This is to certify that the

dissertation entitled

Mass spectrometric studies of molecules modified for negative chemical ionization

presented by

Guo-Rong Wei

has been accepted towards fulfillment
of the requirements for

PhD degree in Chemistry

J. Throck Watson
Major professor

Date 5 August 1985

MASS SPECTROMETRIC STUDIES OF MOLECULES MODIFIED FOR ANALYSIS BY ELECTRON CAPTURE NEGATIVE CHEMICAL IONIZATION

By

Guor-Rong Her

A DISSERATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1985

ABSTRACT

MASS SPECTROMETRIC STUDIES OF MOLECULES MODIFIED FOR ELECTRON CAPTURE NEGATIVE CHEMICAL IONIZATION

By

Guor-Rong Her

The sensitivity and selectivity of gas chromatography-mass spectrometry for certain analytes can be improved if the mass spectrometer is operated under the conditions for electron capture negative chemical ionization. In general, this technique is not suitable for most unmodified steroids due to their low electron affinities and/or small electron capture cross-sections. New procedures which can be used to modify steroids for analysis by electron capture negative chemical ionization are presented in this dissertation. The highly conjugated dicyanomethylene derivatives of C-19 keto steroids can be prepared through a condensation reaction with malonitrile. Alternatively, corticosteroids can be oxidized to 1,4-androstadien-3,11,17-trione or 4-androsten-3,6,17-trione structural analogs; under electron capture negative chemical ionization conditions, these oxidized corticosteroids show over two orders of magnitude higher response than many endogenous steroids. In addition, the detection limit of oxidized corticosteroids have been measured to be approximately 10 pg which is at least 10 times more sensitive than

existing gas chromatography-mass spectrometry methods. Based on these observations, a sensitive and specific methodology for the quantitative analysis of corticosteroids from biological fluids was develpoed and demonstrated in the quantitative analysis of horse urine for dexamethasone.

During the course of studying dicyanomethylene(DCM) derivative for keto compounds, unusual electron impact mass spectra were observed for DCM derivative of benzophenone analogs. Careful investigation of fragmentation pathways using a deuterium labelled compound led to the proposal of a mechanism involving rearrangement of two hydrogen atoms to rationalize the cleavage of a double bond for DCM derivative of benzophenone analogs. In addition, unusual methane negative chemical ionization mass spectra were observed for DCM derivatives of both benzophenone and 9-fluorenone. In this case, following investigation by positive chemical ionization mass spectrometry and mass spectrometry / mass spectrometry techniques, a molecule / radical reaction prior to electron capture is proposed to account for the unusual behavior observed in the negative ion mass spectra of benzophenone-DCM and 9-fluorenone-DCM.

ACKNOWLEDGEMENTS

I would like to sincerely thank my preceptor, Professor J. T. Watson, for his guidance, support, and friendship during the course of my graduate training at Michigan State University.

I appreciate the counseling and help given me by Professor J. H. Holland, Brain Musselman, Manager of the Mass Spectrometry Facility, and Mr. Mike Davenport concerning the modification of an HP 5985A GC/MS for negative ion detection. Many thanks go to the members of Dr. Watson's laboratory and the MSU/NIH Mass Spectrometry Facility for their friendship and advice.

Finally, I wish to thank my wife Chi-Ping and my parents for their support and encouragement which made the accomplishment of this dissertation possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Negative Ion Mass Spectrometry

Introduction:

It is a historical fact that low pressure negative ion mass spectrometry has never been used as much as its positive ion counterpart. This is partly due to a lack of the instrumental capabilities necessary for negative ion detection, but mainly due to the fundamental difference in the nature of negative ion production. Under electron-impact conditions, most organic compounds do not form stable anions in conventional mass spectrometer sources, and when negative ions are formed, the ion currents are often weak when compared with the positive ion currents. The efficient production of negative ions in the high pressure(e.g., chemical ionization) ion source has stimulated a resurgence of interest in negative ion mass spectrometry(1-3). The development of this field has been particularly rapid since the demonstration in 1976 by Hunt et al.(4) of the large sensitivity increase for compounds which possess a positive electron affinity and a large cross section for electron capture in the negative ion CI mode.

The level of interest in negative ion work is indicated by the fact that most current generation commerical instruments are now available with both positive and negative ion capabilities.

1. Formation of Negative Ions

Negative ions can be formed via interaction of a molecule and an electron(5-7) and via interaction of a molecule and a negative reagent ion(8). These are shown below:

a. Nondissociative resonance capture

$$AB + e^- \rightarrow AB^- \qquad (E_e = \sim 0 \text{ eV})$$

b. Dissociative resonance capture

$$AB + e^- \rightarrow A^- + B \qquad (E_e = 0\text{-}15 \text{ eV})$$

c. Ion pair formation

$$AB + e^- \rightarrow A^- + B^+ + e^- \qquad (E_e \geq 10 \text{ eV})$$

d. Negative ion / molecule reaction

Ex: $AB + C^- \rightarrow AB^- + C$

Nondissociative resonance capture is a prerequisite for the formation of a molecular anion capable of detection by mass spectrometer. Figure 1 shows the potential energy curves which describe the resonance electron capture process. Molecule AB captures an electron and undergoes a vertical electronic transition within the cross-hatched area, in accordance with the Frank-Condon principle, to the upper curve.

