduction of a ketone group occurs at fairly negative potentials, it is imperative that oxygen be removed before analysis, which is difficult to do when using HPLC. In order to efficiently oxidize ketosteroids, derivatization is necessary (11,12), usually with phenylhydrazine. Detection limits are on the order of 5 ng.

Because the electrochemical oxidation of alcohols occurs at fairly high oxidation potentials, some researchers have designed ingenious methods of overcoming this problem. One such method involves the use of iodonium ion as the catalytic electron carrier (13). The low anode potentials used (0.6-0.8 V vs. SCE) are sufficient for the oxidation of the iodine, which serves as both a catalyst and an electron carrier. Secondary aliphatic linear and cyclic alcohols have been converted to their corresponding ketones in yields typically ranging from 72 to 100%.

Electrosynthesis on Planar Electrodes

In these experiments, steroids were directly oxidized using platinum electrodes. After problems with extracting the products from the supporting electrolyte were solved, bulk electrolysis was used for synthesis. Because the oxidization product

adhered to the electrode, direct desorption off an electrode in a mass spectrometer source was an interesting possibility.

Experimental

Chemicals. All solvents used for electrochemistry were HPLC grade and purified before use. All solvents were transferred under vacuum to ensure they were pure and oxygen-free. The supporting electrolyte, tetrabutylammonium fluoroborate (TBAF), $(n\text{-}C_4H_9)_4NBF_4$, was purchased from Southwestern Chemicals (Austin, TX) or Aldrich (Milwaukee, WI). Steroids were purchased from Sigma (St. Louis, MO) or Steraloids (Wilton, NH).

Electrochemical Cell. For cyclic voltammetry studies, a specially designed one-compartment cell was used (see Figure 2.1). The cell is designed for use with three electrodes: a platinum wire auxiliary electrode, a silver wire reference electrode, and either a platinum, glassy carbon, or gold disk working electrode (BAS, W. Lafayette, IN). The design of the cell allows solvent transfer under vacuum. A "dumpster" side arm of the cell holds the compound of interest; this allows a blank analysis before the sample is added.

The silver wire reference electrode is really a quasi-reference electrode (QRE). If the silver wire is placed in nitric acid, it undergoes the following reaction:

$$AgNO_3 + e^- \rightleftharpoons Ag^0 + NO_3^-.$$

Because nitric acid will not be used in these analyses, no equilibrium will be established. Also, the silver wire electrode potential will change with time. To determine a reference point, an internal standard that has a well-known reduction potential should be added to the cell. Ferrocene has been used for this purpose. All the other peaks in the cyclic voltammagram (CV) then can be compared to the reference peak.

Electrochemical synthesis was performed in the three-compartment cell shown in Figure 2.2. These three compartments are separated by frits, but all three

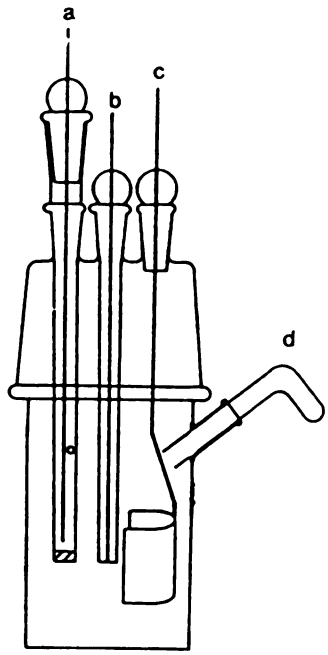

Figure 2.1: One-compartment electrochemical cell used for cyclic voltammetry. All solvent transfer is done under vacuum; the connection to vacuum is not shown. (a) Silver wire reference electrode, (b) Platinum working electrode, (c) Platinum counter electrode, (d) Sample reservior (adapted from reference 14).

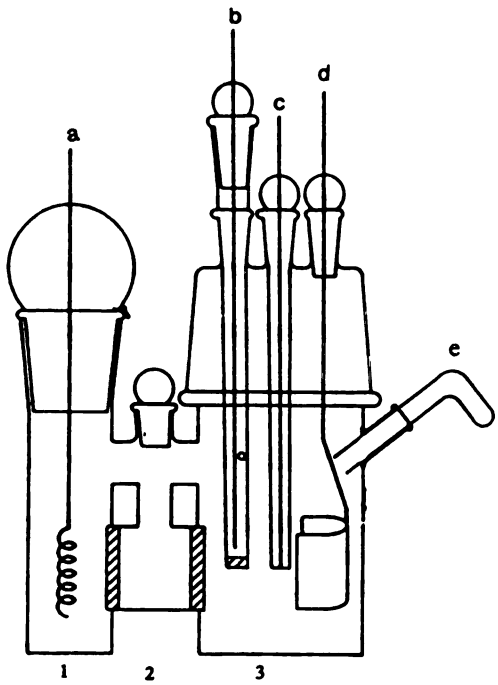

Figure 2.2: Electrochemical cell used for bulk electrolysis. All solvent transfer is done under vacuum; the connection to vacuum is not shown. (a) Platinum counter electrode, (b) Silver wire reference electrode, (c) Platinum working electrode, (d) Platinum gauze electrode, (e) Sample reservoir (adapted from reference 14).

compartments contain supporting electrolyte and solvent. The actual conversion of analyte using a platinum gauze electrode occurs in compartment 3. The silver wire reference electrode, which is encased in glass with a small frit on the bottom, is also in this chamber. The platinum auxiliary electrode is located in compartment 1 to prevent any undesired side reactions. The auxiliary electrode in compartment 3 is used only for the preliminary CV before beginning the electrolysis. All solvent transfer is done under vacuum.

Cyclic Voltammetry (CV). In CV, the potential of a stationary working electrode is increased linearly to a point and then decreased back to its starting point. After scanning to a potential where one or more electrode reactions take place, the direction of the scan is reversed and the reactions of the intermediates and products formed during the forward scan can sometimes be detected. CV is not a routine quantitative analysis technique, but has been used to study rates and mechanisms of oxidation-reduction processes (15, 16). In a reversible reaction, the equilibrium conditions between the oxidized and reduced material are maintained even with a rapidly changing potential. To be a reversible reaction, the difference in peak potentials between the anodic and cathodic peaks must be ΔEp = Ep(anode) - Ep(cathode) = 57/n mV where n equals the number of electrons involved in the electrochemical process. Reversibility is dependent on the scan rate used. The forward and reverse rate constants for the expression

$$O + e^- \rightleftharpoons R$$

are finite; therefore at high scan rates the equilibrium will not be maintained as the potential changes. Quasi-reversible refers to reaction where the forward and reverse rate constants are of the same magnitude. Irreversible refers to reactions where $k_b >> k_f$ for the anodic peak. In this case, $\Delta Ep > 57/n$ mV. Often only the anodic peak is seen because the material is not rereduced at a sufficient rate.

In this work, cyclic voltammetry was done using a one-compartment cell with about 2-5 mg of steroid, 0.2 *M* TBAF, and 3-4 mL solvent. The potential was scanned from 0 to 2.5 V at 100 mV/s and then reversed. All analyses were run on a BAS 100-A Electrochemical Analyzer (W. Lafayette, IN).

Bulk Coulometry. Coulometry is a constant potential technique; the current changes to keep the potential constant relative to a reference electrode. Bulk coulometry was used for the oxidation of steroids on a synthetic scale. About 20 mg of steroid were placed in compartment 3 of a three-compartment electrolysis cell (illustrated in Figure 2.2). The solvent and 0.2 *M* TBAF were placed in all three compartments. An EG&G Princeton Applied Research Potentiostat/Galvanostat Model 273 (Princeton, NJ) was used in the potentiostat mode. Synthetic oxidation was accomplished at a constant potential 200 mV above the anodic peak in the CV because at the peak potential only 50% of the sample can be converted.

Thin-Layer Bulk Electrolysis (TBLE). Thin-layer bulk electrolysis is an option on the BAS-100A Electrochemical Analyzer system. It differs from bulk coulometry in the way data are gathered and displayed. In bulk electrolysis, at minute-long intervals, the ratio of the average current of that minute to that of the first minute is computed. The charge ratio is also computed at one-minute intervals. In TLBE, the current and charge ratios are computed every second; therefore, a decision about the fate of the electrolysis can be made every second, rather than every minute. TLBE is useful for electrolysis of short duration; that is, only a few minutes.

Preliminary Cyclic Voltammetry

Choice of Solvent and Electrolyte. The solvent for oxidation must be capable of dissolving the analyte as well as having the appropriate potential range. The accessible potential ranges for several solvents are listed in Table 2.1. Note that these figures can only be taken as a guide because the actual potential limit is dependent on

Table 2.1: Accessible Potential Range for Some Solvents (Adapted from reference 10)

Solvent	Cathodic Limit V vs. SCE	Anodic Limit V vs. SCE
Water	-2.7	1.5
Methanol	-2.2	1.8
Acetonitrile	-3.5	2.4
Dimethylformamide	-3.5	1.5
Tetrahydrofuran	-3.6	1.8
Methylene chloride	-1.7	1.8

Table 2.2: Accessible Potential Ranges in Dimethylsulfoxide Containing Different Supporting Electrolytes (0.1 *M*) (Adapted from reference 10)

Electrolyte	Cathodic Limit V vs. SCE	Anodic Limit V vs. SCE
$LiClO_4$	-2.68	2.10
$KClO_4$	-2.33	2.10
$NaClO_4$	-2.08	2.10
KNO_3	-2.33	2.10
KBF_4	-2.33	2.10
$K_2S_2O_8$	-2.33	2.10
LiCl	-2.68	1.52
Me_4NCl	-2.40	1.52
Et_4BClO_4	-2.30	2.10
Bu_4NBr	-2.40	1.45

solvent purity, electrode material, and electrolyte. The potential range of some of the common electrolytes is listed in Table 2.2.

In this work, methylene chloride, acetonitrile and tetrahydrofuran were used as solvents for cyclic voltammetry because steroids are soluble in these solvents. TBAF was the supporting electrolyte because tetrabutylammonium salts have a high anodic limit. Prednisolone, prednisone, and 4-androsten-17β-ol-3-one were used as the test compounds.

Results from Preliminary Oxidation Studies. Of all the commonly used electrochemical solvents, steroids are generally more soluble in methylene chloride. Using methylene chloride, both prednisolone and 4-androsten-17β-ol-3-one were oxidized, and the oxidation was not reversible. However, the CV was not reproducible on a day-to-day scale; the next time the experiment was attempted, no oxidative peak was seen for either compound.

Table 2.3: Oxidation Potentials of Various Steroids in Acetonitrile
N. B.: All oxidation potentials were obtained using a quasi-reference electrode. Because these data were obtained on different days and no internal standard was used, the values cannot be directly compared.

Compound	Oxidation Potential (V vs. QRE)
Prednisolone	2.4
Prednisone	Did not work
Cortisol	2.1
Cholesterol	2.1
Dexamethasone	2.3
6β-Hydroxycortisone	2.4
4-Androsten-6β-ol-3,17-dione	2.1

Acetonitrile has a larger potential window than methylene chloride, which allows more steroids to be oxidized. As shown in Table 2.3, many steroids were

oxidized using acetonitrile with TBAF as the supporting electrolyte. Prednisolone has prominent oxidative peaks (see Figure 2.3), and the oxidation reaction was not reversible so that the products will not be reconverted into starting material. Prednisolone was chosen as the model compound for the bulk coulometry studies.

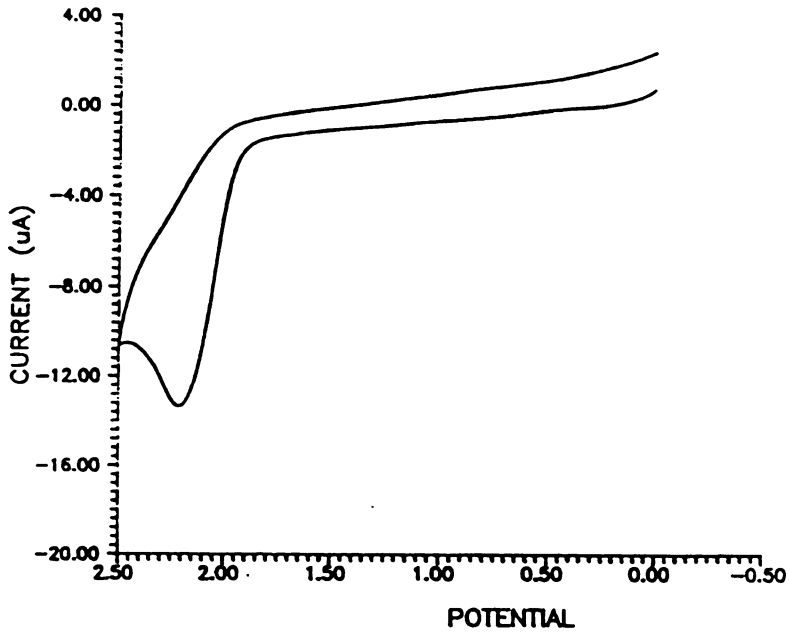

Figure 2.3: Cyclic voltammagram showing that electrochemical oxidation of prednisolone is irreversible. The oxidation potential of prednisolone is 2.3 V vs. QRE (quasi-reference electrode).

Steroids with 6β-hydroxy groups have large oxidative peaks. 6β-Hydroxycortisone had a very large oxidative peak, but because it was very expensive (about $8000 per gram), all further work was done using the much less expensive 4-androsten-6β-ol-3,17-dione.

Electrosynthesis

Removal of the Supporting Electrolyte. In order to determine the efficiency and the products of the oxidation using GC-MS, first the supporting electrolyte must be removed. About 99% (w/w) of the solute is supporting electrolyte. Many methods were developed to remove the TBAF.

The steroids are soluble in benzene, but not in water. TBAF is insoluble in benzene and slightly soluble in water. A trial solution containing prednisolone, 1,4-androstadien-3,11,17-trione, TBAF, and acetonitrile was evaporated under nitrogen, then redissolved in benzene. A cloudy solution resulted. Then, the salt was extracted with water, because the salt is more easily dissolved in water than the steroid. After five to six extractions, the benzene is evaporated and the residue is dissolved in ethyl acetate. Using ECD-GC, the extraction efficiency was calculated to be 2.1% for prednisolone and 14% for the expected oxidized product, 1,4-androstadien-3,11,17-trione. Another extraction that works just as well as benzene/water is ethyl acetate/water. This extraction follows the same procedure as benzene/water with about the same yield. Many other methods of separation of salt and steroid were tried, most unsuccessfully. One reason for the limited success is that TBAF and the steroids used have approximately the same solubility in a variety of organic compounds.

C18 solid-phase extraction columns have been used for desalting matrices because salts pass through unretained (17). A C18 column was conditioned with methanol and then water. Then 1 mL of trial solution containing TBAF, 1,4-androstadien-3,11,17-trione, and acetonitrile was placed on column. After washing with water and then hexane, ethanol was used as the eluent. Less than 0.5% of the steroid was recovered, and a great deal of noise was present in the GC chromatogram. This desalting procedure is probably most effective with salts that are more water-soluble then TBAF.

Because the methods of salt separation have very poor efficiency, "benzene recrystallization" was used to separate out the salt. The electrochemical solution, dissolved in acetonitrile, is evaporated under nitrogen. A small amount of benzene is added, and the solution is cooled in an ice bath. After several hours of cooling, crystals of salt form and are filtered. The filtrate, containing the steroid, is evaporated and reconstituted in ethyl acetate for further analysis. A trial electrochemistry solution

containing the supporting electrolyte tetrabutylammonium tetrafluoroborate (TBAF) and 1,4-androstadien-3,11,17-trione went through this procedure. TBAF was recovered with an 85% yield and the steroid was recovered with a 90% yield.

The benzene recrystallization method was also tried with a real sample, a prednisolone electrochemical oxidation. The approximate recovery of TBAF was 93%. The recovery of the oxidized product (1,4-androstadien-3,11,17-trione) was less than 1%. The recovery of starting material, prednisolone, was 25%. This suggests that the extraction procedure works, and the low yield of product is due to something other than poor extraction efficiency.

UV Studies of Steroids. Ultraviolet spectroscopy (UV) studies were undertaken for two reasons. One was to determine the maximum wavelength of selected steroids for use with HPLC-UV detection. The other was to see if the differences between the unextracted and the salt-extracted electrooxidation products of prednisolone were observable by UV spectroscopy.

All studies were done using a Varian DMS 200 UV-Vis Spectrophotometer. The maximum wavelengths for a series of steroids in methanol are shown in Table 2.4. Most steroids have a maximum wavelength between 235-250 nm, which justifies the use of 240 nm for the HPLC detector. Cholesterol, with a wavelength below 220 nm, is one exception. The supporting electrolyte, TBAF, also has a maximum wavelength under 220 nm.

The actual electrochemical reaction products, all dissolved in acetonitrile, were also studied. The maximum wavelengths for the standards in acetonitrile were less than those in methanol. Prednisolone had a maximum wavelength of 233 nm, while 1,4-androstadien-3,11,17-trione had one of 240 nm. The unextracted electrooxidation of prednisolone had two maxima, one at 236 nm and the other at less than 200 nm. The maximum wavelength of the benzene recrystallized product is 236 nm, just the same as one of the unextracted peaks. The extra peak in the unextracted sample is

probably not due to TBAF. An almost saturated solution of TBAF in acetonitrile was analyzed and no response was noted in the 190-300 nm range.

Table 2.4: UV Data for Selected Steroids in Methanol

Sample	Maximum Wavelength (nm)	Molar absorptivity ($L\ mol^{-1}cm^{-1}$)
1,4-Androstadien-3,11,17-trione	237.8	14500
Prednisolone	242.4	9770
Cortisol	241.0	13400
4-Androsten-3,11,17-trione	236.3	13800
Cholesterol	< 220	----
4-Cholesten-3,6-dione	250.1	7870
TBAF	< 220	----

High Performance Liquid Chromatography. In order to identify and quantitate both the starting material and the oxidized products from the electrochemical oxidation, a reverse phase HPLC method was developed. A Waters 600 multisolvent delivery system coupled with a Waters 712 WISP autosampler, a Waters 740 data module, and a Kratos Spectroflow 783 absorbance detector set at a wavelength of 240 nm with a range of 0.2 AUFS. Either 20 μL of the steroid standard or 10 μL of the real reaction solution was injected into a 150 x 4 mm Biorad ODS-55 C18 column. The flow rate was 1 mL/min.

The major objective of the HPLC method development was to separate TBAF from prednisolone and 1,4-androstadien-3,11,17-trione. Isocratic systems consisting of varying amounts of water/methanol and water/methanol/acetic acid were not successful. A gradient system which varied the methanol concentration from 100% to 80% in 20 minutes also did not work. An acetonitrile/water solvent system seemed

most promising. The results from this study are shown in Table 2.5. The solvent system used for the analysis of the real electrochemical samples was 70% water/30% acetonitrile.

Table 2.5: Reverse Phase HPLC of Electrochemical Solution

% Water	% Acetonitrile	Results
40	60	TBAF and prednisolone were not separated
60	40	TBAF and prednisolone were not separated
70	30	TBAF and prednisolone separated
80	20	Did not work

Bulk Coulometry of Prednisolone

Experimental. About 35 mg of prednisolone was placed in the third compartment of a three-compartment cell (see Figure 2.2) with acetonitrile as the solvent and TBAF as the supporting electrolyte. Controlled potential coulometry was run at 2.1 V. The decay of current was monitored. After two and a half hours, it did not quite reach zero, but this may be due to impurities. During the experiment, 7.06 coulombs were passed.

Results. The oxidized solution was analyzed using HPLC with the 70% water/30% acetonitrile solvent system. The chromatogram is shown in Figure 2.4(a). From a comparison with standards, the apparent formation of 1,4-androstadien-3,11,17-trione is 0.9%, and the recovery of the starting material (prednisolone) is 56%. However, if the electrochemical sample is spiked with an authentic sample of 1,4-androstadien-3,11,17-trione, the peaks are separated as shown in Figure 2.4(b).

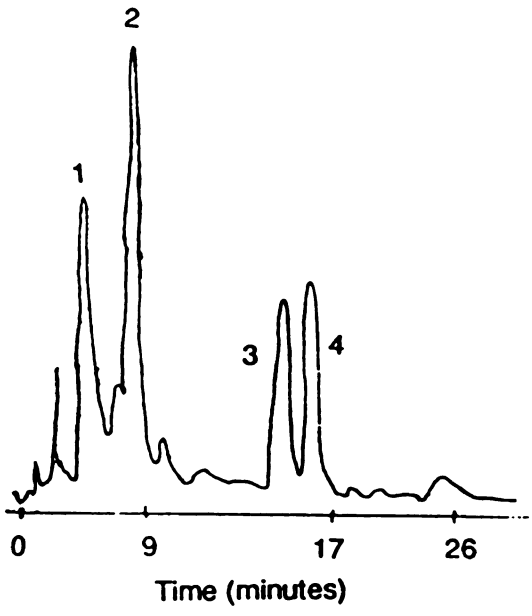

Figure 2.4: HPLC of electrochemically oxidized prednisolone (not extracted). Suspected identity of peaks: (1) TBAF, (2) prednisolone, and (3) oxidized product. (a) Electrochemical oxidation product. (b) Electrochemical oxidation product spiked with 1,4-androstadien-3,11,17-trione. Peak 4 is the spiked compound.

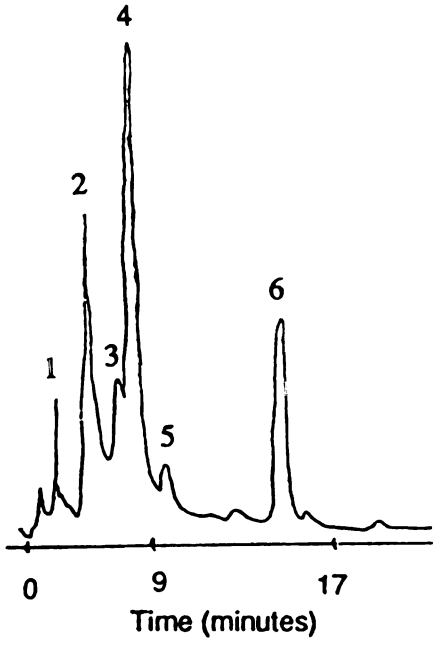

Figure 2.5: Fractions collected from the HPLC analysis of the electrooxidation product of prednisolone.

Table 2.6: Suspected Identity of HPLC Fractions

HPLC Fraction	GC Peak	Identity
1	1	nothing (perhaps solvent front)
2	1	plasticizer
	2	unknown
3	1	unknown
	2	plasticizer
4	1	unknown
	2	plasticizer
	3	*m/z* 282 (corresponds to prednisolone – substituents at C–17 – water)
	4	1,4-androstadien-3,11,17-trione
5	1	nothing
6	1	1,4-androstadien-3,11,17-trione
	2	prednisolone

In order to determine the identity of the compounds represented by these peaks, fractions were collected (see Figure 2.5). Six injections of 50 μL each were made. The fractions were lyopholized, then reconstituted for further analysis by GC-MS. The GC-EI-MS results are summarized in Table 2.6. Note that the supporting electrolyte produced no GC peaks other than baseline noise because it breaks down in the injector. Some fractions may have contained substantial amounts of supporting electrolyte.

The peak previously assigned to the supporting electrolyte was incorrect because a sample of this compound at the concentration in the electrooxidation product did not give a response. Further UV analysis shows that TBAF does not have a response at 240 nm, the wavelength used for all HPLC analyses. This peak, at 4.3 minutes, disappears after the sample has had the supporting electrolyte removed, so somehow whatever is eluting must have some relation to the supporting electrolyte or is just extracted along with the supporting electrolyte.

The other problem is the peak correponding to the suspected oxidation product. This retention time is shifted for all the electrooxidations, whether or not they have had the salt extracted. If prednisolone is oxidized using PCC oxidation, the peak retention times for oxidized product and 1,4-androstadien-3,11,17-trione coincide.

The results from the quantitation of the peaks again show the inefficiency of the electrooxidation. By PCC oxidation, there was a 50% recovery of the oxidized product. When the electrochemical product was run unextracted, 14% of the prednisolone and 11% of what is thought to be the oxidized product were recovered. The prednisolone peak is overlapped by several peaks; therefore, it is hard to quantitate. After the electrochemical oxidation product has had the salt removed by benzene recrystallization, 6% of the prednisolone and 5% of what is thought to be the oxidized product is recovered. This shows that some steroid is lost in the extraction procedure.

Electrochemistry of Steroids Other than Prednisolone

Prednisolone was the first steroid to exhibit any type of electrochemical response. In the search for more steroids that might exhibit an electrochemical response, prednisone was thought to be promising because it has an 11-carbonyl rather than a 11-hydroxy group, thus having one less functionality to oxidize than prednisolone. Prednisone, however, did not give an electrochemical response. This may be because its oxidation potential is greater than that of the solvent (acetonitrile).

Cortisol (4-pregnen-11β,17α,21-triol-3,20-dione) seemed to be a logical next choice because it is structurally similar to prednisolone, lacking only one double bond. Cortisol did display an oxidative response (potential at 2.1 V vs. QRE). For the bulk electrolysis, about 23 coulombs should have been passed if all the steroid had been oxidized; only about 11 coulombs were passed. The resulting solution was yellow, but the color may have been due to an impurity. Analysis by GC-MS showed that the product has two components. One is 4-androsten-3,11,17-trione, the expected

oxidation product. The other was not identified, but could be the breakdown of cortisol in the gas chromatograph. The starting concentration of cortisol was not available because more was added during the course of the reaction; therefore, the percentage oxidized is not known. Pyridinium chlorochromate oxidation (PCC) oxidation of cortisol gave a 30% yield.

Cholesterol was chosen as the next steroid to oxidize because it is relatively inexpensive and has been used for oxidative electrochemical detection in HPLC. Cholesterol has many oxidation products, even when oxidized chemically. For example, chemical oxidization using PCC resulted in the formation of two products. The major product is possibly 4-cholesten-3,6-dione. The minor product did not result in an interpretable spectra and was not identified.

Cholesterol was electrolyzed at a voltage of 2.2 V. After an hour, five coulombs had been passed. About seven coulombs should have been passed if all the substrate had been oxidized. The changes in color during the experiment were interesting. The solution first turned a pink, then a dusty rose, and ended as a forest green solution. Not all of the cholesterol had dissolved, so the resulting solution was filtered to get rid of the remaining crystals of cholesterol. Six products were formed, three of which have been identified as 4-cholesten-3,6-dione, 4-cholesten-3-one, and 5-cholesten-3-one.

6β-Hydroxycortisone had an oxidation potential of 2.3 V vs. QRE, but also had a larger current response (8.4×10^{-5} A) than even prednisolone (5.6×10^{-5} A). This larger response may be due to the ease of oxidation of the 6-hydroxy group. Bulk coulometry studies were not performed on 6β-hydroxycortisone because it is a very expensive steroid. Instead, 4-androsten-6β-ol-3,17-dione was used, which gave the same magnitude of response and was much less expensive. Almost no charge was passed (0.8 coulombs) and, not surprisingly, no product was converted.

Why is the Electrosynthesis of Steroids So Inefficient?

As has been stated before, the recovery of oxidized steroids has been very low, ranging from less than 1 to 10% depending on the analysis method used. One reason for the inefficiency may be that the oxidized material is adsorbed to the electrode after it is formed, thus rendering the electrode useless for the oxidation of the rest of the material in solution. This conclusion was reached because the current decreased on each successive cyclic voltammetric cycle when the voltage is scanned from 0 to 2.5 V (vs. QRE). If the voltage is scanned to -1.5 V, the current stays almost constant (see Figure 2.6). If, after oxidation, the voltage is scanned to -2.0 V, the electrode "cleans" itself; i. e., this procedure reduces the oxidized product back to prednisolone and then the cyclic voltammagram appears normal. This shows that the product of the oxidation appears to remain on the surface of the electrode (see Figure 2.6).

Probable Methods to Overcome Fouling

Fouling of the electrode may depend on the nature of the analyte, the characteristics of the solvent, and the material used for the working electrode. The solvent and working electrode were varied next, using prednisolone as the analyte. When acetonitrile was used as the solvent, prednisolone stuck to the glassy carbon electrode. Benzonitrile has the same potential window as acetonitrile, but different solvent properties. When dissolved in benzonitrile, prednisolone adhered to both the platinum and glassy carbon electrodes. No oxidative peak was present when using the gold electrode.

Desorption Off an Electrode--Direct Probe

After discovering that the electrooxidized products from steroids adhered to the electrode, procedures were developed to advantageously use this phenomenon. If the oxidized product absorbed on a metal surface is placed in a mass spectrometer, the surface compounds may be desorbed and analyzed.

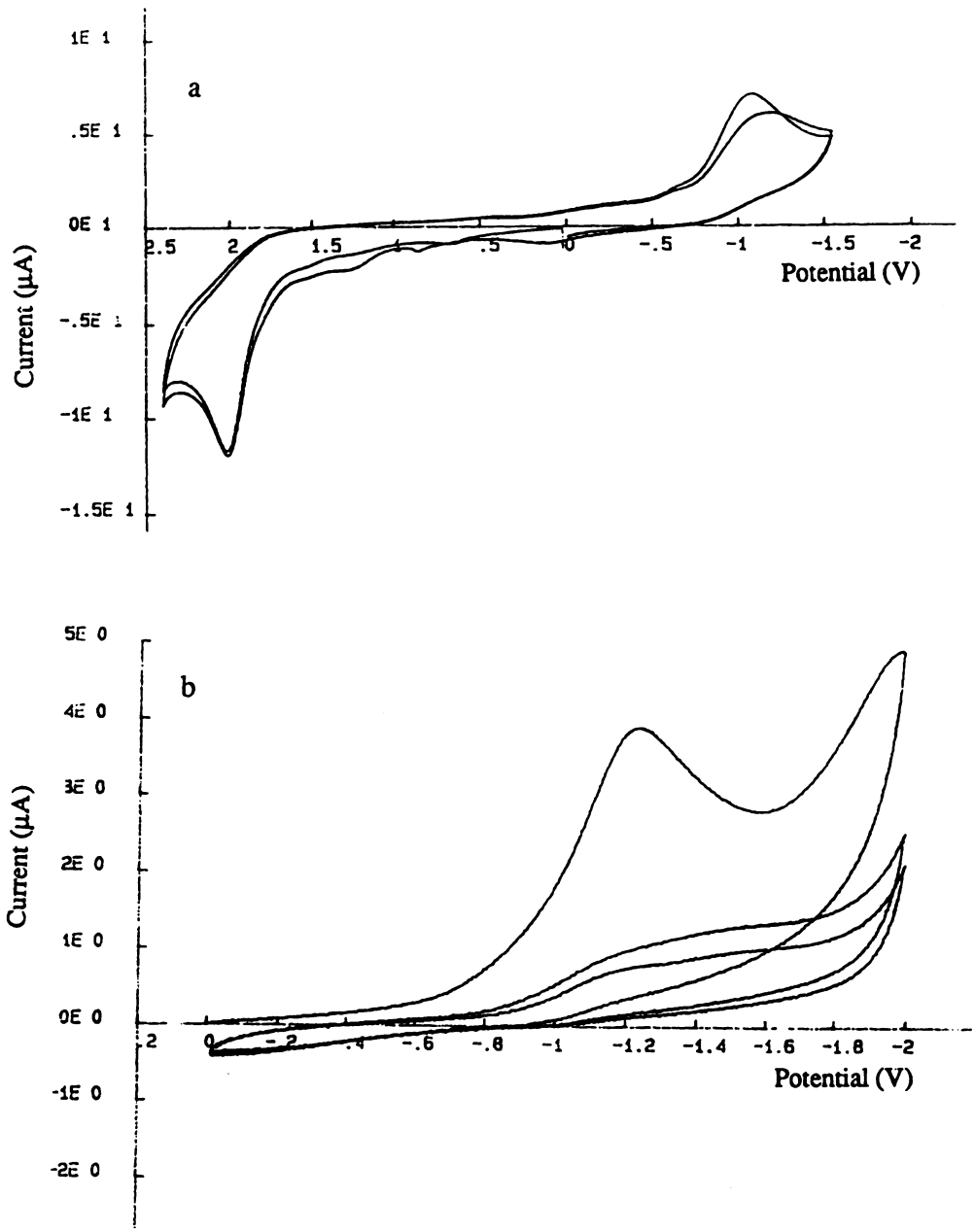

Figure 2.6: Cyclic voltammagrams of prednisolone. (a) The current stays approximately the same if the potential is scanned from -1.5 V to + 2.5 V. (b) However, if the potential is scanned from 0 to -2.2 V repeatably, the amount of current decreases, proving that material is adhering on the surface.

An electrode that can fit into either the probe assembly of a HP5985 or a JEOL AX505 mass spectrometer was designed (see Figure 2.7). A thin sheet of platinum foil was cut into a 2 x 11 mm rectangle and attached to a platinum wire. The wire was attached to the gold nest in the case of the HP5985 assembly with a series of electrical connectors. For the JEOL assembly, the electrical connectors were placed into the direct probe and held by an allen screw. It is estimated that the electrode, with a surface area of 44 mm^2, can possibly hold up to 454 ng of oxidized steroid, which should be more than enough to detect.

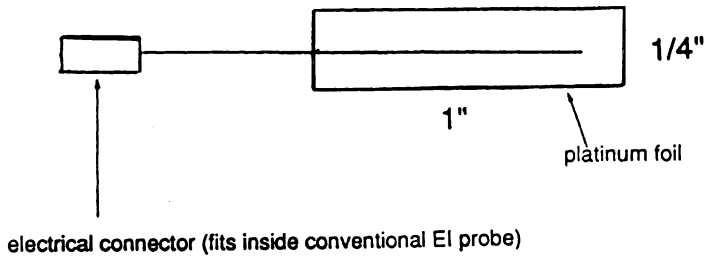

Figure 2.7: A platinum electrode that fits into the direct probe of either a HP5985 or JEOL 505 mass spectrometer.

In order to deposit steroids on the platinum surface, 3-4 mg of steroid were dissolved in acetonitrile with 0.2 *M* TBAF in a one-compartment cell. The TLBE program on the BAS Electrochemical Analyzer was used to deposit the thin films.

If a steroid solution is deposited directly on a platinum electrode using a syringe, the low mass end of the spectrum is the same as if the steroid had been introduced via direct probe (DIP); however, the high mass end is of lower abundance. A quadrupole instrument such as the HP5985 forms steroid molecular ions in low relative intensities; therefore, the JEOL AX505, a magnetic sector instrument, was used for all subsequent experiments.

Several studies were undertaken to determine the optimum probe position in the JEOL. Not surprisingly, it was better to have the sample face the filament than be

opposite the filament. The best reconstructed total ion chromatogram (TIC) and spectrum was obtained when the electrode was perpendicular to the filament. In this case, the TIC and spectrum looked just like the results obtained using a DIP.

From the area under the curve of a cyclic voltammagram, it was determined that approximately 300 ng of material, possibly 1,4-androstadien-3,11,17-trione, the oxidized product of prednisolone, had been deposited on the electrode. From the analysis of the area under the oxidation curve, the oxidation of testosterone on an electrode deposited about 100 ng of material. Only about 50 ng are necessary to detect the oxidized compound by EI-MS if the steroid is placed on the platinum probe directly (no electrochemistry involved). Therefore, enough compound should have been electrochemically oxidized on the probe for detection by mass spectrometry. The supporting electrolyte can also be detected by mass spectrometry. The electrode was usually dipped in acetonitrile before analysis, although sometimes this step was omitted. When this step was omitted, there were a few more ions from the supporting electrolyte, which desorbs at a later time than the steriod, but no other differences were noted.

Two very careful studies were undertaken to determine the feasibility of placing the electrode directly in the source. One experiment used EI as the ionization technique; the other, ECNI.

The contributions to the direct probe EI profile of the electrode from background ions in the source, from the clean electrode, from the supporting electrolyte and from unoxidized material were each determined in separate experiments. No contribution to the background was observed from either the connectors used to hold the electrode in the direct probe apparatus or from the platinum itself. Placed directly on the probe, the spectrum of both 1,4-androstadien-3,11,17-trione (oxidized prednisolone) and 4-androsten-3,17-dione (oxidized testosterone), looked like the spectrum obtained from a normal direct probe experiment

except for a diminished molecular ion. After prednisolone and testosterone were oxidized with the TBLE program, each electrode was dipped in acetonitrile and then placed inside the JEOL 505 ion source. In the case of both the "oxidized testosterone" and the "oxidized prednisolone," no ions indicative of these oxidized products were seen. In fact, the spectra looked surprisingly similar to spectra taken when the electrode is merely dipped in the electrolysis solution (that is, the solution containing just testosterone or prednisolone and supporting electrolyte). Perhaps, the amount of oxidized material on the electrode is below the detection limit of electron impact ionization.

The oxidized material electrochemically adsorbed on a platinum electrode was also analyzed by ECNI. ECNI has less fragmentation, so if the steroid were present, the molecular anion would be detected, thus simplifying the spectrum. Also, if the material on the electrode was an intermediate, such measurements would provide molecular weight information about this intermediate. Samples larger than 50 ng of material can be detected.

In previous studies, an unusual ion isotope pattern was noted. Distinct patterns were repeated at 305/307/309, 339/341/343/345, and 358/360/362/364. These are also observed for clean electrodes when ammonia is used as the reagent gas. What these ions represent is a mystery.

When prednisolone oxidized on a platinum surface is analyzed, only background ions are present. The most intense peaks at *m/z* 233/235 represent rhenium oxides and are present in all backgrounds. Again, if there is material oxidized on the electrode, the amounts are too small to detect. In conclusion, neither EI nor ECNI is a viable technique for detection of electrochemically oxidized steroids on a platinum surface.

Electrochemical Deposition on a Surface--FAB

Although the EI/ECNI desorption studies were not successful, it was thought that fast atom bombardment (FAB) could possibly desorb a steroid that had been electrochemically oxidized on a platinum surface. A FD-FAB probe (see Figure 2.8) was modified, replacing the metal originally used with platinum. The only problem was that the repeller contact was not notched into the ceramic used; therefore, the repeller has to be adjusted in a previous analysis.

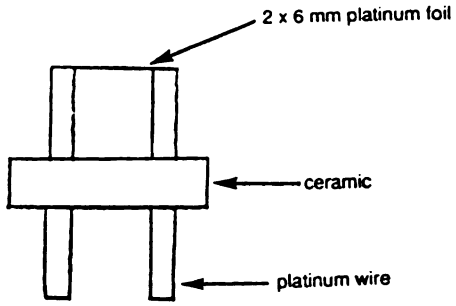

Figure 2.8: A platinum electrode that fits into the FD-FAB probe of the JEOL HX-110 mass spectrometer.

In preliminary studies using a regular FAB probe and the JEOL HX110 double focussing mass spectrometer, 1,4-androstadien-3,11,17-trione was used to determine the best matrix for steroid analysis. Glycerol, thioglycerol, nitrobenzyl alcohol, and 5:1 dithiothreitol:dithioerythritol did not work. With glycerol/HCl (0.1 *M*), a substantial $[M+H]^+$ peak was observed at *m/z* 299, but *m/z* 299 also corresponds to $[3G+Na]^+$. The test compound then was changed to 4-androsten-3,17-dione, which does not have any interferences with glycerol ion. In the positive ion FAB spectrum with glycerol/HCl as the matrix, a substantial $[M+H]^+$ peak was observed at *m/z* 287. The addition of 20% TBAF does not diminish the analyte peak. The detection limit is 150 ng for 4-androsten-3,17-dione. In a study of ketosteroids, DiDonato and Busch had to use derivatization to obtain FAB spectra of 1 μg of steroid (18).

Negative FAB matrices such as glycerol, hexamethylphosphoramide, and triethanolamine were used for some more electrophilic steroids such as 1,4-androstadien-3,11,17-trione and 1,4-androstadien-9α-fluorine-16α-methyl-3,11,17-trione. No response was obtained for 1,4-androstadien-3,11,17-trione, but 1,4-androstadien-9α-fluorine-16α-methyl-3,11,17-trione had a spectrum with major contributions from peaks at *m/z* 329 (30%) corresponding to $[M-H]^-$ and *m/z* 310 corresponding to $[M-HF]^-$.

To test the FAB probe under real electrochemical desorption conditions, testosterone and dexamethasone were each separately oxidized onto the electrode. Neither gave any signal from the analyte, oxidized or unoxidized, but the supporting electrolyte was observed. The amount of dexamethasone oxidized on the electrode was obtained using the electrochemical data. From analyzing the area under the curve, there were 230 ng of material on the electrode. Because this is a two-sided electrode and only one side faces the fast atom beam in the source, effectively only 115 ng of material are available for analysis. Because 115 ng is less than the detection limit of steroids by FAB, no signal could be observed. Therefore, desorption by FAB is not a viable technique.

Modified Nickel Electrodes: An Alternative Synthesis Method

A modified nickel oxide hydroxide electrode has been reported to convert primary alcohols, α,ω-diols, and secondary alcohols into their respective carboxylic acids, dicarboxylic acids, or ketones (8). Because the oxidation rate decreases with increasing steric hindrance, 3-hydroxysteroids can be selectively oxidized. For example, 4-androsten-3β,17β-diol was selectively oxidized to 4-androsten-3-one-17β-ol in a 50% yield. Other products included a 38% yield of 4-androsten-3,17-dione and a 7% yield of unconsumed starting material. Long reaction times (about 24 hours) resulted in a 80% formation of 4-androsten-3,17-dione.

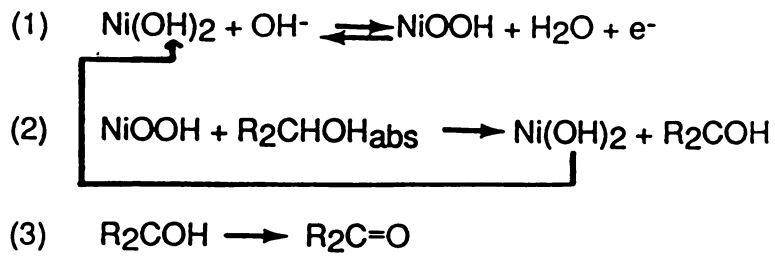

Figure 2.9: Mechanism for the electrochemically regenerated nickel oxide hydroxide electrode.

In this method of oxidation, nickel hydroxide is coated on a nickel foil electrode. The nickel hydroxide reacts with hydroxide ion from the supporting electrolyte KOH in order to form nickel oxide hydroxide on the surface of the electrode (see Figure 2.9 for the appropriate equations). The nickel oxide hydroxide reacts with the absorbed alcohol in the rate-determining step with the transfer of the proton to the surface, thus regenerating the nickel hydroxide on the surface of the electrode. The steroid radical intermediate is easily oxidized to a ketone.