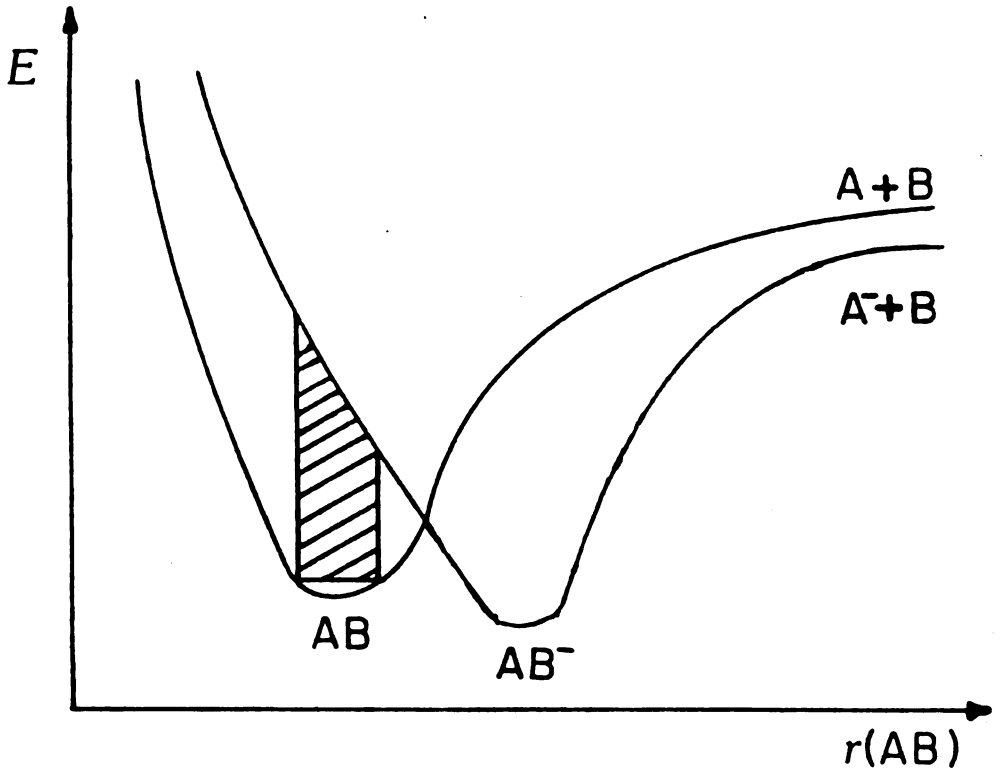


Figure 1. Potential energy curve for non-dissociative resonance capture.

This process requires a thermal electron or a near thermal electron (E_e approximately 0 eV) and is exothermic. That is, the anion formed is more stable than the parent molecule. However, the anion is produced in a vibrationally excited state and some of this energy must be removed or delocalized if the anion is to be stabilized. If no mode of stabilization is available, or if stabilization does not take place rapidly, either dissociation or electron autodetachment will occur. In general, there are four ways to stabilize the molecular anion. In one way, the molecular anion is stabilized by delocalizing the charge through a conjugated system. This is observed for aromatic systems containing electron-withdrawing substituents(9-12). The second way is one in which the excitation energy can be partitioned into degenerate vibrational degrees of freedom, thus, enchancing the stability of the molecular anion. The third way to stabilize the molecular anion is through collisional stabilization. The excited molecular anion can lose some of its excess energy through the collision with other particles which exist in the ion source(2). The forth way is to have the anion emit one or more infared photons.

The second electron capture process, dissociative electron capture, as depicted in figure 2, can occur with higher energy electrons (E_e = 0 - 15 ev).

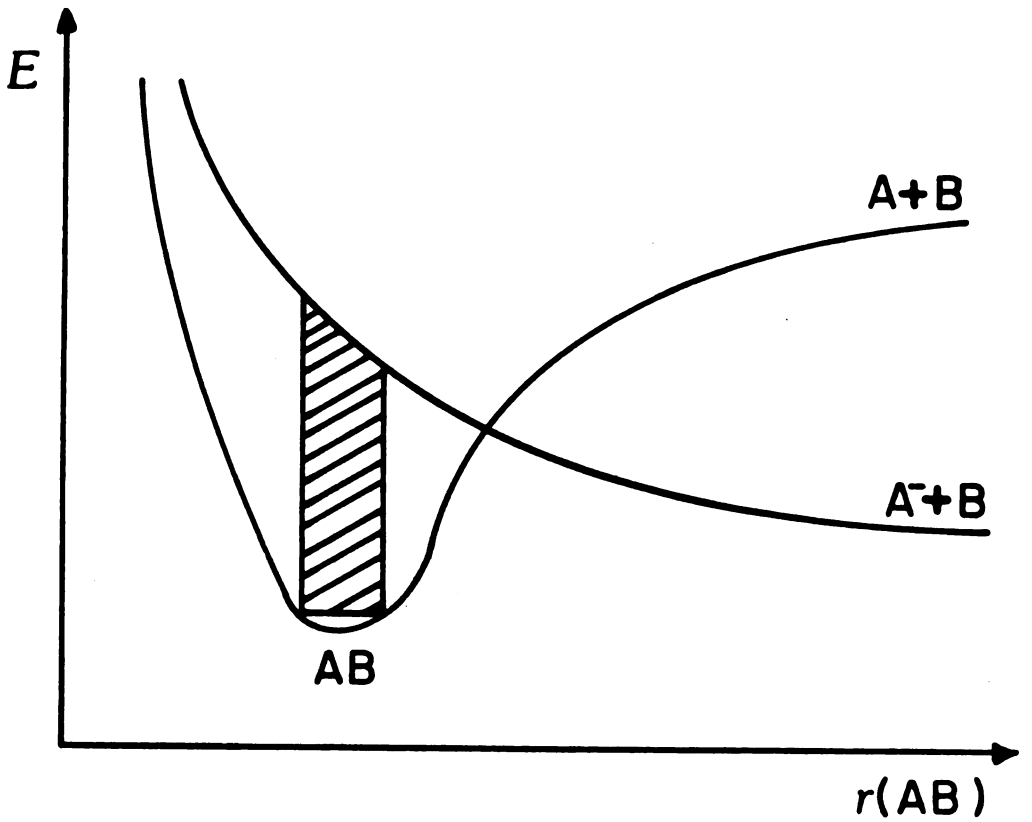


Figure 2. Potential energy curve for dissociative resonance capture

During electron capture, AB^- is formed in a repulsive state, the excited anion fragments in order to dissipate its excess energy.

The third process, ion pair formation, occurs at electron energies above 10 eV and is depicted in figure 3.