Experimental

Preparation of the Electrode. The nickel (II) hydroxide is deposited according to published methods (19). A 2.3 cm x 3.0 cm nickel foil was placed in a 0.1 *N* $NiSO_4 \cdot 6H_2O$, 0.1 *N* sodium acetate, and 0.005 *N* sodium hydroxide solution. A silver wire reference and a platinum gauze auxiliary electrode were connected to the nickel foil with a PAR Potentiostat/Galvanostat Model 273 used in the galvanostat mode. A current density of 0.5 mA/cm^2 was applied in the following manner:

a.) Step to -9 mA

b.) Hold for 60 s

c.) Step to +9 mA

d.) Hold for 60 s

e.) Repeat

This cycle was repeated for 8 to 10 minutes to ensure an even, black coating of the electrode. To clean the electrode after use, it was immersed in a 20% HCl solution for a few seconds.

Electrolysis. About 10-20 mg of steroid were placed in a 30 mL TLC bottle with 20-25 mL 50% *t*-butanol/50% 0.1 *M* KOH. The steroid is sparingly soluble in this mixture, so rapid mixing during the entire electrolysis procedure is necessary. A silver wire reference electrode and platinum gauze auxiliary electrode are used. The PAR Model 273 is used in the galvanostat mode; that is, the current is held constant while the potential changes in order to keep a constant current. Therefore, everything in the solution that can be oxidized will be in order to keep the current constant. A current density of 1.2 mA/cm^2 was maintained. The reaction time was on the order of 24-28 hours.

Isolation procedures. The authors (8) recommended the following isolation procedure. About 0.2 g of NaCl is added. Ether extraction (3 x 25 mL) was followed by drying the ether fraction. The ether was removed, and a solution of the solute reconstituted in ethyl acetate. A practice extraction of 1 mg of pure 4-androsten-3,17-dione proved that the extraction efficiency was only 20%.

A second attempt at isolation was based on the assumption that KOH did not dissolve in ethyl acetate. The 50% *t*-butanol/50% 0.1 *M* KOH solution was evaporated to dryness and then dissolved in ethyl acetate. The KOH crystals did not precipitate as expected, even after hours of cooling in an ice bath. Even so, the ethyl acetate solution

was analyzed by GC. The extraction efficiency was 60%, and no problems due to the KOH were noted.

Results

Prednisolone (1,4-pregnadien-11β,17α,21-triol-3,20-dione) was oxidized using the nickel hydroxide electrode for twelve hours. The solution turned yellow green during the electrolysis in which 7.928 coulombs were passed. About one-fourth of the electrode surface had degraded. Only prednisolone itself was recovered after electrolysis. After all, 3-hydroxy groups are especially amenable to this procedure and prednisolone does not have any 3-hydroxy groups. Also, the substitution at the 17-position may be difficult to oxidize.

In the literature study, 4-androsten-3β,17β-diol was easily oxidized; therefore, it was chosen as a test compound. The current was kept constant at -21 mA. After 24 hours, 1387 Q of charge had been passed. The working electrode had lost all of its coating, and some black material was floating in the green solution. The auxiliary electrode was coated with a green layer, much like the color of the nickel sulfate solution. The yields were:

4-androsten-3β,17β-diol (starting material)	1.3%
4-androsten-3-one-17β-ol	32%
4-androsten-3,17-dione	10%

The extraction procedure has a 60% extraction efficiency for 4-androsten-3,17-dione.

Another attempt at oxidation was conducted with a constant current of -18 mA. After 26 hours, 1881 coulombs were passed. The nickel oxide surface on one side of the electrode degraded, but the other side remained intact. The yields were:

4-androsten-3β,17β-diol (starting material)	85%
4-androsten-3-one-17β-ol	13%
4-androsten-3,17-dione	3%

All the starting material is accounted for this time.

The nickel oxide hydroxide electrode is not a good method to prepare oxidized steroids. The reaction times are long, there is no selectivity (everything in solution is oxidized, including supporting electrolyte and solvent, in order to keep the current constant), and too many side products are formed. The diketone (4-androsten-3,17-dione) is very difficult to obtain; it is perhaps obtainable if a high current density is maintained, the electrode surface is redeposited many times during the reaction, and the reaction is run for several days.

Conclusions

Electrochemical oxidation had been proposed as a more selective and more efficient method of preparing ketosteroids. Synthetic oxidation on a planar electrode had a low yield as the electrode was fouled by the reaction. If the fouled electrode was placed directly inside a mass spectrometer source, the material on the electrode could not be detected by either EI, ECNI, or FAB. A modified electrode, the nickel oxide hydroxide electrode, was used to oxidize steroids. This electrode has found use in preparing 3-keto steroids but, even under harsh conditions, synthesizing fully oxidized steroids was very inefficient. Pyridinium chlorochromate oxidation, even with its problems of low yield and lack of selectivity, is still the best choice for preparing ketosteroids.

The Determination of Reduction Potentials

Reduction potentials were determined for the comparison with ECNI studies described in Chapter 4. In this section, the electrochemical aspects of the reduction potential study will be undertaken.

Reduction potentials of selected ketosteroids have been obtained by various researchers using polarographic techniques. Reduction potentials obtained in different labs cannot be directly compared, even if the solvent and the supporting electrolyte are the same. Small differences in the purity of solvents used, the amount of moisture in a

system, or the difference in the liquid junction potentials can result in large differences in reduction potential. As a basis for comparison between two sets of data, at least one (and preferably more) of the compounds must be repeated in each set. This criterion was not observed in previous work on ketosteroids in the literature; therefore, reduction potentials had to be obtained in this laboratory. Table 2.7 summarizes the findings of the literature survey.

The effect of functional groups on the reduction potentials of steroids has been extensively studied (25). It had been generally accepted that substitution in a position remote from the electrophilic group has little effect on the half-wave potential. However, in the case of steroid compounds, substituent effects can be transmitted from other rings and from remote positions. It was assumed that in most cases a potential difference of 0.01 V is significant, but in all cases, reduction potentials measured by only one research group were compared.

Substitutents at C-17 on the steroid nucleus affect a carbonyl group at C-3. For example, in the comparison of the last three compounds in Table 2.7, 5α-androstan-3,17-dione is much more difficult to reduce than 5α-cholestan-3-one. The influence of the 17-substituent depends on the solvent system; in 50% ethanol at pH 10.5, the effect of substitution at the 17-position on 4-en-3-one steroids is minimal.

Substitution at C-11 had much less effect on the carbonyl group at C-3; however, the author was comparing the half-wave potentials of two steroids that differed by the presence or absence of an 11-hydroxy group. However, if the substitution at C-11 is a carbonyl group, the steroid is more easily reduced than if a hydroxyl group at C-11 is present. A carbonyl substitution at C-11 is more easily reduced than a double bond at the same position, but the reduction potentials differ by only 0.1 V.

Table 2.7: Literature Values for the Reduction Potentials of Ketosteroids

Compound	Conditions	$E_{1/2}$ (V vs. SCE)	Reference
4-Androsten-3,17-dione-16α-fluoro	DMF, 0.1 *M* TBAI, 0.1 *M* TEAI	-2.10, -2.23	24
4-Androsten-3,17-dione-16β-fluoro		-2.10, -2.23	
4-Androsten-3,17-dione-16β-chloro		-1.74, -2.22	
4-Androsten-3,17-dione	50% DMF, 0.1 *M* LiCl	-1.74	23
1(5β)-Androsten-3,17-dione		-1.63	
1(5α)-Androsten-3,17-dione		-1.60	
1,4-Androstadien-3,17-dione		-1.54	
4,6-Androstadien-3,17-dione		-1.37	
1,4,6-Androstatrien-3,17-dione		-1.31, -1.66	
3,5-Androstadien-7,17-dione		-1.46	
4-Androsten-3,6,17-trione		-0.81, -1.48	
4-Androsten-3,11,17-trione		-1.70	
Androstan-3,17-dione	90% EtOH, 0.1 *M* TBACl	-2.38	22
Androstan-3,11,17-trione		-2.37	
Pregnan-3,20-dione		-2.44	
Cholestan-3-one		-2.28	
4-Cholesten-3-one	90% EtOH, pH 8.5	-1.55	26
4-Pregnen-3,20-dione		-1.45	
1,4-Pregnadien-3,20-dione-17α-ol acetate	50% EtOH, pH 6.0	-1.22	
4,6-Pregnadien-3,20-dione-17α-ol acetate		-1.06	
Cholestan-3-one-2α-bromo	95% EtOH, 0.05 *M* TBACl	-0.06	27
Cholestan-3-one-2α-fluoro		-2.23	
Cholestan-3-one-2α-chloro		-0.94	
Cholestan-3-one-2α-iodo		-0.02	
4-Androsten-3,17-dione-6α-bromo		-0.05	
4-Androsten-3,17-dione-6β-bromo		-0.02	

Table 2.7 (cont'd)

Androsten-3,17-dione-2α-bromo	80% EtOH, 2% sodium lauryl sulfate, pH 6.7	-0.05	28
Pregnan-3-one-21-bromo		-0.24	
4-Pregnen-3-one-21-bromo		-0.23	

Abbreviations

DMF	Dimethylformamide
EtOH	Ethanol
TBACl	Tetrabutylammonium chloride
TBAI	Tetrabutylammonium iodide
TEAI	Tetraethylammonium iodide

The effect of a substituted methyl group in the 6-position of 3-keto-α,β-unsaturated steroids is again very minimal. Only occasionally do methyl substituents have a significant effect on half-wave potentials.

A linearly-conjugated system is much easier to reduce than a cross-conjugated system. 1,4-Pregnadien-3,20-dione-17α-ol acetate has a half-wave potential of -1.22 V (vs. SCE) in 50% ethanol at pH 6. 4,6-Pregnadien-3,20-dione-17α-ol acetate in the same solvent has a potential of -1.06 V. The position of the linearly conjugated system within the steroid is important. In 90% ethanol at pH 8.5, 4,6-cholestadien-3-one has a half-wave potential 0.06 V more positive than that of 3,5-cholestadien-7-one.

The dependence of reduction potentials on the number of carbonyl groups is slight. Both androstan-3,17-dione and androstan-3,11,17-trione have the same reduction potential.

Halogen subsitutions have been studied within the steroid nucleus. The reduction potentials become more negative in the sequence I < Br < Cl < F. In rigid systems, such as the B and C rings of steroids, it is possible to distinguish the epimers of α-halo ketones from polarographic data.

Cyclic Voltammetry

Experimental

A one-compartment cell (illustrated in Figure 2.1) was used with a platinum wire auxiliary electrode, a silver wire reference electrode, and a platinum disk working electrode. The purified solvent was transferred under vacuum. TBAF (0.2 *M*) was used as the supporting electrolyte. Reduction potentials were obtained on a BAS Electrochemical Analyzer. Typical conditions were the following:

3-4 mg of steroid dissolved in about 7 mL of solvent

Potential range = 0.0 to -2.0 V

Scan rate = 200 mV/s.

Results

Acetonitrile should be a good solvent for the reduction of steroids because it has a large potential window. The CV obtained has no forward or reverse peaks. The cell was open to atmosphere, and more steroid was added because it was thought that the concentration was too low. Now, both forward and reverse waves were noted for the test compounds, 1,4-androstadien-3,11,17-trione and 5α-androstane-3-one.

Silver wire is a QRE. In order to obtain reproducible values for oxidation-reduction potentials, a compound with a known reduction potential must be added and the CV reanalyzed. A good choice for the internal standard is ferrocene, which is soluble in a variety of organic solvents and has well-characterized electrochemical properties. Ferrocene has the following equilibrium (20):

$Fe(C_5H_5)_2 + e^- \leftrightarrow Fe(C_5H_5)_2$ $\quad E_{1/2} = +0.190 \pm 0.007$ V vs. SHE in acetonitrile

After adding more sample, the data obtained for reduction potentials are in Table 2.8.

Table 2.8: Reduction Potentials of Steroids in Acetonitrile

Sample	$E_{1/2}$ Sample V vs. QRE	$E_{1/2}$ Ferrocene V vs. QRE	$E_{1/2}$ Sample V vs. SHE
1,4-A-3,11,17-trione*	-1.35	+0.75	-1.91
Androstane-3-one	-1.48	+0.72	-2.01

*A = androstadien

In a second attempt, about 10-15 mg of steroid was used. Only after the cell was opened and more steroid was added did a reductive peak appear.

Methylene chloride was also tried as a solvent. With 1,4-androstadien-3,11,17-trione, a small peak appeared at -1.82 V (vs. QRE). After the cell was opened and more steroid was added, it took about four reductive-oxidative cycles to get a reproducible peak that by then had shifted to -1.60 V (vs. QRE).

1,4-Androstadien-3,17-dione in methylene chloride was also analyzed. A very small reductive peak that shifted its potential with successive cycles was observed, but this could have been a contaminant. After the cell was opened and more steroid was added, the reduction potential was at -1.6 V.

After the electrochemical cell was opened to air, the peak potential always changed and grew larger. This is due to the oxygen wave. None of the steroids could be analyzed by cyclic voltammetry using a platinum electrode.

Differential Pulse Polarography

As shown in the previous section, cyclic voltammetry does not work for the determination of reduction potentials for steroids. Almost all of the reduction potentials in the literature were determined using polarographic techniques.

Classical polarography involves measuring current as a function of potential at a dropping mercury electrode. The electrode continually regenerates, with each drop corresponding to a new experiment. Therefore, there are no problems with contamination or with electrode fouling.

Because classical polarography involves recording the current continuously over the lifetime of a drop, current fluctuations due to the growth and fall of the drop are also recorded, generating a great deal of noise. In order to decrease the noise level, sampled-dc polarography has been developed. In sampled-dc polarography, the current is measured for the last 5-20 ms of the drop. The resulting polarogram is a smooth curve with a minimum of noise. The detection limit and resolution are the same as for classical polarography.

In differential pulse polarography (DPP), as in classical polarography, a dc potential is increased linearly with time. However, a dc pulse of an additional 20 to 100 mV is applied for 60 ms before the detachment of the mercury drop. The current measured near the end of the pulse is subtracted from the current measured just prior to

the pulse. The resolution and sensitivity of this technique are better than that for classical polarography.

Experimental

All polarograms were generated using a EG&G PAR Model 303A static mercury dropping electrode connected to an EG&G PAR Model 273 Galvanostat/Potentiostat operated by EG&G PAR Model 270 Electrochemical Analysis System software on an IBM computer. The cell had a capacity of about 20 mL of solution. At least 10 mg of steroid were used for each analysis. A platinum wire auxiliary electrode and a Ag/AgCl reference electrode were used. All samples were deaerated with nitrogen for four minutes before analysis. The conditions used for sampled-dc polarography are the following:

drop time = 1 s

drop size = small

scan rate = 0.01 V/s

scan increment = 0.01 V

current range = 10 μA

potential range = -0.2 to -3.1 V (dependent on solvent)

For DPP, all of the above conditions were used as well as:

pulse height = 0.025 V

pulse width = 0.05 s

The reference electrode, Ag/AgCl, was supplied by the manufacturer. An SCE is not available for this model dropping mercury electrode (DME). According to the DME manual (21), the Ag/AgCl electrode has a potential difference of 50 mA from a SCE.

Results

Several solvent systems in the literature provide a basis for comparison of reduction potentials of steroids. Two of these were attempted. It is difficult to

compare the experimental results with the literature values because experimental conditions and reference electrodes make a difference.

The first experiment compared two solvents systems and two methods of data collection. Androstan-3,17-dione was used as the test compound. Solvent system I (22) consisted of 1.5 m*M* steroid made up in a 25 mL volumetric flask. After the steroid was dissolved in a small amount of 95% ethanol, 1.25 mL of 1 *M* tetrabutylammonium chloride (Southwestern Chemical, Austin, TX), 1 drop of Triton X-100 (Aldrich), and 1 mL water were added. The solution in the volumetric flask was diluted to mark with 95% ethanol. Solvent system II consisted of about 1.5 m*M* of steroid in a 50% DMF (Mallinkrodt HPLC grade)/50% water with 0.1 *M* lithium chloride (Mallinkrodt) as the supporting electrolyte (23). One drop of Triton X-100 was added before analysis.

In this experiment, sampled-dc polarography was compared to DPP. Using solvent system I, the reduction potential of androstan-3,17-dione was 2.0 V (vs. Ag/AgCl) under DPP conditions. No reductive wave was seen using sampled-dc. In solvent system II, the reduction potential under DPP conditions was -1.6 V and -1.5 V for sampled-dc polarography. The reductive waves were sharper and larger using solvent system II; therefore, the use of solvent system I was discontinued.

In the next experiment, the reproducibility of dc-sampled and DPP were compared. Both 1,4-androstadien-3,17-dione and 4-androsten-3,17-dione had been analyzed in the literature study. Each compound was analyzed at least twice under both dc-sampled and DPP conditions. DPP values are more reproducible.

Table 2.9: Comparison of Experimental and Literature Half-Wave Potentials

Compound	Literature $E_{1/2}$ V vs. SCE	Experimental $E_{1/2}$ V vs. Ag/AgCl	$\Delta E_{1/2}$ (V) Lit. - Exp.
1,4-Androstadien-3,17-dione	-1.54	-1.63 ± 0.01	0.09
4-Androsten-3,17-dione	-1.74	-1.80 ± 0.015	0.06

Table 2.9 summarizes the findings from this study. The reproducibility of the experimental values is quite good. The difference in $E_{1/2}$ values (column 4 of Table 2.9) should be the same for both compounds if they really track the literature study. As shown by the data, there is a slight difference between them.

A number of steroids and terpenes dissolved in DMF/water were analyzed by DPP. Problems were encountered because not all steroids are soluble in this solvent system. Table 2.10 summarizes the results of this study.

Acetontrile was used as a solvent for the determination of reduction potentials because not all steroids are soluble in DMF/H_2O. As shown in Table 2.1, acetontrile has a larger potential window. Steroids (7-16 mg) were dissolved in 25 mL HPLC-grade acetonitrile (Mallinkrodt) with 0.1 *M* TBAF.

Discussion

The 50% DMF solution was used for the determination of half-wave potentials for terpenes because they all dissolved in this system. To compare these values to those obtained in acetonitrile, it is necessary to subtract 0.44 V from the 50% DMF reduction potentials.

Most terpenes have half-waves potentials greater than that of the solvent (-2.35 V); therefore, they have very little ability to capture an electron in solution. There are some noteable exceptions. In the bicyclononanes (compounds **1-5**), compound **3** which has an α,β-unsaturated site is reducible while compound **1** is not. Compound **5** has an epoxide group which makes it fairly easy to reduce. In fact, compound **5** and compound **12** have the same reduction potential, which shows that a dienone and a en-epoxide have the same electron-capturing ability. Among compounds having a propyl chain (compounds **16-20**), only compound **19** has an observable half-wave potential. The presence of a double bond in both the propyl chain and in the alpha position to the carbonyl in the ring is important for reducibility.

Table 2.10: Reduction Potentials Using the DMF/H_2O Solvent System
NB: -2.35 V is the maximum reduction potential

Compound*	$E_{1/2}$ (V)
Androstane-3,17-dione	nothing
4-Androsten-3,17-dione	-1.84
1-Androsten-3,17-dione	-1.73
4-Androsten-3,11,17-trione	-1.81
4-Androsten-3,6,17-trione	-0.93, -1.55
1,4-Androstadien-3,17-dione	-1.67
1,4-Androstadien-3,11,17-trione	-1.62
1,4,6-Androstatrien-3,17-dione	-1.44
4-Pregnen-3,20-dione	-1.82
4-Pregnen-3,11,20-trione	-1.82
4-Pregnen-3,6,20-trione	-0.93, -2.08
4-Pregnen-16α,17α-epoxy-3,20-dione	-1.84
Pregnane-4α,5α-epoxy-3,20-dione	-1.89, -2.17
1	nothing
3	-1.80
5	-0.94
7	nothing
10	-1.83
12	-0.93
13	-1.55
14	-1.82
16	nothing
17	nothing
18	nothing
19	-1.72
20	nothing
21	nothing
22	nothing
23	nothing
24	-2.13
25	-1.72
26	nothing

*The structures of compounds 1-27 are presented in Figure 4.9.

Table 2.11: Polarography of Steroids Using Acetonitrile
NB: -2.97 V is the maximum reduction potential

Compound	$E_{1/2}$ (V)	ECNI Response*
Androstane-3-one	nothing	1.0
2-Androsten-17-one	nothing	1.1
Androstane-3-one-4β,5β-epoxy-17-ol Acetate	-2.41	26
1-Androsten-3-one-4β,5β-epoxy-17-ol Acetate	-1.04, -2.05	200
16-Androsten-17-cyano	-2.59	1.2
Androstane-2α-bromo-3,17-dione	-0.69	0.35
Androstane-2α-iodo-3,17-dione	-0.72	0.35
Androstane-3,17-dione	nothing	1.4
4-Androsten-3,17-dione	-2.27	1.2
1-Androsten-3,17-dione	-1.03, -2.12	0.98
4-Androsten-3,11,17-trione	-2.21	3.2
4-Androsten-3,6,17-trione	-1.26, -2.26	300
1,4-Androstadien-3,17-dione	-2.03	2.1
1,4-Androstadien-3,11,17-trione	-1.95	360
1,4,6-Androstatrien-3,17-dione	-1.75	300
4-Pregnen-3,20-dione	-2.23	1.4
4-Pregnen-3,11,20-trione	-2.22	9.1
4-Pregnen-3,6,20-trione	-1.28	280
Pregnane-16α,17α-epoxy-3,20-dione	-2.41	24
4-Pregnen-16α,17α-epoxy-3,20-dione	-2.25	29
Pregnane-4α,5α-epoxy-3,20-dione	-2.41	21
4,6-Cholestadiene	nothing	0.47
4,6-Cholestadien-3-chlorine	nothing	0.35
4,6-Cholestadien-1α,2α-epoxy-3-one	-1.50, -1.82	58
4,6-Cholestadien-1α,2α-epoxy-3-ol Acetate	-1.80	---
5-Cholesten-3-one	-2.31	0.17
Cholestane-3,6-dione	nothing	1.6
4-Cholesten-3,6-dione	-1.32, -2.30	280
Cholestane-5α,6α-epoxy-4-one	-2.68	6.1
Cholestane-4β,5β-epoxy-3-one	-2.41	9.2

*5α-Androstan-3-one has been assigned a relative response of 1.0.

Steroids were chosen for reduction potential studies based on several factors. A representative selection of various structural features was included. Very expensive steroids and those obtained from the Steroid Reference Collection (Queen Mary College, London) could not be used because not enough was available to make 20 mL of an 1.5 m*M* solution. Simple steroids without many structural features, such as androstan-3-one, 2-androsten-17-one, and androstan-3,17-dione did not have a response; i. e., the half-wave potential of these compounds is greater than -2.97 V vs. Ag/AgCl. In the literature, it has been observed that the group at the 17-position sometimes affects the reduction potential. In this study, the effect of changes at the 17-position is small. 4-Androsten-3,17-dione has a $E_{1/2}$ of -2.27 V as compared to 4-pregnen-3,20-dione with a $E_{1/2}$ of -2.23 V. The addition of a 6-carbonyl group to a 4-en-3-one compound makes it much easier to reduce. 4-Androsten-3,11,17-trione has half-wave potentials at -1.26 and -2.26 V. In this case, the substitution at the 17-position makes a difference. An alkyl chain at the 17-position makes the steroid more difficult to reduce, as in the case of 4-cholesten-3,6-dione with $E_{1/2}$ at -1.32 and -2.30 V. The compound with the pregnane substitution at the 17-position, 4-pregnen-3,6,20-trione, has only one half-wave potential at -1.28 V.

Depending on the other substituents in the steroid, the presence of an 11-carbonyl may or may not affect the reduction potential. The reduction potential of 4-androsten-3,11,17-trione is 0.06 V lower than the reduction potential of 4-androsten-3,17-dione. Similarly, the reduction potential of 1,4-androstadien-3,11,17-trione is 0.08 V lower than that of 1,4-androstadien-3,17-dione. However, the difference in reduction potentials between 4-pregnen-3,20-dione and 4-pregnen-3,11,17-trione is only 0.01 V. A 11-carbonyl group in conjunction with the 17-carbonyl group often results in a more easily reduced compound.

The presence of an α-halogen results in a higher reduction potential. For example, androstan-3,17-dione has no response; however, if a α-halogen such as

bromine or iodine is present alpha to the C-3 carbonyl, the half-wave potential is on the order of -0.7 V. The iodine has a more negative potential than the bromine; the opposite trend is expected.

The presence of an epoxide group has a small effect on the reduction potential. 4-Pregnen-3,20-dione has almost the same reduction potential as 4-pregnen-16α,17α-epoxy-3,20-dione. The position of the epoxide does not seem to be important; both pregnan-4α,5α-epoxy-3,20-dione and pregnan-16α,17α-epoxy-3,20-dione have an $E_{1/2}$ of -2.41. This is the same reduction potential as cholestan-4,5-epoxy-3-one and androstan-3-one-4β,5β-epoxy-17-ol acetate. The substitution at the 17-position is not very important.

The reduction potentials will be compared to electron capture negative ionization mass spectrometric responses in Chapter 4.

Conclusions

Cyclic voltammetry did not allow the determination of reduction potentials for steroids because the electrodes used do not allow the more negative potentials needed. Polarographic analysis on a mercury electrode was a much better technique. Although the 50% DMF/50% water solvent system allowed the determination of reduction potentials of bicylic compounds, many steroids were not soluble in this system and thus could not be analyzed. These steroids were much more soluble in acetonitrile with TBAF as the supporting electrolyte. Certain structural features are important for ease of reduction. Carbonyl groups in the 6-position, a 11-carbonyl group in combination with a 17-carbonyl group, a halogen alpha to a carbonyl group, and an epoxide group all result in less negative reduction potentials.

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Chapter 3: The Analysis of Anabolic Steroids by ECNI-MS

In today's society, with the emphasis on physical fitness and "win-at-all-costs" philosophy, it is no surprise that "miracle drugs" such as anabolic steroids are a widespread problem. A glance at the headlines over the past few years shows a range of abuse of steroids, from that of superstars such as Ben Johnson who was stripped of a gold metal in the 1988 Summer Olympic Games, to studies showing that an estimated 8% of the high school students in Michigan used steroids in the past year (1). Even lawmakers have not ignored this problem. In Michigan, the sale of steroids is now a felony, rather than a misdemeanor. At the federal level, steroids are Schedule III controlled substances (section 202C of Controlled Substance Act, Congressional Record, November 2, 1990).

Even with this recent interest in steroids, scientific debate over the effectiveness and the dangers of anabolics remains unsettled, even though it is generally agreed that the disadvantages outweigh any probable advantages. The main thrusts of the campaign against steroids have been education and enforcement by random drug testing during sporting events. Drug testing today is hampered by several problems. There is a need for better sensitivity, in order to detect drugs taken long periods of time before an athletic event, and for long-term research projects. There also is a need for better selectivity to avoid false positive results with other compounds such as oral contraceptives and endogenous steroids.

Some analytical methodologies for anabolic steroids rely on conventional gas chromatography/electron impact mass spectrometry (GC/EI-MS). Electron capture negative ionization mass spectrometry (ECNI-MS) has been proven to have better sensitivity than EI for compounds that are highly electrophilic (2-4). Because most biological compounds are neither highly halogenated nor highly conjugated, fluorinated derivatives that have been developed for GC/electron capture detection

(ECD) also have been used for ECNI-MS. This chapter will concentrate on the development of suitable MS techniques for the quantitative detection of anabolic steroids and metabolites in urine. Selective and sensitive methodology was sought via two avenues involving ECNI-MS: a) one approach involved the use of conventional ECD derivatization prior to analysis by ECNI-MS, and b) the other was patterned after the novel oxidative approach developed in this laboratory for dexamethasone.

Profile of Steroid Abuse

Why Take Steroids?

Anabolic steroids are derivatives of testosterone which have been structurally altered to retain the anabolic effect while minimizing the androgenic effect. This alteration is usually done by adding a 17α-methyl group. Conventional medical uses of anabolics include correcting deficient testosterone levels, anemia due to bone marrow failure, and hereditary angioneurotic edema (5). Medical uses of anabolics also exist in sports; for example, anabolics can help improve recuperation of muscles after strain and may help injuries heal more quickly (6).

There is conflicting scientific evidence for improvement of athletic performance following administration of anabolic steroids. In a survey by Haupt and Rovere (7), reports in the literature only agreed on one point: there is no improvement in the aerobic performance of athletes treated with anabolic steroids. When comparing the effect of anabolic steroids on strength, the literature was not conclusive; fourteen of twenty-four studies reported significant strength increases with anabolic use. In all cases, strength increase depended on a combination of having previous weight-training and continuing this training during the period of steroid use. A high protein diet also helps because anabolic steroids increase protein biosynthesis in muscles, which results in an increase in muscle mass and strength (8). Interestingly, studies which showed

increases in body size and weight while using anabolics were the only ones which showed increases in strength.

Why Not Take Steroids: Possible Side Effects

The side effects of anabolic steroids also have been hotly debated. They range from "part of the price you pay" discomfort to those which are possibly life threatening. Unfortunately, long-term effects have not been studied. One of the most common side effects is mood shifts, usually in the form of aggressiveness. In fact, double-blind studies are being discontinued (7) because athletes can discern whether they are on anabolic steroids based on mood shifts alone. About 30% of the athletes using anabolic steroids report subjective side effects (7). These side effects can include aggressiveness, changes in libido, muscle spasms, irritability, headache, dizziness, and nausea (9). These effects are reversible upon discontinuation of steroids.

Endocrine functions are also impaired (9). These include decreased spermatogenesis (which may result in temporary infertility), testicular atrophy, acne, gynecomastia, and decreased amounts of luteinizing hormone, follicle-stimulating hormone, and testosterone. Again, all these effects are reversible upon discontinuing steroid use. Hepatic and cardiovascular side effects are not as widely observed, but they may be linked to possible long-term effects (5). For example, HDL-cholesterol sometimes decreases in plasma and low-density lipoproteins increase with sustained steroid use, a factor which could increase the probability of a subsequent heart attack. Elevated liver-function test values have occurred, but these are reversible, and long-term liver damage appears to be rare (7). Both peliosis hepatitis and liver tumors have occurred in fewer than 60 cases, all in patients treated with anabolic steroids for an extended period (usually over two years) for medical purposes.

Of greater concern are the irreversible side effects observed in early adolescent boys and in women of all ages (8). Young boys may experience deepening of the voice, male-pattern baldness, and acne. Full adult height may not be obtained due to premature epiphyseal closure. Women may experience the following irreversible masculinizing side effects: deepening of the voice, hirsutism, growth of facial hair, increased body hair, enlarged clitoris, and alopecia. Some side effects such as irregular menstruation and decreased breast size can be reversed.

Who are the Users of Anabolic Steroids?

The actual extent of steroid abuse is difficult to determine, given the reluctance of athletes to participate honestly in such studies. The number of Olympic athletes who used steroids in training for the 1988 Summer Games has been estimated from 10% to 90% (10). In 1988, six percent of the National Football League's players tested positive for steroid use (10). A survey of athletes in the 1987 National Championship of US Powerlifters, 33% of the respondents admitted to steroid use (11). Even among younger people, reported steroid use is high. A survey of high school juniors, both athletes and nonathletes, found 11% of the boys and 0.5% of the girls admitted to steroid use (12). Anabolics are not just a problem in competitive sports; some use is directed toward improving personal appearance. In a 1988 survey of 1010 men at three US colleges, 2% admitted steroid use, a gross underestimate by the authors' standards (13). Of those who admitted steroid use, 24% were not competitive athletes.

What Steroids are Most Commonly Abused?

The International Olympic Committee (IOC) has forbidden the use of drugs in five categories: stimulants, narcotic analgesics, beta blockers, diuretics, and anabolic steroids (6). The list of forbidden anabolic steroids is found in Table 3.1. Many studies agree that the most commonly self-reported abused steroid is

methandrostanolone (tradename Dianabol) (11, 13, 15-16). Next in popularity are the injectable testosterone esters. Even though these steroids retain an androgenic effect, they are popular because detection is more difficult. The use of testosterone is suspected if the testosterone-to-epitestosterone ratio in urine is above 6:1 (14). Other popular steroids among powerlifters include oxandrolone, nandrolone, stanozolol, and oxymetholone (11, 15).

In a study of weight trainers (16), common patterns of anabolic steroid use were obtained. Eighty percent of the athletes "stacked", or took two or more steroids at a time. Typically, this consisted of an injectable testosterone ester and a fast-acting oral formulation. After a cycle of six to ten weeks taking steroids, the minimum time off was four weeks. Often, dosages of steroids were varied during the cycle, and athletes usually took four to eight times the recommended medical dose of steroids. Since steroids taken orally or as water-based injections can be detected for about two weeks after discontinuation and oil-based injections can be detected for about two weeks after administration (8), athletes carefully time cycles to maximize performance and minimize their chances of being caught.

Table 3.1: IOC Forbidden Anabolic Steroids
(Adapted from reference 6)

Bolasterone	Methyltestosterone
Boldenone	Nandrolone
Clostebol	Norethandrolone
Dehydrochloromethyltestosterone	Oxandrolone
Fluoxymesterone	Oxymesterone
Mesterolone	Oxymetholone
Methandienone	Stanozolol
Methenolone	Testosterone

--And other chemically and pharmacologically related compounds.

Commonly Used Detection Techniques

Radioimmunoassay

Radioimmunoassay (RIA) techniques for detecting anabolic steroids have been in use for a number of years (17). Three groups of steroids can be detected by RIA techniques: (a) nandrolone analogs, (b) norethandrolone analogs, and (c) 17α-substituted analogs. Before RIA analysis can be performed, an extraction step is necessary. The major advantage of this technique is its sensitivity, but RIA is not routinely used in screening because of its many disadvantages. Cross-reactivity is observed, especially with normal urinary steroids and oral contraceptives such as norethindrone and norgestrel. The testosterone-to-epitestosterone ratio cannot be measured without using another RIA (14). One anabolic steroid, methenolone, cannot even be detected. During the 1976 Summer Olympic Games, RIA was used for screening of urine samples with confirmation by GC/MS (18). By the time of the 1984 Summer Games, GC/MS was used for both screening and confirmation.

High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) has been used mainly for the analysis of anabolics in the veterinary, pharmaceutical, and forensic fields, rather than human urine screening (17). Recent studies using HPLC include the analysis of nandrolone in biological samples using on-line immunoaffinity sample pretreatment (19) and the analysis of 17-hydroxy steroid esters (20).

One major problem with HPLC is in establishing absolute confirmation of the analyte. Suggested means for identification include: (1) same retention time as that of a standard, (2) the nearest peak in the chromatogram is separated by at least one full peak width at half maximum height, and (3) reanalysis with a standard, proving co-elution (21). More promising techniques based on HPLC are LC/MS and LC/MS/MS (22,23). The mass spectrometer provides confirmation of the analyte, while the LC

can separate even underivatized steroids. Also, the lengthy (often 3 to 48 hours) hydrolysis step for conjugated steroids can be eliminated (24). The digestive juice most often used for the hydrolysis step, *Helix pomatia*, has been observed to convert free 3β-hydroxy-5-en-pregnanes into their 4-en-3-oxo analogs as well as to convert androstenedione, androstenediol, and dehydroepiandosterone into their 4-en-3-oxo analogs, which can lead to false positives.

Gas Chromatography/Electron Impact Mass Spectrometry

By far the most common and the most trusted analytical technique for detecting anabolic steroids is GC/MS. Because good sensitivity (< 10 ng/mL of urine)(17) is needed for the screening of anabolic steroids or their metabolites, selected ion monitoring (SIM) is used. The few characteristic ions of a steroid or metabolite are monitored during a specific retention time window. In one study (14), screening is accomplished by monitoring two characteristic ions of each steroid. Confirmation of positive screens is achieved by reanalyzing the sample while monitoring twenty characteristic ions or, if possible, obtaining a full scan. Some anabolic steroids have more than one major metabolite (see Table 3.2), the identification of which can help in the establishing whether a particular steroid was used.

Extraction and Hydrolysis of Urine

To prepare urine for analysis by GC/MS, about 5 mL of urine is extracted using a XAD-2 column or, more commonly, a C18 solid-phase extraction column. If desired, the steroids can be separated into free and conjugated fractions at this point (see Table 3.3). Before analyzing the conjugated steroids, they are hydrolyzed with *Helix pomatia* or *E. coli* β-glucuronidase for 3 to 24 hours.

Table 3.2: Urinary Metabolites of Anabolic Steroids (references in parenthesis)

Parent Anabolic Steroid	Urinary Metabolites
Methanedienone (31)	Major--6β-hydroxymethanedienone 6β-hydroxyepimethanedienone epimethanedienone Minor--17β-methyl-5β-1-androsten-3β,17α-diol and epimer 17α-methyl-5β-androstane-3-one 17α-methyl-5β-androstane-3α,17β-diol 18-nor-17α,17β-dimethyl-1,4,13(14)-androstane-3-one 17α-methyl-6β,17β-diol-androstane-3-one and isomer 17α-methyl-6β,17α-diol-1-androsten-3-one 6β,16-dihydroxymethanedienone
Boldenone (24)	Major--boldenone Minor--1-androsten-3-ol-17-one Others not identified
Stanozolol (24)	Major--stanozolol Minor--3'-hydroxystanozolol 4-hydroxystanozolol 16-hydroxystanozolol 3',16-dihydroxystanozolol 4,16-dihydroxystanozolol
Nandrolone (29)	19-norandrosterone 19-noretiocholanolone 19-norepiandrosterone
Norethandrolone (87)	3α-17β-dihydroxy-19-nor-5α-pregnane and epimers 19-nor-5α-pregnane-3α,17β,21-triol
Oxymesterone (24)	5α-androstane-17α-methyl-3α,17α-diol (four isomeric forms)

Table 3.2 (cont'd)

Chlorotestosterone (29)	4-chloroandrosterone 4-chloroetiocholanolone
Fluoxymesterone (24)	6β-fluoxymesterone 4-en-fluoxymesterone
Oxymetholone (24)	Major--3-hydroxyoxymetholone Minor--6β-hydroxylation hydroxymethylene group reduction
Methenolone (30)	17-epimethenolone 6-hydroxymethenolone (plus epimers) 1,4,6-androstatrien-3-one-17α-methyl-17β-ol 18-nor-17α,17β-dimethyl-1,4,13(14)-androstatrien-3-one
Oxandrolone (32)	Major--unchanged (35.8%) Minor--17-epioxandrolone 16α and 16β-hydroxyoxandrolone δ-hydroxy acid
4-Chloromethandienone (28)	Major--6β-hydroxy-4-chloromethandienone 6β,12-dihydroxy-4-chloromethandienone 6β,16-dihydroxy-4-chloromethandienone Minor--many more metabolites

Table 3.3: Anabolic Steroids Excreted in Urine* (from reference 17)

Conjugated Fraction	Free Fraction
Bolasterone	Dehydrochloromethyltestosterone
Boldenone	Fluoxymesterone
Chlorotestosterone	Methandienone
Dromostanolone	Oxandrolone
Ethylestrenol	Stanozolol
Mesterolone	
Methandriol	
Methenolone	
Methyltestosterone	
Nandrolone	
Norethandrolone	
Oxymesterone	
Oxymetholone	
Stanozolol	
Testerosterone/Epitestosterone	

*"Anabolic steroid" means either the parent steroid or one of its metabolites.

Derivatization

In order to improve chromatographic properties and possibly sensitivity, derivatives must be made of anabolic steroids before analysis by GC. Many different derivatives have been used for analysis by EI/MS; the most popular of these will be described.

Methoxime-trimethylsilyl (MO-TMS) derivatives were among the first to be used in the analysis of anabolic steroids. The methoxime moiety protects the carbonyl functionality while the trimethylsilyl reacts with the hydroxyl groups. Reports of detection limits for individual steroids or metabolites range from 3 ng/mL to 20 ng/mL of urine (25-27). The ketone and hydroxyll groups are derivatized differently, a process that can help in structure identification, by providing unique ions and retention

times for certain steroids. Problems associated with the MO-TMS derivative include long reaction times, formation of multiple products in the form of syn/anti isomers, acid lability, and the need for a liquid chromatography step after derivatization.

Trimethylsilyl (TMS) derivatives are now more widely accepted than MO-TMS derivatives (17, 24, 27-33). Selective derivatization can be accomplished by selecting the silanizing reagent and catalyst. For example, a 100:2 mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide:trimethylchlorosilane (MSTFA:TMCS) will form primarily TMS-ethers. If the derivatizing reagent is *N*-methyl-*N*-trimethylsilyltrifluoroacetamide:trimethylsilylimidazole, MSTFA:TMIS, (1000:2 with 2 mg/mL dithioerythritol), TMS-ether/TMS-enol derivatives are formed. MSTFA is usually chosen for derivatization because it promotes derivative stability by preventing loss of TMS groups from the steroid.

Electron Capture Negative Ionization Mass Spectrometry

In order to detect trace levels of anabolic steroids long after administration, analytical techniques with better sensitivity and selectivity are needed. Electron capture negative ionization (ECNI) mass spectrometry has the capacity to meet some of these analytical needs. ECNI has the attractive features of high sensitivity and high selectivity for molecules that are sufficiently electrophilic to capture thermal electrons. Despite its attractive attributes, ECNI has not been as widely used as had been anticipated; this is partially due to problems associated with finding proper derivatives of the analyte which are amenable to the ionization technique. Successful applications of the technique in the biomedical area have been limited principally to those reported for selected drugs (34-36), biogenic amines (37, 38), metabolites of arachidonic acid (39-42), and steroids (43).

Electron capture techniques have seen limited use in anabolic steroid analysis. Heptafluorobutrate (HFB) derivatives of testosterone and nandrolone have been

analyzed using GC/electron capture detector (GC/ECD) (44). More recently, Ginkel, *et al.* analyzed nandrolone in meat using a di-HFB derivative and GC/ECNI/MS (45). The HFB derivative was chosen because the ECNI response to MO-TMS derivatives was inadequate.

Electron-capturing derivatives also have been useful in EI/MS analyses. In fact, one of the most common methods of analyzing stanozolol involves the use of the HFB-TMS derivative (14, 24, 46) because complete TMS derivatization leads to unstable products. The HFB reagent derivatizes the nitrogen in the pyrazole ring, while the TMS reagent reacts with the 17β-hydroxyl group. The EI/MS detection limit of 1 ng/mL may be improved by using ECNI-MS. One significant problem in the analysis of stanozolol, as well as in the analysis of many other anabolics, is that only a small proportion of the steroid and/or metabolite is excreted in the urine. For stanozolol, about 16% is excreted in urine, while 40-60% is excreted in feces.

Commonly-Used Electron Capture Derivatization Agents

Many electron capture derivatives of steroids have been developed (47), most originally for use with GC/ECD.