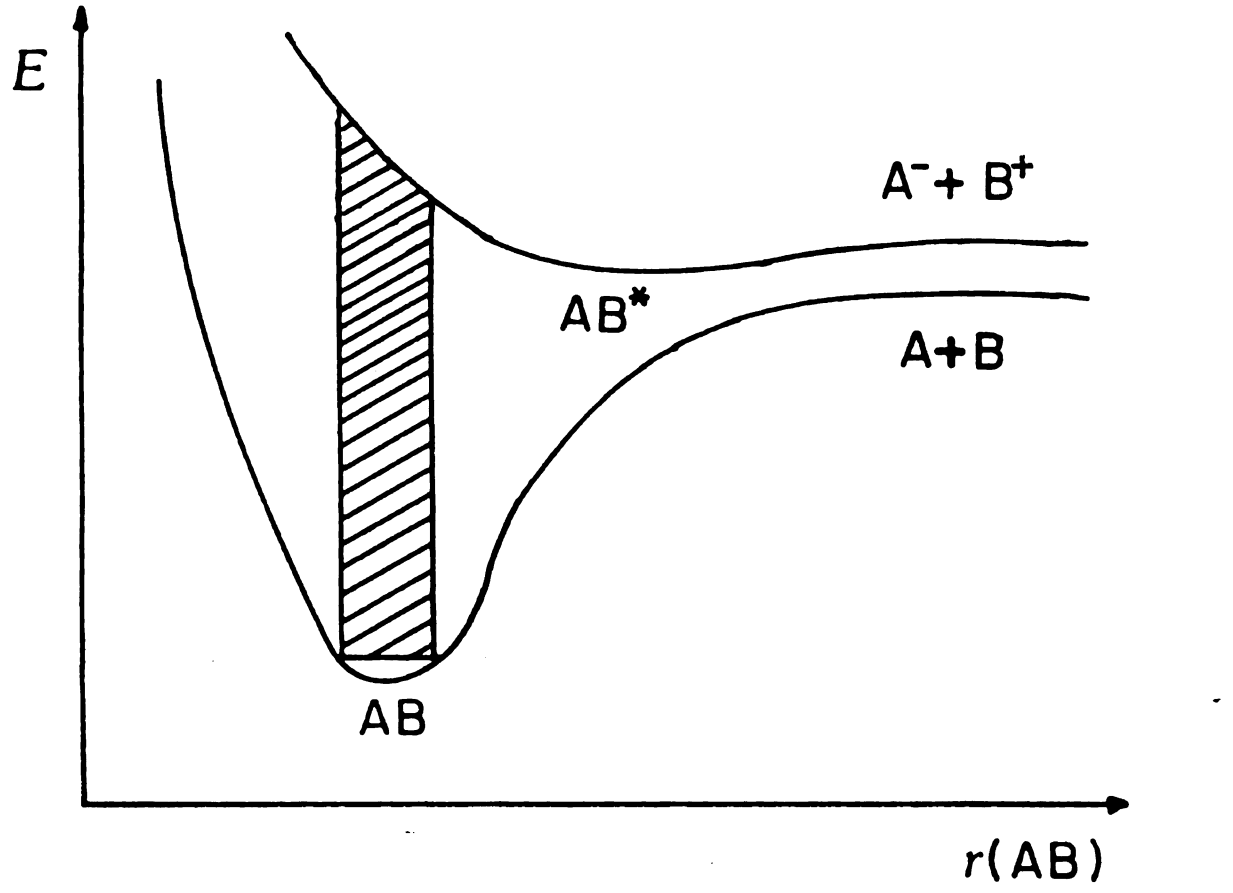


Figure 3. Potential energy curve for ion pair formation.

This is a nonresonant process. The electron is not captured, but merely acts as a source of energy. The collision between the incident electron and the molecule imparts sufficient energy to the molecule to excite it to a dissociative state corresponding to production of both a positive and a negative ion from the parent molecule.

The last process, the ion / molecule reaction, occurs between ions and neutrals in the ion source. At higher source pressures, a large number of ion / molecule reaction types can also occur, including anion / molecule adduct formation, charge transfer, displacement, abstraction, etc. The reactions vary depending on the negative reagent ion used(13).

2. Conventional Electron Impact Negative Ion Mass Spectrometry (Negative EI)

With the negative ion production processes in mind, it is easy to see why the condition under which an EI mass spectrometer normally is operated does not favor negative ion analysis. Because the production of a large number of electrons in a restricted low energy range at low pressure is a difficult experimental task, the EI mass spectrometer is normally operated at high vacuum conditions (10^{-5} - 10^{-7} torr) using an electron beam with an energy on the order of 10 to 70 eV. With these high energy electrons, the primary negative ion formation processes are dissociative electron capture and ion - pair formation, leading to fragmentation of a target molecule. Hence, Most of the negative ion sample current is carried by low mass species such as H^- , OH^- , Cl^- , CN^-, NO_2^-, etc.(14). Obviously, very few thermal electrons are present in the normal EI source and thus, molecular anions formed by the interaction of molecules and thermal electrons are rarely seen. The condition discussed above: the small concentration of molecular ions verses the large concentration of low mass fragment ions, is one of the major disadvantages of negative EI. The other major disadvantage of negative EI is that negative ion currents produced in negative EI have

been up to 10^3 times smaller than currents of its positive ion counterpart. This results in poor sensitivity.

Despite the above difficulties, negative EI has found limited utility in the analysis of polynitroaromatic(15-16), organometallic(17), organoboron(18), polyfluoro(19-20), organosulfur and organophosphorous compounds(21). Spectra of these compounds often exhibit both molecular anion and fragment ions. Interaction of sample molecules with a small population of low energy electrons produced from a surface is assumed to be responsible for the generation of molecular anions.

In spite of the valuable work done in negative EI, it seems unlikely that negative EI will be widely used.

3. Electron Capture Negative Chemical Ionization Mass Spectrometry (EC-NCI)

The capture of electrons by polyatomic molecules is a resonance process which normally requires electrons of near-thermal energy. The first instrument which effectively produced thermal electrons was designed by Von Ardene(22), who employed a low pressure (10^{-4} torr) argon discharge to generate a plasma containing positive argon ions and a large population of low energy electrons. The discharge plasma was constrained by a strong magnetic field and the sample was placed inside this plasma(23). The cost and the extensive nature of the modification required to facilitate attachment of the dual plasma source to a commerically available mass spectrometer are the main reasons for its

limited use.