1. Trimethylsilyl (TMS) ethers have been used in methods that rely on detection by electron capture techniques. The TMS-ether moiety in itself does not affect electron capture (48), but after forming TMS ethers, thermally-labile steroids survive transit through the GC and are more likely to reach the detector intact, thus increasing sensitivity. The benefit of ECNI in this case is realized as a result of discrimination against background as the TMS derivative has no significant electron-capture activity. A comparison of the TMS derivative of dexamethasone to other derivatives is shown in Table 4.10.

2. Methoxime-trimethylsilyl (MO-TMS) derivatives have been used for ECNI/MS analysis of many corticosteroids with much success (49, 50). A common

problem with the conventional approach to derivatization for ECNI analysis is that many of the derivatives produce abundant ions that relate only to the derivatizing agent, and not to the parent structure of the analyte. For MO-TMS derivatives, major high-mass ions are based on the parent molecule, not the derivatizing agent, which allows for monitoring of specific ions with good sensitivity. Different reaction times and conditions are used to derivatize hindered ketone and hydroxyl groups, which can be used to help elucidate the structure of the original molecule. By using MO-TMS derivatives, however, one is not taking advantage of any added electron capturing moiety that possibly could increase the detector response.

MO-TMS derivatives also were studied as part of the comparison study of dexamethasone derivatives under ECNI conditions. MO-TMS and TMS derivatives have the same response as dexamethasone (when it is analyzed using the direct probe inlet). Again, no additional electron capture ability is imparted.

3a. Silyl ethers with halogen substitution provide an improvement in sensitivity over that available with conventional TMS techniques. Among halogens, electron capture response increases with increasing atomic weight, but volatility decreases (51). If these derivatives have the same volatility and ease of preparation as conventional TMS derivatives, these derivatives can be very useful in ECNI.

3b. Silyl ethers with pentafluorophenyl substitutions (known as *flophemesyl* derivatives) are a unique variation on a TMS derivative that also has an electron-capturing functionality (52-54). The pentafluorophenyl group is more sensitive to GC/ECD than a chloromethyl group, and is more thermally stable than a HFB derivative. The flophemesyl derivatives can be made to selectively react with hydroxyl and/or ketone groups. For example, flophemesylamine reacts with certain hydroxyl groups in the presence of ketones, but not with 17β- or 11β-hydroxyls. For anabolic steroids, the most useful reagent seems to be flophemesylamine:flophemesyl chloride (10:1) which will react with unhindered secondary hydroxyl groups and the

tertiary 17β-hydroxyl groups. This reagent will not react with the 11β-hydroxyl group; the only anabolic steroid that exhibits this functionality is fluoxymesterone.

4. Lesser known derivatives, including pentafluoroctanoate (55), 9-H-hexadecafluorononoate (56), chloroacetate (57), benzoate (58), and pentafluorobenzoate (59), have all been used to derivatize steroids for analysis by GC and detection by ECD. Their success with anabolic-type steroids or ECNI/MS analysis is unknown.

5. Perfluoroarene derivatives result from promising new reactions for steroids (60). The perfluorotolyl derivative in particular is volatile, can be prepared in good yields, and results only from reaction with hydroxyl groups. The major disadvantage, from the anabolic steroid point of view, is that this derivative will not react with 11β-hydroxyl or tertiary 17-hydroxyl groups. For urinary metabolites without these groups, this could be a good derivative.

6. Pentafluorophenylhydrazone is used to derivatize ketones (47). In this case, the disadvantages far outweigh the advantages. The derivative itself is not very volatile, and is acid-labile and unstable when exposed to air and direct light. Better sensitivity can be achieved using another reagent specific for ketones, *o*-pentafluorobenzyloxime.

7. *o*-Pentafluorobenzyloxime (sold as *Florox* by Pierce) reacts with ketone groups for complete derivatization. It has been used successfully for both ECD (61) and ECNI/MS (62) analyses of ketones. Sometimes, any remaining hydroxyl groups must be protected (usually as TMS-ethers) to improve chromatographic properties. In the ECNI spectrum, the base peak corresponds to the derivatizing agent, but there is a prominent ion representing the parent molecule.

8. *bis*-Trifluoromethylbenzoyl derivatives react exclusively with hydroxyl groups (63, 64). Under ECNI conditions, the molecular ion is advantageously represented by the base peak, and the detection limit is 1 pg. The major disadvantage

is the possible formation of multiple products. Up to five different products can be observed for a typical diol. For certain steroids, this problem can be compensated by reaction time. Hindered hydroxyl groups, such as at C-11, are difficult to derivatize; a tertiary 17β-hydroxyl also may not be derivatized.

9. Haloacyl derivatives include trifluoroacetyl (TFA) (65), pentafluoropropionyl (PFP) (66), and heptafluorobutryl (HFB) (45, 62, 67, 68) groups. In a study comparing the relative ECD responses of various haloacyl derivatives of testosterone (66), the HFB derivative had the best response, about fifty times that of the TFA derivative. Not surprisingly, the most common haloacyl derivative used for ECNI is HFB (45, 62). The base peak in any HFB derivative ECNI spectrum is representative of the derivative; any peaks representative of the parent molecule are very small. For example, the peaks representing the parent molecule of derivatized nandrolone are less than 5% (45). Other problems with HFB derivatives include the fact that hindered hydroxyl groups may not react quantitatively and that some products are chemically unstable.

10. Chemical oxidation has been used in this laboratory for the determination of dexamethasone in plasma (69) and 6β-hydroxycortisol in urine (70). This methodology involves chemical oxidation of the analyte to a modified product with extraordinarily high electrophilic properties. The synergism in selectivity available from the combination of a selective chemical reaction, selective ionization conditions, and selective mass-to-charge monitoring techniques by mass spectrometry offer the potential for a uniquely simple and sensitive quantitative assay for particular steroids.

Preliminary Results

TMS Derivatives of Anabolics

TMS derivatives do not add any electrophilic character to a molecule, but they do allow thermally labile steroids to pass through the GC intact; thus, steroids that

have other electron-capturing features can be detected. The only anabolics which possibly could benefit from TMS derivatization are stanozolol and fluoxymesterone. The chemically oxidized product of fluoxymesterone (1,4-androstadien-17α-methyl-17β-ol-9α-fluorine-3,11-dione) was also used for this study.

The steroids were derivatized with MSTFA:TMCS (100:2) at 60°C for 5 minutes. Approximate concentrations were determined by using GC-FID and the effective carbon number concept (72). The ECD and ECNI responses for these compounds are summarized in Table 3.4.

Table 3.4: ECD Relative Responses of TMS Derivatives of Anabolic Steroids

Compound	Relative Response*
1,4-Androstadien-3,11,17-trione	1.0
4-Androsten-3,17-dione (ox. testosterone)	0.003
Fluoxymesterone	0.004
Oxidized fluoxymesterone	0.63
Fluoxymesterone-TMS	0.027
Oxidized fluoxymesterone-TMS	1.2
Stanozolol-TMS	0.0002

*1,4-Androstadien-3,11,17-trione has been assigned a relative response of 1.0.

In Table 3.4, 1,4-androstadien-3,11,17-trione is a representative high-response compound; that is, any compound with a response at this level will be suitable for ECNI assay development. 4-Androsten-3,17-dione is the oxidized product of the endogenous steroid testosterone; any compound with a response at this level will not be easily detectable by ECNI. Even as its TMS derivative, stanozolol is a very poor electron capturer, much worse than oxidized testosterone. Underivatized fluoxymesterone is a poor-response compound because it is thermally labile. The

response of the TMS derivative of fluoxymesterone is slightly better. To obtain a reasonable response, fluoxymesterone must be oxidized. The presence of the 11-carbonyl in conjunction with the 9-fluorine is significant for response. The TMS derivative of the hindered 17-hydroxyl group in oxidized fluoxymesterone does help the response slightly.

Selected Fluorinated Derivatives

Three steroids, stanozolol, methyltestosterone, and fluoxymesterone (see Figure 3.1), were chosen for a preliminary evaluation of derivative formation and detector response because they have a variety of functional groups. These steroids were derivatized using published (but not optimized) methods. Derivatives tested include those prepared by using reagents such as TFA, HFB, flophemesyl, Florox, *bis*-trifluoromethylbenzoyl, and pyridinium chlorochromate (for chemical oxidation). If the reaction proceeded in sufficient yield, the approximate concentration of the desired product was calculated using the GC/flame ionization detector (GC/FID) and the effective carbon number concept for assessing the detector response (72). Relative responses of the derivatives were determined using GC/ECD.

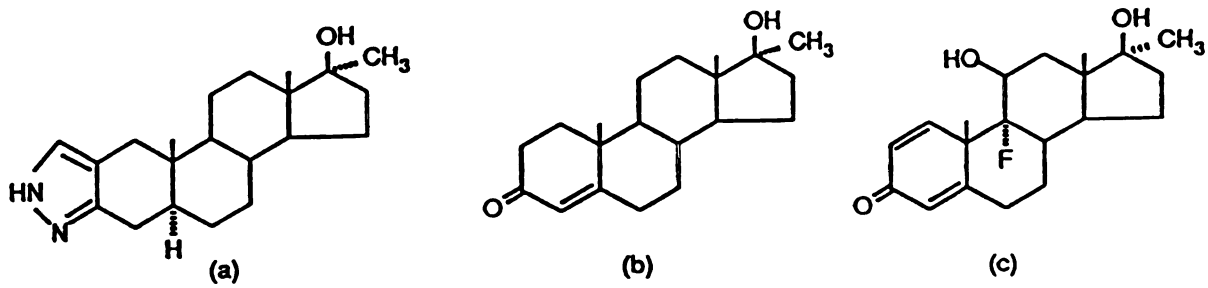

Figure 3.1: Steroids used for pilot study on derivatization: (a) stanozolol, (b) methyltestosterone, and (c) fluoxymesterone.

In the procedure attempted (73), TFA derivatization reactions proceeded with a very low yield. Only fluoxymesterone reacted in any significant amount, and even

then only one of two hydroxyl groups was protected. Problems with this procedure may be solved by the choice of catalyst and clean-up procedure.

Many procedures for the formation of HFB derivatives have been published. The two procedures attempted here used heptafluorobutyric acid anhydride (68) and heptafluorobutrylimidazole (73). Both methods suffered from the formation of multiple reaction products. Stanozolol was derivatized only at the nitrogen in the pyrazole ring as expected (14). Methyltestosterone formed only the HFB derivative at the ketone, leaving a free hydroxyl group. A future possibility is to form the di-HFB derivative (45), which should be easier to detect than a mono-HFB derivative.

As stated previously, flophemesyl derivatives can selectively react with a variety of functional groups depending on the choice of reagent and reaction conditions. For the anabolic steroids used in this experiment, a 10:1 mixture of flophemesyldiethyamine:flophemesylchloride did not work very well. Stanozolol did not react at all. For fluoxymesterone, one hydroxyl group was derivatized, as would be expected because the 11β-hydroxyl group does not react under the conditions used. Mono- and di-flophemesyl derivatives were formed for methyltestosterone.

The *bis*-trifluoromethylbenzoyl chloride derivatized testosterone which has only one unhindered hydroxyl group in one hour at 60°C. After a five-hour reaction, the hindered hydroxyl group on methyltestosterone barely reacted. A study of reaction conditions and times should be undertaken to optimize this yield.

Florox is a ketone-specific reagent. Not unexpectedly, stanozolol did not react. Both methyltestosterone and fluoxymesterone were derivatized as expected. The reaction conditions for the formation of Florox derivatives were 60 minutes at 50°C.

Conclusions from Preliminary Studies

Table 3.5 shows the relative ECD responses of anabolic steroid derivatives relative to that of 1,4-androstadien-3,11,17-trione, a very good electron capturing

compound. Any compound with a response higher than that of 1,4-androstadien-3,11,17-trione will have an advantageous detection limit by electron capture detector. The ECD detector was used to estimate the electron capture response of a given derivative. Generally, the trends in responses are the same for either ECD or ECNI-MS; however, the absolute limits of detection may differ. Under ECNI conditions, the derivatives can fragment, which leads to higher detection limits.

Table 3.5: ECD Relative Response of Selected Anabolic Steroid Derivatives

Derivative	Relative Response*
Florox of Methyltestosterone	10
Florox of Fluoxymesterone	4
HFB of Methyltestosterone	0.5
HFB of Fluoxymesterone	0.2
HFB of Stanozolol	2
Oxidized Fluoxymesterone	100

*1,4-Androstadien-3,11,17-trione has been assigned a relative response of 1.0.

As shown by the results in Table 3.5, both mono-HFB and Florox derivatives have a very good electron capture response. The addition of a TMS group to the 17β-hydroxyl group of either fluoxymesterone or methyltestosterone Florox derivatives has no effect on the ECD response.

The most promising procedure for detection of fluoxymesterone involves chemical oxidation because it gives a 100-fold greater response than 1,4-androstadien-3,11,17-trione. This enhancement is achieved by oxidative transformation of the analyte, a process which discriminates against producing chemical noise from the sample matrix.

Along with improving the formation of the above derivatives, other derivatives which are promising include the pentafluorobenzoate and silyl derivatives with halogen substitution. 4-O-Perfluorotolyl ethers do not react with tertiary 17-hydroxyl groups; however, they could be used for steroids and metabolites such as nandrolone or boldenone that do not contain this group.

Future Work

Proposed work with conventional derivatization

Many qualitative screens exist for anabolic steroids; most are based on GC/EI/MS of TMS-ether and/or TMS-ether-TMS-enol derivatives. More selective and sensitive methodology is needed for the confirmation of a positive screen. Also, many physiological and pharmacological studies of long-term steroid use require methodology that can detect trace levels of steroids or metabolites.

The proper derivative for a steroid or a set of steroids must meet several conditions. After appropriate reaction conditions are determined in order to optimize the formation of one product, the derivatives will be evaluated for their sensitivity under GC/ECNI/MS. Derivatives that generate major ions indicative of the parent molecule rather than the derivatizing moiety are most desirable. Tradeoffs between one-step reactions, complete reactions, short reaction times, and short GC analysis times should be evaluated.

Derivatives also should be evaluated for their selectivity. One of the greatest problems in anabolic steroid analysis today derives from the similarity of oral contraceptive metabolite structures to those of certain anabolics. For example, the oral contraceptive norethisterone is partially metabolized to 19-norandosterone, the major metabolite of nandrolone (75). Another problem is the similarity of the major metabolite of norethisterone (see Figure 3.2) to the major metabolite of methenolone (75). During analysis by GC-MS, the TMS-ether-TMS-enol derivatives of these two

metabolites produce the same ions at the same retention time. If one uses a derivative specific for ketones (such as Florox), only the methenolone metabolite will appear. The norethisterone metabolite will not be derivatized; thus, it will not appear in the chromatogram.

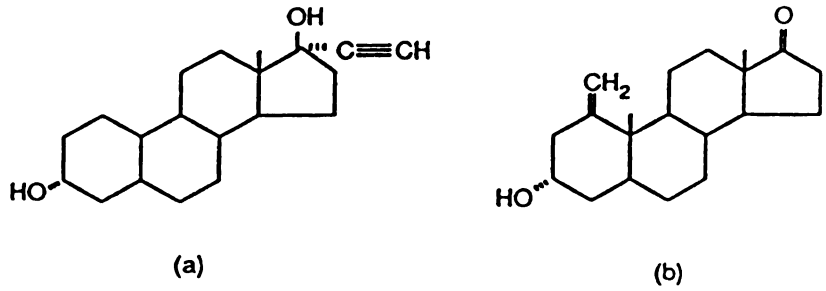

Figure 3.2: Similarity of oral contraceptive metabolites to anabolic steroid metabolites. (a) Major metabolite of norethisterone (an oral contraceptive). (b) Major metabolite of methenolone (an anabolic steroid).

Applications of Chemical Oxidation to the Analysis of Anabolic Steroids

The proposed methodology is extremely promising for the selective detection of fluoxymesterone. From comparison of preliminary data, the steroid most amenable to the oxidation technique is fluoxymesterone which can be chemically oxidized to 1,4-androstadien-17α-methyl-17β-hydroxy-9α-fluorine-3,11-dione. Not only does the oxidized product have the 1,4-en-3,11-one structure that enhances the electron-capture response (74), it also has a 9-fluorine group. In fact, the response of this compound under ECNI conditions is 20 times that of 1,4-androstadien-3,11,17-trione, which is a high-response compound (see Table 3.6). The presence of the 17β-hydroxy does not seem to adversely affect the chromatography.

Many anabolic steroids have a tertiary 17-hydroxy group which cannot be oxidized to the ketone in a simple, one-step reaction. Those anabolics that have secondary 17-hydroxy groups, namely testosterone, nandrolone, and boldenone, are easily oxidized to ketosteroids, but their response is poor in comparison to that of 1,4-

androstadien-3,11,17-trione (see Table 3.6). These results were expected from the preliminary studies because these oxidized anabolic steroids have only one conjugated site. The detection limit of oxidized boldenone is 2 ng/mL, far short of the 0.5 ng/mL minimum needed for effective analysis of urine for anabolics. Also, the advantage of selectivity over the endogenous steroids is lost.

Table 3.6: Relative ECNI Responses of Oxidized Anabolic Steroids

Steroid	Relative Response*
Oxidized testosterone	0.0075
Oxidized nandrolone	0.0075
Oxidized boldenone	0.013
1,4-Androstadien-3,11,17-trione	1.0
Oxidized fluoxymesterone	19

*1,4-Androstadien-3,11,17-trione has been assigned a relative response of 1.0.

Fast Chromatography

The rate-limiting step in anabolic steroid analysis is usually the work-up and derivatization of the urine samples, but long GC analysis times also can be frustrating. Tandem mass spectrometry (MS/MS) methods can be used to shorten analysis times by using shorter columns without substantial loss of chromatography (76). A twenty-minute analysis on a 30-m column can be shrunk to seven minutes by monitoring daughter ion scans on the effluent from a 4-m column (77). As well as shortening analysis times, selectively also can be improved because one can monitor specific daughter ions, even if the parent molecular ions are the same. Negative ion MS/MS can be used to analyze the most promising optimized derivatives for even more selectivity and perhaps better sensitivity (78).

Another method of reducing analysis time is by using narrow bore columns. For example, other GC analysis of urinary steroids usually takes about an hour using conventional 0.25-mm inner diameter columns. If a 50-mm inner diameter column with the same plate number is used, the analysis is complete in only 12 minutes (79).

Validation of Analysis

After a suitable derivative for a certain anabolic steroid or metabolite is identified based on the ECNI sensitivity and reaction conditions, it should be tested in a biological matrix for possible interferences. Extraction procedures from urine should be optimized. Possible interferences from urine of females using oral contraceptives also should be investigated. The use of short column chromatography in conjunction with MS/MS should be evaluated, if indicated; should this approach be necessary, a protocol involving a Finnigan TSQ 70 as previously published by this laboratory (78) should be used. Sensitivity and selectivity should be compared to those of published methods. After a derivatization method passes these tests, it should be evaluated using real samples with parallel analyses by conventional, published methods to establish validity.

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Chapter 4: ECNI-MS Structure-Response Study

The oxidation methodology developed in this laboratory for the analysis of corticosteroids is advantageous, in part, because of its selectivity. The analyte chemically oxidizes to a highly electrophilic species, whearas endogenous steroids do not, thus decreasing the chemical background. In order to predict the relative responses of ketosteroids and similar compounds under ECNI conditions, a study of the effect of structure on response was undertaken as decribed in this chapter. Interactions between double bonds, carbonyl groups, halogens, and epoxides were examined in steroids and other cyclic compounds such as cyclohexanones, terpenes, quinones, and trichothecenes.

Previous work correlating ECNI responses with steroid structure has been limited to only a few compounds (1). More extensive studies of steroid electron-capture responses (2, 3) have been conducted with GC-ECD. These studies have examined combinations of double bonds and carbonyl groups almost exclusively.

The work summarized in this chapter has concentrated primarily on ascertaining the ECNI responses of cyclic compounds. After the determination of optimum instrumental conditions, many sets of compounds were analyzed. The electron-capture properties of ketosteroids were investigated, with special emphasis on epoxidized and halogenated compounds. The responses of conventional electron-capturing derivatives, such as trifluoroacetyl derivatives, were compared to those of compounds obtained by PCC oxidations. Cyclohexanones and bicyclic compounds were analyzed to provide comparisons of compound size to response in addition to providing functional orientation not found within a steroid nucleus. The ECNI response of quinones was compared to published electron affinity values. The reduction potentials of many other compounds were determined to estimate their electron affinities.

Several other studies were undertaken in order to determine other factors affecting electron capture. The responses of several steroids under EI, CI, ECNI, and ECD conditions were compared. The use of different modulating gases for steroids also was studied. ECNI responses of steroids were compared to UV data. Finally, the day-to-day reproducibility of ECNI spectra on the JEOL 505 was assessed.

Determination of Optimum Instrumental Conditions

In studies with ECNI-MS, the control of instrumental parameters, especially source temperature and pressure, is paramount to sensitivity and reproducibility (4,5). Gas chromatographic and mass spectral conditions were optimized for two representative ketosteroids, 1-androsten-3,17-dione and 1,4-androstadien-3,11,17-trione. The former is a low-response compound whose mass spectrum consists of only one peak, that representing $[M-H]^-$. The latter steroid is a high-response compound whose mass spectrum consists of only one peak, that representing $M^{-\cdot}$.

Source Temperature. Because electron capture depends on a collision between a thermal electron and the analyte of interest, source temperature plays an important role in determining the rate of this reaction. Temperature also affects the reagent gas reactions which result in the formation of the thermal electrons. In general, the lower the source temperature, the greater the ECNI response. This is true for "conventional" ECNI compounds (highly halogenated compounds) such as octafluoronaphthalene (OFN), but does not hold true for steroids (see Figure 4.1). In this experiment, chromatograms from full-scan analysis rather than SIM were compared. OFN is a fairly sensitive compound and is about 22000 times more sensitive than 5α-androstan-3-one. The ECNI mass spectrum of OFN consists of two peaks at *m/z* 272 ($M^{-\cdot}$) and *m/z* 238 ($[M-34]^-$). Usually, as the temperature increases, so does the amount of fragmentation; however, the opposite is true for OFN. As is typical for highly

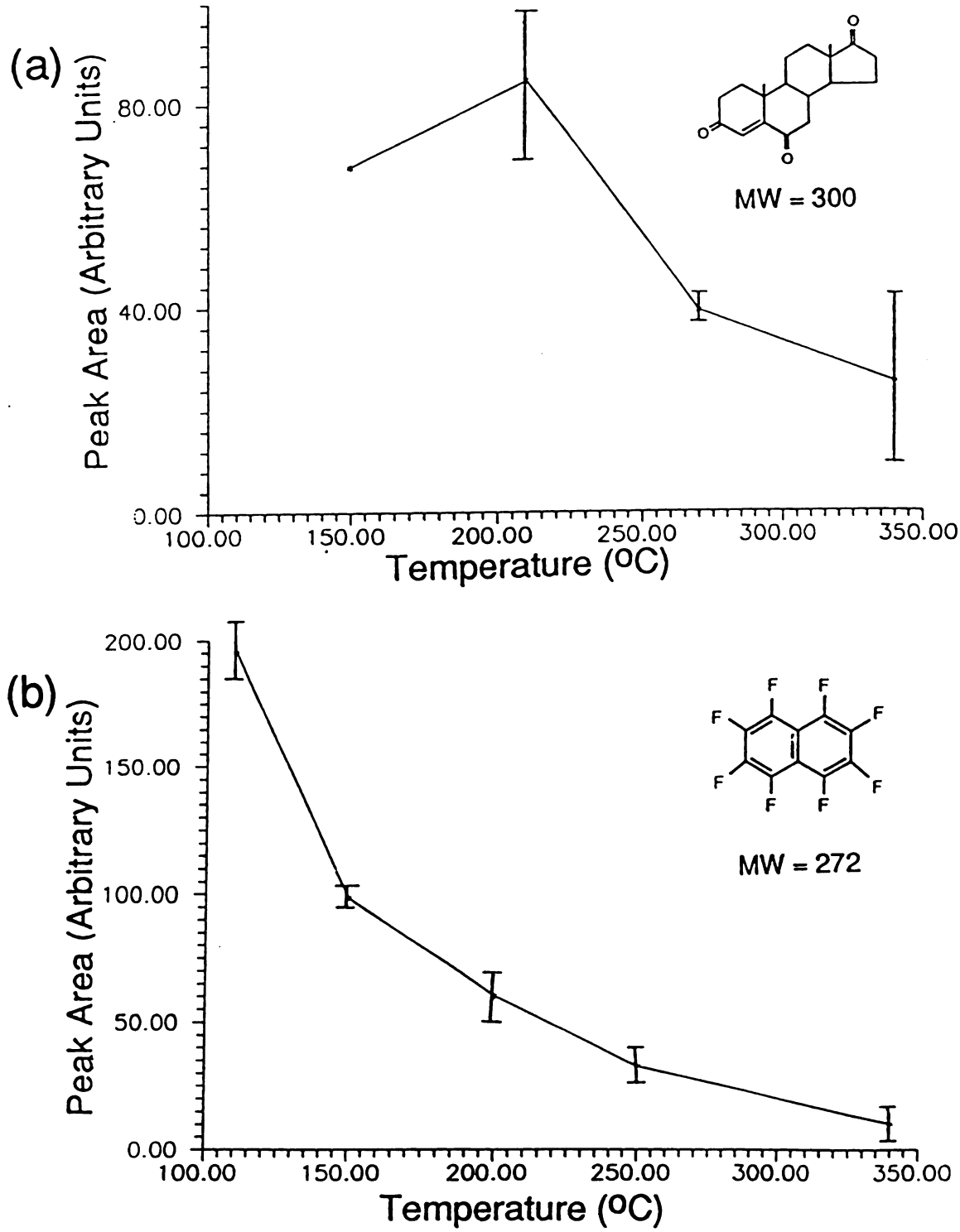


Figure 4.1: ECNI response as a function of source temperature. The source pressure was held constant at 2 x 10^{-5} torr. (a) The response of octafluoronaphthalene decreases with increasing source temperature. (b) The response of 4-androsten-3,6,17-trione is maximized at a source temperature of 200 °C.

halogenated compounds, the sensitivity decreases as the temperature increases. This is true for OFN, as shown in Figure 4.1a. In comparison, the data for 4-androsten-3,6,17-trione (Figure 4.1b) shows an optimum temperature of 200°C. In general, steroids have greater sensitivity at higher temperatures, but the optimum varies from compound to compound. For example, the optimum temperature for the detection of 1,4-androstadien-9α-fluoro-16α-methyl-3,11,17-trione (oxidized dexamethasone) is at 150°C.

Reagent Gas Pressure/Source Temperature. Pressure/temperature studies were performed at four source temperatures and three pressures of methane gas in order to determine the best combination of temperature and pressure for maximum sensitivity. For 1,4-androstadien-3,11,17-trione, a high-response compound that has a peak for the molecular anion at *m/z* 298, the optimum source temperature was relatively high (250°C) with a low reagent gas pressure (1×10^{-5} torr). For 1-androsten-3,17-dione, a low-response compound which has a $[M-H]^-$ anion at *m/z* 285, the best conditions were a low temperature (150°C) coupled with a high reagent gas pressure (4×10^{-5} torr). See Figure 4.2 for a graphical representation of these data. Both high- and low-response compounds were used in this study; therefore, a compromise temperature and pressure was used. The source temperature was set at 200°C, and the reagent gas pressure was held at 2×10^{-5} torr.

Also of interest is the relationship among the peak areas of individual components of the three-compound internal standard. If these ratios are constant over a range of temperatures and pressures, then the internal standard probably will compensate for small variations in instrumental conditions over a series of analyses. The ratio of areas of the ion at *m/z* 298 to the ion at *m/z* 285 were compared using various temperatures and pressures (see Figure 4.3). The ratio was most nearly alike at 150°C, but the ratios at 200°C are within the error bars. Therefore, run-to-run

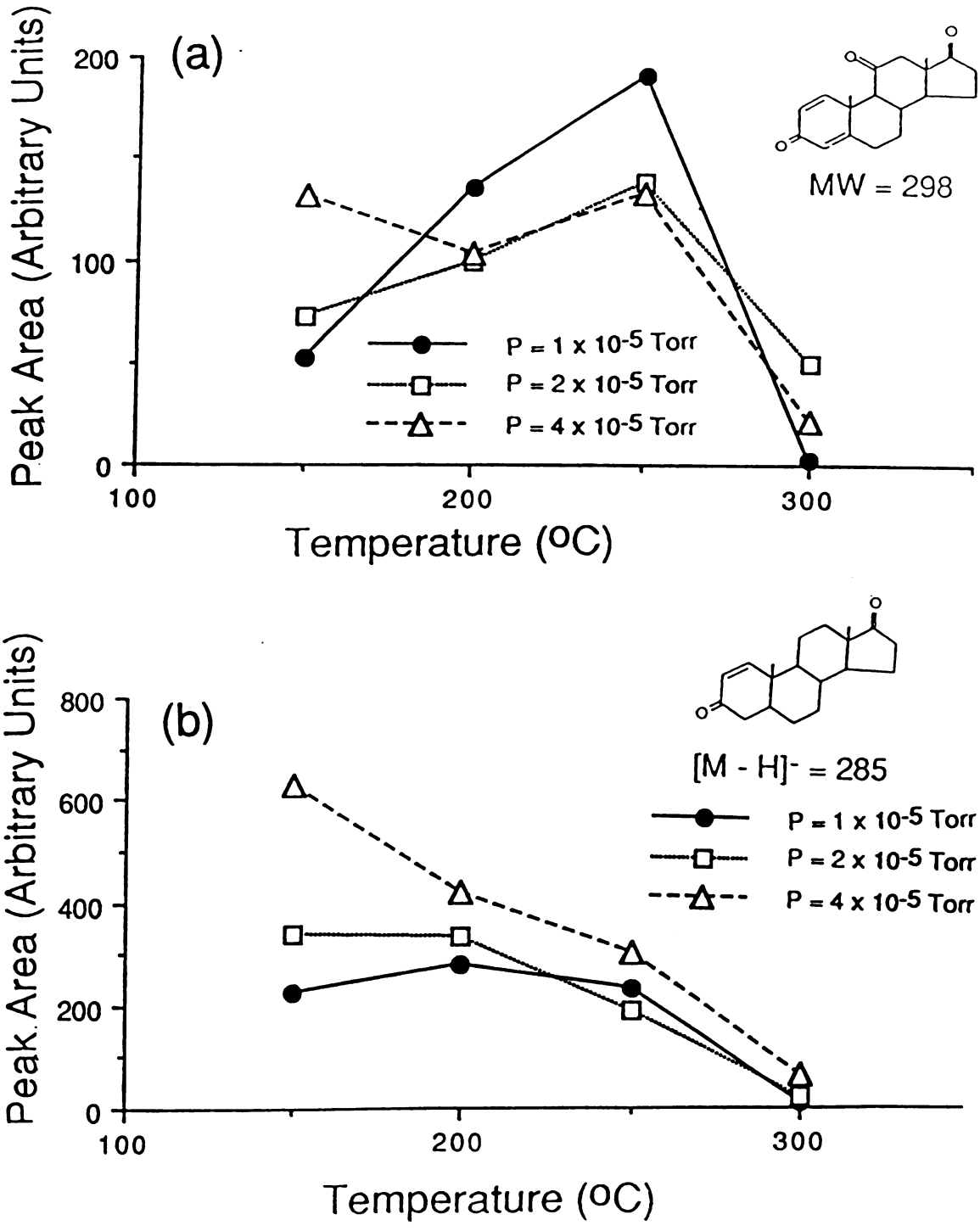


Figure 4.2: ECNI response as a function of source temperature and pressure. (a) The optimum conditions for the analysis of a low-response compound such as 1-androsten-3,17-dione were at a source temperature of 150 °C and a source pressure of 4 x 10^{-5} torr. (b) The optimum conditions for the analysis of a high-response compound such as 1,4-androstadien-3,11,17-trione were at a source temperature of 250 °C and a source pressure of 1 x 10^{-5} torr.

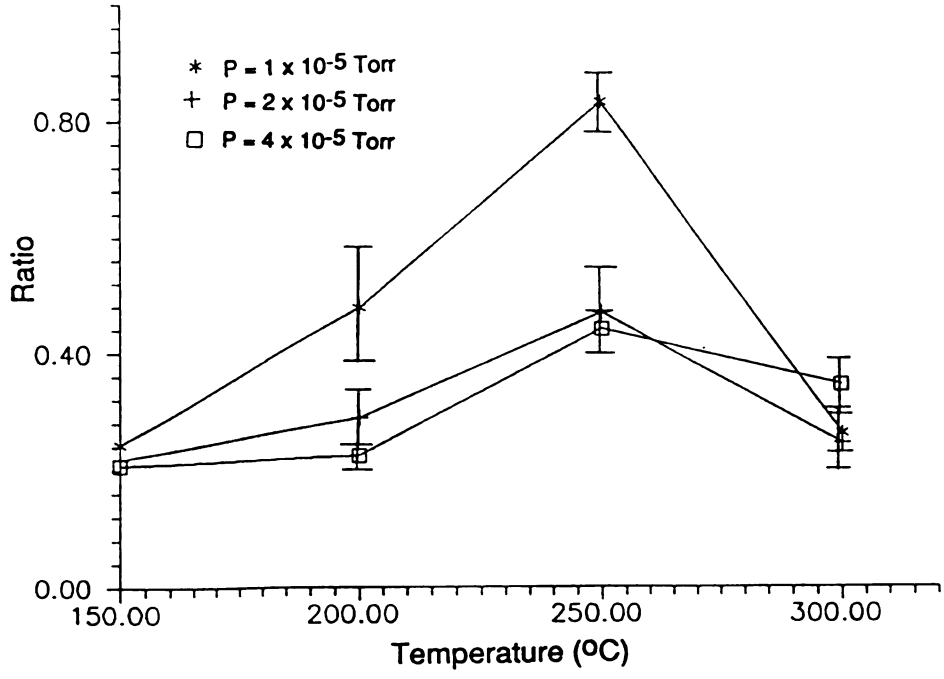


Figure 4.3: The ratio of the peak areas of the ion at *m/z* 298 (from 1,4-androstadien-3,11,17-trione) to ion at *m/z* 285 (from 1-androsten-3,17-dione) as a function of source temperature and pressure.

variations in pressure can be compensated by comparing the analyte area to that of an internal standard.

Reagent Gas Composition. The composition of the reagent gas can also influence sensitivity. A preliminary study of reagent gas composition used methane and ammonia gases; a more involved study with many gases will be presented later. For the high-response steroids (those that exhibit a molecular anion), there was no difference in sensitivity between the two gases. However, for compounds with a $[M-H]^-$ ion, such as 1-androsten-3,17-dione, sensitivity was enhanced by using ammonia as the reagent gas. Methane was chosen as the reagent gas because it is relatively nonselective for the compounds analyzed.

Reagent Gas Inlet. Conventionally, the reagent gas enters the source perpendicular to the introduction of analyte through the GC. Perhaps if the reagent gas and the GC effluent were introduced in the same direction, the sensitivity might be improved. The reagent gas line was attached to the make-up line in the GC. One advantage was a more stable baseline of pressure. The chromatography was not improved, however. Sensitivity was slightly increased for high-response compounds, but not at all for low-response compounds. The make-up line inlet is no longer being used.

Ionization Voltage and Current. Normally, the ionization voltage for ECNI is set by JEOL at 200 eV for the 505 instrument. A modification in the circuit allowed control of this parameter. When the voltage was varied between 100 and 200 eV, but no difference in peak areas was observed. Therefore, the default ionization voltage of 200 eV was used. The ionization current has two possible values on the JEOL 505, 100 and 300 μA. Unquestionably, 300 μA is the recommended setting for ECNI, because at 100 μA ions can barely be observed.

Linear range. ECNI has a narrow range of linearity because of a limited concentration of thermal electrons. "Overloading" the sample on a GC column causes flat-topped reconstructed mass chromatogram peaks under ECNI conditions. A study was undertaken to confirm that the amount of steroid used in the internal standard fell within the linear range for these compounds. 1,4-Androstadien-3,11,17-trione was linear in the region of 0.4 to 20 ng/μL (r^2 = 0.999). If the point at 20 ng/μL was ignored, 4-androsten-3,6,17-trione was linear between 0.3 and 3 ng/μL. In other words, the concentrations of steroid used for the internal standard are within the linear range. In order to assure linearity for the other compounds tested, their peak area should be smaller than the peak area of the largest chromatographic peak in the internal standard.

Data Acquisition. Three methods of data acquisition are available using the JEOL 505: full scan, magnetic field switching selected ion monitoring (MFSIM), and electric field switching SIM (EFSIM). All three techniques gave the same corrected response factors for the same compound analyzed. In a comparison of MFSIM vs. full scan (100-400 u, scan rate = 0.9 s), two compounds were tested. The mass spectrum of 5α-androstan-3-one has only one significent peak, namely that representing $[M-1]^-$; 1,4-androstadien-3,17-dione has three peaks, those representing $[M-H]^-$, $[M-2H]^-$ and $[M-CH_3]^-$. In each case, MFSIM produced data that had lower coefficients of variance (cv). Each compound was spiked with the three-component internal standard (as described below) and analyzed five times. After division by the response of the internal standard, the cv of the full scan data was 15-20%, while the cv of the MFSIM analyses was under 10%. The advantage is that under SIM conditions, the signal-to-noise ratio is increased sufficiently so that smaller peaks can be detected. Another advantage of SIM over full scan is the amount of memory needed to store the data. A typical SIM file has about 130 blocks as compared to 5000 to 10000 blocks (depending on analysis time and noise levels) for a full scan file. Typically, free space on the computer is between 100,000 to 150,000 blocks. A back-up tape has only 180,000 blocks. To make data acquisition and storage much more feasible, SIM was used for all analyses. With respect to sensitivity, EFSIM and MFSIM are not significantly different. MFSIM, however, is the better documented of the two; therefore, it will be used for future analyses. Full scan analyses were extremely important in this study because they were used to determine the retention times of the compounds as well as to verify the peaks chosen for analysis.

Ion Multiplier. To improve sensitivity, the ion multiplier voltage can be increased, but this also increases the noise. At the nominal ion multiplier voltage of 1000 V, the signal was too weak to observe. The ion multiplier was increased to 1200,

1400, and 1600 V. The 1400 V setting was chosen as a compromise between increased signal and increased noise.

Sample Introduction. GC was used as the method of choice for sample introduction. Use of the direct-insertion probe (DIP) was studied in an attempt to shorten the analysis time without affecting reproducibility. To quantify the responses of steroids, a three-component internal standard was used. Upon introduction by DIP, these compounds desorb simultaneously. Assuming the steroid of interest will probably desorb at the same time, there would be four compounds competing for the same thermal electrons. This could introduce variables not found in GC analyses. For example, a compound could have a diminished electron capture response because it cannot compete as well for electrons as a coeluting compound. The reproducibility of the technique is on the order of that of a GC inlet, with a cv of about 10%. However, using the DIP, the reconstructed ion peak shape was very poor. Determining the area under the reconstructed ion curve was difficult because often it was impossible to judge where the peak began and ended.

Injection Volume. One of the most important contributions to reproducibility is control of the injection volume. After trying many different injection techniques, the best one is the solvent-push technique. This involves placing about 1 μL of ethyl acetate in the syringe followed by about 1 μL of air, and finally 1 μL of sample. The sample volume should now be read by placing one end of the liquid at 0 μL and reading the other value. The liquid should be just at the end of the barrel (but not in the tip of the needle) before injection. Injection should be a slow, smooth motion. Also, it is easier to read the volume injected from a 5–μL syringe, which is calibrated to 0.1 μL rather the more commonly-used 10–μL syringe calibrated to 0.2 μL.

GC Chromatography: Temperature Program. With the splitless GC injector, it is recommended that one start well below the boiling point of the lowest-boiling analyte or below the boiling point of the solvent. At first, the analysis was started at

50°C, which is below the boiling point of the solvent. Time was wasted in two ways. First, it takes time for the temperature to ramp up, even at 40 degrees per minute. Secondly, it takes a significant time to cool the GC oven back to 50 degrees because heat loss is slow during the last 30 degrees. Under EI conditions, the chromatography improved when the temperature program was started at 140 or 160 degrees instead of at 50 degrees. At starting temperatures higher than 160°C, the chromatography degraded again. The best sensitivity was obtained when starting at 140°C. This temperature program does not work for all compounds. For example, the EI-MS sensitivity of methyl stearate was greater starting at 50 degrees rather than 140 degrees.

Under ECNI conditions, the best chromatography and sensitivity were obtained using 140 degrees as the starting temperature. Also, if one holds the temperature at 140 degrees for a minute, a bit longer than the time needed for the purge valve to open, the sensitivity is also improved.

GC Chromatography: Literature Study. As mentioned in the previous section, the steroids tail much worse under ECNI conditions than under EI. Reviewing twenty-nine randomly-selected papers in the literature was revealing, as shown in Table 4.1. About 60% of the compounds tail. The type of instrument and the column does not seem to have an effect; for example, PAHs tail on both nonpolar and slightly polar columns. Tailing seems to be an inherent trait of ECNI. As shown by others (10), interactions with free radicals on the ion source surface result in severe tailing.