In chemical ionization mass spectrometry (CI), a plasma of positive reagent ions is generated by electron bombardment of reagent gas. Subsequently, these positive reagent ions ionize the sample molecule via ion / molecule reactions(24). In addition, and of particular interest to negative ion analysis, thermal electrons are formed as a consequence of the ionization of reagent gas. For example, the following reactions are involved in the formation of methane reagent ions:

a. $CH_4 + e^- \rightarrow CH_4^+ + 2\ e^-$

b. $CH_4 + e^- \rightarrow CH_3^+ + H^\cdot + 2\ e^-$

c. $CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3^\cdot$

d. $CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$

of particular interest are reactions a and b.

The bombardment of a methane molecule by a high energy electron produces an excited methane molecule. The energy of the "incident electron" (primary electron) is reduced approximately 30 eV with each ionization event(25). The excited methane molecule emits an electron (secondary electron) to form a positive ion (CH_4^+). The primary electron and secondary electron are either completely stopped or largely stopped by subsequent ionization and collision in approximately 1 torr of gas in the mass spectrometer CI chamber. Thus, a flux of high energy

electrons will produce electrons with thermal energy which can rapidly be captured by the high electron affinity analytes. The high pressure of the gas not only provides a way for the production of thermal electrons but also provides a way for the stabilization of molecular anions.

If high pressure gases which do not produce negative reagent ions are used, negative ions of the analyte are assumed to be produced only by resonance capture mechanisms (dissociative and nondissociative). Since only electron capture processes occur in the production of negative ions, the term electron capture negative chemical ionization(EC-NCI) is used in this research to describe this technique. Although negative chemical ionization (NCI) is the most popular name, it does not describe this technique properly. In this thesis, negative chemical ionization (NCI) will be used only to describe a technique that produces negative ions mainly by negative ion / molecule reactions.

4. Negative Chemical Ionization Mass Spectrometry (NCI)

If the high pressure gas used can produce a high population of negative reagent ions, then true negative chemical ionization becomes the dominant ionization process. Many different types of negative ions have been used in the NCI technique, such as the chloride ion(26), the superoxide ion(27), the atomic oxygen ion(28), the methanolate ion(4), and the popular hydroxy ion(29-30).

5. The Useful Aspects of EC-NCI and NCI

EC-NCI shows advantages over EI and PCI in sensitivity and selectivity in published work. On the other hand, NCI reportedly has high selectivity in some cases but does not show a clear advantage in selectivity.

a. Sensitivity

If one assumes that an ion / molecule reaction proceeds with unit efficiency, then the positive and the negative ion / molecule reaction (PCI and NCI) each has an approximate rate constant of 10^{-9} $cm^3sec^{-1}molecule^{-1}$(31). Because PCI and NCI have the same maximum rate constant, one has no reason to expect <u>a priori</u> that a very large difference in sensitivity will be found between NCI and PCI. In fact, no report has been published which demonstrated a significant difference in sensitivity of these two techniques. For EC-NCI, if it is assumed that the unit efficiency is also correct for the electron capture process. The electron capture process has a approximate rate constant of $4X10^{-7}$ $cm^3sec^{-1}molecule^{-1}$(32), which is 400 times greater than either positive or negative ion / molecule reactions. Thus, EC-NCI should be able to achieve a sensitivity 400 times greater than ion / molecule reactions(PCI or NCI). In fact, by using pulsed positive / negative chemical ionization mass spectrometry, Hunt has reported that negative ion currents can be as much as 1000 times greater than positive ion currents which are produced under the same source conditions(4). His group has subsequently reported detection of various fluoro-substituted

compounds at levels in the range of 25 femtograms(33). This level has never been reported by conventional EI and PCI techniques.

b. Selectivity

EI is considered to be a universal ionization technique. Since most compounds have ionization pontentials less than 15 ev which is much smaller than the 70 eV electron beam used, they show similar ionization efficiency. For EC-NCI, the ionization efficiency is mainly determined by the electron affinity and electron capture cross-section of compounds. Because many organic compounds have negative electron affinities and because the rate constants of organic compounds are strongly structure dependent, EC-NCI can be a highly selective technique for the detection of compounds which have positive electron affinities and high electron capture cross sections.

The selectivity of NCI depends on the negative reagent ion used. For example, hydroxyl ion (OH^-) NCI is not considered to be a highly selective technique because the conjugated bases(M^-) of most organic compounds(MH) have lower basicities than the OH^- ion($D°(M^- - H^+) < D°(OH^- - H^+)$), therefore, it will react with a wide range of compounds by abstraction of protons from those compounds. On the other hand, chloride ion (Cl^-) NCI is considered a selective technique because many organic compounds have higher basicities than the chloride ion, therefore, the most common reaction is chloride attachment. Chloride ions attach strongly with acidic compounds such as carboxylic acids, amino acids etc., but do not react with basic compounds such as tertiary

amines, nitriles etc.

The selectivity cited above for chloride ion is illustrated by Dougherty et al.(34) Under identical ion source conditions, both positive and negative ion mass spectra are generated from an extract of chicken tissue that contained approximately 0.1 ppm Dieldrin. The PCI mass spectrum showed a homologous series of ions corresponding to neutral lipids that came through the Fluorosil cleaning procedure. Although there are also ions correspounding to Dieldrin minus chloride and protonated Diedrin, their prominence in the spectrum is limited. In the negative ion spectrum, because chloride ion cannot react with neutral lipids, the only ions of any prominence corresponded to the chloride adduct of Dieldrin.

6. Major Objective

A large part of recent research in this EC-NCI has been directed at environmental chemistry(35-37). This is because many of the xenobiotic compounds that have been introduced into the environment as agricutural chemicals and industrial waste products are halogenated species that inherently have strong electron capture properties. The remaining organic molecules in the samples are of biological origin and generally have low electron affinities, which makes them transparent to EC-NCI. Thus the EC-NCI effectively discriminates in favor of the halogenated pollutants, allowing their detection in small quantities in the presence of a large quantities of other diverse biological molecules.