GC Chromatography: Columns. For most of the preliminary work, two DB-1 30-m capillary columns with 0.25-micron packing were used. One had an inner diameter of 0.25 mm and the other, 0.32 mm. Tailing was noted on both columns, especially for the later-eluting substances. Sometimes the tailing was so extensive that the later-eluting compounds were completely lost in the noise. Tailing accounts for

Table 4.1: Chromatography Under ECNI Conditions

Compound	Column	Instrument	Peak Shape	Ref.
Bromobenzenes	DB-1	Finnigan 3300	Slight tailing	6
Chlordane	DB-1	Nermag R10-10C	Gaussian	7
Bromobenzenes	SE-54	Finnigan 4000	Fronting	8
Perfluorocarbons	SE-54	Finnigan 4510	Tailing	9
Fluoranil	---	VG 7070 E-HF	Tailing	10
Chloranil	---	VG 7070 E-HF	Gaussian	10
PAH	DB-5	HP 5985B	Slight Tailing	11
PAH	BP-1	Finnigan 3200	Tailing	12
PFB-TMS of Dopamine	OV-1	Finnigan 3300	Gaussian	13
PFB of Diclofenac	DB-5	Finnigan 4500	Tailing	14
HFB of Clebopride	DB-1	Finnigan 4021	Gaussian	15
Methylated Dihydrophyridine Calcium Antagonist	DB-1	JEOL DX-303	Tailing	16
Phenothiazines	SPB-1	JEOL D-300	Tailing	17
Mepirodipine	DB-5	JEOL DX-303	Tailing	18
HFIP of Quinolinic Acid	DB-5	Finnigan 3200	Gaussian	19
s-Triazines	SPB-608	HP5890	Severe tailing	20
PFB of Histamine	DB-1	Nermag R10-10C	Slight tailing	21
Abscisic Acid Methyl Ester	DB-5	Finnigan TSQ-70	Gaussian	22
Bistrifluoromethylbenzyl of Quinoxaline	DB-5	Finnigan 4500	Gaussian	23
HFB of Quinoline	DB-5	JEOL DX-303	Tailing	24
Lorazepam	DB-1	VG 12-250	Severe tailing	25
PFB of Indomethacin	BP5	Finnigan TSQ-46	Gaussian	26
HFB of Estradiol	OV-1	VG 70-70E	Tailing	27
MO-TMS of Corticosteroids	OV-1	HP 5998A	Gaussian	28
MO-TMS of Corticosteroid Acetates	BP1	HP 5998A	Gaussian	29
Trifluoromethylbenzoyl of Steroids	SE-54	Finnigan 4500	Tailing	30
Pregnane Fatty Acyl Esters	OV-1	VG 7070H	Gaussian	31
Oxidized Dexamethasone	DB-1	TSQ-70	Slight tailing	32
TMS of Dexamethasone	OV-1701	HP 5985	Gaussian	33

some of the problems with precision because it is more difficult to determine where the peaks begin and end. Both of these columns developed a contaminant that coeluted with 4-androsten-3,6,17-trione, a component of the internal standard.

The source of contamination was sought by testing other columns. Another DB-1 column was tested for contamination. This DB-1 30-m x 1.0-μm x 0.25-mm inner diameter column was fairly new and had only been used for ABA (not also for service work like the other two columns mentioned previously). In Figure 4.4, the comparison between the old column (0.25-μm packing) and the new column (1.0-μm packing) is evident. Not only does the new column not have the contamination, it also mimimizes the tailing. Unfortunately, the 30-m column, with a thicker film, also doubled the retention time. The 15-m column keeps most analysis times below 10 minutes, an important consideration in replicate analyses.

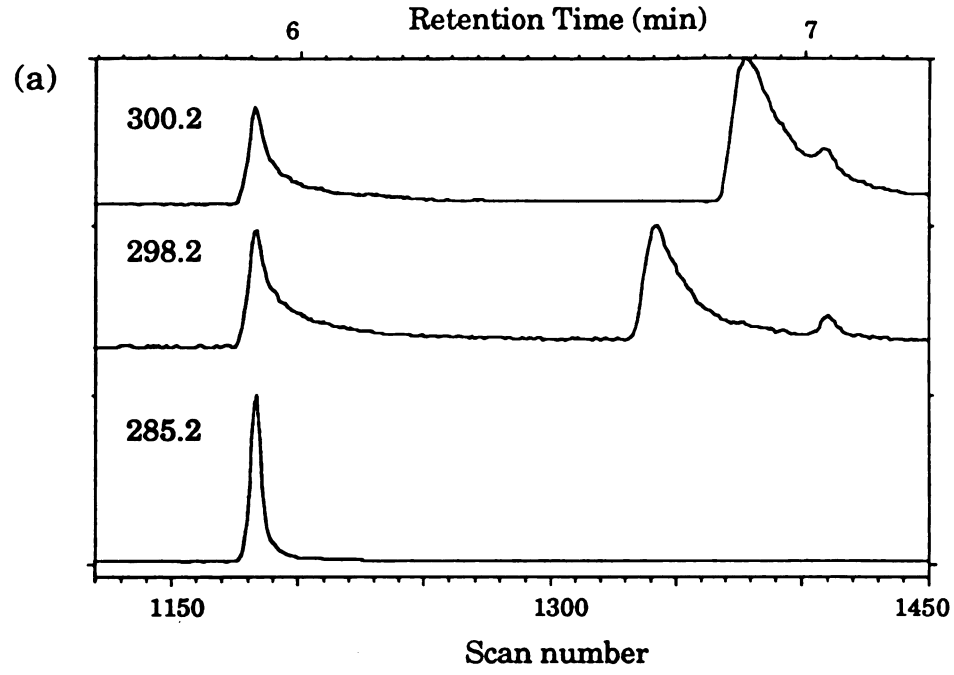
Experimental

Materials

Commercially-available steroids were purchased from Steraloids (Wilton, NH) or Sigma (St. Louis, MO). Quinones were purchased from Aldrich (Milwaukee, WI). Many cholestanes, monocyclics, and bicyclics were a gift from Dr. William Reusch of the Department of Chemistry, Michigan State University. The other steroids were obtained from the Steroid Reference Collection (Queen Mary College, London, D. N. Kirk, Curator). HPLC-grade ethyl acetate was from Mallinckrodt (Paris, KY) or J. T. Baker (Phillipsburg, NJ). All samples were stored in silanized vials.

Internal Standard

To minimize variances between replicate analyses, a three-component internal standard was added to each sample. Androstanes were chosen because pregnanes and cholestanes have longer retention times. Compounds with three different responses were used. The standard was made of 2525 ng/μL 1-androsten-3,17-dione, 14.2 ng/μL



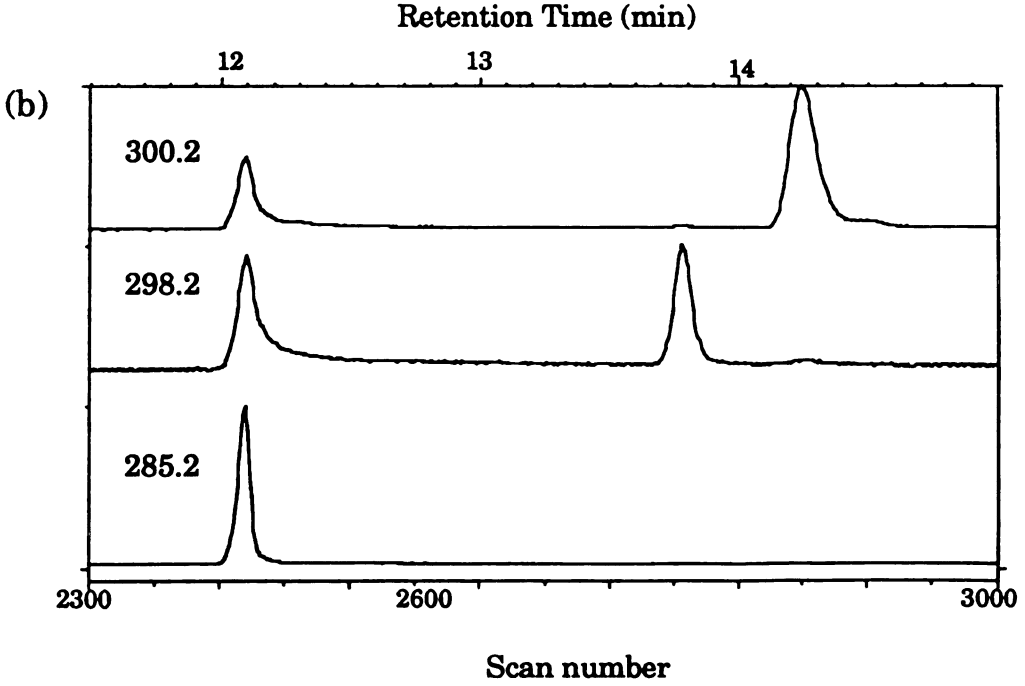


Figure 4.4: Reconstructed mass chromatograms of the major ions of the three components of the internal standard. (a) Note the tailing on the DB-1 30-m x 0.32-mm x 0.25-μm column. (b) The tailing is minimized on a DB-1 30-m x 0.25-mm x 1.0-μm column, but the retention time is doubled.

1,4-androstadien-3,11,17-trione, and 17.7 ng/μL of 4-androsten-3,6,17-trione. To prepare a sample for analysis, 50 μL of the internal standard stock solution was added to 500 μL of the sample solution. If the analyte of interest coeluted with one of the internal standards, a two-component internal standard was used.

Gas Chromatography-Mass Spectrometry

ECNI mass spectral data was obtained on a JEOL JMS-AX505H mass spectrometer (Peabody, MA) interfaced to a Hewlett Packard 5890 gas chromatograph (Palo Alto, CA). The following parameters were used: source temperature, 200°C; ionization current, 300 μA; electron energy, 200 eV; modulating gas, methane; source pressure (measured at the source housing), 2 x 10^{-5} torr; conversion dynode voltage, 10 keV; and accelerating voltage, 3 keV. A J&W 15-m x 0.25-mm id x 1.0-μm DB-1 column (Folsom, CA) was directly inserted into the mass spectrometer source. Helium was used as the carrier gas. The splitless injector was held at 260°C and the transfer line at 260°C. For steroids, the GC oven was held at 140°C for 1 min, then ramped to 260°C at 40°/min, followed by a 4°/min ramp to 290°C. For quinones, the GC oven was ramped at 40°/min from 50 to 160°C, then at 15°/min to 270°C, and finally at 4°/min to 290°C. For cyclohexanones and other compounds that elute at less than 130°C, the following program was used: the GC oven was ramped at 10°/min between 50 and 140°C, then at 40°/min to 270°C, and finally at 4°/min to 290°C. Selected ion monitoring (SIM) in the magnetic field switching mode (with an open collector slit) was used to analyze the most abundant ions in each sample. To increase the signal, the electron multiplier was held at 1400 V, regardless of the age of the multiplier.

Electron impact spectra were obtained to check the composition and purity of each sample. Either the JEOL 505 or the HP Mass Selective Detector (MSD) Model 5970 was used. The JEOL was used under identical conditions as above, except the ionization current was lowered to 100 μA. The scan rate was 1.0 s for a mass range

from 50 to 500 u. The MSD was connected to a HP 5890 GC. Source temperature was 200°C, interface temperature was 280°C, and the mass range was 50 to 500 u.

Gas Chromatography

All GC chromatograms were obtained on a Varian 3700 gas chromatograph (Walnut Creek, CA) that had been modified to accept megabore (0.53-mm inner diameter) columns. The following instrumental conditions were used for all modes of operation: injection temperature, 260°C; detector temperature, 360°C; flow rate, 15 mL/min of nitrogen; 15-m x 0.53-mm id x 1.5-μm DB-1 column from J&W. The temperature program for steroids started at 200°C and ramped at 5°/min to 280°C with a 5-minute hold at the upper temperature. The temperature program for other compounds varied. The integrator used was a HP 3393A. For FID analyses, the range was 10-11 amp/mV with an instrumental attenuation of 1. When using the ECD mode, the range was changed to 10 amp/mV, the output to negative, and the attenuation to 32 through 128 depending on sample response and the integrity of the detector. The make-up gas was adjusted to 40 mL/min total flow.

The response for 1,4-androstadien-3,11,17-trione was optimized on the Varian GC. The detector temperature should be about 50°C above the highest temperature of the ramp program. In this experiment, the detector temperature was varied from 300 to 380°C in steps of ten degrees. The relative responses at each temperature were similar. No advantage of higher or lower temperature was observed, so a temperature of 360°C was used. The optimal flow rate was also determined. The make-up flow was adjusted to a total flow varying between 12 and 86 mL/min (measured using a bubblemeter at the ECD outlet). Three different compounds with three vastly different responses were used as probes: 1,4-androstadien-3,11,17-trione, 1,4-androstadien-3,17-dione, and 5α-androstan-17-one. In each case, the response was highest at 12 mL/min and decreased almost linearly until 55 mL/min. Any higher value of flow resulted in almost a constant response. The responses to flow track each other;

therefore, the relative responses should be independent of flow. Flow rates under 20 mL/min are difficult to achieve in practice because the separation suffers. Therefore, a total flow of 30 mL/min was chosen.

Sample Preparation

Samples were prepared at concentrations ranging from 0.01 ng/μL to 2 ng/μL, depending on the electron-capture response. The ECD was used to estimate electron-capture response and concentration needed for mass spectrometry. All samples were analyzed by GC-EI-MS to determine structure and purity. The concentrations of impure samples were determined using the GC-FID and the effective carbon number technique described below.

The samples were analyzed by full scan ECNI-MS to determine the major ions. Then, the internal standard was added to the sample. After replicate (n = 3-5) SIM analyses of the most abundant peaks, the sum of all the ion current monitored was divided by the ion current for the internal standard.

PCC of Selected Steroids

Some of the steroids that were not available from other sources were prepared using chemical oxidation. The procedure used is described in Chapter 5 under the experimental procedure for mouse plasmas. The oxidized steroids were analyzed by EI to confirm structures. Those with sufficient yields were used in the ECNI response study. Effective carbon number (ECN) procedures described below were used to determine concentration in every case save one. Oxidized dexamethasone was synthesized in sufficient quantity and recrystallized to give a highly accurate primary standard. Those compounds prepared by the PCC method are listed in Table 4.2.

Preparation of Derivatives

Trifluoroacetyl (TFA) and Heptafluorobutyric (HFB). These derivatives were prepared based on the method of Wilson and co-workers (34). About 100-150 μg of steroid were transferred to a silanized reacti-vial (Pierce) and dissolved in 50 μL of

benzene. Then, 50 μL of trifluoroacetic acid anhydride (for the TFA derivative) or heptafluorobutyric anhydride (for the HFB derivative) were added, and the mixture was heated at 60°C for 30 min. After the reagents were evaporated under nitrogen, the residue was dissolved in 50 μL benzene.

Table 4.2: Steroids Prepared by Chemical Oxidation

Starting Material	Product
Dexamethasone	1,4-Androstadien-3,11,17-trione-9α-F-16α-methyl
Flumethasone	1,4-Androstadien-3,11,17-one-9α,6α-F-16α-methyl
Fludrocortisone	4-Androsten-3,11,17-trione-9α-F
Fluoromethalone	1,4-Androstadien-3,11,17-trione-9α-F-6α-methyl
Fluoxymesterone	4-Androsten-9α-F-17α-methyl-17β-ol-3,11-diol
6β-Hydroxydexamethasone	1,4-Androstadien-3,6,11,17-tetraone-9α-F-16α-methyl
Cholestan-5α,6α-epoxy-3-ol	Cholestan-5α,6α-epoxy-3-one
4-Androsten-16α,17β–diol-3-one	4-Androsten-3,16,17-trione
1,4-Pregnadien-11,17,21-triol-3,20-dione-6α-F	1,4-Androstadien-3,11,17-dione-6α-F
4-Pregnen-11-ol-9α-F-3,20-dione	4-Androsten-9α-F-3,11,17-trione

o-Pentafluorobenzyloxime (Florox). The procedure for this derivatization is based on the method suggested by the Pierce Catalog (35). To 100 μg of steroid in a silanized 1-dram vial was added 0.1 mL Florox. The reaction mixture was heated at 65° for 1 h. After cooling, the solvent was evaporated under nitrogen. Then 1 mL cyclohexane and 1 mL water were added to the vial and vortexed. The cyclohexane layer was removed to another vial and dried with Na_2SO_4.

Flophemesyl (pentafluorophenylsilyl ethers) will selectively react depending on the conditions used (36-37). A 10:1 flophemesyldiethylamine:flophemesyl chloride

solution will react with unhindered secondary hydroxy groups and tertiary 17β-hydroxy groups. This reagent will not react with a 11β-hydroxy group. To about 100 μg of steroid in a reacti-vial, 20 μL of the 10:1 mixture plus 20 μL of pyridine were added and vortexed.

MO-TMS. These derivatives (38) were made by adding 100 μL of 100 μg/μL methoxyamine-HCl (Aldrich) in dry pyridine to about 40 μg of steroid. After heating at 60°C for 4 h, the solvent was evaporated under nitrogen until glassy (about 30-45 min). Then 200 μL of Sylon BTZ (Supelco) were added, and the solution was heated to 80°C for 24 h.

TMS. These derivatives were synthesized using the method of Kilts and co-workers (39). In a reacti-vial, 10 μg of potassium acetate and 10 μg of steroid were pre-dried. After 50 μL of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) were added, the mixture was heated at 80°C for 5 h. After the solvent was evaporated under nitrogen, the residue was reconstituted in 50 μL hexane.

Acetylation. The acetylation is based on the method of Wortic and co-workers (40). To the sample was added 50 μL of pyridine and 130 μL of acetic anhydride. The reaction mixture was heated for 2 h at 60°C. After evaporation under nitrogen, the residue was dissolved in 50 μL of ethyl acetate.

Determination of Concentration Using Effective Carbon Number

Many compounds obtained for this study did not have accurately-known concentrations due to the presence of impurities or incomplete synthesis in this laboratory. To determine concentration, the GC-FID was used as a carbon counter. The environment that the carbon group is in will determine its contribution to the total carbon number of the molecule, commonly referred to as "effective carbon number" (ECN). For example, an aromatic carbon will contribute 1.0 to the ECN, but a carbonyl carbon will not contribute anything. Steinberg and co-workers (41) have

developed ECN for a variety of substituents. Scanlon and Willis (42) added several derivatives to the list. Table 4.3 summarizes the findings of both studies.

In preliminary work with the ECN concept, a standard curve was made with standards that had the same ECN as the analyte with unknown concentration. This is a tedious process, and many compounds of interest do not have pure standards available.

Table 4.3: Contributions to the Effective Carbon Number

Atom	Type	ECN Contribution
C	Aliphatic	1.0
C	Aromatic	1.0
C	Olefinic	0.95
C	Acetylenic	1.30
C	Carbonyl	0.0
C	Nitrile	0.3
O	Ether	-1.0
O	Primary alcohol	-0.6
O	Secondary alcohol	-0.75
O	Tertiary alcohol, ester	-0.25
Cl	2 or more on a single aliphatic carbon	-0.12 each
Cl	On olefinic carbon	0.05
N	Amine	as in alcohols
	H-C-O-TMS (alcohol)	3.69-3.78
	CO_2-TMS (acid)	3.0
	CH=N-O-TMS (silyl oxime)	3.3
	CH=N-O-CH_3 (methoxime)	0.92-1.04

The ECN concept has been very underutilized, but it has been used in the quantification of PAHs (43,44), steroids (45), TMS of alcohols, and MO-TMS of carbohydrates in complex mixtures without the use of an authentic standard for each compound. In general, internal standard(s) with accurately-known ECN were added to

the sample. To determine the concentration, the following calculations were performed. The relative weight response factor, F(R-wt), is defined by (42):

$$F(R\text{-}wt) = \frac{\text{MW of compound x ECD-ref.}}{\text{MW of reference x ECN-comp.}}.$$

The weight of the compound thus will be given by:

$$\text{weight of compound} = \frac{\text{area counts for ref. x weight of compound}}{\text{area counts for compound x F(R-wt)}}.$$

If the amount injected is known, the resulting concentration is easily calculated. This method has an accuracy of 2-3% (46).

The choice of reference compound is very important. Its ECN should be accurately known, independent of response tables. The reference compound also should be very pure and should be chromatographically resolved from the compound(s) of interest. Naphthalene, with an ECN of 10, has been used in one impressive study (46) and will be used for this work.

Table 4.4: Estimated Concentration Using the ECN Method

Compound*	Actual Concentration (ng/μL)	Estimated Concentration (ng/μL)	Percent Difference
1,4-A-3,11,17-trione	188	180	4.0
1,4-A-3,11,17-trione	104	100	3.9
4-A-3,6,17-trione	177	180	2.0

*A=Androstane parent molecule.

A 434 ng/μL solution of scintillation-grade naphthalene (99+% pure, Aldrich) was made in ethyl acetate. 100 μL of this stock solution was added to 400 μL of the sample solutions. All samples were analyzed in triplicate on the Varian GC-FID with

Table 4.5: Concentrations Estimated by ECN Method

Compound	Estimated Concentration (ng/μL)
PCC A^4-16,17-ol-3-one	39
C^5-3-one-4,4-Me	319 ± 4
C^5-3-one	45 ± 5
αA-2,4-bromo-3,17-one	303 ± 10
A^5-17-one	390 ± 2
C^5-3,4-one	61
C^5-3,7-one-4,4-Me	27 ± 1
αA-11-one	630 ± 10
αA-1,6,17-one	179 ± 20
αA-11,17-one	3300 ± 600
αA-3-F-7,11,17-one	550 ± 40
αA-17-F-3-one	370 ± 20
αA-2,17-one-9-F	111 ± 20
PCC $P^{1,4}$-11,17,21-triol-3,20-dione-6α-F	23 ± 0.1
PCC P^4-11-ol-9α-F-3,20-dione	21 ± 2
αC-4-one-5,6-epoxy	430 ± 10
αP-3,20-one-4β,5β-epoxy	390 ± 10
Isophorone oxide	4900 ± 100
PCC C-5,6-epoxy-3-ol	37 ± 3
Compound **3**	20 ± 0.4
Compound **4**	36 ± 1
Compound **5**	19 ± 0.6
Compound **6**	550 ± 20
Compound **9**	940 ±8
Compound **12**	68 ± 8
Compound **14**	51 ± 1
Compound **22**	32 ± 1

a DB-1 megabore column. The temperature program started with a 2-min hold at 50°C, then a ramp at 10°/min to 280°C and another 5-min hold.

The procedure was attempted on compounds whose concentrations were accurately known. From the results of this study (Table 4.4), the accuracy of this method was found to be about 2-4%. The list of compounds whose concentrations was determined by this technique is in Table 4.5.

ECNI Response Studies of Various Classes of Compounds

Preliminary ECD Study

A preliminary response study was performed on the Varian GC with electron capture detection. The results are presented in Table 4.6. 1,4-Androstadien-3,11,17-trione was clearly the compound with the highest response. The absence of either an 1-one or an 11-one decreased the response by two orders of magnitude. The 4-en-3,6-dione structure had a response one order of magnitude less than 1,4-androstadien-3,11,17-trione. The other compounds tested had poor responses (<50 arbitrary units).

Table 4.6: Relative ECD Responses of Steroids

Compound	Exp. Rel. Response	Lit. Rel. Response (2)
5α-Androstane	1	1
5α-Androstan-3-one	4.3	---
5α-Androstan-17-one	3.2	---
5α-Androstan-3,17-one	1.3	10
4-Androsten-3,11,17-trione	18	21
1,4-Androstadien-3,17-dione	123	450
2-Androsten-17-one	6.1	---
5α-Androstan-3,11,17-trione	26	---
4-Androstene-3,11,17-trione	340	24800
1,4-Androstadien-3,11,17-trione	15000	53500
4-Androsten-3,6,17-trione	2600	---
4-Pregnen-3,20-dione	36	2220
5α-Pregnan-3,11,20-trione	45	---

The results from this study were compared to those obtained by Lovelock and co-workers (2). The trends in response are the same, but the absolute values differ greatly, especially for medium-response compounds like 4-pregnen-3,20-dione. This may be due to differences in the electron-capture mechanism. Lovelock used a ^{3}H-foil while modern electron capture detectors are equipped with ^{63}Ni-foils. Also, the Lovelock study used 5% methane in argon as the carrier. This carrier gas is far superior in its ability to facilitate electron capture processes than the nitrogen carrier gas used in this study.

ECNI Responses of Ketosteroids

Ketosteroids with cross or linear π-π conjugation, as well as steroids not so highly conjugated, were analyzed to determine their ECNI response. The effect of halogen substituents in steroids with and without extensive conjugation was also studied. Epoxides can increase the ECNI response; therefore, the relative responses of epoxides within a steroid nucleus were determined. The results from these response studies are presented in Tables 4.7-4.9. All molar responses are corrected for internal standard and reported relative to that of 5α-androstan-3-one. The major ions are those monitored by SIM; they are listed in order of intensity.

Some functional groups do not contribute to the response; methyl groups and cyano groups are two examples. An ethynyl group on the same atom as a hydroxyl does not affect the response. For example, contraceptive steroids with the 17α-ethynyl, 17β-ol functionality are not amenable to ECNI analysis without the use of fluorinated derivatives.

An unfunctionalized steroid skeleton, e.g., 5α-androstane, has an extremely low electron-capture cross section. The presence of isolated π-electron functional groups (such as carbonyls, olefins, and epoxides) enhances electron capture by more than a hundred-fold, but the effective relative response is still low. Relative to 5α-androstan-3-one, these responses range from 0.5 to 1.2.

Table 4.7: ECNI Relative Responses of Steroids

Compound	Major Ions	Relative Response*
Androstanes		
αA	$[M-2H]^-$	0.0040 ± 0.0008
αA-3-one	$[M-H]^-$	1.0 ± 0.07
αA-17-one	$[M-H]^-$	1.2 ± 0.1
αA-11-one	$[M-H]^-$	0.95 ± 0.22
αA^{2}-17-one	$[M-H]^-$	1.1 ± 0.2
A^{5}–17–one	$[M-H]^-$	0.21 ± 0.03
A^{4}-3-one	$[M-H]^-$	1.2 ± 0.1
$A^{4,16}$–3–one	$[M-H]^-$	0.94 ± 0.09
$A^{3,5}$–17–one	$[M-H]^-$	0.67 ± 0.07
αA-3,17-one	$[M-H]^-$	1.4 ± 0.1
A^{1}-3,17-one	$[M-H]^-$	0.98 ± 0.11
A^{4}-3,17-one	$[M-H]^-$	1.2 ± 0.1
$A^{4,6}$-3,17-one	$[M-H]^-$	18 ± 2
$A^{1,4,6}$-3,17-one	M^-,$[M-CH_3]^-$	300 ± 50
A^{4}-3,16,17-one	M^-	27 ± 1
A^{4}-3,6,17-one	M^-	300 ± 15
nor-$\alpha A^{2,7}$-1,4-one-14α-Me	M^-	100 ± 6
$A^{1,4}$-3,17-one	$[M-H]^-$,$[M-CH_3]^-$	2.1 ± 0.1
αA-3,11,17-one	$[M-H]^-$	0.89 ± 0.11
A^{4}-3,11,17-one	$[M-2H]^-$,$[M-16]^-$	3.2 ± 0.3
A^{5}-3,11,17-one	$[M-H]^-$	0.24 ± 0.01
$A^{1,4}$-3,11,17-one	M^-	360 ± 40
$\alpha A^{9(11)}$-3,17-one	$[M-H]^-$	0.59 ± 0.1
$A^{1,4,9(11)}$-3,17-one	$[M-CH_3]^-$,M^-	150 ± 20
aA^{16}-17-CN	$[M-H]^-$	1.2 ± 0.1
αA^{4}-17α-ethynyl-17β-ol-3-one	$[M-H]^-$	0.37 ± 0.06
Pregnanes		
αP-3,20-one	$[M-H]^-$	1.2 ± 0.1
P^{4}-3,20-one	$[M-H]^-$	1.4 ± 0.3
P^{4}-3,11,20-one	$[M-2H]^-$,$[M-16]^-$	9.1 ± 0.5
P^{4}-3,6,20-one	M^-	280 ± 23
Cholestanes		
$C^{3,5}$	$[M-H]^-$,$[M+O_2-H]^-$	0.58 ± 0.19
$C^{4,6}$	$[M-H]^-$,$[M+O_2-H]^-$	0.47 ± 0.03
$C^{4,6}$-3-Cl	$[M-H]^-$	0.35 ± 0.03
αC-2-one	$[M-H]^-$	0.12 ± 0.01
αC-3-one	$[M-H]^-$	0.27 ± 0.02
C^{1}-3-one	$[M-H]^-$	0.05 ± 0.003
C^{5}-4-one	$[M-H]^-$	0.91 ± 0.11
C^{5}-3-one	$[M-H]^-$	0.17 ± 0.05
C^{5}-3-one-4,4-Me	$[M-H]^-$	0.22 ± 0.04
C^{5}-3,7-one-4,4-Me	$[M-H]^-$	7.2 ± 0.8
C^{4}-3-one-2,2-Me	$[M-H]^-$	3.5 ± 0.5
C^{4}-3-one	$[M-H]^-$	0.25 ± 0.02
C-3,6-one	$[M-4]^-$	1.6 ± 0.5
C^{4}-3,6-one	M^-	280 ± 24

*A-3-one has been assigned a relative response of 1.0; n = 3 to 5.

Table 4.8: ECNI Relative Responses of Epoxysteroids

Compound	Major Ions	Relative Response*
Androstanes		
αA-3-one	$[M-H]^-$	1.0 ± 0.06
αA-1α,2α-epoxy-3-one	$[M-H]^-$	25 ± 4
αA-9α,11α-epoxy-3-one	$[M-H]^-$	5.0 ± 0.4
A^4-3-one	$[M-H]^-$	1.2 ± 0.1
A^4-16α,17α-epoxy-3-one	$[M-H]^-$	1.8 ± 0.04
αA-3-one-17-ol acetate	$[M-H]^-$	1.8 ± 0.04
A-4β,5β-epoxy-3-one-17-ol acetate	M^-	26 ± 1
A^1-4β,5β-epoxy-3-one-17-ol acetate	M^-	200 ± 18
$A^{1,4}$-3,17-dione	$[M-H]^-$, $[M-CH_3]^-$	2.1 ± 0.1
αA-3,17-dione	$[M-H]^-$	1.0 ± 0.05
A^4-3,17-dione	$[M-H]^-$	1.2 ± 0.1
A-4α,5α-epoxy-3,17-dione	M^-	39 ± 1
Pregnanes		
αP-3,20-one	$[M-H]^-$	1.2 ± 0.1
P-4α,5α-epoxy-3,20-one	M^-	21 ± 0.9
αP-16α,17α-epoxy-3,20-dione	$[M-H]^-$	24 ± 0.2
P^4-16α,17α-epoxy-3,20-dione	$[M-H]^-$	29 ± 3
P^4-8β-19-epoxy-3,20-dione	$[M-H]^-$,M^-,$[M-O_2]^-$	33 ± 6
P^4-3,11,20-trione	M^-	9.1 ± 0.5
αP-16α,17α-epoxy-3,11,20-trione	$[M-H]^-$	63 ± 2
Cholestanes		
C-5α,6α-epoxy	$[M-H]^-$	0.13 ± 0.01
C-3-cyano-5α,6α-epoxy	$[148]^-$, $[M-H]^-$, M^-	6.8 ± 0.03
C-3-Cl-5α,6α-epoxy	$[M-Cl]^-$, M^-, $[M-H]^-$	6.4 ± 0.6
C-5α,6α-epoxy-4-one	$[M-H]^-$	6.1 ± 0.1
C-5β,6β-epoxy-4-one	$[M-H]^-$	5.2 ± 0.6
C-4β,5β-epoxy-3-one	M^-	9.2 ± 1.2
$C^{4,6}$-1α,2α-epoxy-3-one	$[M-H]^-$	58 ± 6

*A-3-one has been assigned a relative response of 1.0; n = 3 to 5.

Table 4.9: ECNI Responses of Halogenated Steroids

Compound	Major Ions	ECNI Relative Response*
αA-3-one	$[M-H]^-$	1.0 ± 0.1
αA–3–one–17α–F	$[M-H]^-$	0.41 ± 0.02
αA^{16}-3-one-17α-F	$[M-H]^-$	1.6 ± 0.08
αA-3-one-17α,17β-F	$[M-H]^-$	2.1 ± 0.1
αA-2,17-one	$[M-H]^-$	3.5 ± 0.3
αA-2,17-one-9α-F	$[M-H]^-$	0.75 ± 0.05
αA-11,17-one	$[M-H]^-$	3.8 ± 0.5
αA-11,17-one-3α-F	$[M-H]^-$,$[M-F]^-$	0.09 ± 0.01
αA-7,11,17-one-3α-F	$[M-HF-2H]^-$,$[M-H]^-$	4.6 ± 0.4
αA–3,17–one	$[M-H]^-$	1.0 ± 0.05
αA-2α-Br-3,17-one	$[M-Br]^-$, Br^-	0.35 ± 0.05
αA-2α,4α-Br-3,17-one	$[M-2Br]^-$, Br^-	0.80 ± 0.37
αA-2α-I-2,17-one	$[M-I]^-$, I^-	0.35 ± 0.05
A^4-3,11,17-one-9α-F	$[M-HF]^-$,M^-,$[M-HF-CH_3]^-$	2600 ± 300
A^4-17β-ol-3,11-one-9α-F-17α-Me	$[M-HF]^-$,M^-, $[M-HF-H_2O]^-$	3000 ± 150
$A^{1,4}$-3,11,17-one-9α-F-16α-Me	$[M-HF]^-$,M^-,$[M-HF-CH_3]^-$	4100 ± 420
$A^{1,4}$-3,11,17-one-9α,6α-F-16α-Me	$[M-HF]^-$,M^-,$[M-HF-CH_3]^-$	5400 ± 250
$A^{1,4}$-3,11,17-one-9α-F-6α-Me	$[M-HF]^-$,M^-,$[M-HF-CH_3]^-$	3900 ± 470
$A^{1,4}$-3,6,11,17-one-9α-F-16α-Me	$[M-HF]^-$,M^-,$[M-HF-CH_3]^-$	7400 ± 500

*A-3-one has been assigned a response of 1.0.

When two or three isolated π-functions are present in a steroid, little enhancement of ECNI response is observed. Even if two such functions are conjugated, as in the common 4-en-3-one moiety, the relative response remains low. For example, note the responses of 5α-androstan-3,11,17-trione (with a relative response of 0.89), 4-pregnen-3,20-dione (1.4), and 4-androsten-16α,17α-epoxy-3-one (1.8).

The introduction of a fourth unconjugated π-function appears to give a modest enhancement of response, as observed for 4-androsten-3,11,17-trione (3.2) and 4-pregnen-3,11,20-trione (9.1). Interestingly, the α-diketone, 4-androsten-3,16,17-trione, has an unusually high response (27) relative to similar compounds. This characteristic of α-diketones is expected from their low reduction potential (for 3,3,6,6-tetramethyl-1,2-cyclohexanedione, $E_{1/2}$ = -0.71 V vs. SCE (47)). This provides a useful reference for evaluating the results for α,β-epoxyketones discussed below.

From the data in Table 4.8, it is clear that simple α,β-epoxyketones have ECNI responses at least an order of magnitude greater than those of their corresponding α,β-unsaturated ketones. For example, 5α-androstan-1α,2α-epoxy-3-one has a relative response 25 times greater than that of 1(5α)-androsten-3,17-dione. When compared to other cyclic ethers, epoxides are exceptionally reactive with various nucleophilic reagents. Indeed, organic chemists have described this behavior as "carbonyl-like," just as cyclopropane is referred to as "olefin-like." Clearly, this is a helpful analogy for this work, because α,β-epoxyketones then become "α-diketone-like," and the enhanced response is expected.

The greatest ECNI responses result from polyunsaturated ketosteroids that have more extensive conjugation than is present in the previous cases. The ECNI responses of compounds with cross conjugation differ from those with extended (or linear) conjugation. For instance, the linearly-conjugated dienone, 4,6-androsten-3,17-dione,

has nine times the response of its cross-conjugated isomer, 1,4-androstadien-3,17-dione. Extending the 4-en-3-one conjugation by the introduction of a carbonyl group at C-6 results in a dramatic enhancement of response, as noted for 4-androsten-3,6,20-trione (relative response of 300), 4-pregnen-3,6,17-trione (280), and 4-cholesten-3,6-dione (280). For these linearly-conjugated steroids, the substitution at the C-17 position does not affect the overall ECNI response. The enedione moiety in the 4-en-3,6-dione compounds is a vinylog (a system extended by the placement of an allyl group between the functional groups, as shown in Figure 4.5) of an α-diketone, which has been previously noted for its enhanced electron-capture response.

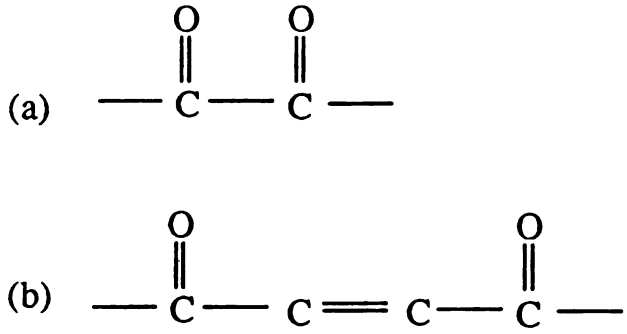


Figure 4.5: Structure (b) is a vinylog of structure (a).

An intriguing aspect of this study is the synergistic effect that cross-conjugated or homo conjugated π-functions have on the higher-response groupings such as α-ketoepoxides and linearly-conjugated enediones. Examples of this synergistic effect include the C-1 double bond in 1,4,6-androstatrien-3,17-dione (300) and 1-androsten-4β,5β-epoxy-3-one-17-ol acetate (200). The epoxide in 4,6-cholestadien-1α,2α-epoxy-3-one (58) is another example. This synergistic effect is particularly dramatic in cases of 1,4-dien-3-one cross-conjugated systems combined with a 9(11)-double bond or a C-11 carbonyl function, such as 1,4,9(11)-androstatrien-3,17-dione (150) and 1,4-androstadien-3,11,17-trione (360). The presence of a carbonyl group at C-11 enhances the response greater than the corresponding double bond.

Because similar ECNI responses were observed for the 4-en-3,6-dione and the 1,4-dien-3,11-dione systems in androstanes, we attempted to estimate the contribution of a polar substituent on the overall response by using the Taft equation (48). Analogous to the Hammett equation, the Taft equation applies linear free-energy relationships to aliphatic systems. Using a modified Taft treatment, the effects of a polar substituent, designated by σ^*, were calculated assuming that the inductive effects would be transmitted to C-3 by all possible bond paths in the steroid. Also, it was assumed that each bond would attenuate the effect of any substituent by a factor of 0.5 per carbon atom (48). Ethylene bonds were assumed to attenuate the effect by a factor of 0.508. Thus, the net inductive effect was calculated for all reasonable bond connections by the following equation:

$$\Sigma\sigma_t^* = \sigma^*(a^l + b^m + c^n + ...)$$

where a, b, and c are the attentuation factors for either single or double bonds and l, m, and n are the number of bonds in that pathway. By this treatment, the inductive effect for the 4-en-3,6-one system was 3.4 times greater than that for the 1,4-dien-3,11-dione system. As shown in Table 4.7, the relative responses of 4-androsten-3,11,17-trione (300) and 1,4-androstadien-3,11,17-trione (360) are similar. Therefore, the marked influence of an 11-carbonyl appears to be an example of a through-space nonclassical conjugation effect on the A-ring.

ECNI Fragmentation of Ketosteroids

The ECNI spectra of the 1,4-dien-3-one steroids include a peak for a $[M\text{-}CH_3]^-$ ion as well as a $M^{-\cdot}$ or $[M\text{-}H]^-$ ion with the exception of 1,4-androstadien-3,11,17-trione whose spectrum consists of only a peak for $M^{-\cdot}$. In all of these compounds, the 18-methyl group is ideally oriented for fragmentation. After capture of an electron and loss of the 18-methyl group as shown in Figure 4.6, a very stable phenolate ion is formed. There is one exception to this pattern. Loss of the 18-methyl group from

1,4-androstadien-3,11,17-trione should be less favorable because the electron-rich oxygen atom of the 11-carbonyl group crowds or has a nonbonding interaction with the C-1 hydrogen which, as part of the phenolate ring, will have a negative charge. This crowding is more severe in the $[M-15]^-$ phenolate ion than in the $M^{-\cdot}$ parent ion.

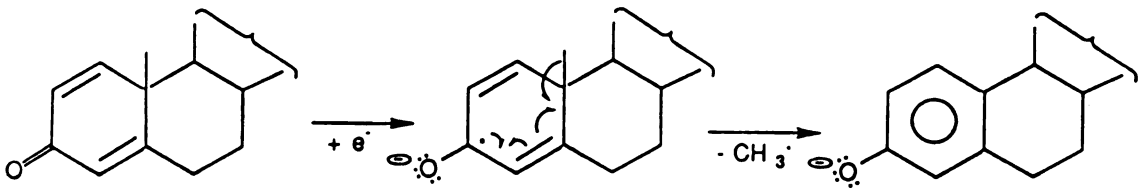


Figure 4.6: Proposed mechanism for the formation of a phenolate ion in 1,4-dien-3-one steroids.

The ECNI spectrum for 4-androsten-3,11,17-trione is interesting. The base peak represents $[M-2]^-$; a less intense peak represents $[M-16]^-$. The formation of these unusual ions may involve an interaction between the 11-carbonyl group and the A-ring. The 11-carbonyl oxygen is close to C-1 and may be able to extract a hydrogen atom. The resulting radical at C-1 may, in turn, induce fragmentations leading to formations of these ions (see Figure 4.7).

ECNI Responses of Epoxysteroids

For enhanced ECNI response, an epoxide group must be alpha to an electrophilic group. An isolated epoxide group, such as the one in 5α,6α-epoxycholestane, has a poor response. If epoxide and carbonyl groups are located at opposite ends of a molecule, as in 4-androsten-16α,17α-epoxy-3-one, no enhancement of ECNI response occurs. However, by positioning an electrophilic group, such as a chlorine, a cyano, or a carbonyl, in proximity to or in conjugation with an epoxide, an enhanced response is observed. Results for substituted cholestanes are reported in Table 4.8. The combination of an epoxide in the 9,11-position with a carbonyl function at C-3, as in androstan-9α,11α-epoxy-3-one, also showed enhanced response.

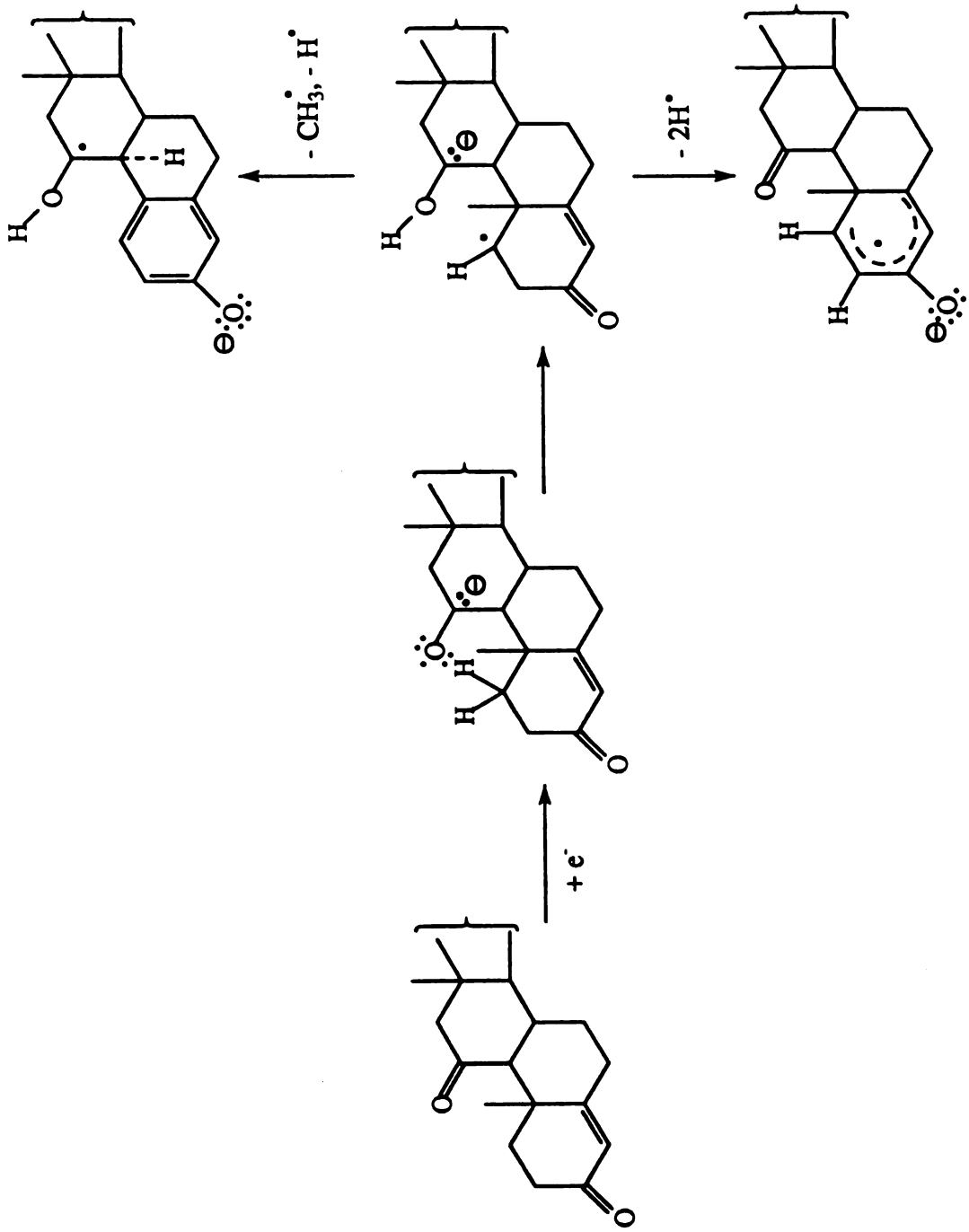


Figure 4.7: Proposed mechanism for the formation of $[M-2]^-$ and $[M-16]^-$ anions in the ECNI spectrum of 4-androsten-3,11,17-trione.

In general, α,β-epoxyketones have an enhanced response at least an order of magnitude greater than that of the equivalent enones. For example, 5α-pregnen-4α,5α-epoxy-3,20-dione has a response 15 times greater than that of 4-pregnen-3,20-dione. Other functional groups in the molecule have a little effect on the overall response. This is illustrated by the fact that 5α-pregnan-16α,17α-epoxy-3,20-dione and 4-pregnen-16α,17α-epoxy-3,20-dione have almost the same response.

ECNI Responses of Halogenated Steroids

If a halogen is added to an already low-response steroid (see Table 4.9), the relative response will decrease further. For example, the presence of a 17-fluorine atom in 5α-androstan-3-one decreases the response. Another electrophilic group near the fluorine will increase the response slightly, as in those of 5α-androstan-17α,17β-difluoro-3-one vs. 5α-androstan-17α-fluoro-3-one.

Varied responses have been noted for α-halo ketones. Both 5α-androstan-2α-bromo-3,17-dione and 5α-androstan-2α-iodo-3,17-dione have 0.35 times the response of the unhalogenated 5α-androstan-3,17-dione. The major peak in the ECNI spectrum in each case is $[M\text{-halogen}]^-$; the halogen anion is a minor contributor (< 5%). Even in the EI spectrum, $[M\text{-halogen}]^+$ is the highest mass peak. Two processes must be occuring in these compounds: the loss of a halogen followed by the capture of an electron. Because the fragmentation preceeds the electron capture, the efficiency is probably not as good as that of resonance or dissociative capture processes, and this could contribute to the lowered responses.

In contrast, fluorine atoms in some highly-conjugated systems induce impressive responses. A fluorine added alpha to an 11-carbonyl group in a cross-conjugated system (e.g., 1,4-androstadien-9α-fluoro-16α-methyl-3,11,17-trione) increases the response by a factor of 10. However, a second fluorine substituent located at C-6 does not significantly affect the relative response. In the case of a less conjugated system,

such as 4-androsten-9α-fluoro-3,11,17-trione, 9α-fluorine substitution increases the relative response by a factor of 200. It is likely that 1,4-androstadien-16α-methyl-3,11,17-trione has such powerful electron-capturing conjugated π-functions that the addition of a fluorine atom has a smaller relative contribution to the response than the addition of a 9α-fluorine to 4-androsten-3,11,17-trione. The compound with the best response of all those tested in this study is 1,4-androstadien-9α-fluoro-16α-methyl-3,6,11,17-tetraone. This compound has all the functions associated with a high response, including a cross-conjugated 1,4-dien-3,11-dione system and a 9α-fluorine as well as a linearly-conjugated 4-en-3,6-dione.

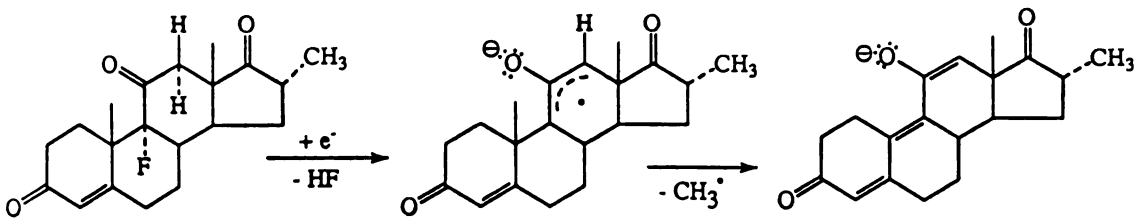


Figure 4.8: Fragmentation of 4-androsten-9α-fluoro-16α-methyl-3,11,17-trione.

An explanation for the different ECNI responses of α-haloketones lies in the orientation of the halogen. If the halogen is axial, as in the ketosteroids containing the 9α-fluorine, the carbon:halogen bond overlaps the π-electron orbital of the carbonyl group at C-11, providing a higher electron-capture response. In addition, the $[M\text{-}HF]^{-\cdot}$ and $[M\text{-}HF\text{-}CH_3]^-$ fragments are exceptionally stable anions. For the loss of HF, it is assumed that the hydrogen is from the C-12 position on the other side of the carbonyl group (see Figure 4.8). This type of HX loss has its basis in the well-characterized Favorskii reaction (49). In the Favorskii reaction, α-haloketones in the presence of base lose hydrogen from one side of the carbonyl and halogen from the other. A carbanion is formed which rearranges to an ester. After the loss of the 18-methyl group, the resulting anion has the very stable 4,8,10-trien-3-one structure (see Figure 4.8). In contrast, an equatorial halogen, such as that in 5α-androstan-2α-bromo-3,17-dione, does

not overlap well with the adjacent C-3 carbonyl group, and provides little or no enhancement of response.

ECNI Responses of Derivatized Steroids

Most steroids must be derivatized before GC-ECNI-MS analysis because they are not inherently electrophilic and/or are thermally labile and cannot survive the GC intact. The oxidation methodology has been compared to a variety of commonplace GC derivatives for the analysis of dexamethasone and 5α-androstan-3β,17β-diol.

Dexamethasone has a thermally-labile 17-substituent. TMS and MO-TMS derivatives have been often used in assay development of dexamethasone under ECNI conditions (28, 33). These two derivatives were compared to underivatized dexamethasone as well as the chemical oxidation product of dexamethasone, 1,4-androstadien-9α-fluoro-16α-methyl-3,11,17-trione. Because the underivatized dexamethasone does not survive the GC intact, thus decreasing sensitivity, it was analyzed by direct probe. The other derivatives were analyzed by GC-MS to insure that any electrophilic by-products would be separated from the analyte. Internal standard was added to all samples before analysis.

As shown in Table 4.10, the tetra-TMS and MO-TMS derivatives have about the same ECNI response as the underivatized dexamethasone. Therefore, the advantages gained using these derivatives are merely chromatographic; no additional electron-capture properties are added to the molecule. In contrast, the oxidized dexamethasone has a response 40 times greater than unoxidized dexamethasone. Not only are the chromatographic properties improved, but the electron-capture properties were also enhanced.

The chemically-oxidized dexamethasone was also compared to more conventional electron-capturing derivatives, such as TFA, HFB, and flophemesyl, in a separate study (see Table 4.11). All three of these derivatives have significant fragmentation. Only in the case of the flophemesyl derivative is the most abundant

peak indicative of the parent ion ([M-504]$^-$ corresponds to the loss of the 17-substitution). The response of the TFA derivative was disappointing, on the order of a low-response ketosteroid. The HFB and flophemesyl derivatives had a large enough response to be beneficial for assay development; however, chemical oxidation has a response an order of magnitude higher. Therefore, for compounds that can be oxidized to highly-electrophilic species, chemical oxidation is the best derivative.

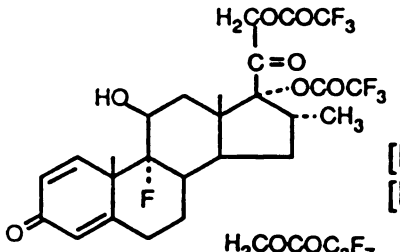
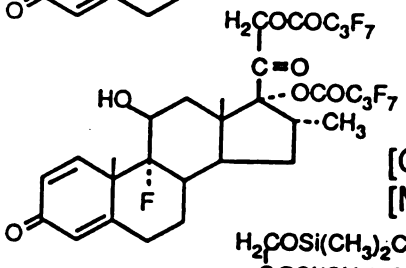
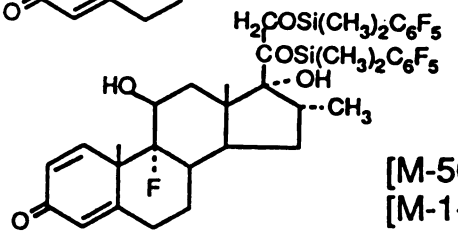
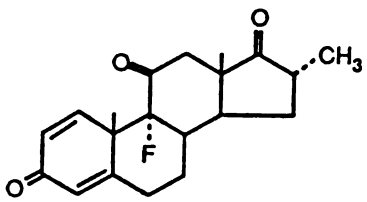
Table 4.10: Relative ECNI Responses of Dexamethasone Derivatives

Derivative	Relative Response*
None (DIP)	1.0 ± 0.2
Tetra-TMS	1.3 ± 0.1
MO-TMS	1.5 ± 0.06
Chemical oxidation	41 ± 5

*Underivatized dexamethasone has been assigned a relative response of 1.0.

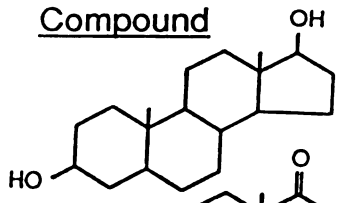
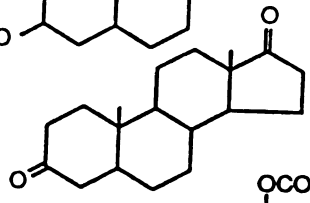
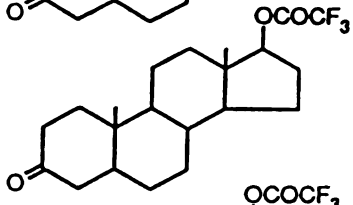
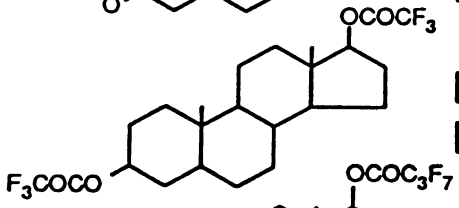
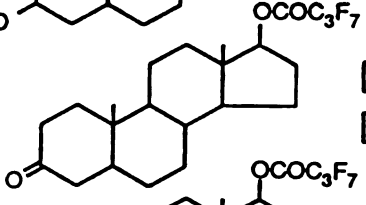
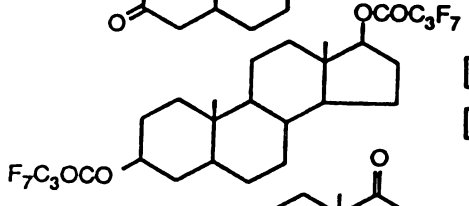
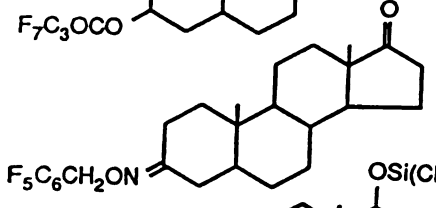
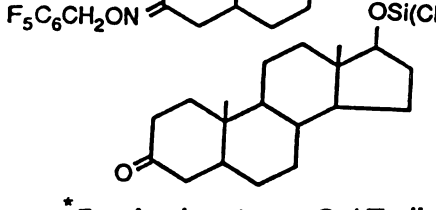
The chemical oxidation protocol does not work as well for compounds that are oxidized to low-response species. For example, 5α-androstan-3,17-diol oxidizes to 5α-androstan-3,17-dione, a fairly low-response compound. The oxidized product has a response 500 times that of 5α-androstan-3,17-diol (see Table 4.12), but only because the hydroxylated compound is lost on the injection liner or the column packing. The fluorinated derivatives all provide better response than chemical oxidation. Mono- and di-TFA derivatives have the TFA substituent as their most abundant peak; however, the response is fair. The ions at *m/z* 178 and *m/z* 194 in the HFB and TFA derivatives are most likely from wall reactions because of their non-Gaussian mass chromatograms. The mono-flophemesyl derivative has a better response, but is very difficult to synthesize. The Florox derivative has a relative response of 1700 which makes it a good candidate for assay development, but the pentafluorobenzyl moiety is the most

Table 4.11: ECNI Relative Responses of Halogenated Derivatives of Dexamethasone

Compound	Major Ions	Relative Response*
	$[M-101]^-$, $[M-2HF]^{-\cdot}$, $[M-80]^{-\cdot}$, $[M-1]^-$, $[OCOCF_3]^{-\cdot}$	0.94 ± 0.05
	$[OCOC_3F_7]^-$, $[M-COC_3F_7]^-$, $[M-HF]^{-\cdot}$	260 ± 10
	$[M-504]^{-\cdot}$, $[M-336]^{-\cdot}$ $[M-1-C_6F_5]^{-\cdot}$, $[M-C_6F_5]^-$	120 ± 80
	$[M-HF]^{-\cdot}$, $M^{-\cdot}$, $[M-HF-CH_3]^-$	4200 ± 30

*5α-Androstane-3,17-dione has been assigned a relative response of 1.0.

Table 4.12: ECNI Responses of Derivatives of 5α-Androstane-3,17-diol

Compound	Major Ions	Relative Response*
	$[M-H]^-$	0.0018 ± 0.0003
	$[M-H]^-$	1.0 ± 0.27
	$[OCOCF_3]^-$,$[2COCF_3]^-$, $[M-H]^-$	8.1 ± 2.1
	$[OCOCF_3]^-$,$[2COCF_3]^-$, $[M-OCCF_3]^-$,$[M-H]^-$	31 ± 8
	$[M-HF]^{-\cdot}$,$[M-2HF]^{-\cdot}$, $[COC_3F_7]^-$,$[OCC_3F_6]^-$	2100 ± 200
	$[M-HF]^{-\cdot}$,$[M-2HF]^{-\cdot}$, $[COC_3F_7]^-$,$[OCC_3F_6]^-$	5500 ± 1500
	$[OCHC_6F_6]^-$,$[OCH_2C_6F_6]^-$, $[178]^{-\cdot}$,$[M-HF]^{-\cdot}$	1700 ± 300
	$[124]^{-\cdot}$,$[C_6H_5]^-$, $M^{-\cdot}$,$[C_6F_4]^{-\cdot}$	65 ± 16

*5α-Androstane-3,17-dione has been assigned a value of 1.0.

abundant anion. All steroids treated with Florox would have this anion in their spectrum; this would make for a non-selective assay. By far the best derivatives were the mono- and di-HFB derivatives, which combine both high responses and abundant peaks indicative of the parent molecule (in this case, $[M-HF]^-$).

For dexamethasone and other highly conjugated steroids, the best ECNI derivative is achieved through chemical oxidation. The highly fluorinated derivatives as well as the non-electrophilic silylated derivatives all have poor responses, corresponding merely to enabling the molecule to survive the GC intact. However, for steroids without electrophilic groups, such as androstan-3β,17β-diol, the HFB derivative is the best choice for optimum sensitivity.

ECNI Spectra of Bicyclics

Bicyclic compounds with α,β-unsaturation were available courtesy of Dr. Reusch. The compounds were either bicyclononanes (fused five- and six-membered rings) or bicyclodecanes (two fused six-membered rings). The compounds will be referred to as compound **1**, compound **2**, etc. The structures of these compounds are presented in Figure 4.9. Many of these compounds were of interest because features not found in steroids were available.

The spectra of the bicyclic compounds are slightly more complicated than those of ketosteroids. More fragmentation is noted, and the losses are not all simple. The major ions found in the spectra of these compounds are listed in Table 4.13.

Eight of the twenty-six compounds display a molecular anion. Most of these compounds have more than one conjugated group, such as compounds **12** and **19**. Compound **15** also has a triple bond; $[M-2H]^-$ and $[M-H_20]^-$ ions are also noted due to fragmentation involving the hydroxyl group. The $[M-2]^-$ ion appears due to thermal degradation and is often present in the EI spectra of alcohols. Compound **5** and **6** are similar, except for a carbonyl or an epoxide group. Compound **5**, with the epoxy group, has M^- as the base peak in its spectrum. The base peak of compound **6** is an oxygen

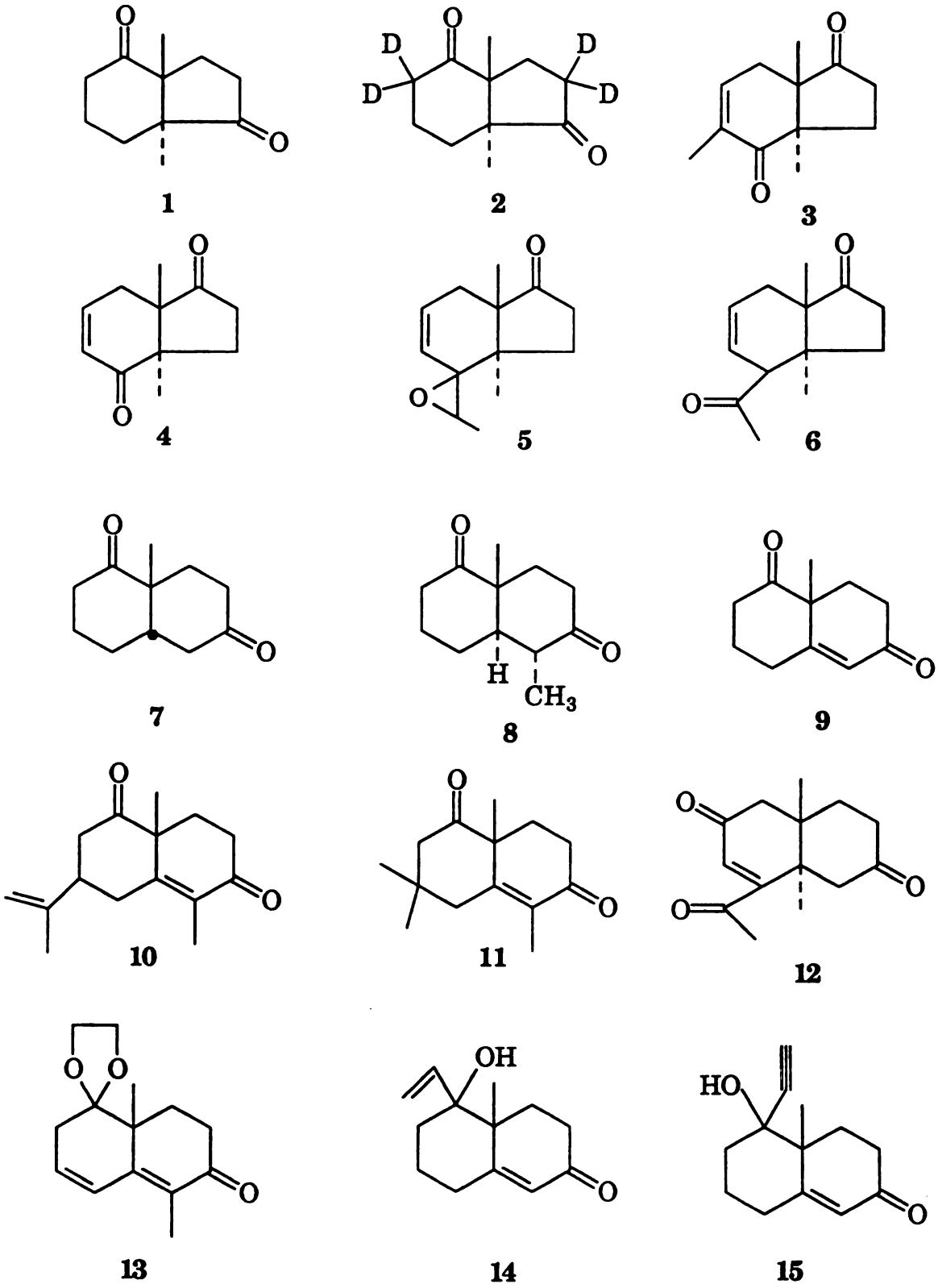
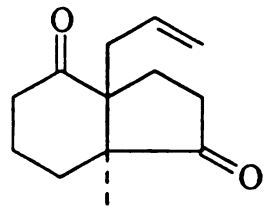


Figure 4.9: Structures of the bicyclic compounds analyzed in this dissertation.



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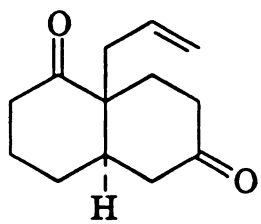

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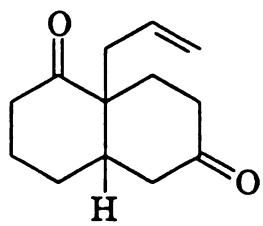

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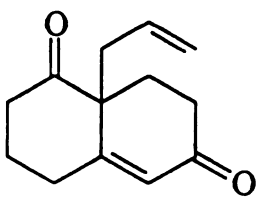

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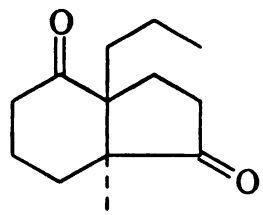

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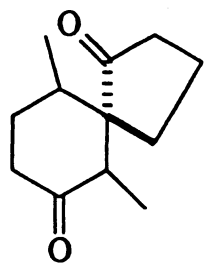

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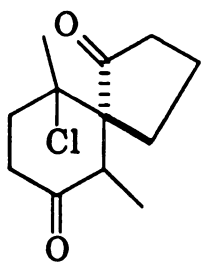

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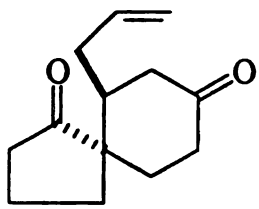

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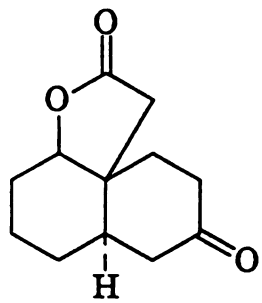

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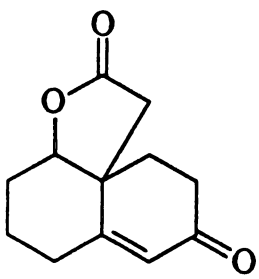

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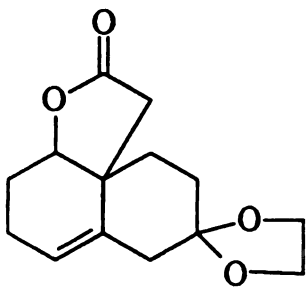

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Figure 4.9 (cont'd).

Table 4.13: ECNI Mass Spectra of Bicyclic Compounds

Compound*	[Anion]$^-$, (Relative Intensity)
1	$[M-H]^-$, (100); $[M-CO-H]^-$, (15)
2	$[M-D]^-$, (100); $[M-27]^-$, (26); $[M-D-H]^-$, (22)
3	$[M-H]^-$
4	$[M-H]^-$, (100); $[M-2H]^-$, (13); $[M+CH_3]^-$, (20)
5	M^-, (100)
6	$[M+O]^-$, (100); M^-, (8)
7	$[M-H]^-$, (100)
8	$[M-H]^-$, (100)
9	$[M-H]^-$, (100); $[M-2H]^-$, (49); $[M-3H]^-$, (22); $[M-CH_3]^-$, (29)
10	$[M-H]^-$, (100); $[M-C_6H_8O]^-$, (29)
11	$[M-H]^-$, (100); $[M-2H]^-$, (23); $[M-H-CH_3]^-$, (19); $[M-2H-CH_3]^-$, (48)
12	M^-, (100)
13	M^-, (100); $[M-H]^-$, (75); $[M-44]^-$, (31); $[M-45]^-$, 27
14	$[M-H]^-$, (100); $[M-2H]^-$, (84); $[M-H_2O]^-$, (32); $[M-H_2O-CH_2]^-$, (18)
15	M^-, (100); $[M-H]^-$, (50); $[M-2H]^-$, (55); $[M-H_2O]^-$, (6)
16	$[M-H]^-$, (100); [M-propenyl]$^-$, (17); $[M-56]^-$, (28)
17	$[M-C_3H_4]^-$, (51); $[M-C_3H_5]^-$, (100)
18	$[M-H]^-$, (100); $[M-C_3H_7]^-$, (65); $[M-78]^-$, (14)
19	$[M-C_3H_4]^-$, (100); $[M-C_3H_5]^-$, (99); M^-, (3)
20	$[M-H]^-$, (100)
21	$[M-H]^-$, (100)
22	M^-, (100); $[M+Cl]^-$, (19)
23	$[M-H]^-$, (100)
24	$[M-H]^-$, (100)
25	M^-, (100)
26	$[M-H]^-$, (100); $[M-46]^-$, 75

*The compounds are illustrated in Figure 4.9.

containing adduct, M^- being only a minor contributor. The addition of a halogen group forces resonance capture as opposed to $[M-H]^-$ formation (compare compounds **21** and **22**); however, a significant $[M+Cl]^-$ peak is also noted. This unusual ion may be due to a contaminant.

Compounds that have ECNI spectra consisting of the $[M-H]^-$ anion, tend to be simple structures with two carbonyl groups and little or no other substituents. Compounds **3**, **7**, **8**, **21**, **23**, **20**, and **24** all are in this category. Many compounds have spectra with $[M-H]^-$ as the base peak, but also produce significant $[M-2H]^-$ peaks as well as other peaks resulting from fragmentation or adduct-formation. These compounds, **4**, **9**, **11**, **14**, **16**, **18**, and **26**, tend to have an enone structure with another carbonyl group three bonds away. Some of these compounds have other interesting fragments. Compounds with methyl substitutions, like **9** and **11**, exhibit $[M-CH_3]^-$ or $[M-H-CH_3]^-$ ions in their spectra. The spectrum of compound **4** has an adduct ion at $[M+CH_3]^-$; however, the structurally similar compound **3** does not produce this adduct. In molecules with propene chains, the chain is sometimes lost, as in compounds **16** and **17**. Some ring fissure is also possible. Compound **10** has a loss of C_6H_8O from one ring (see Figure 4.10). This must be a very facile loss because it is not observed for any other bicyclic compounds.

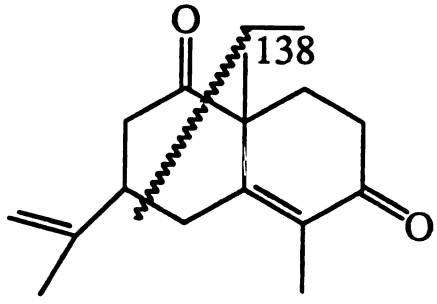


Figure 4.10: Fragmentation of C_6H_8O from Compound **10** results in an ion at *m/z* 138.

ECNI Responses of Bicylic Compounds

The relative ECNI responses of bicyclics are presented in Table 4.14. The ions monitored are also noted in this table. Structures of the bicyclic compounds are shown in Figure 4.9.

The size of the bicyclic rings have no effect on the response. Both bicyclononanes and bicyclodecanes with two carbonyl groups have the same response (cf. compounds **1** and **7**).

Several functional groups do not affect the response. As shown for other classes of compounds, the addition of a methyl group does not affect response. Deuterium substitution in compound **2** decreases the response slightly. One site of α,β-unsaturation, such as in compound **11**, does not increase the response for bicyclics. For the compounds which also have a lactone ring moiety (compounds **14-26**), the presence of a double bond in conjugation with a carbonyl group increases the response by a factor of ten. If the double bond is not conjugated with the carbonyl, the response is similar to the compound without a double bond.

Compound **6**, which has a carbonyl bonded to a ring incorporating a β,γ-double bond, has an enhanced response over a comparable α,β-unsaturated compound. Compound **12**, with an additional site of unsaturation, has a response 30 times greater than compound **6**.

A linearly conjugated enedione has a far better response than a linearly-conjugated dienone. Compound **12**, an enedione, has a response 60 times greater than compound **13** with its dienone.

The presence of an epoxide enhances the relative ECNI response. In compound **5**, the spiroepoxide is in conjugation with a double bond. The relative response is 2000, the largest response for any compound in this set.

A neighboring group is important for bicyclic compounds. Compounds **10** and **11** are similar, except that **10** has a side-chain double bond located three bonds away

Table 4.14: ECNI Relative Responses of Bicyclic Compounds

Compound	Ions Monitored	Relative Response*
1	$[M-H]^-$	1.0 ± 0.2
2	$[M-D]^-$	0.67 ± 0.06
3	$[M-H]^-$	1.6 ± 0.1
4	$[M-H]^-$	1.1 ± 0.06
5	M^-	2000 ± 400
6	M^-	41 ± 4
7	$[M-H]^-$	1.0 ± 0.2
8	$[M-H]^-$	0.46 ± 0.06
9	$[M-H]^-$, $[M-2H]^-$, $[M-CH_3]^-$	1.9 ± 0.2
10	$[M-H]^-$, $[M-2H]^-$	6.7 ± 0.9
11	$[M-16]^-$, $[M-H]^-$	1.3 ± 0.07
12	M^-, $[M-16]^-$	1500 ± 200
13	$[M-44]^-$, $[M-H]^-$, M^-	17 ± 0.9
14	$[M-2H]^-$, $[M-H]^-$, $[M-H_2O]^-$	7.9 ± 0.2
15	M^-, $[M-2H]^-$	22 ± 5
16	$[M-H]^-$	1.8 ± 0.2
17	$[M-H]^-$	0.49 ± 0.11
18	$[M-H]^-$	0.53 ± 0.06
19	$[M-41]^-$, M^-	330 ± 30
20	$[M-H]^-$, $[M-58]^-$	1.1 ± 0.1
21	$[M-H]^-$	0.84 ± 0.04
22	M^-	7.3 ± 0.8
23	$[M-H]^-$	0.14 ± 0.04
24	$[M-H]^-$	3.6 ± 0.6
25	M^-, $[M-2H]^-$	36 ± 5
26	$[M-H]^-$, $[M-44]^-$	4.4 ± 0.7

*Compound 1 has been assigned a relative response of 1.0.

from the β-carbon of an enone. Compound **10** has a response seven times greater than compound **11**.

A triple bond substituent one carbon removed from the ring has a response greater than a double bond. This response is larger than for the unsubstituted bicyclodecanedione. A comparable increase in response for a triple bond is not noted for steroids; for example 4-androsten-17α-ethynyl-17β-ol-3-one does not show an increase in response.

Compounds **16-20** have a propenyl or propyl substitution at C-6. If the only double bond is in the C_3 chain, as in compounds **16-18**, the response is similar and low. If two double bonds are present, one in the propyl chain and the other alpha to the carbonyl, the response increases by a factor of 300. This increase in response may be due to the proximity of the propyl double bond to the α,β-unsaturation in the ring.

Addition of a halogen and a methyl group to compound **21**, as in compound **22**, increases the relative response by a factor of eight. From other studies, it is known that the methyl group does not affect the response; therefore, all increases in response are probably due to the chlorine substitution alpha to the carbonyl group.

ECNI Responses of Cyclohexanones

Cyclohexanones, being single-ring compounds, are not able to internally distribute excess energy and thus are not as sensitive to ECNI as steroids. Cyclohexanone, with one carbonyl group, is one-tenth as sensitive as its steroid counterpart, 5α-androstan-3-one. The responses of cyclohexanones are presented in Table 4.15.

Linear free-energy relationships such as the Hammett equation have been used in ECNI-MS to compare substituents on aromatic compounds based on the log of the ratio of the major ions. One comparison involves the ratio of a substituent fragment to the molecular anion (50). Taft σ* values are used in aliphatic linear-free energy

Table 4.15: Relative ECNI Responses of Cyclohexanones

Compound	Major Ions	Relative Response*
Cyclohexan-1-one	$[M-H]^-$	1.0 ± 0.05
Cyclohexan-1-one-4-methyl	$[M-H]^-$	0.89 ± 0.06
2-Cyclohexen-1-one	$[M-H]^-$	0.91 ± 0.09
Cyclohexan-1,3-dione	$[M-H]^-$	1.7 ± 0.7
Cyclohexan-1,3,5-trione-2,2,4,4,6,6-methyl	M^-,$[M-CH_3]^-$	310 ± 46
Cyclohexan-2-one-1,1-dimethyl-3-benzylidine	M^-	70 ± 15

*Cyclohexan-1-one has been assigned a relative response of 1.0; n= 3 to 4.

relationships. However, if one calculates the Taft number for the substituted cyclohexanones, there is no correlation with the ECNI responses. For example, the $\Sigma\sigma_t$* value for cyclohexan-1,3-dione is 0.877, and its ECNI relative response is 1.7. For 2,2,4,4,6,6-hexamethyl-cyclohexan-1,3,5-trione, the Taft number increases to 1.75, while the relative response increases to 310. In the case of cyclohexan-2-one-1,1-dimethyl-3-benzylidine, the Taft number falls to 0.318, but the response increases to 70. Other factors must be involved in generating the ECNI response and not just the polar effects measured by the Taft equation.

ECNI Mass Spectra of Quinones

The ECNI-MS relative responses of quinones were of interest because quinones are biologically important, easily available in halogenated and non-halogenated forms, and their electron affinities, reduction potentials, and UV values known from the literature. Quinones are instrumental in electron-transfer reaction in biological systems (51) Ubiquinone (also known as coenzyme Q_{10}), found in mitochondria, is important because it is widely-distributed, lipid-soluble, and participates in oxidation-reduction reactions involving the release and uptake of protons. A number of quinones, including plastoquinone, are the electron carriers between photosystems I and II during photosynthesis. Vitamin K_1, a 1,4-naphthoquinone analog, helps maintain the

coagulative properties of blood. In this work, simple quinones, such as benzoquinones, naphthoquinones, and anthraquinones, were studied.

In the EI mass spectra of quinones, the molecular ion often is accompanied by an abundant ion at $[M+2H]^+$. This ion probably corresponds to the reduction of the quinone to the dihydroquinone, possibly in the ion source (52). The $[M+2H]^+$ ion is usually present for compounds with high redox potentials and is more pronounced in *ortho* quinones than *para* quinones. In FAB-MS, this ion is also observed, but it is dependent on the choice of sample matrix (53). Other prominent ions in EI include the losses of CO and 2CO.

In ECNI spectra (see Table 4.16), the most abundant peak is usually $M^{-\cdot}$. Very little fragmentation is noted and no losses of CO are present in any quinone. Prominent ions at $[M+H]^-$ and $[M+2H]^{-\cdot}$ are also present in these spectra. These ions probably correspond to the dihydroquinone formation noted in EI spectra. The peak at $[M+H]^-$ is most likely the result of proton abstraction from the dihydroquinone. Typically, if $[M+2]^+$ is enhanced in EI spectra, it will also be enhanced for ECNI spectra. For example, anthraquinone has a very small $[M+2]^+$ peak in EI, whearas phenanthraquinone has a $[M+2]^+$ ion peak the same size as the molecular ion. In ECNI-MS, the spectrum of anthraquinone has a $[M-H]^-$ ion at a relative abundance of 20%. The phenanthraquinone molecule, which exhibited a larger hydroquinone ion in its EI spectrum, also was found to have a larger $[M+H]^-$ ion (relative abundance of 33%) in ECNI.

Adduct ions are much more common in quinone spectra than in the spectra of ketosteroids. Oxygen adducts are present in half of the compounds tested. Most of these oxygen adducts also have a $[M+O+14]^-$ ion, probably from additional adduct formation with CH_2 from the reagent gas. 1,4-Benzoquinone has only the $[M+14]^-$ adduct peaks without any contribution from $[M+O]^-$. Interestingly, this adduct is the base peak.

Table 4.16: ECNI Spectra of Quinones

Compound	[Anion], (Relative Intensity)
1,4-Benzoquinone	$[M+14]^-$, (100); $[M+H]^-$, (38); M^-, (67)
Tetrachloro-1,4-benzoquinone	$[M-Cl]^-$, (100); $[M-2Cl]^-$, (29)
1,2-Naphthoquinone	M^-, (100); $[M+H]^-$, (99); $[M+O]^-$, (60); $[M+O+14]^-$, (24)
1,4-Naphthoquinone	M^-, (100); $[M+2H]^-$, (42); $[M+O]^-$, (22); $[M+O+14]^-$, (10)
2,3-Dichloro-1,4-naphthoquinone	M^-, (100); $[M-Cl]^-$, (17)
2-Methyl-1,4-naphthoquinone	M^-, (100); $[M+2H]^-$, (38); $[M+O]^-$, (22); $[M+O+14]^-$, (12)
9,10-Anthraquinone	M^-, (100); $[M+H]^-$, (20)
1-Chloro-9,10-anthraquinone	M^-, (100); $[M-Cl]^-$, (50)
β-Methyl-9,10-anthraquinone	M^-, (100); $[M+H]^-$, (20); $[M+O]^-$, (3)
9,10-Phenanthraquinone	M^-, (100); $[M+H]^-$, (33); $[M+O]^-$, (76); $[M+O+14]^-$, (37)
Benzophenone	M^-, (100); $[M+H]^-$, (32); $[M-2H]^-$, (25); $[M+O]^-$, (5)

Relative Responses of Quinones

The conjugated structure of quinones account for their enhanced ECNI responses. Several quinones studied have chlorine substituents which also contribute to their ECNI responses. Full scan ECNI, SIM ECNI, GC-ECD, EA, $E_{1/2}$, and UV data are compared in Table 4.17.

ECNI SIM. Quinones were analyzed by SIM in the same manner as the steroids; that is, the major ions were monitored. SIM is a poor method for the comparison of the responses of quinones because the spectra are so complicated that the monitoring of a few ions is not representative of the response of the whole molecule. After several

Table 4.17: Relative Responses of ECNI-MS and GC-ECD of Quinones

Compound	ECNI Response Full Scan	ECNI Response SIM (a)	ECD Response (b)	EA (eV) (c)	$E_{1/2}$ (V) (d)	UV λ_{max} (nm) (e)
1,4-Benzoquinone	0.027	2.3×10^{-5}	0.018	1.91	-0.51,-1.14	242
Tetrachloro-1,4-benzoquinone	1.1	0.019	0.41	2.78	+0.01,-0.71	287
1,2-Naphthoquinone	0.14	0.026	0.16	----	-0.56,-1.02	249
1,4-Naphthoquinone	1.0	1.0	1.0	1.81	-0.71,-1.25	245
2,3-Dichloro-1,4-naphthoquinone	4.5	0.43	1.3	2.21	----	---
2-Methyl-1,4-naphthoquinone	2.4	0.22	0.54	1.74	----	246
9,10-Anthraquinone	0.19	0.16	0.17	1.50	-0.94,-1.45	242, 252
1-Chloro-9,10-anthraquinone	0.18	0.11	0.48	1.71	----	---
β-Methyl-9,10-anthraquinone	0.20	0.42	0.17	----	----	252
9,10-Phenanthraquinone	0.16	0.064	0.16	----	-0.66,-1.22	256
Benzophenone	0.049	0.013	0.00031	----	----	---

Table 4.17 (cont'd)

Key to Table

(a) Selected ion monitoring of largest two or three peaks.

(b) ECD data collected on a Perkin-Elmer Sigma 3B gas chromatograph with a DB-1 megabore column.

(c) Electron affinity data from reference (54).

(d) Half-wave reduction potential from Peover, P. E. *J. Chem Soc.* **1962**, 4540.

(e) UV spectra for benzoquinones have three major transitions; for the fused quinones, there are four. The transition used fot comparison is the $\pi \rightarrow \pi^*$ (benzenoid) transition. The spectra were obtained in either ethanol or methanol. From Berger, St.; Rieker, A. In *The Chemistry of Quinoid Compounds Part 1*; Ed., S. Patia; Wiley: New York, 1974.

attempts at SIM, the method was abandoned, and full scan ECNI was used for the comparison study.

Full Scan Studies. Full scan ECNI studies resulted in much more accurate quantitation and much better correlation with electron affinities. Several trends are noted for these quinones. The unsubstituted parent quinones have relative responses in the order 1,4-benzoquinone < 1,2-naphthoquinone ~ 9,10-phenanthraquinone ~ 9,10-anthraquinone < 1,4-naphthoquinone. Usually, *ortho* quinones have higher redox potentials (and presumably higher ECNI responses) than *para* quinones (55), but this is opposite to the naphthoquinone data. The electron affinities of these parent molecules are anthraquinone < 1,4-naphthoquinone < 1,4-benzoquinone. The molecular orbital energies of the π^* LUMOs increase for quinones in the order benzoquinone < naphthoquinone < anthraquinone. The negative charge is presumed to be concentrated on the oxygen, rather than spread over the whole molecule; therefore, EAs for these three compounds are not very different (54). The ECNI responses do not follow this trend of electron affinities.

The number of chlorine atoms in a quinone correlates with the magnitude of ECNI response. The addition of one chlorine in the 1-position of anthraquinone does not affect the ECNI relative response at all. Two chlorines added to the 2- and 3-positions of 1,4-naphthoquinone increase the response by a factor of five. Tetrachloro-1,4-benzoquinone has a relative response almost 100 times greater than the parent quinone. The addition of more chlorines will increase the response, but the nature of the parent quinone determines which compound has a higher response. For example, the two-ring 2,3-dichloro-1,4-naphthoquinone has a higher response than the one-ring tetrachloro-1,4-benzoquinone. This does not agree with the trend expected from electron affinity data.