In general, biological molecules are highly reduced and have low electron affinity. Therefore most biological compounds are generally not suitable for detection by EC-NCI. The key to the application of EC-NCI for detection and quantitation of such compounds is the development of chemical derivatization procedures that enchance the electron affinity of the molecules. The major objective of this research was to look for appropriate high electron affinity derivatives for steroids. Because the existing GC/MS methods for the analysis of corticosteroids are poor compared to other types of steroids, special effort was put on finding appropriate derivatives for the analysis of corticosteroids with EC-NCI.

Chapter 2: Dicyanomethylene(DCM) and Pentafluorophenyl acetonitrile(PFA) Derivatives for Keto-Steroids

Introduction

Steroid hormones are important metabolites in human beings and animals. They are generally divided into five major classes: progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens. Progesterone, a progestagen, prepares the lining of the uterus for implantation of an ovum. Progesterone is also essential for the maintaining of pregnancy. Androgens (such as testosterone) are responsible for the development of male secondary sex characteristics, whereas estrogens(such as estradiol) are required for the development for female secondary sex characteristics. Glucocorticoids (such as cortisol) promote the formation of glycogen and enchance the degradation of fat and protein. Mineralcorticoids (such as aldosterone) increase reabsorption of Na^+, Cl^-, and HCO_3^- by the kidney, which leads to an increase in blood volume and blood pressure.

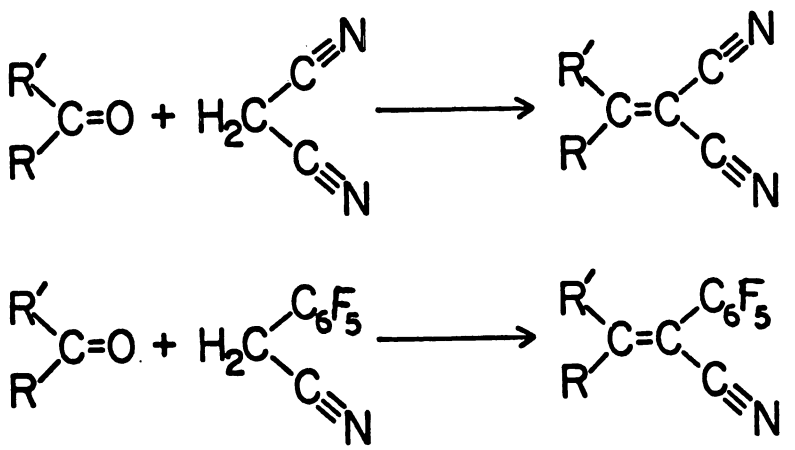
Many steroids have been studied by gas chromatography with electron capture detection(ECD)(38-40). Because most steroids have little or no intrinsic electron capture properties, electrophores must be introduced in to the molecules by derivatization in order to increase their responses to ECD. Steroids with polar functional groups such as hydroxyl, phenolic, or carbonyl groups, often require prior

derivatization to improve their thermal stability and chromatographic performance. In such cases, derivatization is not an extra chemical step but an integral part of the analytical scheme. A reagent is selected not only based on its ability to increase electron affinity of the analyte but also based on its ability to increase thermal stability and volatility of the analyte. Because of the long history of ECD, many derivatization reagents suitable for steroid analysis with GC/ECD have been developed(41-47). The halocarbonacylating compounds, pentafluorophenyl dimethylsilyl (Flophemesyl), and o-(pentafluorobenzyl) hydroxylamine (Florox) are the most widely used derivatization reagents. Steroid derivatives prepared with these reagents generally show good response and good chromatographic performance under ECD conditions.

ECD and electron capture NCI are considered to be similiar analytical techniques because the mechanisms of negative ion formation by these two techniques are very similar. Because of the similarity between these two techniques, it is expected that the electrophilic derivatives which are widely used in ECD can also be used in EC-NCI. However, quite often, the use of these ECD reagents in EC-NCI produce negative ion mass spectra with intense ions whose origins are largely dictated by the fluorinated derivatization reagent(48,49). For example, in an analysis of testosterone by electron capture NCI, the pentafluorobenzyl oxime (Florox) derivative was used(49). The negative ion mass spectrum of this derivatized testosterone is characterized by the absence of a distinguishable molecular ion and with the presence of the most intense ion($C_6F_5CH_2^-$), which represents the derivative

moiety(Figure 4). Selected ion monitoring of ion current corresponding to the $C_6F_5CH_2^-$ ion may show very good sensitivity. However, the specificity is poor. The major problem for the application of these "standard" electrophilic derivatives is that the derivatization moiety fragments so easily under EC-NCI conditions. These observations point to the need to develop new electrophilic derivatives which would resist fragmentation under EC-NCI.

The new derivatives must meet two criteria. First, they must contain a high electron affinity functional group in order to enhance the electron affinity of the analyte. Second, they must form two bonds rather than one bond with the analyte so that it will be less likely to cleave from the analyte during the ionization process. Two derivatives which meet these criteria are the dicyanomethylene(DCM) and the pentafluoroacetonitrile(PFA) derivatives(Scheme 1). Both derivatives contain high electron affinity functional groups, such as cyano and/or pentafluorophenyl groups, and both derivatives form a double bond with the ketonic analyte through a condensation reaction.