ECD. The trend in ECD responses mimics the trend in ECNI full scan responses with the exception of tetrachloro-1,4-benzoquinone and benzophenone.

Even the magnitude of the responses is similar. For quinones, ECD is a good indicator of ECNI relative response.

Electron Affinity. Electron affinity is a fair indicator of ECD and full scan ECNI response, with the exceptions of 1,4-benzoquinone and tetrachloro-1,4-benzoquinone. The discrepancies in response might be explained by the chromatographic properties of these two compounds. Both have much poorer peak shape on a DB-1 column than condensed quinones like naphthoquinone and anthraquinone. Tetrachloro-1,4-benzoquinone has especially bad tailing. The mass spectra for this compound are hard to replicate even under electron impact conditions. The stability of these two compounds in dilute solution may be suspect.

For compounds where both are available, the electron affinity and the reduction potential data show the same trend. Even though the reduction potential data was obtained in an aprotic solvent, the Chen and Wentworth (56) equation relating electron affinity to reduction potential does not work for these quinones.

UV. Benzoquinones generally have three absorption bands, attributed to $\pi \rightarrow \pi^*$, $\pi \rightarrow \pi^*$, and $n \rightarrow \pi^*$ transitions. The spectra of condensed quinones are more complex because both quinoid and benzenoid absorptions may be present. Four absorptions are possible, corresponding to benzenoid $\pi \rightarrow \pi^*$, quinoid $\pi \rightarrow \pi^*$, a second quinoid $\pi \rightarrow \pi^*$, and a $n \rightarrow \pi^*$. Not all of these are found in every quinone. The first $\pi \rightarrow \pi^*$ band was used for comparison in Table 4.17.

The UV data do not correlate with the EA data very well. Perhaps the electron occupies different orbitals in these two cases. The ECD and ECNI data do not correlate well to UV data either.

Relative Responses of Arene Epoxides

Although steroid epoxides have excellent responses, not all epoxides have such useful ECNI responses. The arene epoxides (see Table 4.18) are examples of low-

response epoxides. The lowest response is found for compound A, which has a response approximately one-half that of the steroid 5α-androstan-3-one.

A few conclusions can be drawn from this study. Methyl groups do not contribute to the relative response, as shown in the comparison of compounds A and B. The tetraphenyl-substituted compound C has a slightly greater response. Substitution of a carbonyl group for a phenyl group, as in compound D, slightly enhances the response.

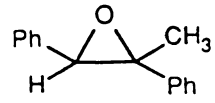
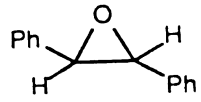
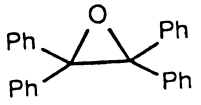
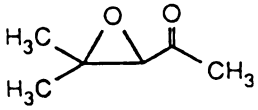
ECNI Responses of Mycotoxins and Trichothecines

Mycotoxins are fungal metabolites found as contaminants of various grains, feeds, milk, and peanut butter (57). These multi-ringed compounds often have extended conjugation and epoxide groups. All mycotoxins were acetylated to facilitate GC analysis. The responses are shown in Table 4.19.

The trichothecines DON and DOM-1 differ by a double bond or an epoxy group in the same position, yet they have similar responses. In steroids, the epoxide-containing molecule exhibited higher responses than comparable molecules containing a double bond. In DON, however, the epoxy group is not located near a carbonyl group, so the response is not enhanced. Structural features other than the epoxide must be responsible for enhanced response. For example, zearalenone, which does not contain an epoxide, but has extended conjugation in the form of an aromatic ring, has a better relative response.

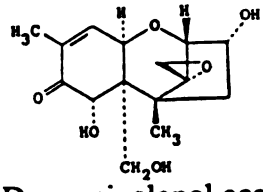
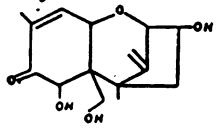
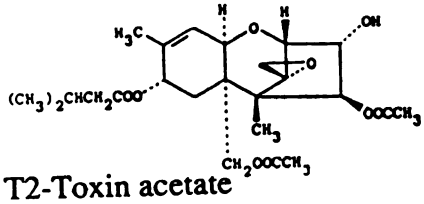
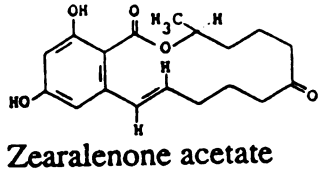
In conclusion, these mycotoxins have enhanced ECNI responses due to extended conjugation, not because of contributions from epoxides. Even with the extended conjugation, the response is an order of magnitude less than that of a highly conjugated steroid like 1,4-androstadien-3,11,17-trione.

Table 4.18: ECNI Relative Responses of Arene Epoxides

Compound		Major Ions	Relative Response*
	A	$[M-CHO]^-$,$[M-CH_3]^-$, $[M-H]^-$	1.0 ± 0.04
	B	$[M-H]^-$,$[M-Ph]^-$	1.3 ± 0.1
	C	$[M-Ph]^-$,M^-	5.0 ± 0.3
	D	$[101]^-$,M^-, $[M+O-CHO]^-$	8.1 ± 1.0

*Compound A has been assigned a relative response of 1.0; n = 3-5.

Table 4.19: ECNI Relative Responses of Mycotoxins and Trichothecines

Compound	Major Ions	Relative Response*
 Deoxynivalenol acetate	$[193]^-$,$[M-H]^-$	9.6 ± 0.8
 DOM-1 acetate	$[M-OAc-H]^-$,$[M-2OAc-2H]^-$, $[M-3OAc-3H]^-$,$[M-H]^-$	12 ± 0.6
 T2-Toxin acetate	$[119]^-$,$[182]^-$,$[226]^-$, $[M-OAc-H]^-$,M^-	16 ± 1
 Zearalenone acetate	$[M-Ac-H]^-$,$[M-Ac]^-$,$[M-H]^-$	37 ± 6

*A-3-one has been assigned a relative response of 1.0; n = 3-4.

Other ECNI Studies

[M-H]⁻ Formation: An Indication of Low Response?

As shown in Table 4.7, the ECNI mass spectra of steroids are very simple, usually consisting of either the molecular anion or simple losses of groups such as H, CH_3, or HF. For ketosteroids, the presence of a $[M-H]^-$ ion usually indicates low relative response (value less than 10). From studies with deuterium-labelled steroids, it was determined that the hydrogen is lost from the α-carbon to the carbonyl.

Proton abstraction is thought to be due to an ion-molecule reaction with OH^-, O_2, or O^- present in the source. In one case, the ECNI spectrum of the methyl ester of abscisic acid (ABA) shows a $[M-H]^-$ ion and an ion at *m/z* 141 only if oxygen is present in the source (22). On the JEOL 505 mass spectrometer, the ECNI mass spectrum of ABA always had a contribution from $[M-H]^-$ as well as the peak at *m/z* 141. This suggests that oxygen is always present in the JEOL 505 source. At times it was possible to achieve conditions on the TSQ 70 when these ions were not present in the spectrum of ABA. When the $[M-H]^-$ ion was not present in the ABA spectrum, the spectra of both 1-androsten-3,17-dione and 2-androsten-17-dione still exhibited only the $[M-H]^-$ ion. Two possible conclusions can be drawn. Perhaps proton abstraction by oxygen in the source is not the mechanism responsible for the $[M-H]^-$ ion formation in steroids. Alternatively a trace of oxygen in the source may be sufficient for proton abstraction from low-response steroids but not for abstraction from ABA.

To determine the position of proton abstraction, several steroids were labelled with deuterium alpha to the carbonyl group. About 1-3 mg of steroid was dissolved in 1 mL CH_3OD (KOR Isotopes, Cambridge, MA). A small chunk of sodium was added, and the reaction proceeded for 1, 6, or 24 h. One hour seemed sufficient for the labelling procedure. The reaction was stopped with the addition of 1 mL of methylene chloride. One mL of water was added and vortexed. The methylene chloride layer

was extracted and dried with Na_2SO_4. A small amount of this layer was dried under nitrogen and reconstituted in acetonitrile for ECNI-MS analysis.

ECNI-MS analysis of deuterated 5α-androstan-17-one proved that the major peak had shifted to $[M-2]^-$. Therefore, the hydrogen lost is alpha to a carbonyl in a steroid without double bonds. If more than one carbonyl is present in a molecule, it is not known from which group the hydrogen is abstracted. Proton abstraction alpha to a carbonyl was also demonstrated for low-response bicyclic compounds through the analysis of the deuterated bicyclic compound **2**.

A possible mechanism for the formation of more stable anions by the loss of a hydrogen is shown in Figure 4.11. After loss of a hydrogen, resonance structures can be drawn, showing stabilization of the ion. Perhaps this type of stabilization is necessary to elicit any type of response from these compounds.

The general rule that $[M-H]^-$ formation indicates a low-response ketosteroid is not true for the epoxysteroids listed in Table 4.8. As shown in Tables 4.20 and 4.21, there is no correlation between ECNI response and molecular anion formation. For example, pregnan-4α,5α-epoxy-3,20-dione and pregnan-16α,17α-epoxy-3,20-dione have similar responses, but the spectrum of the former consists of only $M^{-\cdot}$, while the latter produces $[M-H]^-$. For epoxysteroids, the position of the epoxide within the steroid nucleus is the only consideration for $[M-H]^-$ formation, not the electron-capture cross section. Following electron capture, the resulting radical anion may react by opening the epoxide ring with relief of ring strain, as shown in Figure 4.12. At this point, two rearrangements are possible. If a hydrogen is available on the β-carbon, it will be abstracted preferentially, resulting in an $[M-H]^-$ anion. However, if a hydrogen is not available on the β-carbon, as in the 4,5-epoxides, rearrangment involving cleavage of the carbon-carbon bond between the β-carbon and R^2 may occur (Figure 4.12). Because R^2 is part of a ring structure, it will remain attached, resulting in retention of the $M^{-\cdot}$ anion.

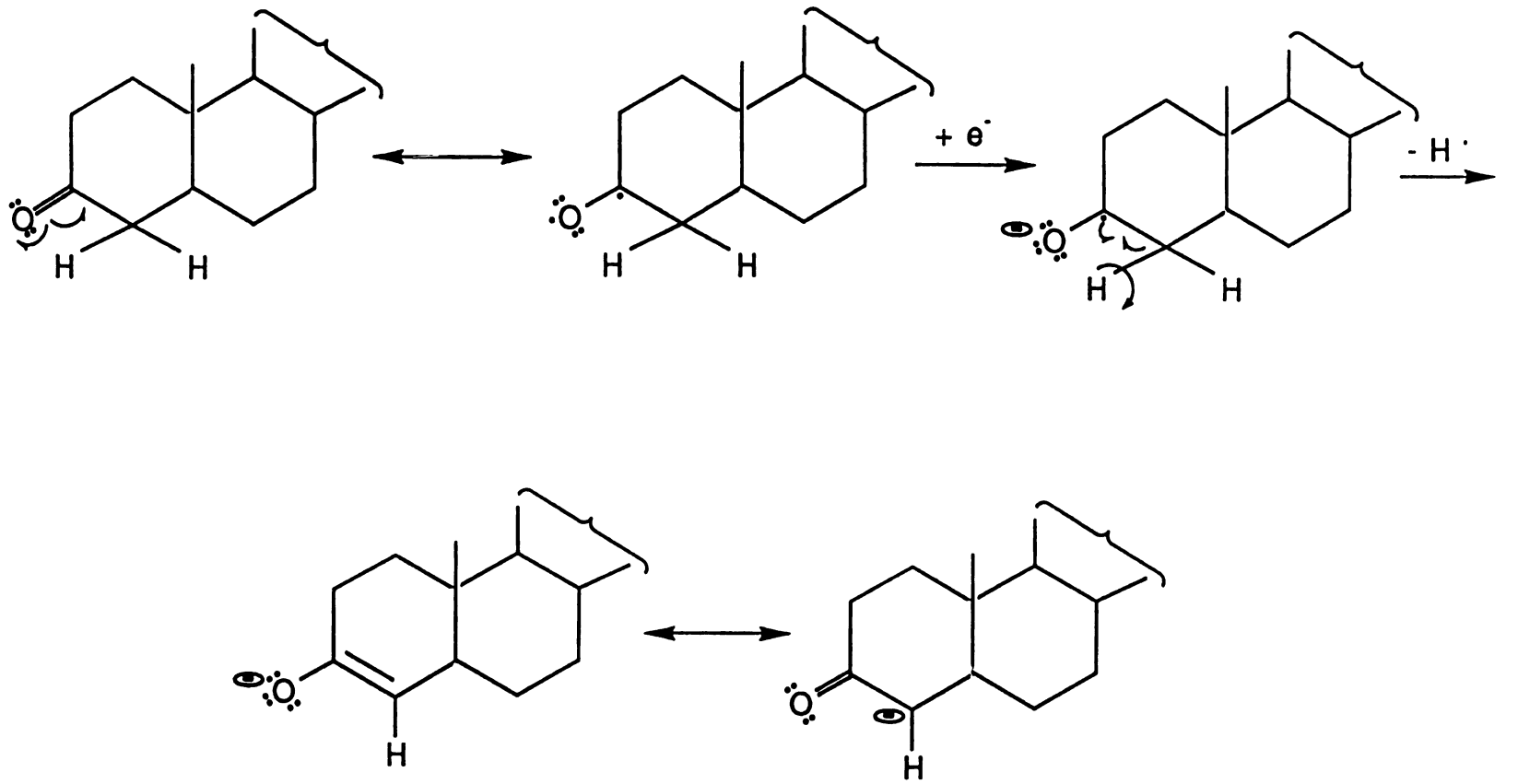


Figure 4.11: Proposed mechanism for the formation of a more stable anion by the loss of a hydrogen alpha to a carbonyl carbon in saturated ketosteroids.

Table 4.20: Steroid Epoxides with Molecular Anions

Compound	Relative Response*
A-4α,5α-epoxy-3,17-dione	39
A-4β,5β-epoxy-3-one-17-ol acetate	26
A^{1}-4β,5β-epoxy-3-one-17-ol acetate	200
P-4α,5α-epoxy-3,20-dione	21
C-4β,5β-epoxy-3-one	9.2

*A-3-one has been assigned a relative response of 1.0.

Table 4.21: Steroid Epoxides with $[M-H]^-$ Ions

Compound	Relative Response*
A-1α,2α-epoxy-3-one	25
A-9α,11α-epoxy-3-one	5
A^{4}-16α,17α-epoxy-3,17-dione	1.8
αP-16α,17α-epoxy-3,20-dione	47
P^{4}-16α,17α-epoxy-3,20-dione	57
αP-16α,17α-epoxy-3,11,20-trione	63
C-5α,6α-epoxy-4-one	61
$C^{4,6}$-1α,2α-epoxy-3-one	58

*A-3-one has been assigned a relative response of 1.0.

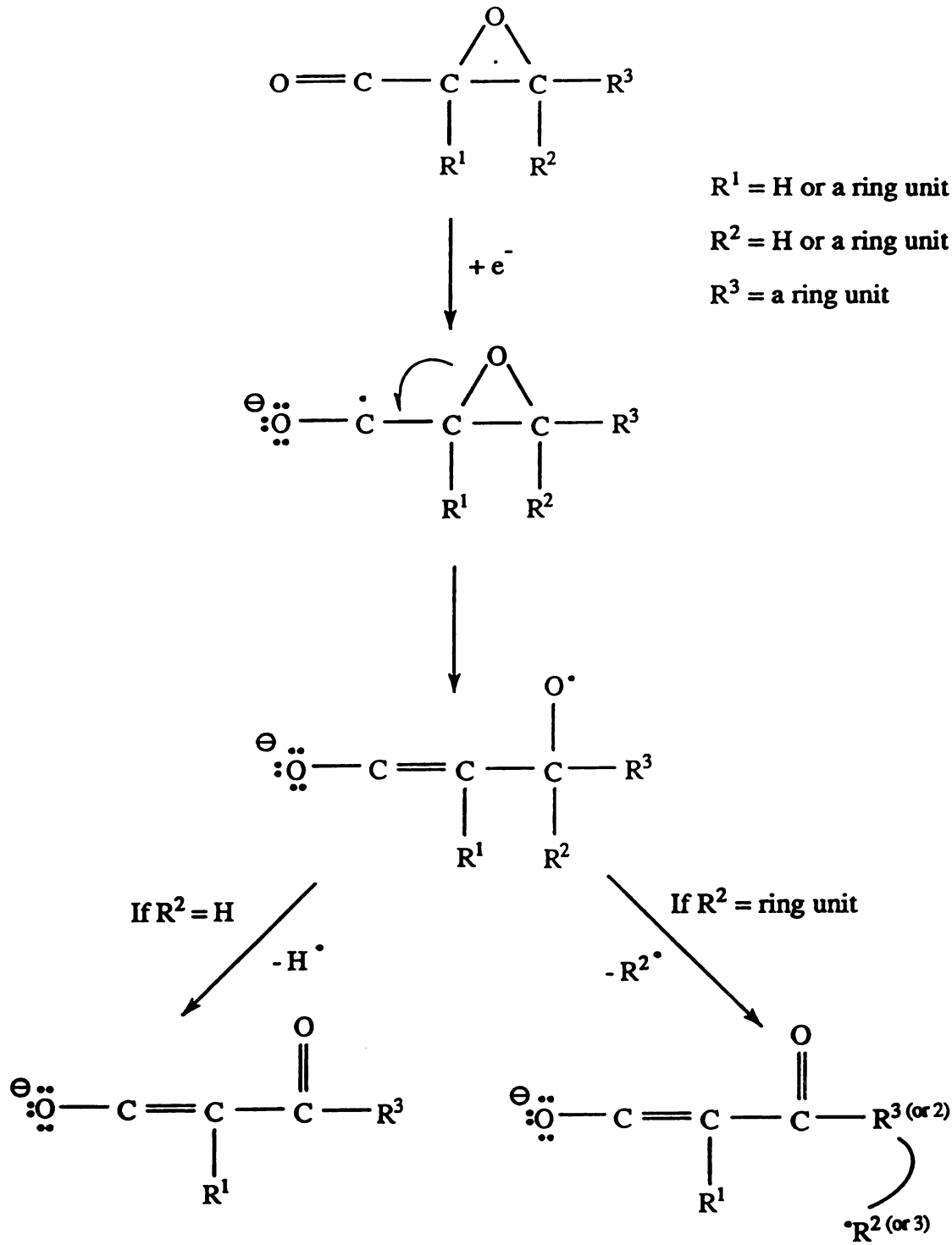


Figure 4.12: Proposed mechanisms for the capture of an electron in an α-ketoepoxide. After the opening of the epoxide ring to relieve strain, two rearrangements are possible. If a hydrogen is available on the β-carbon to the epoxide, a $[M-H]^-$ anion will be formed. Otherwise, the M^- anion will be formed after the breakage of the ring.

Size Comparison Study

Three categories of compounds were analyzed for the size comparison study: steroids, bicyclics, and cyclohexanones. A comparison of the three types of compounds is presented in Table 4.22. Each of these compounds had only one functional group or, in the case of bicyclics, two isolated functional groups. The relative responses are indicative of the size of the molecule. Steroids have a greater relative response because they can distribute the excess energy imparted by the capture of an electron more effectively.

Table 4.22: Size Comparison Study

Compound	**ECNI Relative Response***
Cyclohexanone	1.0
Compound **8**	2.0
5α-Androstan-3-one	10

*Cyclohexanone has been assigned a relative response of 1.0.

Comparison of Reduction Potentials to ECNI Responses

Ketosteroids

Chen and Wentworth (56) correlated half-wave reduction potentials ($E_{1/2}$) in aprotic solvents to experimental gas-phase electron affinities (EA) using the expression $EA = E_{1/2} + 2.49 \pm 0.2$ eV. Therefore, the trend in $E_{1/2}$ data should mimic the trend in electron affinity data.

It is expected that compounds with higher electron affinities (more positive reduction potentials) should have higher ECNI responses. This is generally true for ketosteroids (see Table 2.10). Compounds with $E_{1/2}$ of less than -2.0 V have low responses; the higher-response compounds all show reduction potentials of more than -2.0. No exact correlation between ECNI response and reduction potential is possible.

This is not an unusual occurrence; other researchers have noted that EA values and ECNI responses do not correlate well for a series of polyaromatic hydrocarbons (58).

Bicyclic Compounds

The electrochemical reduction potentials are tabulated in Table 2.11. A half-wave potential of -2.35 V is the value where the solvent reduces. Many bicyclics tested had reduction potentials lower than this value.

Most of the reduction potentials and relative ECNI responses track each other (see graphical representation in Figure 4.13). For example, compounds **11** and **15** have the same $E_{1/2}$ and the same ECNI response. Compound **25** had a lower reduction potential and a lower response than these two.

Many dissimilarities exist between reduction potential and ECNI response. Compounds **20** and **26** have the same reduction potential, but thier ECNI responses differ by one order of magnitude. As was observed for ketosteroids, the reduction potential of bicyclics is not a very good indication of ECNI response.

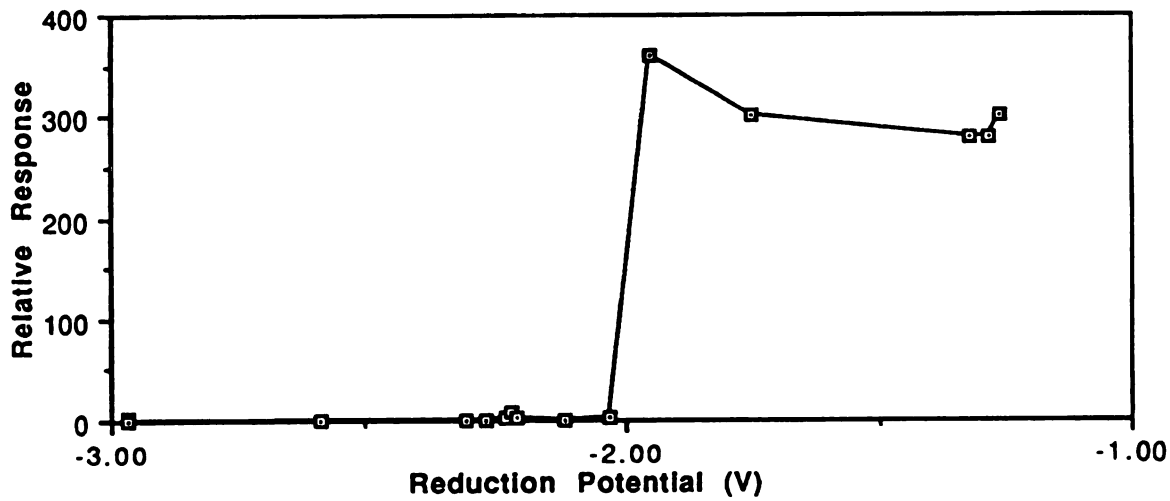


Figure 4.13: Half-wave reduction potentials of ketosteroids as a function of ECNI relative response. All responses are relative to androstan-3-one.

Comparison of UV Spectra to ECNI Response

Field (59) has suggested that electron affinities (and therefore ECNI responses) are affected by the same structural features that affect the wavelength of light absorption. In light absorption, an electron is promoted from an occupied to an unoccupied orbital, as in $n \rightarrow \pi^*$ and $\pi^* \rightarrow \pi^*$ transitions. In electron capture, the electron enters the LUMO, which should be the same orbital that an electron would be promoted to in absorption. Of course, the absolute energies in these two cases are not the same, but the same trends in structural features should be noted. The UV spectra of many steroids in methanol and ethanol have been recorded (see Table 4.23). The maximum wavelength of spectra recorded in either ethanol or methanol can be directly compared (60).

With the exception of the epoxides, all compounds that have a low response (relative response factor less than 3) all have maximum wavelengths of absorption below 243 nm. Epoxides are exceptions to the rules in $[M-H]^-$ formation and electrochemical trends.

As was observed for electrochemical reduction potentials, there are no trends between maximum wavelength and ECNI response. In some cases, there is a correlation, but it does not hold true for several notable exceptions. The steroids with the largest λ_{max}, 4,6-androstadien-3,17-dione, has an ECNI relative response of only 18. The steroid with the largest relative response, 1,4-androstadien-3,11,17-trione, has a λ_{max} of only 238 nm.

The trends in λ_{max} do not correlate to the trends in electrochemical reduction potential (an indication of electron affinity values). In conclusion, UV spectra can be used only as a very rough guess of the ECNI relative response.

Table 4.23: UV Spectra of Steroids

Name	λ_{max} (nm)	ε (L-cm^{-1}mole^{-1})	Ref.*	ECNI Response**
Androstanes				
5α-A-3-one	< 220		a	1.0
5α-A-3,17-one	< 220		a	1.4
5α-A-2α-Br-3,17-dione	244	11800	b	0.35
5α-A-2α-I-3,17-dione	242	14800	b	0.35
5α-A-2α,4α-Br-3,17-one	< 220		a	0.80
5α-A-3,11,17-one	< 220		a	0.89
$A^{3,5}$-17-one	241	18000	b	0.67
$A^{4,16}$-3-one	240	17170	b	0.94
A^{1}-3,17-one	230	10700	a	0.98
A^{4}-3,17-dione	239	16600	a	1.2
A^{4}-3,11,17-trione	238	15400	a	3.2
$A^{1,4}$-3,17-dione	243	15400	a	2.1
$A^{4,6}$-3,17-dione	283	26100	a	18
$A^{1,4}$-3,11,17-trione	238	14500	c	360
A^{4}-3,6,17-trione	253	10800	b	300
Pregnanes				
P^{4}-3,20-dione	241	16400	a	1.4
P^{4}-3,6,20-trione	252	10900	a	280
P^{4}-16β,17-ex-3,20-dione	240	17100	a	57
P-16α,17-ex-3,20-dione	< 220		a	24
Cholestanes				
$C^{4,6}$	235	24000	b	0.47
5α-C-3-one	< 220		a	0.27
5β-C-4β,5-ex-3-one	< 220		a	9.2
C^{4}-3-one	241	16700	a	0.91
C^{5}-4,4-Me-3-one	< 220		a	1.7
C^{4}-3,6-dione	250	7870	c	280

*References are the following:

(a) Data from reference 61; methanol as solvent.

(b) Data from reference 62; ethanol as solvent.

(c) From this work; methanol as solvent; see Chapter 2 for experimental details.

**Androstan-3-one has been assigned a relative response of 1.0

Reproducibility

Even with careful control of the instrumental parameters that directly affect the ECNI ionization process, the reproducibility of spectra is a major problem. The variability in ECNI spectra has been minimized by Stemmler and Hites (63) on an HP5985 quadrupole GC-MS by careful tuning of the drawout and focus lenses. In the Stemmler and Hites study, after tuning with perfluorotributylamine to set the mass axis and peak widths, the spectrum of DFTPP was measured at a source temperature of 250°C and a pressure of 0.55 torr (measured by a capacitance manometer) to meet the following criteria: *m/z* 442 (>60%), *m/z* 365 (>10%), *m/z* 275 (<10%), and *m/z* 167 (<20%). The drawout and ion focus lenses were adjusted to meet these criteria. Over a period of one year, the relative standard deviations for the ion abundances were less then 10%, with the exception of the weak ion at *m/z* 275.

The effect of tuning parameters on the reproducibility of ECNI spectra obtained on the JEOL 505 were studied. The source temperature was held at 200 °C and the source pressure (measured at the source housing) was 2 x 10^{-5} torr. About 0.4 ng of DFTPP (Ultra Scientific, Hope, RI) was injected, and ions at *m/z* 442, 365, 275, and 167 were monitored using magnetic field switching SIM. The areas under the peaks were used to calculate relative intensities. Replicate injections at the same set of conditions had a cv of less than 1.8%.

"Perfect" conditions were determined by the optimum tuning of perfluorokerosene (PFK), the usual tuning compound. The voltages for various lenses were varied one at a time. The spectrum of PFK does not change markedly with the change in lens potential, but the spectrum of a test compound like DFTPP does. Two settings different from the "perfect" setting were used for each parameter. Voltage measurements corresponding to these settings are not available. In all cases, the base peak was at *m/z* 442 ($M^{-\cdot}$); all data were reported relative to this base peak.

The intensity of the *m/z* 275 ion (corresponding to $[M-C_6F_5]^-$) was usually small and undependable. Variations from "perfect" conditions uielded ion intensities between 20 and 100%. This ion also caused troubles in the Stemmler and Hites study; in fact, it was not part of the claim for good reproducibility over the span of a year. The 275 ion will not be studied further.

The relative abundance of the ions at *m/z* 365 ($[M-C_6H_5]^-$) and *m/z* 167 ($[C_6F_5]^-$) varied depending on the parameter. Deviations from "perfect" were calculated; any relative standard deviation greater than 10% was considered to be significant.

Focus. On the JEOL 505 source there are two focus lenses, right and left. Each lens was varied separately. For all positions, there was a significant (20-30%) variation from "perfect" for *m/z* 365 and a large (60-90%) variation for *m/z* 167. As in the case of the Stemmler study, the adjustment of the focus lens is significant for the determination of ion abundances.

Electron Energy. The electron energy is typically set at 200 eV; for this experiment, it was varied between 50 and 100 eV. Minor differences were noted at 50 eV for *m/z* 167. The Stemmler study also noted small differences with the variation of electron energy.

Emission Current. The literature study revealed that the emission current had an effect on certain compounds. On the JEOL 505, the ionization current has two settings. The emission current is a function of the ionization current setting and the position of the filament (a misaligned filament will cause the emission current to be higher than normal). The lower setting of ionization current resulted in a great loss of sensitivity. For these two reasons, variation of ion abundance due to emission current was not studied.

Repeller. The change in repeller potential had a minor effect on ion abundances. This was also the case for the literature study.

Deflector. Variations in the deflector potential had a minor effect on the *m/z* 167 ion abundance. Other ions were not affected.

Accelerating Voltage. The voltage on the accelerating lens can be adjusted. The differences were important only for the *m/z* 167 ion. At very high adjustments of the accelerating voltage, the ions may shift almost an entire mass unit; therefore, any measurements at these voltages would obviously result in skewed abundances.

Octapole. The octapole determines the peak shape more than any other lens potential. Changes to the octapole lowered ion abundances in almost every case. This could be due to the distortion of the peak shape so that the correct mass value was obliterated.

DFTPP was monitored at a source pressure of 2×10^{-5} and a temperature of 200 °C for a three-month period. The peak at *m/z* 442 was always the most abundant. The average relative abundance of the ion at *m/z* 365 was 51% with a cv of 15%. The peak at *m/z* 167 usually did not meet the tuning criteria. Adjusting the focus to meet tuning criteria often decreased the signal to levels that were not sensitive enough for ECNI analyses. The relative abundance of *m/z* 167 under conditions favorable to ECNI usually was between 20 and 30%. This high value could also be attributed to the reagent gas pressure used; 0.55 torr was used in the literature study, but there is no method of measuring the CI volume pressure directly on the JEOL 505. The average abundance for the ion at *m/z* 167 was 25% with an rsd of 40%.

Reagent Gas Study

The choice of reagent gas is very important for enhanced responses under ECNI conditions. The actual responses are dependent on the type of compound (64-66). In general, heavier mass gases have the best sensitivities.

A test mixture consisting of the following steroids was used in this study:

androstan-3-one at 189 ng/μL

1-androsten-3,17-dione at 214 ng/μL

1,4-androstadien-3,11,17-trione at 2.48 ng/μL

4-androsten-3,6,17-trione at 7.90 ng/μL

oxidized dexamethasone at 0.62 ng/μL (determined by ECN).

Each sample was analyzed in replicate at two different gas pressures, 2×10^{-5} and 1×10^{-5} torr, measured at the gauge on the source housing. The only gases that showed significant differences between the two pressure levels were methane and carbon dioxide, both of which were enhanced at higher source pressures. Helium was used only at a source pressure of 1×10^{-5} torr due to problems with regulation. Surges in pressure are normal for helium; therefore, helium is more likely to overpressurize the instrument when utilizing higher source pressures. All gases, with the exception of helium, were used from the cylinder without purification. Helium was passed through a heated oxygen scrubber before use.

Different steroids respond differently to various reagent gases. In general, 1-androsten-3,17-dione and androstane-3-one, both of which have only one peak in their spectra, corresponding to $[M-H]^-$, behave in a similar fashion to various reagent gases. Both 1,4-androstadien-3,11,17-trione and 4-androsten-3,6,17-trione, both of which have $M^{-\cdot}$ as the only peak in the ECNI spectrum, also track each other. Oxidized dexamethasone, which dissociates plus produces a small molecular anion, behaves as a steroid that produces $M^{-\cdot}$ exclusively. Because the behavior changes with reagent gas, it is difficult to choose an internal standard. An ideal internal standard must not alter its response under a variety of conditions. This is not possible when dealing with different reagent gases. In fact, if one wants to prove the superiority of a reagent gas over another for a particular compound, one only needs to choose the correct internal standard. For example, the relative responses for 4-androsten-3,6,17-trione using

different reagent gases differs widely depending on the internal standard chosen. Remember, the same data is used for all comparisons presented. If androstan-3-one is chosen as the internal standard, the relative response of 4-androsten-3,6,17-trione is: air < ammonia < helium < methane < carbon dioxide < isobutane < nitrogen. If the internal standard is changed to 1,4-androstadien-3,11,17-trione, the relative responses shift to: nitrogen < helium < ammonia < methane < carbon dioxide ~ isobutane < air. In this case, all gases except nitrogen had approximately the same response, with air generating the lowest response. In the previous case, air produced the best response. Caution must be exercised in interpreting any data in the literature that compares relative responses of reagent gases using an internal standard (for example, data in reference 64). Other literature studies have compared the responses under different reagent gases without the use of an internal standard. There is no method of compensating for variations in response from such that factors change between analyses, such as cleanliness of the source.

Table 4.24: Reagent Gas Study*

Compound	Air	NH_3	CO_2	He	Isobutane	N_2	Methane
A-3-one	0.18	2.8	0.25	0.12	1.2	0.20	1.1
A^1-3,17-one	0.18	2.0	0.31	0.078	0.86	0.26	1.0
$A^{1,4}$-3,11,17-one	0.69	68	21	0.80	230	170	170
A^4-3,6,17-one	0.40	20	9.6	0.24	90	36	70
Ox. dexamethasone	4.7	420	140	9.1	1400	770	1000

*A^1-3,17-one analyzed using methane ECNI was assigned a relative response of 1.0.

Without the use of internal standard, the relative responses for seven reagent gases are shown in Table 4.24. The trend for low-response compounds is helium < air ~ nitrogen ~ carbon dioxide < methane ~ isobutane < ammonia. For high-response steroids, the trend is helium ~ air < carbon dioxide < ammonia < nitrogen < methane < isobutane.

Several differences between the high- and low-response steroids are noted. Ammonia is a much better reagent gas for low-response steroids than is methane. Even though ammonia does have a better K_f (electron thermalization rate, see Table 1.2), the increase in response for this certain set of compounds must be due to the ease of proton abstraction. Nitrogen is better for high-response steroids than for low. Isobutane is slightly better than methane as a reagent gas, probably because it has a higher mass. Isobutane may be worth pursuing in assay development. Helium is a poor choice for both low- and high-response compounds. For helium, both K_f and collisional stabilization are poor, resulting in loss of electron capture ability.

Carbon dioxide has been mentioned in the literature as comparable or superior to methane as a reagent gas (65). This was not found to be true for the analysis of steroids, even though relatively pure (99.99%) carbon dioxide was used.

Air, with its oxygen content, should enhance the formation of $[M-H]^-$ ions in compounds which form this ion primarily. This was not observed for either androstan-3-one or 1-androsten-3,17-dione. The presence of O_2 in the source must not be the cause of the $[M-H]^-$ formation, even though the possibility of abstraction by OH^- was not ruled out by this experiment.

Fragmentation patterns also varied using different reagent gases. In this experiment, oxidized dexamethasone was the only compound studied that produces observable fragments. As shown in Table 4.25, fragmentation patterns are dependent on the nature of the reagent gas (<1% means that the peak was not detected). Under all reagent gas conditions, the peak at *m/z* 310 ($[M-HF]^-$) was the most abundant. The peak at *m/z* 295 ($[M-HF-CH_3]^-$) varies greatly with reagent gas. This peak is enhanced when using air or isobutane. The molecular anion at *m/z* 330 is typically small at the source temperature used (ca. 200°C); however, some enhancement was noted when using ammonia as the reagent gas.

Table 4.25: ECNI Spectra of 1,4-Androstadien-9α-fluoro-16α-methyl-3,11,17-trione (Oxidized Dexamethasone) Using Different Reagent Gases

m/z	Air	NH_3	CO_2	He	Isobutane	N_2	Methane
295	34	11	11	7.2	30	16	16
310	100	100	100	100	100	100	100
330	<1*	2.4	0.81	<1	0.64	0.51	0.47

* <1 means that the peak was not detected.

Comparison of Ionization Techniques

ECNI is a selective technique for electrophilic compounds. The response under ECNI conditions was compared to the other commonly used GC-MS techniques of EI and methane CI as well as to GC-ECD for both electrophilic and non-electrophilic compounds.

Samples were made in concentrations amenable to the technique in question. The internal standard was 1-androsten-3,17-dione, added to each sample at a concentration of 165 ng/μL regardless of the concentrations of the other compounds. All compounds were analyzed by full scan in each ionization technique to choose the most abundant peaks for further SIM analysis. SIM was chosen over full scan for the comparison analysis because any quantitation of these compounds would normally be done using this technique.

Under full scan EI conditions (see Table 4.26), all compounds tested had almost identical molar responses. All steroids should be ionized and detected by EI in a similar fashion. Striking differences were noted when the SIM of M^+ ions of each compound was performed. The differences in response may be due to variations in mass spectra. The EI spectra of 1,4-androstadiene-3,11,17-trione and 3,5-cholestadiene exhibit M^+ as the base peak. The spectra of 1-androsten-3,17-dione and 4-pregnen-3,6,17-trione produce M^+ in 90% relative abundance. The spectrum of androstane-3-one has a 60% relative abundance of M^+. The differences in abundances of the molecular ion do not

account for the differences in the EI full scan and EI SIM responses. These differences could result from the relative amount of fragmentation in the entire mass spectrum.

Table 4.26: Comparison of Various Ionization Techniques

	Ionization Method				
Compound	**EI Full Scan**	**EI SIM**	**CI SIM**	**ECNI SIM**	**GC-ECD**
A-3-one	1.3 ± 0.2	1.6 ± 0.3	1.1 ± 0.2	2.9 ± 1.3	0.3
A^1-3,17-one*	1.0 ± 0.09	1.0 ± 0.09	1.0 ± 0.1	1.0 ± 0.4	1.0
$A^{1,4}$-3,11,17-one	0.88 ± 0.12	0.73 ± 0.08	0.56 ± 0.18	370 ± 70	240
P^4-3,6,20-one	0.75 ± 0.07	0.36 ± 0.04	0.39 ± 0.06	320 ± 50	130
$C^{3,5}$	1.1 ± 0.05	1.6 ± 0.08	0.68 ± 0.07	0.43 ± 0.05	0.22

*This compound was the internal standard.

The relative response of the MH^+ ion monitored under CI conditions had a similar response to the M^+ ion monitored in EI, with the exception of 3,5-cholestadiene. CI is based on the collision of the analyte with a gas molecule and then transfer of a proton. There is no dependence of this mechanism on structure; therefore, no differences were noted in the CI response. The exception is 3,5-cholestadiene whose CI response is lower than its EI response. Under CI conditions, $[MH-2H]^+$ ions are also prominent in the spectrum of 3,5-cholestadiene. This fragmentation may account for its lowered CI response.

Unlike CI responses, ECNI responses are dependent on the structure of the compound. ECNI responses for 1,4-androstadien-3,11,17-trione and 4-pregnen-3,6,20-trione were higher than their corresponding EI or CI responses by three orders of magnitude. Androstan-3-one, a low-response compound with $[M-H]^-$ as its prominent ECNI peak, did not show any enhancement of response. 3,5-Cholestadiene has two ECNI peaks, one at $[M-H]^-$, the other at $[M+O_2]^-$. Even so, the electron capture processes are so inefficient that the ECNI response was lower than that of EI or CI.

GC-ECD responses are enhanced similarly to those found for ECNI, but the actual numbers differ. The poorest responses for androstan-3-one and 3,5-cholestadiene were obtained using GC-ECD.

Of the three mass spectral ionization techniques tested, ECNI is superior for electrophilic compounds. For compounds that have poor ECNI responses, similar (or at times better) relative responses could be obtained by either EI or CI.

Conclusions

ECNI-MS is a selective technique for certain steroids. "Low-response" steroids, that is, those with no functional groups, isolated functional groups, or only one site of unsaturation, have responses on the order of those obtainable by EI or CI. For enhanced ECNI response within a steroid nucleus, the following structural features are important: a cross-conjugated system with a neighboring group, such as in 1,4-dien-3,11-dione; a linearly-conjugated system, such as 4-en-3,6-dione; a fluorine atom alpha to a carbonyl group in a highly-conjugated steroid; or an epoxide alpha to a carbonyl group. Usually, the presence of a $[M-H]^-$ peak indicates a low-response compound, except for epoxysteroids. In epoxysteroids, if a hydrogen on a β-carbon is present, an $[M-H]^-$ ion will be preferentially formed.

The reduction potentials in acetonitrile can be assumed to be indicative of the trends of electron affinity. In a series of ketosteroids, a half-wave reduction potential more positive than -2.0 V indicates a high-response compound, except in the cases of epoxysteroids. UV maximum wavelengths have been postulated as correlating to ECNI responses; however, UV data can only be taken as a very rough estimate of ECNI response.

Table 4.27: Comparison of ECNI Relative Response to Literature Values

Compound	ECNI Response Experimental*	ECNI Response Literature**
A^4-3,17-one	1.2	1
A^4-3,6,17-one	300	350
$A^{1,4}$-3,17-one	2.1	1
A^4-3,11,17-one	3.2	6
$A^{1,4}$-3,11,17-one	360	350
$A^{1,4}$-3,11,17-one-9α-F-16α-Me	4100	700
$A^{1,4}$-3,11,17-one-9α,6α-F-16α-Me	5400	700
$A^{1,4}$-3,11,17-one-9α-F-6α-Me	3900	700
A^4-3,11,17-one-9α-F	2600	525
P^4-3,20-one	1.4	1
P^4-3,11,20-one	9.1	4

*A-3-one has been assigned a relative response of 1.0
**A^4-3,17-dione has been assigned a relative response of 1.0; from reference 1.

Several of the compounds used for the ECNI response study were the chemical oxidation products of steroidal drugs. The responses of the chemically oxidized derivatives were compared to other electron-capturing derivatives. For compounds that have extensive conjugation, chemical oxidation is the best derivative; for steroids that oxidize to low-response moieties, the HFB derivative is far superior.