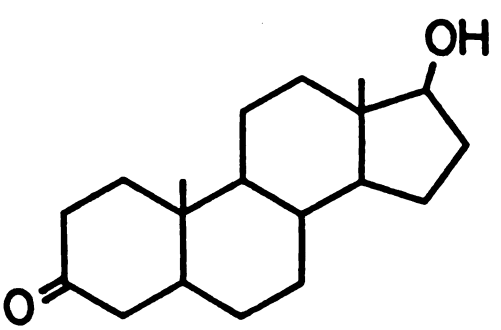


Scheme 1

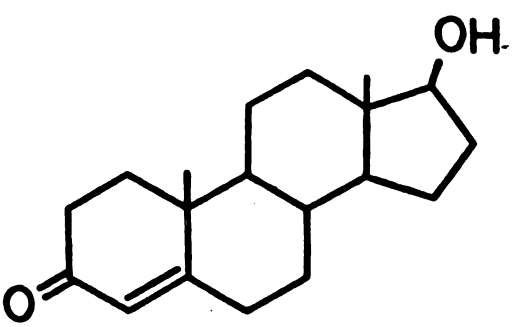
Experimental section:

1. Materials

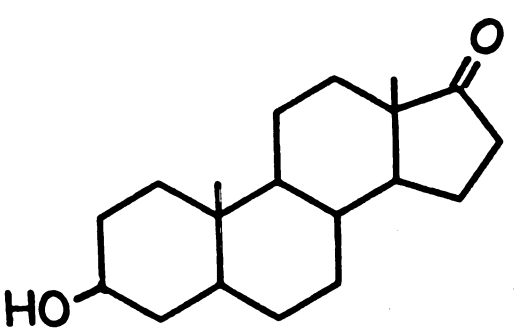
Dihydrotestosterone(5α-androstan-17β-ol-3-one)(I), testosterone(4-androsten-17β-ol-3-one)(II), androsterone(5α-androstan-3β-ol-17-one)(III) were purchased from Steraloids Inc.; dicyanomethane, ammonium acetate, acetic acid, piperidine,β-alanine from Aldrich Inc.; pentafluoroacetonitrile from PCR Research Chemical Inc.; bis(trimethyl-silyl)-trifluoroacetamide(BSTFA) from Pierce Inc.



(I)



(II)



(III)

2. Instrumentation

The gas chromatography(GC) was performed on a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector(FID) or on a Sigma 3A gas chromatograph equipped with an FID and electron capture detector(ECD). FID is used for monitoring the chemical reactions and establishing retention times which are necessary in preparing for a GC/MS run. Because of the similarity between ECD and EC-NCI, the ECD response is used as a guide for those derivatives expected to have high electron affinity in EC-NCI.

A Hewlett Packard 5985A GC-MS was modified for negative ion detection based on the conversion dynode concept. The details of the modification are discussed in chapter 5. The instrument was modified so that a capillary column on- column injector(J&W Scientific, Inc.) and a GMCC/90 open split interface (Scientific Instrument Services, Inc.) can be mounted on the instrument. The GC-MS conditions were as follows: (1) Methane was used as the modification gas, source pressure approximately 0.4 torr, source temperature 200°C; (2) A 3m 3% OV-101 packed column or a 25m SE-54 cross-linked widebore open-tubular capillary column was used. Helium was used as carrier gas. Head pressure for the capillary column was 20 psi; (3) The packed column was operated at 230°C isothermally and the capillary column was used with temperature programming from 200°C to 250°C at 20°C/min, then from 250°C to 300°C at 5°C/min.

3. Synthesis of DCM derivative

2.5 mg of steroids and 40mg dicyanomethane were dissolved in 0.5 ml ethyl acetate. 56 mg ammonium acetate and 9 drops acetic acid were added as catalysts. The solution was heated at 90°C for 1 hour and then separated by preparative TLC. Toluene:ethyl acetate(1:1) was used as the elution solvent. The DCM derivative band in the preparative TLC was scraped off the plate and washed with ethyl ether. The crude product was recrystallized from ethyl ether and n-hexane.

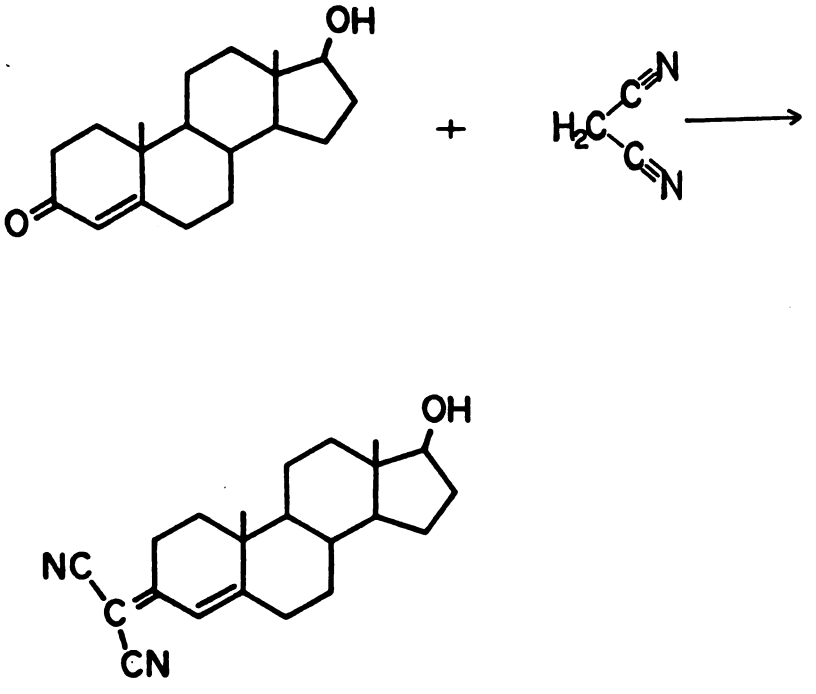
4. Synthesis of PFA derivative

2.5 mg of dihydrotestosterone and 40mg PFA were dissolved in 0.5 ml ethyl acetate. 56 mg ammonium acetate and 9 drops acetic acid were added as catalyst. The solution was heated at 90°C for 10 hours. It was then separated by preparative TLC as described above.

Results and Discussion:

1. Dicyanomethane (DCM) Derivative

Several DCM derivatives were synthesized by the condensation reaction of keto-steroids with dicyanomethane as shown in Scheme 2.



Scheme 2

The highly conjugated electrophilic functional group is believed to increase the response of those steroids under ECD and electron capture NCI conditions. Furthermore, the electrophilic functional group is connected through a double bond to the parent molecule. Hence, when the molecule is ionized in the ion chamber, the electrophilic functional group is more firmly bonded to the parent molecule than are many of the "standard" ECD derivatives.

A. The Choosing of A Catalyst System

Several different catalyst systems such as ammonium acetate/acetic acid(50), piperidine(51), and β-alanine(52) have been used in the condensation reaction between dicyanomethane and ketones. The choice of catalyst system and appropriate solvent for derivatizing steroids was evaluated by the condensation reaction of testosterone and dicyanomethane. The study showed that using ammonium acetate/acetic acid as the catalyst and ethyl acetate as the solvent gave the best result. The desired product was produced with 90% yield after refluxing the reaction mixture for 40 minutes.

B. The Negative Ion Mass Spectra of DCM Derivatives

The negative ion mass spectra of DCM keto-steroids are characterized by a base peak which respresents the molecular ion and a major fragment ion which respresents $(M-27)^-$, presumably due to the loss of HCN. The negative ion mass spectrum of DCM testosterone is shown in Figure 5. The base peak at m/z 336 respresents the molecular ion. The only other significant peak at m/z 309 respresents the loss of HCN. The negative ion mass spectrum of free testosterone(molecular weight 288) also shown in Figure 5, shows no discernible peak representing M^-, but instead a base peak at m/z 287 which is $(M-H)^-$.

C. The Advantage in Response

The improvement in response due to DCM derivatization was evaluated by coinjection of one microgram of derivatized and free testosterone into a 3m 3% OV-1 column for EC-NCI detection. The EC-NCI response to the DCM derivative of testosterone is approximately 40 times greater than the response to free testosterone under the same conditions. This factor was determined by comparing the area in the mass chromatogram at m/z 336, (M^-), for the DCM derivative with the area in the mass chromatogram at m/z 287, $(M-1)^-$, for free testosterone(see figure 6).

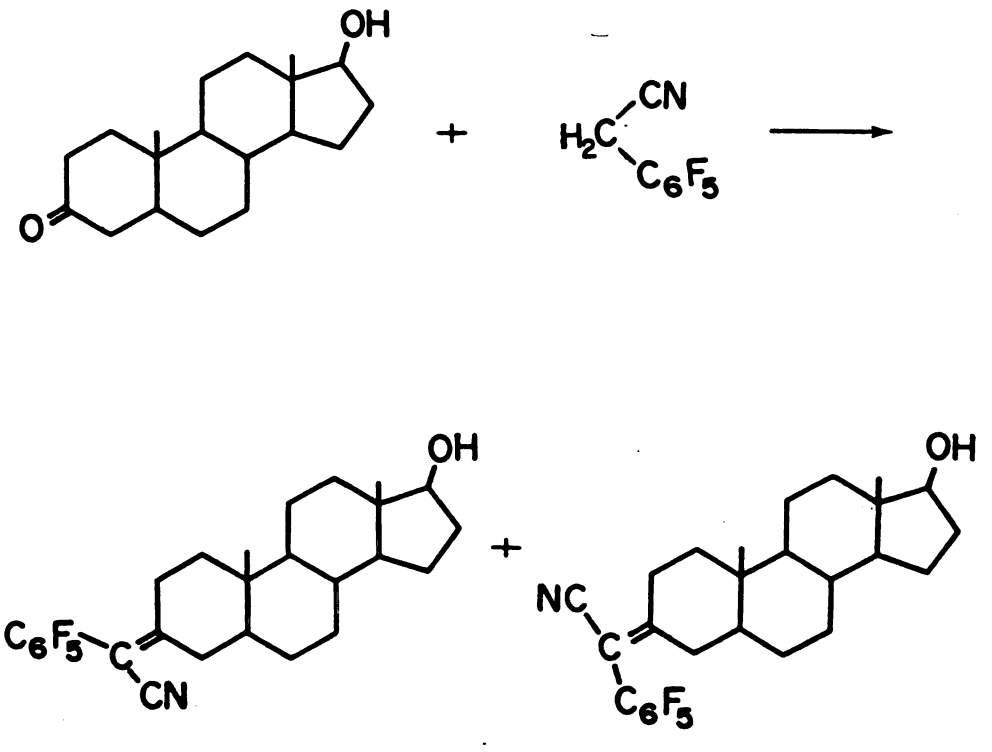
D. The Advantage in Chemical Selectivity

Three steroids were chosen as model compounds: dihydrotestosterone(I)(a saturated ketone), testosterone(II) (a conjugated ketone), and androsterone(III)(a sterically hindered ketone). Under identical conditions, the unconjugated ketone(I) exhibited the fastest reaction. For example, under reaction conditions of a two-fold stoichiometric excess of dicyanomethane with ammonium acetate/acetic acid catalyst at 80°C for 20 minutes, compound I(dihydrotestosterone) showed approximately 100% conversion to the DCM derivative, compound II(testosterone) showed approximately 50% conversion, and compound III showed only a 5% conversion. The 17 keto-steroid(compound III) showed slower DCM formation rate than the other two model compounds due to the steric hindrance of the 17-ketone group. Because the 11-ketone group is considered to have greater steric hindrance than 17-ketone group, the relative high rate for the formation of DCM derivative of 3-ketone

provides a means to selectively detect 3-keto-steroids in the presence of 11- or 17- keto-steroids.

2.Pentafluorophenyl Acetonitrile (PFA) Derivative

DCM derivatives of steroids showed good potential in selectivity against free steroids, however, the detection limit of DCM derivatives is in the order of 300pg under EC-NCI conditions. In order to increase the sensitivity of keto-steroids to EC-NCI, a modified DCM derivative, PFA, in which a cyano group in DCM was replaced by a high electron affinity group(C_6F_5) was studied. The formation of PFA derivative is shown in Scheme 3.



Scheme 3

It was hoped that the pentafluorophenyl group would increase the response of derivatized steroids and at the same time form a double bond with parent molecules to resist fragmentation under electron capture NCI as did the DCM derivatives.

A. The Negative Ion Mass Spectrum of PFA Dihydrotestosterone

After the PFA of dihydrotestosterone was converted to its 17-TMS derivative, it was analyzed by capillary column EC-NCI. Four peaks appear in the reconstructed total ion current as shown in Fig 7, they all have the same molecular weight and similar EI spectrum, so, it is possible that they are isomers. Because only cis and trans isomeric products are expected for the formation of the PFA derivative, it is not clear why four isomeric products are produced. The EC-NCI mass spectra of these four isomers are shown in Figure 8. For peak 1 and peak 3, the EC-NCI mass spectra are characterized with two major ions, M^-(m/z 551), and $(M-HF)^-$(m/z 531). Two more fragment ions, $(M-TMSH)^-$(m/z 477) and $(M-TMSH-HF)^-$(m/z 457) are observed in the EC-NCI mass spectra of peak 2 and peak 4.