Steroids, being large molecules, capture electrons more efficiently than one- or two-ring compounds. In general, the relative ECNI response of steorids is ten times greater than that of cyclohexanones and five times greater than that of bicyclic compounds.

Results from this ECNI study were compared to literature values in Table 4.27. The correlation is very good, except for steroids with fluorine substitutions. The literature study was done on a different mass spectrometer (quadrupole HP5985) under different conditions. This comparison shows that these responses hold under different instrumental conditions.

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Chapter 5: Methodology for the Determination of Steroids in Biological Fluids

The determination of steroids by chemical oxidation followed by analysis of the oxidized extract by GC-ECNI-MS was pioneered in this laboratory (1-3). Dexamethasone in plasma and 6β-hydroxycortisol in urine have been successfully determined. Due to this success, other researchers have asked our assistance in the analysis of steroids in biological samples.

Dr. Stephen Bromley of the Department of Zoology at Michigan State University had been studying the regeneration of limbs of the newt, *Notophthalmus viridensens*. In previous work using radiolabelled compounds (4), he found that cortisol and corticosterone accumulate in the regenerating portions of the limb; the maximum accumulation occurred at seven days. Aldosterone, testosterone, glucose, cholesterol, and leucine do not accumulate in newt limbs, but estradiol is found. To verify that corticosteroids such as cortisol, corticosterone, and 18-hydroxycorticosterone are found in higher concentrations in regenerating portions of newt tissue, a GC-MS method was desired. Because the corticosterones in this study oxidize to poorly electrophilic products, other derivatization methods were investigated.

Dr. Clinton Kilts of the Department of Psychiatry at Duke University has collaborated with this laboratory in the past (3) and has decided to continue the collaboration. His interests include the pharmacokinetics of dexamethasone in the dexamethasone suppression test (DST), a method for distinguishing Cushing's syndrome and a probable indicator of endogenous depression. The RIA method currently used for the determination of dexamethasone may suffer from problems with cross-reactivity with 6β-hydroxydexamethasone, the most common metabolite of dexamethasone, especially at low dexamethasone concentration.

Dr. Pamela Fraker of the Department of Biochemistry at Michigan State University has been studying the effects of endogenous and synthetic corticosterones on the immune system of mice. The fluorescent-labelling technique currently used in her laboratory to quantify steroids did not work with prednisolone, but the chemical oxidation methodology was sensitive and selective enough for this determination.

Along with applying the chemical oxidation methodology to new problems, attempts were made to improve the methodology. Another oxidant, tetra-*n*-propylammonium perruthenate, was compared to pyridinium chlorochromate.

Alternative Oxidation Methods

Pyridinium chlorochromate (PCC) has been the oxidation reagent of choice for the chemical oxidation procedure. Advantages of PCC include fairly efficient yields and easy clean up. Problems with the reagent are long reaction times and toxicity (PCC is a suspected carcinogen). Recently, tetra-*n*-propylammonium perruthenate (TPAP) was proposed as a mild oxidant for alcohols (5,6). This reagent converted primary and secondary alcohols into their corresponding aldehydes and ketones, generally with short reaction times and very high yields. For example, 5α-androstan-17β-ol-3-one was converted into 5α-androstan-3,17-dione in 99% yield in 1.5 h. Other functional groups such as silyl ethers, indoles, pyrroles, acetals, sulfones, esters, epoxides, double bonds, lactones, and halides did not react with TPAP. The TPAP reaction occurs at room temperature with reaction times between ten minutes to one hour. The reagent works well for small quantities (on the order of one milligram).

The TPAP oxidation method was compared to the PCC oxidation method for 5α-androstan-17β-ol-3-one, 5α-androstan-3β,17β-diol, and prednisolone. The first steroid was chosen because it was efficiently converted to the diketone by TPAP (5). Prednisolone was chosen to determine if the TPAP could effectively oxidize a 17-substituted steroid.

For the TPAP oxidation, about 0.1 eq steroid (0.05 mmol) and 1.5 eq *N*-methylmorpholine *N*-oxide (Aldrich, Milwaukee, WI) were dissolved in 2 mL/mmol dry HPLC-grade methylene chloride (Mallinckrodt, Paris, KY). The *N*-methylmorpholine *N*-oxide was the co-oxidant. To improve the rate and efficiency of the reaction, 500 mg/mmol of powdered 4 Å molecular sieves (Aldrich) were added. Griffith and Ley (5) have noted that in dichloromethane some oxidations did not go to completion; the addition of 10% acetonitrile overcame this problem. Addition of acetonitrile was not necessary in this case. The methylene chloride mixture was stirred at room temperature for 10 min, and then 5 mole percent TPAP was added. After stirring at room temperature for 1.5 h (6.5 h for prednisolone), the mixture was filtered through a 200-mg silica solid-phase extraction column (Burdick & Jackson) with methylene chloride as the eluent.

For the PCC oxidation, methodology developed for the determination of prednisolone was used. The reaction was allowed to proceed 1.5 h for the androstanes and 6.5 h for prednisolone.

TPAP oxidation of prednisolone had very disappointing results. Two compounds were observed after the reaction, one being the starting material (34% recovery) and the other being 1,4-androstadien-3,11,17-trione, the expected oxidation product in a 40% yield.

Table 5.1: Comparison of PCC and TPAP Oxidations

Compound	Percent Yield
PCC of Androstan-3-one-17β-ol	84
TPAP of Androstan-3-one-17β-ol	92
PCC of Androstan-3β,17β-diol	50
TPAP of Androstan-3β,17β-diol	44

The yields for the other model compounds are summarized in Table 5.1. Steroids with one hydroxyl group appear to be more efficiently oxidized than those with two hydroxyl groups. The yields for both oxidation methods are about the same; consequently, there is no advantage to switching to the TPAP oxidant.

Determination of Corticosteroids in Newts

To quantify the amount of corticosteroids in newt tissue and plasma, sensitive mass spectral techniques are necessary due to the small sample size. The chemical oxidation methodology that was previously used effectively for synthetic corticosteroids in this laboratory will not work for the corticosteroids of interest here. Both cortisol (4-pregnen-11β,17α,21-triol-3,20-dione) and corticosterone (4-pregnen-11β,21-diol-3,20-dione) oxidize to the same product, 4-androsten-3,11,17-trione. This steroid has a fairly low ECNI response; nanograms of compound are necessary for detection. 18-Hydroxycorticosterone (4-pregnen-11β,18,21-triol-3,20-dione) does not react with PCC. Methoxyamine-trimethylsilyl (MO-TMS) and trimethylsilyl (TMS) derivatives have been used extensively for the analysis of steroids using GC and GC-MS techniques, and were used for the analysis of corticosterones.

Many procedures for the TMS derivatization of steroids have been published. Three of these methods were attempted using a relatively large amount (ca. 50 μg) of cortisol and corticosterone. Efforts to prepare the TMS-ether-TMS-enol derivative using a mixture of MSTFA:TMIS (*N*-methyl-*n*-trimethylsilyltrifluoroacetamide:*N*-trimethylsilylimidazole) (7) gave poor results. If 100 μL of MSTFA:TMCS (TMCS = trimethylchlorosilane) (100:2) are used at 60°C for 10 min (7), both cortisol and corticosterone react. Upon analysis of the reaction mixture by GC-EI-MS, many peaks are noted; the primary chromatographic peak corresponded to the tri-TMS moiety. If 50 μL BSTFA with 10 μg potassium acetate as the catalyst are reacted with the steroid for 5 h at 80°C (3), two major products are observed for both cortisol and

corticosterone. These products were identified as the di- and tri-TMS moieties of cortisol and corticosterone. Because TMS derivativization resulted in multiple products, it was discontinued.

MO-TMS derivatives were prepared based on the urinary steroid procedure from the metabolic profiling lab (8). To the steroid residue were added 100 µL of methoxyamine-HCl (100 mg/mL in dry, redistilled pyridine). After heating at 60 °C for 4 hr, the sample was dried under a stream of nitrogen for 45 min (until glassy). Then 200 µL Sylon BTZ (Supelco, Bellefont, PA) were added and heated at 80 °C for 24 h. Sylon BTZ is a 3:2:3 mixture of BSA *(N,O-bis*(trimethylsilyl) acetamide), TMCS, and TMIS. A Lipidex column was used to separate the products from the excess reagents. In preliminary studies, the Lipidex clean-up step contaminated the sample further. Several other clean-up procedures were then attempted, including the use of a C-18 solid-phase extraction, a methanol/hexane extraction, and a hexane extraction. The hexane procedure resulted in the best extraction efficiency, even for low levels of sample.

The MO-TMS derivatization process was applied to real samples. Newt tissue (from a limb) and newt blood were extracted using C-18 solid-phase extraction columns and the mouse plasma extraction procedure described later in this chapter. Before extraction, each sample was spiked with the internal standard, dexamethasone. GC chromatograms of the MO-TMS products of the real samples are shown in Figure 5.1. After analysis by GC-EI-MS, no evidence of ions indicative of dexamethasone, cholesterol, cortisol or corticosterone were found. Two explanations are possible. One is that the extraction procedure was not very efficient. The other is that the MO-TMS derivative is not very sensitive to detection by GC-EI-MS.

At this point, all further research ceased because Dr. Bromley was too busy with other commitments.

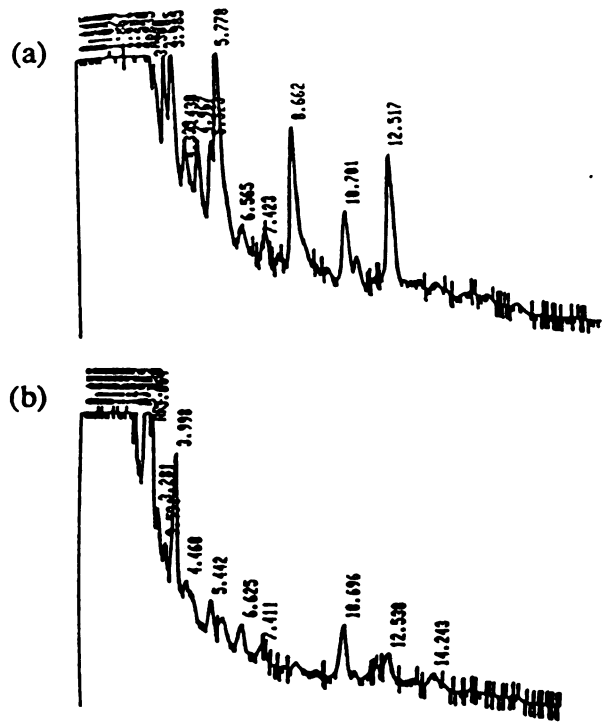

Figure 5.1: GC Chromatograms of (a) newt plasma and (b) newt limb.

The Determination of Dexamethasone in Human Plasma

The dexamethasone suppression test (DST) has been shown to differentiate endogenous depression and depression caused by neurotic, reactive, or characterological factors (9). In this test, a patient is given a 1-mg dose of dexamethasone. Plasma cortisol levels are determined at 8, 16, and 24 h following administration. If the plasma cortisol levels are suppressed for a 24-h period, the patient is classified as non-endogenously depressed; however, if the plasma cortisol levels return to normal after 3-4 h, the patient is diagnosed as being endogenously depressed. Studies have indicated that the bioavailability of dexamethasone is important in interpreting the DST (10). The DST may be improved by simultaneous monitoring of the plasma cortisol and dexamethasone levels.

Plasma dexamethasone concentrations for the DST are in the 0.1 to 4 ng/mL range. RIA has been successfully used in this range; however, the Kilts' group wished to independently validate their RIA method. A major concern was at the end of the 24-h test, when dexamethasone levels are low, the levels for the major metabolite of dexamethasone, 6β-hydroxydexamethasone, may be interfering with the RIA analyses.

In this study, RIA methods were compared to the chemical oxidation method. After the concentration of practice samples was validated, values obtained by GC-ECNI-MS were compared to those obtained by RIA for a large set of samples to determine coefficients of variance. Finally, the amounts of dexamethasone were determined for a 24-h collection of plasma for a normal patient undergoing the DST.

Experimental

Extraction of Plasma. The general scheme for the extraction and oxidation of human plasma samples is shown in Figure 5.2. Steroids were extracted from plasma by James Ritchie who works for Dr. Kilts at Duke University. All samples were spiked with 2.5 or 5 ng/mL of flumethasone (Sigma, St. Louis, MO). Sep-Pak 200-mg solid-phase extraction cartridges (Waters, Milford, MA) were preconditioned as follows. First, 5 mL 8 *M* urea were applied to react with the active sites. Then, 5 mL methanol were washed through the column. Organic solvents such as methanol open up the hydrocarbon chains and increase the surface area available for interaction with the analyte as well as remove residues from the packing material that may interfere with the analysis (11). If this step is not properly done, poor recoveries of analyte due to reduced retention on the column and interference peaks are possible. Next, 10 mL water were placed on the column. This step removes excess methanol as well as prepares the surface for the sample. To be most effective, this wash should be as close as possible to the sample in polarity, ionic strength, and pH. After the sample was applied, it was washed with 4 mL water to remove any polar material. A 38%

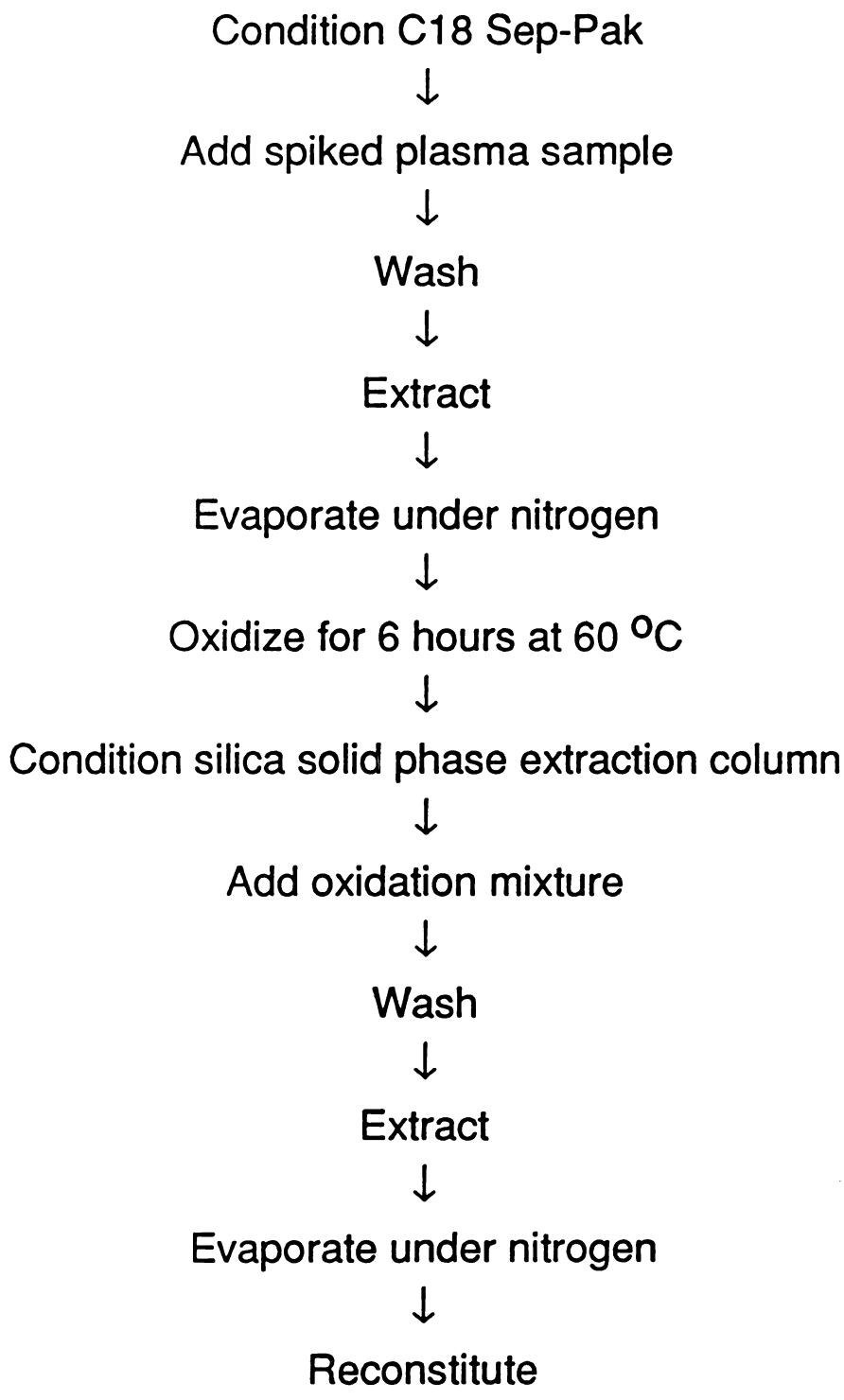

Figure 5.2: Sample preparation scheme for the determination of dexamethasone or prednisolone in plasma.

methanol/62% water wash (4 mL) was used to remove polar endogenous steroids and other polar interferences. The dexamethasone was eluted with 4 mL of 70% methanol/30% hexane into a silanized 1-dram vial. The extracts were dried under nitrogen, frozen, and shipped to MSU overnight under dry ice. All samples were frozen at -4°C until use.

Oxidation. To each residue, 1 mL methylene chloride (HPLC grade, Mallinckrodt, Paris, KY) was added, and the mixture was vortexed. About 10 mg anhydrous sodium acetate (J. T. Baker, Phillipsburg, NJ) were added to buffer the acid liberated during the oxidation. PCC (Aldrich, Milwaukee, WI) and Celite™ analytical filter aid (Johns-Manville, Lompoc, CA) were ground together in a 1:2 ratio with a mortar and pestle. The reaction, with PCC as the oxidant, occurs on Celite, a solid support. An excess of the PCC/Celite mixture was added to each vial. The reaction mixture was stirred and heated to 60°C for 6 h.

Removal of the oxidation reagents. The 200-mg silica solid-phase extraction columns (Burdick & Jackson, Muskegon, MI) were conditioned with several column volumes of methylene chloride. Because they are more easily wetted with the consumption of significantly less solvent and no loss of efficiency, 200-mg silica columns were substituted for the 500-mg columns used in previous work. The oxidation mixture was added and then the column was washed with 1.5 mL methylene chloride. The oxidized dexamethasone was eluted with 4 mL of 5% acetone in methylene chloride. After evaporation under nitrogen, the sample was reconstituted in 75 µL of ethyl acetate.

GC-MS Conditions. ECNI mass spectral data were obtained on a JEOL JMS-AX505H mass spectrometer (Peabody, MA) interfaced to a Hewlett Packard 5890 gas chromatograph (Palo Alto, CA). The following parameters were used: source temperature, 150°C; ionization current, 300 µA; electron energy, 200 eV; modulating gas, methane; source pressure, 2×10^{-5} torr; and accelerating voltage, 3 keV. A J&W

15-m x 0.25-mm x 1.0-μm DB-1 column (Folsom, CA) or a J&W 15-m x 0.25-mm x 0.25-μm DB-1701 column was directly inserted into the mass spectrometer ionization chamber. Helium was used as the carrier gas. The GC oven was held at 140°C for 1 min, then ramped to 260° at 40°/min, followed by a 4°/min ramp to 280°C. The splitless GC injector was held at 260°C, and the transfer line at 260°C.

The mass spectrometer was operated in the selected ion monitoring (SIM) mode to analyze the plasma extracts. Ion currents at *m/z* 310.2 (corresponding to $[M\text{-}HF]^-$ for oxidized dexamethasone) and 328.2 (corresponding to $[M\text{-}HF]^-$ for oxidized flumethasone) were monitored using magnetic field switching SIM.

Changes to the Oxidation Methodology

In previous collaborative work with the Kilts' group analyzing dexamethasone in plasma, several parameters were different than in the work now undertaken. Previously, the plasma samples were sent unextracted to MSU; now, samples are extracted at Duke. The internal standard, GC column, and GC-MS instrument were also changed. To be certain that the methodology worked before analyzing real patient samples, practice standard curves and unknowns were extracted and sent to MSU for further analysis.

Stable isotope dilution is the premier technique for the quantitation of biological compounds by MS. Not only are stable isotope-labeled analogs useful as tracers in the body (12), they are the best choice for internal standards (13). Evidence suggests that stable isotope dilution does not make an assay more accurate; however, the precision is improved (14). The structure of the analyte and standard are the same; therefore, they behave identically under extraction and chromatographic analyses. Correct choice of isotopically-labelled standards is important to avoid errors with loss of label and problems with equilibration of the analyte in the biological sample. In previous studies, the internal standard was (1,2,3,4,10,19-$^{13}C_6$, 19,19,19-$^{2}H_3$)

dexamethasone from Dr. J. P. Freeman of the National Center for Toxicological Research in Jefferson, AR. The major problem encountered with this internal standard was that it was not isotopically pure, resulting in a higher limit of detection. In this work, it was decided to change to a structurally-similar analog, flumethasone, which has one more fluorine group than dexamethasone.

Other changes from the previous procedure include the type of column and, more importantly, the type of mass spectrometer. Because the JEOL 505 at one point did not have the sensitivity and also had problems with hot/cold spots in the transfer line, the Finnigan TSQ-70 triple quadrupole mass spectrometer was used in the single quadrupole mode for previous work on dexamethasone plasma samples. Since then, the sensitivity of the JEOL 505 has been improved by cleaning the flight tube, realigning the magnet, and using one particular source for ECNI (the source designated as the non-collision cell source). The detection limit for the JEOL 505 is not as good as that for the TSQ-70. For the JEOL 505, the detection limit is 3 pg (S/N = 4; *m/z* 310) for a pure sample of oxidized dexamethasone. When using the TSQ-70, the detection limit is 160 fg (S/N = 7, *m/z* 310). The higher detection limit for the JEOL 505 was not a problem for this assay because the interferences of the matrix are greater than 3 pg.

Preliminary work with authentic samples of oxidized flumethasone and oxidized dexamethasone on a DB-1 15-m x 0.25-mm x 1.0-μm column resulted in the overlap of the minor isotope peak of flumethasone with the major isotope peak of dexamethasone (see Figure 5.3). The most intense peak in the ECNI mass spectrum of oxidized dexamethasone is at *m/z* 310. Unfortunately, oxidized flumethasone also gives a small contribution from *m/z* 310 (see Figure 5.4); therefore, even in the analysis of the blank, a peak representing oxidized dexamethasone was present. Quantitation will be affected because the limit of detection (LOD) will be higher.

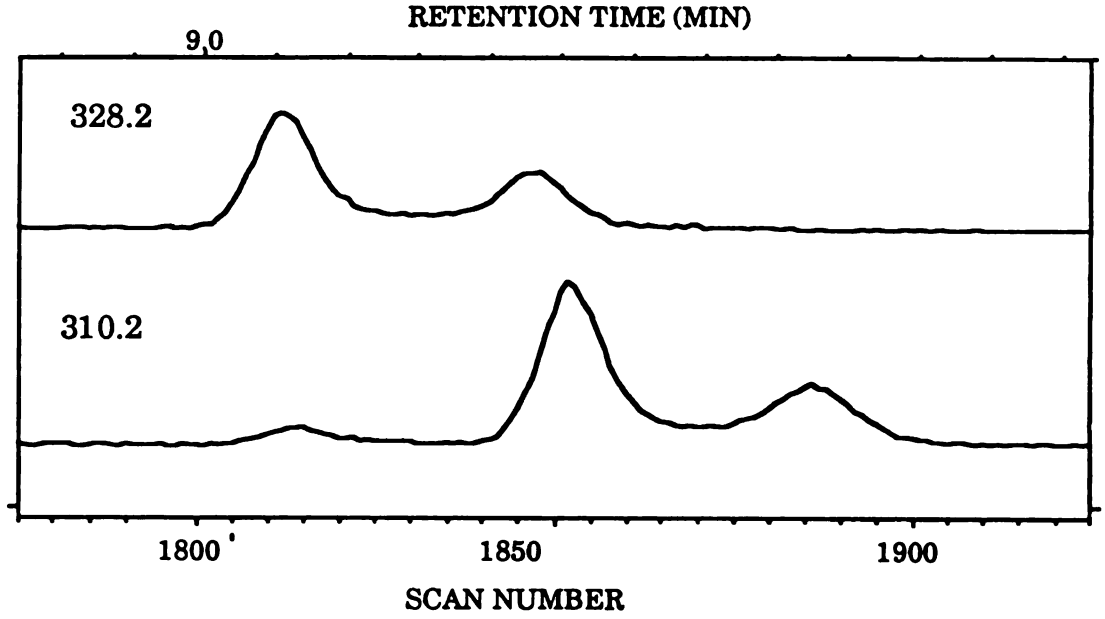

Figure 5.3: On a DB-1 15-m x 0.25-mm x 1.0-μm column, the minor isotope peak of oxidized flumethasone (top mass chromatogram) overlaps with the major isotope peak of oxidized dexamethasone (bottom mass chromatogram).

Different columns were used in an attempt to separate the oxidized flumethasone isomer peak from the oxidized dexamethasone peak. A longer DB-1 column did not work. Neither did a slightly more polar DB-5 30-m x 0.25-mm x 0.25-μm column (Hewlett Packard, Avondale, PA). Polar columns such as OV-17 (50-m x 0.32-mm x 0.25-μm, Quadrex, New Haven, CT) and DB-1701 (10-m x 0.2-mm x 0.15-μm, Chrompack, Raritan, NJ) were tried unsuccessfully. Changing the internal standard to fludrocortisone would not have interfered with the dexamethasone analysis. However, the Kilts' group was unwilling to use to fludrocortisone because the extraction procedure would also have to be changed. They were willing to accept the higher LOD rather than redo the extraction procedure.

The fragment ion peak at *m/z* 310 in the ECNI mass spectrum of flumethasone cannot be explained by any logical loss, so MS/MS was used to elucidate the source of this fragment. The TSQ-70 triple quadrupole mass spectrometer was operated by Tim

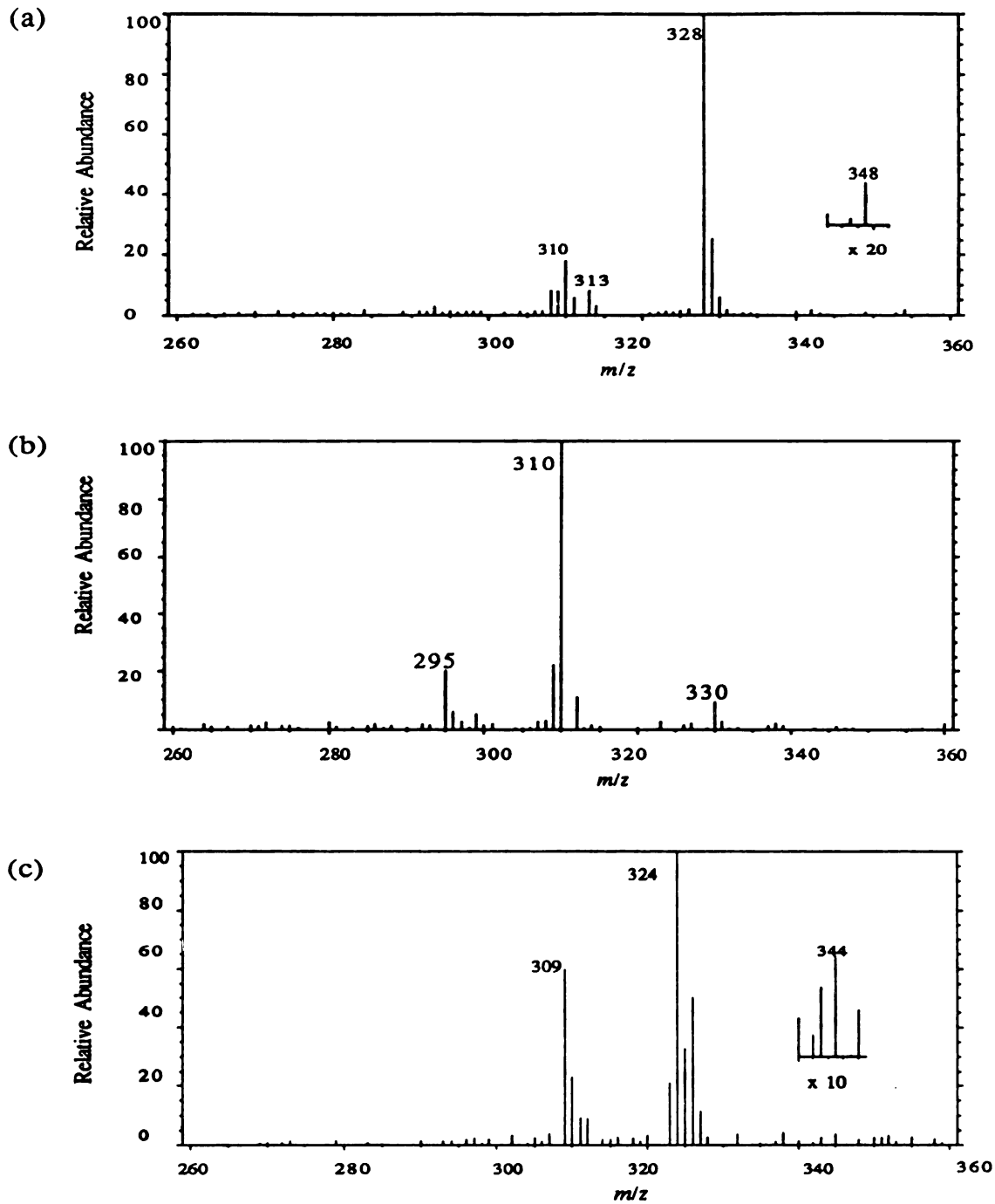

Figure 5.4: ECNI mass spectra of (a) 1,4-androstadien-6α,9α-difluoro-16α-methyl-3,11,17-trione (oxidized flumethasone), (b) 1,4-androstadien-9α-fluoro-16α-methyl-3,11,17-trione (oxidized dexamethasone), and (c) 1,4-androstadien-9α-fluoro-16α-methyl-3,6,11,17-tetrone (oxidized 6β-hydroxydexamethasone).

Heath in the ECNI mode with ammonia as the modulating gas and argon as the collision gas.

A full scan spectrum of oxidized flumethasone has a peak of 16.4% relative intensity at *m/z* 310 and 13.8% at *m/z* 330 under the conditions used on the TSQ-70. The peak at *m/z* 330 is also an isotope peak of *m/z* 328 but, as an isotope peak, the percent intensity should have been only 2.4%. The daughter spectrum of the molecular ion of oxidized flumethasone included peaks at *m/z* 328, 313, 302, and 293. No peak at *m/z* 310 or 330 is derived from the molecular ion. The parent of peak at *m/z* 310 in the spectrum of oxidized flumethasone is *m/z* 330. The peak at *m/z* 350 is also enhanced in the ECNI spectrum of flumethasone. The daughters of *m/z* 350 include *m/z* 330, 315, 310, 286, and 267. The peak at *m/z* 350 is not just an isotope peak of the molecular anion at *m/z* 348, if the relative intensities are compared (see Table 5.2).

Table 5.2: Isotope Peaks of the Molecular Anion of Flumethasone

m/z	% Relative Intensity from Spectrum	% Relative Intensity Calculated	Difference (%)
348	27.16	---	---
349	5.60	5.63	0.5%
350	1.41	0.59	140%

Obviously, *m/z* 349 is an isotope peak, but the peak at *m/z* 350 has a higher abundance than it should. The peak at *m/z* 350 could correspond to 4-androsten-6α,9α-difluoro-16α-methyl-3,11,17-trione, which has one less double bond than oxidized flumethasone. This coeluting electrophilic compound could easily fragment to $[M-2HF]^-$ represented at *m/z* 310. The contaminant could be a by-product of the PCC reaction, or the precursor could be a contaminant in commercially-available flumethasone.

The Contamination Problem

In order to validate the method, practice samples consisting of 1 mL of steroid-stripped plasma spiked with 5 ng/mL flumethasone as the internal standard and 0, 0.1, 0.75, and 5.0 ng/mL dexamethasone were analyzed using the oxidation methodology. The ratio of the peak area of the selected ion current profile at *m/z* 310.2 (corresponding to oxidized dexamethasone) to that at *m/z* 328.2 (corresponding to oxidized flumethasone) was nearly the same for all samples. In fact, the average of the peak ratios for all samples was 0.23 ± 0.05, which suggests that there is no difference between the spiked samples. Obviously, all samples were contaminated with similar amounts of either dexamethasone or another compound with peaks at *m/z* 310 and 295 in its mass spectrum, a highly unlikely combination. This contamination could be from a variety of sources including: (1) the use of commercially-available steroid-stripped plasma, (2) the commercial flumethasone used as the internal standard may have residual dexamethasone, (3) the rinses used by the Kilts' group in the extraction step may be contaminated, (4) cross-contamination of samples may occur during spiking or extraction, (5) the oxidation reagents and the rinses used in this laboratory may be contaminated, and (6) some type of interconversion was happening to change flumethasone to dexamethasone in the plasma sample. Option (6) is highly unlikely. Option (4) is plausible, only if some of the samples were contaminated, not all of them. The other possible sources of contamination were addressed in the next few experiments.

When the reagents (PCC, sodium acetate, Celite, and methylene chloride) were oxidized alone, no dexamethasone (or anything else) was observed. Authentic samples of flumethasone were oxidized in high concentrations, but no dexamethasone contamination was noted.

The next set of samples from Duke University consisted of the following spiked in 1 mL plasma: blank (no steroids added), 5 ng flumethasone, 5 ng

dexamethasone, and both flumethasone and dexamethasone. The steroid-stripped plasma was thought to be the source of the problem, so the samples were extracted from plasma that had never been touched by dexamethasone, i. e., plasma donated by Jim Ritchie himself. The contamination was noted in all samples, which proves the internal standard is not the source of the problem. The contamination had to occur during the plasma extraction or the PCC clean-up steps, but other PCC reactions that were being used for different experiments did not have contamination problems.

The extraction step in question did not occur in this lab. To prove that the plasma extraction step indeed was causing the problem, a set of plasma samples that had already been extracted as well as the corresponding unextracted plasma samples were prepared at Duke University. The unextracted plasmas were worked up in this laboratory as well as a set of blood-bank plasmas that was spiked and extracted in this laboratory. All samples extracted in this laboratory were not contaminated, but those prepared in the Kilts' lab were. After the Kilts' group learned that the source of contamination was in the extraction step, they decided to use freshly-prepared solvents. Thus, the contamination problem was solved.

Practice Standard Curves

Now that the contamination problem was solved, a practice standard curve and five unknowns were prepared to ascertain the viability of this technique. To prepare the standard curve, samples containing 0, 0.1, 0.75, and 5.0 ng/mL of dexamethasone were prepared in duplicate and analyzed by GC-ECNI-MS in duplicate. Before extraction, each of the points on the standard curve as well as each of the unknowns had been spiked with 5 ng flumethasone. The standard curve was linear with the equation of the line $y = 0.0795 + 0.150x$ with a correlation coefficient (r^2) of 0.999. The limit of detection (LOD) has been defined as $y_B + 3\ s_B$ where y_B is the value of the y-intercept and s_B is the standard deviation of the blank. If this definition for LOD

is used, the probability of either a false positive or a false negative is 7% (15). The LOD is 0.25 ng/mL, which is unacceptable because patient samples will have concentrations as low as 0.1 ng/mL. The high LOD made it difficult to distinguish between the blank values and the 0.1 ng/mL values on the standard curve; this made quantification of the unknowns difficult (see Table 5.3).

Table 5.3: Unknowns from Practice Standard Curve

Sample	ECNI-MS Estimate	RIA Estimate
A	2.3 ng	3 ng
B	0 to 0.1 ng	0.15 ng
C	1.8 ng	0.70 ng
D	0 to 0.1 ng	0.11 ng
Patient	2.3 ng	1.9 ng

In order to improve the LOD, the amount of internal standard was decreased by half, to 2.5 ng/mL. The final volume of the reconstituted reaction mixture was decreased from 100 µL to 75 µL. The standard curve for this practice set was excellent, with a correlation coefficient of 1.000. Because only one blank value was used, the LOD cannot be determined. However, there was no agreement between the values for dexamethasone determined by ECNI-MS as compared to the real values and values obtained RIA analysis (see Table 5.4).

Table 5.4: Unknowns from Practice Standard Curve with Less Internal Standard (all concentration values are in ng/mL)

Sample	Real Concentration	RIA Estimate	ECNI-MS Estimate
E	0.60	0.66	1.0
F	0.25	0.25	0.63
G	2.1	3.0	2.6

Another practice standard curve with unknowns was prepared. This time, six blanks were analyzed in order to determine the LOD more precisely. The rest of the samples were prepared in duplicate and analyzed by GC-MS in duplicate. The standard curve was not as linear as in the previous analysis (correlation coefficient of only 0.990), but the LOD has decreased to 0.11 ng/mL. This is a lower LOD than previously reported for the chemical oxidation of dexamethasone using an isotopically-labelled internal standard (3). The values determined by ECNI-MS were much closer to the RIA values (see Table 5.5) than previous practice sets; therefore, the assay was validated.

Table 5.5: Unknowns from Practice Standard Curve

Sample	RIA Concentration (ng/mL)	ECNI-MS Concentration (ng/mL)
17a	0.80	0.90 ± 0.18 (n = 2)
18b	1.8	2.0 ± 0.1 (n = 2)
19c	0.34	0.41 ± 0.12 (n = 2)
Patient	1.44	1.08

Dexamethasone Precision Study

Now that the standard curve was linear and the values for the practice samples agreed with those obtained by RIA, a precision study was undertaken. In this study, a six-point standard curve was prepared in duplicate. To determine a precise LOD value, the six blank samples were prepared. Six unknown samples at four different concentration values were analyzed by both GC-MS and RIA. This was a double-blind study; the operator for each technique did not know either the concentration values or which samples were in which pool. For ECNI-MS, the standard curve was linear (see Figure 5.5) if the 0.3 ng/mL points were rejected. These points were discarded due to cloudy solutions, probably occurring from faulty PCC clean-up

procedures. The equation of the line was y = 0.0646 + 0.299x with a correlation coefficient of 1.000. The LOD ($y_B + 3\ s_B$) was 0.096 ng/ml.

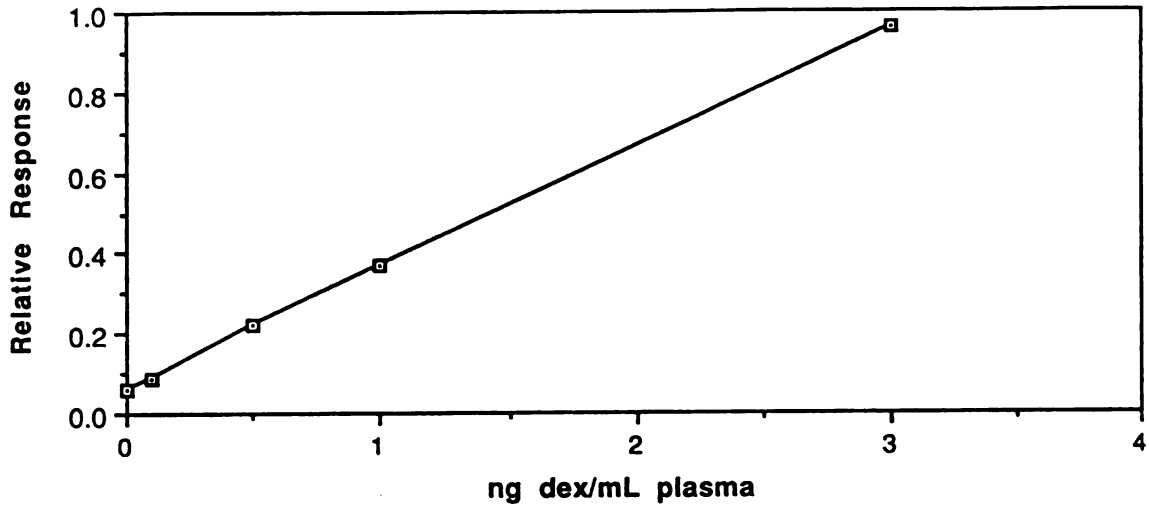

Figure 5.5: Standard curve for the determination of dexamethasone in human plasma. The equation of the line was y = 0.0646 + 0.299x with a correlation coefficient of 1.000. The LOD ($y_B + 3\ s_B$) was 0.096 ng/mL.

The values for each set of data obtained by RIA as well as the means and coefficients of variation (cv) are presented in Table 5.6. The results from the chemical oxidation study are in Table 5.8. It was concluded that three samples were not extracted and/or oxidized efficiently because no response for oxidized dexamethasone and a very diminished response for the internal standard were noted. To improve the variances for the ECNI-MS data, statistical outlier tests (16) were applied to each pool of data. Dixon's r_{11} statistic is a test for a single upper outlier, x_n, in a normal sample with s^2 unknown. The test statistic is

$$T = \frac{x_n - x_{n-1}}{x_n - x_2} \qquad (1)$$

where x_n is the upper outlier, x_{n-1} is its next nearest neighbor, and x_2 is the second lowest value . The Dixon r_{22} statistic tests for an upper outlier pair, x_{n-1}, x_n, in a normal sample with s^2 unknown. The test statistic is

$$T = \frac{x_n - x_{n-2}}{x_n - x_2} \qquad (2)$$

where x_{n-2} is the third highest value. To test for a lower and upper outlier pair, x_1, x_n, in a normal sample with the mean and s^2 unknown, the test statistic is

$$T = \text{internally studentized range} = \frac{x_n - x_1}{s}. \qquad (3)$$

The appropriate tabulated significance levels can be found in reference 16. Using the test statistic in equation 3, the upper and lower values for pool #1 were rejected. The test statistic in equation 2 was used to reject the upper value in pool #2 and the lower value in pool #3. The two upper outliers in pool #4 were rejected using equation #1. The modified means and standard deviations are found in Table 5.8.

When the RIA and GC-MS data are compared to the true values (Table 5.7), several conclusions can be drawn. At low levels of dexamethasone, RIA values were closer to the real value; however, the GC-MS data were better at high values. In general, the coefficient of variance was lower for RIA than for GC-MS. This may be due to the fact that no extraction steps are necessary for the RIA determination, while two are necessary for the oxidation technique. These two extractions were done in two different labs, which may contribute to the variance. The samples spiked with 0.45 ng/mL dexamethasone as well as 0.15 ng/mL 6β-hydroxydexamethasone resulted in high values for both the RIA and GC-MS determinations. At first, this was attributed to a mistake in the spiking step; however, further evidence suggests that the interference of 6β-hydroxydexamethasone is to blame, as will be explored further in the next section.

Table 5.6: Dexamethasone Precision Study--RIA Values for Replicate Analyses of the Indicated Sample Pools (all concentrations in ng/mL)

	Pool #1	Pool #2	Pool #3	Pool #4
	0.10	0.38	1.46	6.99
	0.08	0.35	1.37	8.49
	0.13	0.40	1.34	11.63
	0.09	0.37	1.43	28.47
	0.12	0.40	1.37	6.08
	0.14	0.37	1.67	6.24
n	6	6	6	6
mean	0.11	0.38	1.42	11.3
sd	0.02	0.02	0.14	8.6
cv (%)	22	5.1	9.7	76

Table 5.7: Comparison of Dexamethasone Concentrations Experimentally Obtained by RIA and ECNI-MS. All results expressed in ng/mL.

Real Value	RIA Results	GC-MS Results DB-1 Column	GC-MS Results DB-1701 Column
0.15	0.11 ± 0.02	0.31 ± 0.04	0.26 ± 0.08
0.45	0.38 ± 0.02	0.42 ± 0.04	0.42 ± 0.05
1.20	1.4 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
0.45 + 0.15 6β-OH dex	11 ± 4	2.7 ± 0.2	0.48 ± 0.13

Table 5.8: Dexamethasone Precision Study--ECNI-MS Values for Replicate Analysis of the Indicated Sample Pools (all concentrations in ng/mL)

	Pool #1	Pool #2	Pool #3	Pool #4
	0.28	0.57	1.13	3.9
	0.30	0.44	0.91	4.1
	0.37	0.44	1.37	2.8
	0.39	0.37	1.04	2.9
	0.16		1.13	2.6
			0.45	2.3
n	5	4	6	6
mean	0.28	0.45	1.0	3.1
sd	0.07	0.07	0.3	0.6
cv (%)	24	16	28	21
After statistical outlier tests				
n	4	3	5	4
mean	0.31	0.42	1.1	2.7
sd	0.04	0.04	0.2	0.2
cv (%)	12	8.5	14	7.3

The Interference of 6β-Hydroxydexamethasone

The reproducibility of the GC-MS method, although not as precise as the RIA method, was deemed sufficiently satisfactory to continue the project. At this point, the importance of the suspected interference from the 6β-hydroxydexamethasone on both the RIA and GC-MS assays was stressed. Six samples containing 0.25 ng/mL flumethasone and 0.2 ng/mL 6β-hydroxydexamethasone (and no dexamethasone) underwent analysis by both RIA and the oxidation ECNI-MS methods. By RIA, no response from dexamethasone was noted; however, by GC-MS on the DB-1 column previously used for all analyses, there was a response.

An authentic sample of 6β-hydroxydexamethasone, a gift from the Kilts' group, was oxidized by PCC. 6β-Hydroxydexamethasone was synthesized by Dr. E. Cook of Research Triangle Institute (Research Triangle Park, NC). After oxidation for 6 h and the usual clean-up procedure, the oxidized product, 1,4-androstadien-9α-fluorine-16α-methyl-3,6,11,17-tetrone (MW = 344) had the spectrum shown in Figure 5.4 (c). The peak at *m/z* 309 corresponds to $[M\text{-}HF\text{-}CH_3]^-$; the peak at *m/z* 310 is the isotope peak. Unfortunately, *m/z* 310 also corresponds to the peak monitored in the analysis of oxidized dexamethasone. On a DB-1 15-m x 0.25-mm x 1.0-μm column, the oxidized dexamethasone and the oxidized 6β-hydroxydexamethasone chromatographic peaks overlap.

Several other columns were used in an attempt to separate oxidized dexamethasone from oxidized 6β-hydroxydexamethasone. The two compounds overlapped on a longer DB-1 column, 30-m x 0.25-mm x 0.25-μm (J&W, Folsom, CA). In previous work (3), it was stated that the two compounds separated on a slightly more polar DB-5 column. When a 30-m x 0.25-mm x 0.25-μm DB-5 column (Hewlett Packard, Avondale, PA) was used, the two compounds separated by 0.04 minutes, but because both compounds have alpha and beta isomers at the 16-methyl

position, this will not be enough resolution. On a DB-1701 (15-m x 0.25-mm x 0.25-μm, J&W, Folsom, CA) column, the two separated by more than a minute.

When a DB-1701 column was used to analyze the six samples containing only flumethasone and 6β-hydroxydexamethasone, only a small contribution from dexamethasone was present. The dexamethasone level was 0.074 ng/mL. The LOD for this analysis was 0.059 ng/mL; therefore, the level of dexamethasone in the 6β-hydroxydexamethasone samples is slightly above the detection limit. Perhaps there is residual dexamethasone in the authentic sample of 6β-hydroxydexamethasone.

The precision study was redone using the DB-1701 column. Again, all samples were analyzed randomly. The standard curve again was very good, with an equation of $y = 0.0320 + 0.188x$ and a correlation coefficient of 0.999. The limit of detection was 0.059 ng/mL. The peak at *m/z* 324.2, corresponding to the $[M\text{-}HF]^-$ peak for oxidized 6β-hydroxydexamethasone, was also monitored to determine the contribution from this metabolite. In general, there was very little to no contribution from *m/z* 324. From the data summarized in Table 5.7, the coefficient of variance for this GC-MS analysis is higher than that from the previous analysis, but the accuracy is much better. In fact, the value for pool #4 is almost the same as the real value. From the contribution at *m/z* 324, one suspects that there was more than 0.15 ng/mL 6β-hydroxydexamethasone spiked into these samples; the amount was probably 10-100 times as much.

Post-Dexamethasone Nighttime Study

The oxidation methodology was used to validate the RIA values from a 24-hour post-dexamethasone study. Of special interest were the values at low concentrations of dexamethasone. At long collection times, the amount of dexamethasone should decrease and the relative amount of 6β-hydroxydexamethasone increase. It is suspected that the RIA results may not be valid at this point.

A dose of dexamethasone was given to a normal male aged 22 years. Blood was drawn every half hour for the first two hours, then every 15 min for the next seven hours. Eight hours after this point, the last sample was drawn.

For the oxidation methodology, each sample was spiked with 2.5 ng of flumethasone no matter what the starting volume of plasma (some were below 1 mL). A standard curve consisting of 6 blank samples and two each of 0.1, 0.3, 0.5, 1.0, and 3.0 ng/mL dexamethasone was prepared. Each point on the standard curve except for the blanks were analyzed in duplicate; selected patient samples also were analyzed in duplicate.

The peak at *m/z* 324 corresponding to oxidized 6β-hydroxydexamethasone was monitored along with the peaks at *m/z* 310 for oxidized dexamethasone and at *m/z* 328 for oxidized flumethasone. Only about 40% of the patient plasma samples had any contribution from 6β-hydroxydexamethasone, and there was no pattern as to which samples contained the metabolite. The signal for the metabolite was weak in all instances. Perhaps better results for 6β-hydroxydexamethasone could be obtained if the extraction and oxidation steps were also optimized for the determination of this metabolite.

As shown in Table 5.9, all values obtained from the GC-MS method were higher than those obtained from RIA. No reasons for the discrepancy are inherently obvious; therefore, another sample set was analyzed.

Dexamethasone Precision and Patient Study

Another sample set was analyzed in response to the poor correlation of the post-dexamethasone nighttime study RIA data to GC-MS results. All plasma samples were 1-mL volumes. The standard curve was prepared as before, with the addition of a 4.8 ng/mL point. A standard curve for 6β-hydroxydexamethasone, consisting of plasma samples spiked with 0.2, 0.4, 0.8, and 1.5 ng/mL analyte, was also analyzed.

Table 5.9: Post-Dexamethasone Nighttime Study. Experimentally Determined Values for Dexamethasone in Plasma by Indicated Methods at the Indicated Times.

Sample Number	Time Drawn	RIA Conc. ng/mL	GC-MS Conc. ng/mL
11844	23:30	<0.02	3.43
11845	23:00	<0.02	0.005
11846	23:30	2.51	4.18
11847	24:00	3.89	4.54
11848	00:30	2.64	4.59
11849	01:00	3.81	4.41
11850	01:15	2.65	5.35
11851	01:30	2.35	-----
11852	01:45	1.58	3.59
11853	02:00	2.18	4.17
11854	02:15	2.19	2.66
11855	02:30	3.28	4.23
11856	02:45	2.52	4.03
11857	03:00	2.56	4.01
11858	03:15	2.55	5.12
11859	03:30	2.26	3.48
11860	03:45	2.08	3.65
11861	04:00	1.89	3.22
11862	04:15	2.15	3.43
11863	04:30	1.98	3.58
11864	04:45	2.06	3.61
11865	05:00	2.00	2.96
11866	05:15	1.54	3.13
11867	05:30	2.25	3.39
11868	05:45	2.13	4.34
11869	06:00	1.90	2.93
11870	06:15	1.69	3.12
11871	05:30	1.65	3.45
11872	05:45	2.63	4.69
11873	07:00	1.57	2.71
11874	07:15	2.48	-----
11875	07:30	0.81	2.25
11876	07:45	2.21	3.11
11877	08:00	2.08	0.14
11878	16:00	0.63	5.16

The precision study consisted of three pools of eight samples each. The patient study consisted of 16 samples. All samples were extracted on the same day. The analysis of all samples by GC-MS also occurred on one day. The standard curve for oxidized dexamethasone was linear, with a correlation coefficient of 0.993. The LOD for this analysis was 0.034 ng/mL.

Table 5.10: Precision Study for Dexamethasone. Values for the Replicate Analysis of the Indicated Sample Pools Expressed in ng/mL.

	Pool #1	Pool #2	Pool #3
	0.24	1.46	0.52
	0.31	1.53	0.40
	0.20	1.65	0.44
	0.27	1.60	0.52
	0.25	1.55	0.51
	0.27	1.49	0.37
		1.66	
		1.40	
n	6	8	6
mean (ng/mL)	0.26	1.54	0.46
sd	0.03	0.09	0.06
cv (%)	13	5.6	13
Spiked (ng/mL)	0.34	1.91	0.58
RIA (ng/mL)	0.33	2.32	0.68

The results for the precision study are presented in Table 5.10. The samples with poor extraction efficiencies were not reported. Without using any statistical outlier tests to reject data, the precision of the GC-MS data was far better than that of the study summarized in Table 5.7. Correlations with the spiked value and the RIA values were not as good. On the average, the RIA values were higher than the spiked values, but the GC-MS values were lower than the spiked values.

The patient study results did not follow a consistent pattern when compared to the RIA (see Table 5.11). Sample 22729 had problems with overlapping chromatographic peaks which may account for its low GC-MS value. In general,

plasmas with high RIA values of dexamethasone (above 1 ng/mL) had lower GC-MS values. At lower levels of dexamethasone, the RIA values were lower than the GC-MS values. In the precision study analyzed on the same day, all the GC-MS values were lower than their corresponding RIA values.

Table 5.11: Dexamethasone Patient Study. Comparison of RIA and ECNI-MS Experimental Results.

Sample Number	RIA Concentration ng/mL	GC-MS Concentration ng/mL
22733	2.56	-----
22734	1.68	-----
22735	2.50	-----
22736	1.82	1.60
22737	1.91	1.77
22739	3.09	1.73
22740	2.02	1.36
22743	2.24	1.99
22749	0.86	0.86
22752	0.84	-----
22755	0.75	0.70
22759	0.70	0.85
22764	0.45	-----
22767	0.39	0.47
22773	0.21	0.32
22776	0.15	-----

A rough 6β-hydroxydexamethasone curve was analyzed (see Figure 5.6). The concentration of the standards were low as compared to the amounts found in the patient samples. Many spiked standards had poor extraction efficiencies and were subject to poor chromatographic peak shape. The correlation for the points that did work was not good ($r^2 = 0.974$). In Table 5.12, the estimated concentrations of 6β-hydroxydexamethasone in patient samples was tabulated. Values range from 0.6 to 1.6

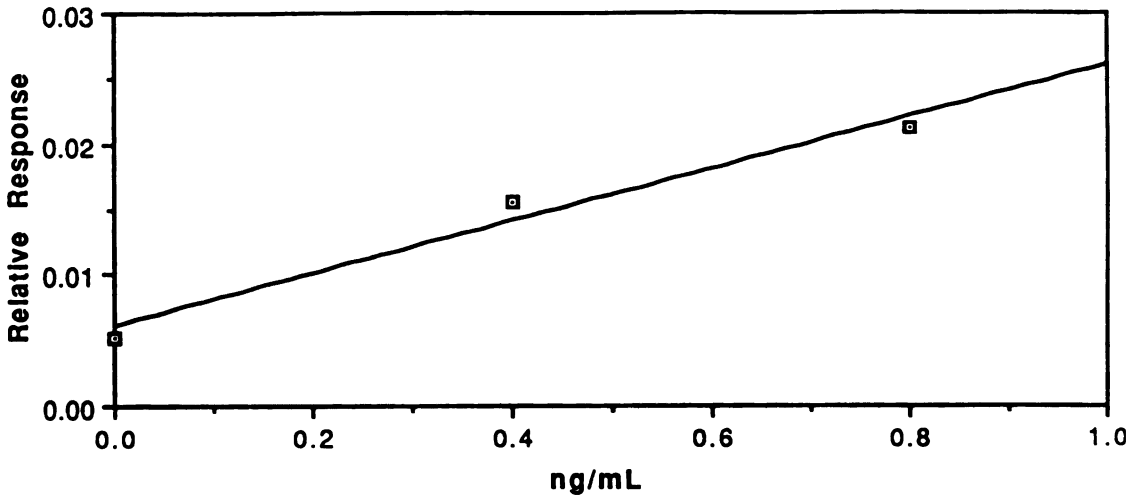

Figure 5.6: Standard curve for the determination of 6β-hydroxydexamethasone in human plasma. The equation of the line was y = 0.0605 + 0.0199x with a correlation coefficient of 0.974.

Table 5.12: Estimated 6β-Hydroxydexamethasone Concentrations for Patient Samples

Sample Number	6β-Hydroxydexamethasone Concentration (ng/mL)
22737	1.12
22739	1.03
22740	1.41
22743	1.67
22749	1.06
22759	0.61
22767	0.66

ng/mL. No correlation of 6β-hydroxydexamethasone concentration with dexamethasone concentration was noted. This assay is not ready for simultaneous detection of dexamethasone and its major metabolite, 6β-hydroxydexamethasone.

Conclusions

Many changes have been made to this assay, but it still remains a valid method of determining dexamethasone in human plasma. The JEOL 505, after improving the sensitivity, worked just as well as the TSQ-70 for the ECNI-MS analyses. The use of flumethasone rather than an isotopically-labelled internal standard resulted in better detection limits. To avoid any overlap with the major metabolite of dexamethasone, 6β-hydroxydexamethasone, a polar column such as a DB-1701 column must be used.

Using this method, the level of the 6β-hydroxydexamethasone was low. If this compound is to be quantitated in conjunction with dexamethasone, the assay must be improved. Perhaps better choices of the temperature and pressure of the mass spectrometer source may increase the sensitivity of the peak at *m/z* 324. The 38% methanol in water wash used to wash steroids more polar than dexamethasone off the column also elutes 6β-hydroxydexamethasone (17). 6β-Hydroxydexamethasone was more effectively extracted if a 15% methanol wash was used instead (17). The 5% acetone in methylene chloride eluent used after the PCC oxidation was optimized for dexamethasone, but not tested for 6β-hydroxydexamethasone. In six hours, dexamethasone is converted to its oxidized product in a 77% yield, but the optimum oxidation time of 6β-hydroxydexamethasone has not been determined.

In the first precision study comparing RIA and the GC-MS method proposed here, the coefficient of variance is lower for RIA, but in most cases, the accuracy is usually better for the GC-MS method. It has been suggested (18) that a rsd of about 16% should be sufficient for the mass spectral analysis of trace components in food

and drugs. The reproducibility study meets this criterion only if the outliers are ignored. Also, if possible, a calibration curve should cover the limit of quantitation to one order of magnitude above trace level. The correlation coefficient of this line should be no less than 0.94 (18). In this work, all standard curves had correlation coefficients of 0.990 or above.

In the second precision study, the coefficient of variance of the GC-MS method decreased, but the accuracy was not as good. The values obtained by GC-MS were lower than the spiked values or the values obtained by RIA.

The analysis of actual patient data gave very different results than the RIA assay. In the first study, the GC-MS results were higher than the RIA results. In the second study, the GC-MS results were lower at high concentrations of dexamethasone and higher at low concentrations of dexamethasone. The lack of correlation between results from RIA and GC-MS analyses for steroid hormone such as testosterone, progesterone, estradiol, and cortisol has been documented (19). Immunoassays were usually positively biased in comparison with the GC-MS value. This observation was generally true for the second patient study.

Determination of Prednisolone in Mouse Plasma

Dr. Pamela Fraker's group is interested in the effects of the immunosuppression of prednisolone on the mouse immune system. Prednisolone is commonly used for the treatment of inflammation due to injury or a disease such as arthritis, allergy and asthma. While it is well documented that pharmacologically-active glucocorticoids cause thymic atropy due to induction of apoptosis (programmed cell death) in immature T-cells (cells that direct cell-mediated immunity), the effects of prednisolone on normal B-cell (cells that synthesize and secret antibodies to introduced antigens) development is virtually unknown. Using an *in-vitro* murine bone marrow culture system, it was found that 10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M* prednisolone caused 36%, 73%,

and 85% inhibition of the bone marow B-cell response to the T-cell independent antigen, trinitrophenol-lipopolysaccharide (TNP-LPS). Modest elevation of plasma prednisolone (2 ng/mL, determined by the method described below) in mice 10 days after implantation of a prednisolone pellet caused splenic and thymic atrophy, as well as decreased white blood cell counts. In addition, flow cytometric data revealed that there was a 30% decrease in proportion of B220 and surface immunoglobin-bearing cells. This was accompanied by a 60% reduction in bone marrow response to TNP-LPS. Taken together, these data indicate that low levels of prednisolone significantly alters bone marrow B-cell function by depletion of B-lineage cells. Further, this depletion appears to be caused in part by prednisolone induction of apoptosis (20).

HPLC has been the method of choice for the determination of prednisolone in clinical samples (21-25). Using a silica HPLC column, prednisolone and prednisone could be easily separated. Detection limits are on the order of 4-10 ng/mL. RIA has been used for the determination of prednisolone; however, interferences from other steroids, especially cortisol and prednisone, are problematic (25). GC-MS techniques have been used to determine levels of prednisolone after preparation of the MO-TMS derivative (26, 27). The chemical ionization method is not as sensitive as the ECNI method. The latter can detect 0.25 ng of pure material, but no information was available for detection in a biological matrix.

Originally, the collaborators roughly estimated the amount of prednisolone in a typical sample of mouse plasma to be between 10-20 ng. This was a very high estimate; actual values were between 1-5 ng per sample. The sample size is small; depending on how quietly the mouse is bled, 30-150 μL of plasma can be obtained. The amount of prednisolone in these samples is below the detection limit of current HPLC methods. The ECNI-MS method using MO-TMS derivatives may work, but MO-TMS derivatives can be difficult to prepare and suffer losses due to the two-step reaction procedure and possible formation of multiple products.

The oxidation of prednisolone followed by analysis by GC-ECNI-MS was chosen for the determination of prednisolone in mouse plasmas. The oxidation methodology is based on converting prednisolone to 1,4-androstadien-3,11,17-trione using the PCC oxidant. A pure sample of this compound has a detection limit of 170 pg (*m/z* 298, S/N = 11) on the JEOL AX505 mass spectrometer. The ECNI spectrum of this compound consists of only one peak at *m/z* 298. Unfortunately, another corticosteroid drug, prednisone, is also oxidized to the same product under the same conditions (see Figure 1.7), making it impossible for the assay to distinguish between the two corticosteroids. To be pharmacologically active, prednisone must first be converted to prednisolone by the reduction of the 11-carbonyl group in the liver (28). This interconversion is thought to occur rapidly (29), so the bioavailability of the two drugs is thought to be similar. There is no evidence that prednisolone is converted to prednisone *in vivo*; however, the possibility cannot be disregarded in assay development. The inability to distinguish between prednisolone and prednisone was communicated to the Fraker group. Because they were interested in the total amount of prednisolone dosed, the distinction between the two corticosteroidal drugs *in vivo* was not important.

Isotopically-labelled prednisolone was not available for the internal standard, so a structurally-similar compound was substituted instead. A corticosteroid drug was used because there is no possibility of its occurring naturally in the mouse. Because oxidized dexamethasone coelutes with 1,4-androstadien-3,11,17-trione on a DB-1 column, it was not used. Flumethasone had no coelution problems, so it was chosen as the internal standard.

Experimental

The extraction procedure is based on the work of Kayganich and co-workers (3) with a few modifications. The manufacturer of the C-18 solid-phase extraction

columns was changed to Burdick & Jackson because these columns were already available. For the PCC clean-up step, 200-mg silica solid-phase extraction columns were used rather than the 500-mg columns because the smaller amount of silica are more easily wetted with less solvent and less time with no loss of efficiency. In the initial plasma extraction step, the selective wash (30% methanol in water) and the eluent (38% methanol in hexane) were changed to the more general water wash and methanol eluent to recover all steroids. For the same reason, ethyl acetate was substituted for the 5% acetone in methylene chloride eluent in the PCC clean-up step.

Instrumentation. ECNI mass spectral data were obtained on a JEOL JMS-AX505H mass spectrometer (Peabody, MA) coupled with a Hewlett Packard 5890 gas chromatograph (Palo Alto, CA). The following parameters were used: source temperature, 200°C; ionization current, 300 μA; electron energy, 200 eV; modulating gas, methane; source pressure, 2×10^{-5} torr; and accelerating voltage, 3 keV. A J&W-15 m x 0.25-mm id x 0.25-μm DB-1701 column (Folsom, CA) was directly inserted into the mass spectrometer ionization chamber. Helium was used as the carrier gas. The GC oven was held at 140°C for 1 min, then ramped to 260° at 40°/min, followed by a 4°/min ramp to 280°C. The splitless injector was held at 260°C, and the transfer line at 260°C.

Selected ion monitoring (SIM) was used to analyze the plasma extracts. Ion currents at *m/z* 298.2 (corresponding to M^- for oxidized prednisolone) and 328.2 (corresponding to $[M\text{-}HF]^-$ for oxidized flumethasone) were monitored using magnetic field switching SIM.

Extraction of plasma samples. Mouse plasma samples were spiked with 3 ng of flumethasone in 10 μL of methanol. The 200-mg C18 solid-phase extraction columns were conditioned with 5 mL of 8 *M* aqueous urea, followed by washes with 5 mL of methanol and 10 mL of distilled water. The plasma samples were applied, followed by 4 mL of distilled water. Prednisolone was eluted with 4 mL of methanol.

Oxidation of plasma extracts. Following the removal of the methanol under nitrogen, 1 mL of methylene chloride, approximately 10 mg of anhydrous sodium acetate, and an excess of pyridinium chlorochromate finely ground with Celite (about 1:3 w/w) was added to the dried residue. The reaction mixtures were stirred and heated to 60°C for 6 h.

Removal of oxidation reagent. Silica solid-phase extraction columns (200 mg) were conditioned with several column volumes of methylene chloride. After the addition of the reaction mixtures, the columns were washed with 2 mL of methylene chloride. The oxidation product was eluted with 4 mL of ethyl acetate. After evaporation under nitrogen, the residue was reconstituted in 75 μL of ethyl acetate; 4-6 mL of this solution were required for analysis by GC-ECNI-MS.

Preparation of the calibration curve. To blank mouse plasma was added 3 ng of the internal standard, flumethasone. Following this, additions of 0, 1, 5, 10, and 20 ng of prednisolone were made. Each standard was prepared in duplicate. The extraction and oxidation steps were the same as above.

Preliminary Study

In order to verify the extraction and oxidation steps for the determination of prednisolone in mouse plasma, spiked mouse plasma samples and one dosed mouse sample were analyzed. Four aliquots of mouse plasma (110 μL) were spiked with no steroids, 55 ng prednisolone, 11 ng prednisolone, or both 11 ng flumethasone and 11 ng prednisolone. The real mouse sample was spiked with 11 ng flumethasone only. All samples were analyzed with a nonpolar DB-1 column (15-m x 0.25-mm x 1.0-μm, J&W, Folsom, CA). All samples were easily detected by this method. Using a one-point estimation, the concentration of the real sample was 17 ng.

Mouse Plasma Sample Set #1

Two sets of mice were studied for the immunosuppression effect. The first set did not result in as great an effect as the second. The first set of mouse plasmas was used to assess the oxidation methodology.

Blank mouse samples from other experiments were spiked with 0, 10, 20, and 50 ng of prednisolone. All samples, included those to be analyzed, were spiked with 7.5 ng of flumethasone. After analysis by GC-ECNI-MS with a DB-1 column, the results are as summarized in Table 5.13, column 3. Mice numbers one through eight were controls; the rest were dosed with prednisolone.

Table 5.13: Determination of the Amount of Prednisolone in Mouse Plasma Sample Set #1

Mouse #	Amount of Plasma µL	Amount of Prednisolone ng, DB-1 column	Amount of Prednisolone ng, DB-1701 column
1	65	1	0.15
2	100	<LOD	NA
3	90	<LOD	<LOD
4	100	11	NA
7	65	<LOD	<LOD
8	100	4	0.9
9	40	<LOD	0.1
12	60	<LOD	<LOD
13	85	6	3.9
15	100	<LOD	0.05

The standard curve was fairly linear with a correlation coefficient of 0.994. The problem with the analysis was the real samples were at very low concentrations where there were no points on the standard curve. The standard curve needed some improvement with respect to reproducibility and detection limits.

Possible Interferences

The blank value for 1,4-androstadien-3,11,17-trione was higher than expected. At very low concentration values, the contamination in the blank at the retention time

of 1,4-androstadien-3,11,17-trione becomes very important. Possible biological interferences were investigated. Corticosterone, an endogenous steroid, oxidizes to 4-androsten-3,11,17-trione, which has a molecular weight of 300. Under ECNI conditions, 4-androsten-3,11,17-trione is a low-response compound. Usually, the mass spectrum of low-response compounds is dominated by a peak at $[M-H]^-$. In this case, $[M-H_2]^-$ is the most intense peak in the spectrum. This peak occurs at *m/z* 298, the same mass as oxidized prednisolone. On a nonpolar DB-1 column, these compounds coelute. Corticosterone has a level of 10 µg/dL in mouse plasma. Interestingly, the peak area of the blank corresponded to the response expected from about 10 µg/dL of 4-androsten-3,11,17-trione.

To remove the corticosterone contamination, two methods are possible. One is to use a selective extraction procedure by choosing the washes, eluents, and column type of the plasma extraction step and/or PCC clean-up step to exclude corticosterone. This should be possible because corticosterone and prednisolone differ by a 1,2-double bond and a 17-hydroxy group. However, optimizing the chromatography can be a very time-consuming procedure; in fact, several different extraction procedures attempted were unsuccessful. The other, easier method of overcoming the contamination problem is to separate 4-androsten-3,11,17-trione (oxidized corticosterone) and 1,4-androstadien-3,11,17-trione (oxidized prednisolone) chromatographically on the GC. More polar columns should separate these closely-related compounds with better resolution. A 50-m OV-17 column (50% phenyl/50% methyl) of unknown (and therefore questionable) history did not separate the two steroids, but reasonable separation was obtained on a 10-m DB-1701 (14% cyanopropylphenyl/86% methyl) column. For the majority of the mouse samples, background from corticosterone was not a problem. The high blank value still persisted.

Other endogenous steroids in the mouse were examined as possible interferences. The biosynthesis of adrenocorticosteroids is shown in Figure 5.7.

Progesterone, deoxycorticosterone, 17α-hydroxyprogesterone, and 11-deoxycortisol all oxidize to 4-androsten-3,17-dione (MW = 286), which has a different retention time and mass spectrum than 1,4-androstadien-3,11,17-trione. Cortisol and corticosterone oxidize to 4-androsten-3,11,17-trione, which has been proven not to cause the interference. Aldosterone oxidizes to 4-androsten-3,11,17,18-tetrone, which has a molecular weight of 314, so this is not a potential interference. 18-Hydroxycorticosterone could possibly oxidize to 4-androsten-18-ol-3,11,17-trione. If this compound loses a water molecule, it could have a peak at *m/z* 298 in its ECNI mass spectrum. To check this possibility, a sample of 18-hydroxycorticosterone (Ikapharm, Ramat-Gan, Israel) was oxidized using PCC for 6 h. The molecular weight of the reaction product was 305, possibly corresponding to the loss of the group at C-17 from the starting material. 18-Hydroxycorticosterone is not an interference.

After the reanalysis of the spiked prednisolone samples and the mouse sample set #1 using the DB-1701 column, the standard curve did not improve in linearity, but the LOD was now 0.04 ng. The results from this study are in Table 5.13, column 4. Lower concentration values can now be detected.

Mouse Plasma Sample Set #2

The second set of mouse samples consisted of eight control mice (#9-16) and eight mice dosed with prednisolone (#1-8). This sample set was considered to be the one which proved the immunosuppression theory. For the standard curve, blank plasma samples were spiked with 0, 1, 5, 10, and 20 ng of prednisolone. All samples were spiked with 3.0 ng of flumethasone before extraction. Each point on the standard curve and each of the actual samples were analyzed by GC-ECNI-MS in duplicate. The standard curve had an equation of $y = 0.0535 + 0.0604x$ with a correlation coefficient of 0.998 (see Figure 5.8). The LOD was 0.3 ng. The results from this study are summarized in Table 5.14.

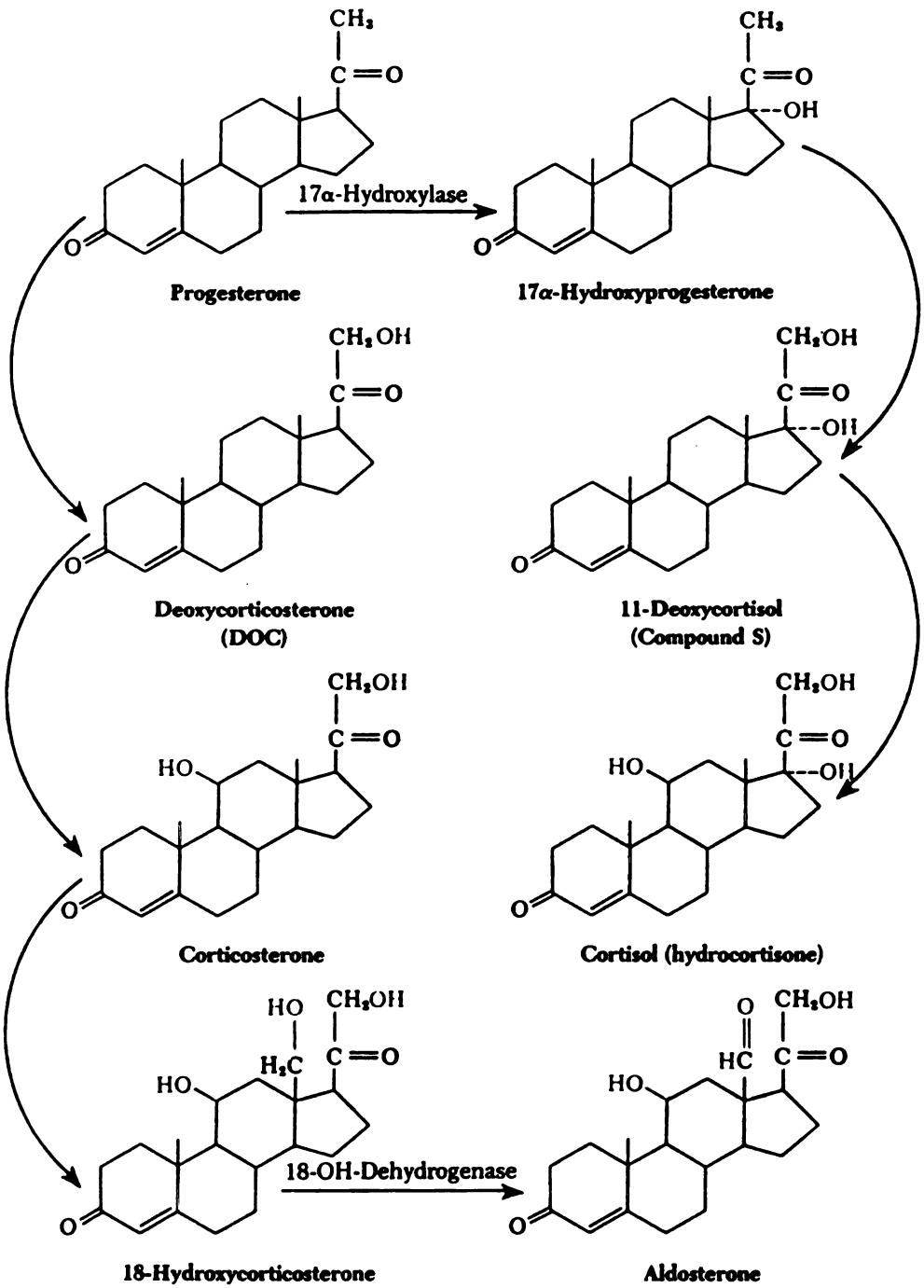

Figure 5.7: The biosynthesis of andrenocorticosteroids. Adapted from Orten, J. M.; Neuhaus, O. W. *Human Biochemistry*, Mosby: St. Louis, 1982, p. 618.

Table 5.14: Determination of the Concentration of Prednisolone in Mouse Plasma Sample Set #2

Mouse #	Amount Plasma μL	Amount Prednisolone ng	Concentration Prednisolone ng/mL
1	95	7.4	78 ± 2
2	75	0.34	4.5 ± 0.2
3	30	<LOD	<LOD
4	135	<LOD	<LOD
5	40	0.055	1.4 ± 0.6
6	100	0.81	8.1 ± 3.5
7	55	<LOD	<LOD
8	85	<LOD	<LOD
9	120	<LOD	<LOD
10	45	<LOD	<LOD
11	45	<LOD	<LOD
12	114	<LOD	<LOD
13	165	<LOD	<LOD
14	90	<LOD	<LOD
15	95	<LOD	<LOD
16	65	0.064	0.98 ± 0.12

Of the eight mice dosed with prednisolone, only four gave a response above background, with an average of 4.7 ± 3.4 ng/mL plasma (disregarding the contribution from mouse #1). The large standard deviation could occur from differences in bioactivity of prednisolone between individual mice. Mouse #1 had an abnormally high concentration of prednisolone (78 ng/mL) in its bloodstream. Most of the control mice had no prednisolone in their bloodstream, with the exception of mouse #16. This could be a false positive, a problem inherent in the design and execution of the assay.

Four more mouse plasma samples were analyzed separately. These samples were drawn 42 hours after prednisolone pellet implantation. Another standard curve, spiked as previously described, was analyzed along with the four samples. One of the samples did not have a measureable amount of prednisolone. The average amount of prednisolone in the remaining three samples was 8.3 ± 0.9 ng/mL. This result was low as compared to the experimenters' expected value of on the order of 100 ng/mL.

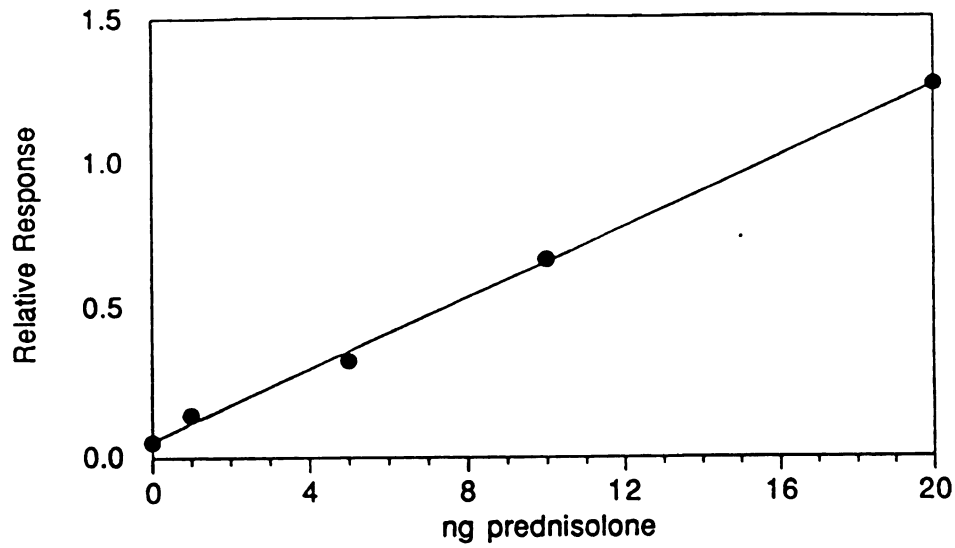

Figure 5.8: Standard curve for the determination of prednisolone in mouse plasma. The equation of the line is y = 0.0535 + 0.0604x with a correlation coefficient of 0.998. The LOD was 0.3 ng.

Conclusions

Even though this assay cannot distinguish between prednisolone and prednisone, it can detect prednisolone at levels found in mouse plasma. The limit of detection of this assay was between 0.3 and 0.5 ng/mL, which is just slightly better than competing HPLC methods that can distinguish between prednisolone and prednisone.

The oxidation methodology for the determination of prednisolone could be improved by improving the LOD. An isotopically-labelled internal standard may help. More selective plasma extractions and/or more selective PCC clean-ups could decrease the biological background sufficiently to allow more sensitive detection of prednisolone. The yield of the PCC reaction also could be improved. After a 6-h reaction time, the yield is only 47%. Perhaps longer reaction times or a different oxidant are needed.

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Chapter 6: Conclusions

One possible method for the analysis of steroids is by chemical oxidation followed by analysis by GC-ECNI-MS. In this work, the ECNI characteristics of various cyclic molecules was studied, in addition to improving the oxidation methodology for steroidal drugs.

Electron capture techniques improve the sensitivity of certain compounds. For enhanced ECNI response within a steroid nucleus, the following structural features are important: a cross-conjugated system with a neighboring group, such as 1,4-dien-3,11-dione; a linearly-conjugated system, such as 4-en-3,6-dione; a fluorine atom alpha to a carbonyl group in a highly-conjugated steroid; or an epoxide alpha to a carbonyl group. The trends in ECD response mimic those for ECNI; however, the actual values differ. Both high- and low-response ketosteroids have similar EI and CI responses. For low-response steroids, the ECNI response is on the order of the corresponding EI response.

Size considerations are also important. The same structural features that increase the response in steroids also increase the response for cyclohexanones and bicyclic compounds. The response for steroids is greater than those for either of these classes of compounds because steroids, being larger molecules, can more effectively distribute excess energy imparted by electron capture.

Usually, the presence of a peak for $[M-H]^-$ indicates a low-response compound, except for epoxysteroids. For saturated ketosteroids, the hydrogen alpha to a carbonyl is lost. In the case of epoxysteroids, an $[M-H]^-$ peak will be present if a hydrogen on the carbon beta to the epoxide is available.

The reduction potentials in acetonitrile can be assumed to be indicative of the trends of electron affinity. In a series of ketosteroids, a half-wave reduction potential

more positive than -2.0 V indicates a high-response compound, except in the cases of epoxysteroids.

The choice of reagent gas can enhance the electron-capture response. Ammonia selectively increases the responses of compounds with $[M-H]^-$ peaks. A better choice for reagent gas for ECNI studies is isobutane, which is nonselective and has a better response than methane.

PCC oxidation is the method of choice for the chemical oxidation of steroids; alternative oxidative methods were also investigated. The TPAP oxidation had a comparable yield and reaction time as the PCC oxidation. Electrochemical oxidation had a very poor yield due to fouling of the electrode. Subsequent experiments that place the electrode directly into the mass spectrometer source also did not work.

An improvement of the oxidation technique may be possible using HPLC-electrochemistry-thermospray mass spectrometry. Using this technique (1), the oxidation products of oxymethalone were analyzed. Not many steroids were amenable to this technique; for example, testosterone did not oxidize at all. Perhaps the highly conjugated steroids like dexamethasone or prednisolone could be successfully analyzed by this technique.

The oxidation methodology was extended to the analysis of other real-world samples. Dexamethasone in human plasma was analyzed and compared to RIA values. The GC-MS values did not correlate with the RIA values and also tended to be less precise. Using a structurally-similar internal standard, the limit of detection was 0.03 ng/mL, which is better than the limit of detection (0.25 ng/mL) obtained in a study using an isotopically-labelled internal standard.

The oxidation methodology is a good method for determining prednisolone content of mouse plasma if prednisolone is not present. The LOD is 0.51 ng/mL, which is on the order of competing methodologies such as HPLC. For the samples analyzed, the dosed mice had about 2 ng/mL prednisolone in their bloodstream.

Based on the ECNI response studied and what is known about the oxidation methodology as well as other electron-capturing derivatives, predictions can be made about the extension of ECNI methodologies to other compounds. Some of these predictions are based on preliminary work contained in this dissertation.

The presence of epoxides increases the relative responses of ketosteroids. Many trichothecine molecules also contain epoxide groups. However, the presence of an epoxide group does not seem important for the overall ECNI responses of trichothecines. The utility of epoxides for enhanced response may be confined to the steroid nucleus and is not as applicable to other biological molecules.

Sensitive assays are needed for anabolic steroids because of problems with abuse. Most anabolic steroids are not amenable to oxidation techniques because they are analogs of testosterone. Testosterone and its analogs oxidize to 4-androsten-3,17-dione, a compound with poor electron-capture response. Other anabolics have a 17α-methyl group, which prevents the oxidation at this position. The only anabolic that seems promising for assay development is fluoxymesterone. This steroid can be oxidized to a compound with the 1,4-dien-9α-fluoro-3,11-dione system, which has excellent electron-capture properties. Other anabolic steroids must be derivatized for ECNI analysis. Based on the ECNI studies of 5α-androstan-3β,17β-diol, HFB derivatives are probably the best choice.

Many steroidal drugs containing fluorine can be oxidized to structures for which assay development seems promising. The oxidized products of flumethasone, fluorandrenolone, and fluocinolone will have the 1,4-dien-6α,9α-fluoro-3,11-dione system. Triamcinolone will oxidize to a steroid containing the 1,4-dien-9α-fluoro-3,11-dione system as well as two adjacent carbonyls at C-16 and C-17 in the D ring. Preliminary studies of the oxidation of triamcinolone have suggested that the PCC conditions described in this dissertation do not work well; perhaps either a different oxidant or more stringent conditions are necessary.

Other candidates for advantageous assay development are steroids with the 5-en-3-ol moiety. This functionality oxidizes to 4-en-3,6-dione, which, regardless of the other substituent on the molecule, captures an electron readily. Compounds with the 5-en-3-ol structure include sterols such as jervine (an azasteroid), cholesterol, and ergosterol (5,7,22-cholestatrien-3-ol).

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