B. The Advantage in Response

The PFA-TMS dihydrotestosterone showed approximately 1/4 the response of that of oxidized dexamethasone(a standard compound normally used in this research which is discussed in chapter 3) under EC-NCI(Figure 7). Because of the low detection limit for oxidized dexamethasone(10pg by SIM), a good detection limit is expected for PFA derivatives.

C. The Advantage in Chemical Selectivity

The condensation reaction was evaluated with three different types of steroids (saturated 3-ketone(I), conjugated 3-ketone(II), and saturated 17-ketone(III)). It was found that only the saturated 3-ketone(I) could react with PFA. Either the steric hindrance of 17-ketone(III) and the conjugated 3-ketone(II) make the condensation reaction difficult. For compound I(dihydrotestosterone), the reaction produced 30% of PFA product when the solution was refluxed for 10 hours at 80°C. Because PFA can selectively derivatize nonconjugated 3-keto-steroids, a selective and sensitive method could be developed for the analysis of nonconjugated 3-keto-steroids from biological sources without extensive sample clean up procedures.

Conclusion

Although the sensitivity of DCM derivative is not as good as expected, it shows good potential in selective detection of 3-keto-steroids in the presence of other steroids. The DCM derivative frequently has a retention time twice that of the free compound under the same GLC conditions. However, the DCM testosterone elutes before free testosterone from an analytical HPLC column packed with 10 micron Si-60(35% EtOAc/hexane is used as the eluent). Thus, whereas the DCM derivative may have some drawbacks in the vapor phase analysis of high molecular weight ketonic compounds, it may offer advantages for mass spectrometric analysis of these compounds using an HPLC inlet.

The PFA derivative shows good potential both in sensitivity and in selectivity for the analysis of unconjugated 3-keto-steroids. The major problem for this derivative is the relatively long reaction time(30% yield for 10 hours at 80°C).

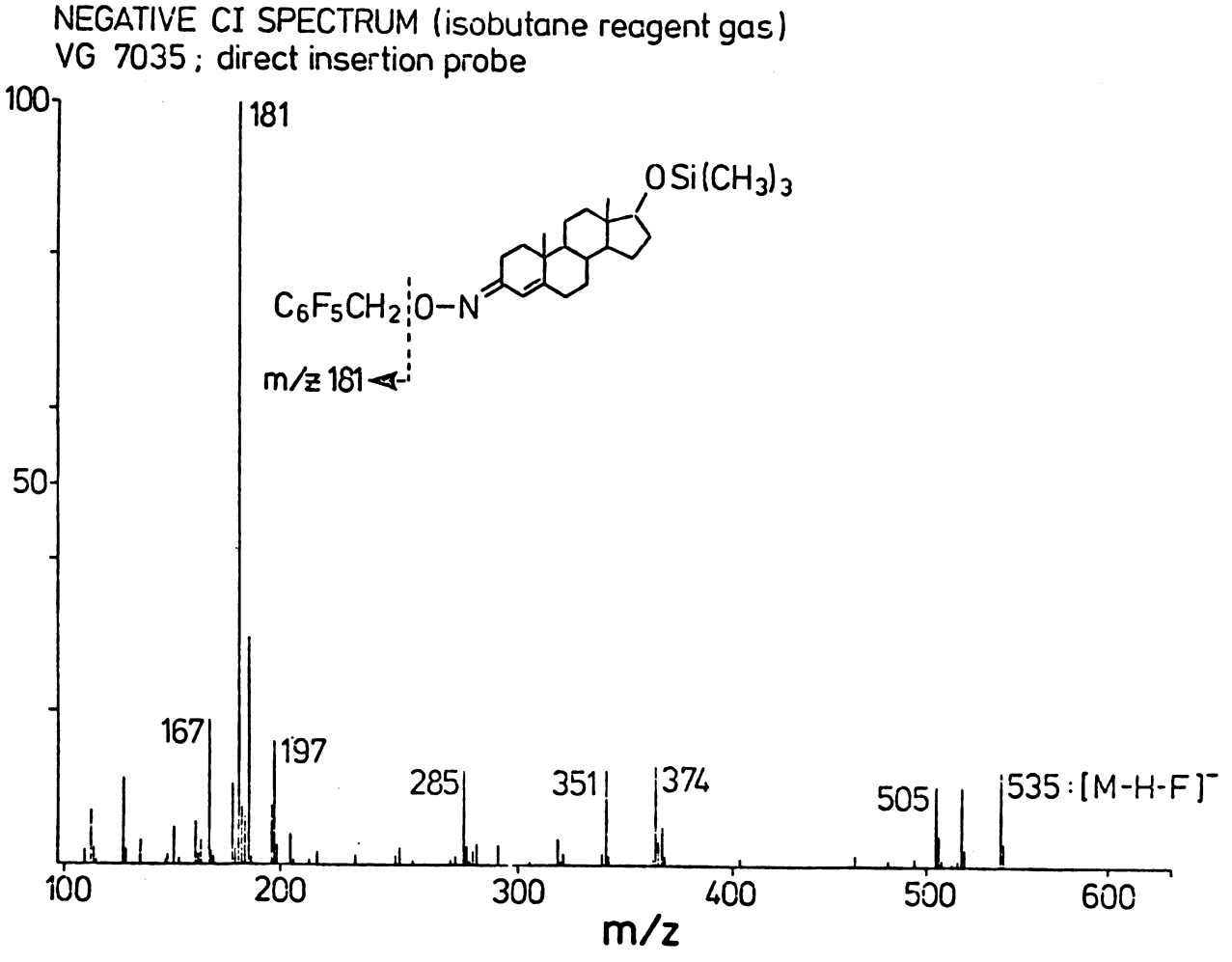


Figure 4: EC-NCI mass spectrum of testosterone pentafluoro benzyl oxime TMS ether.

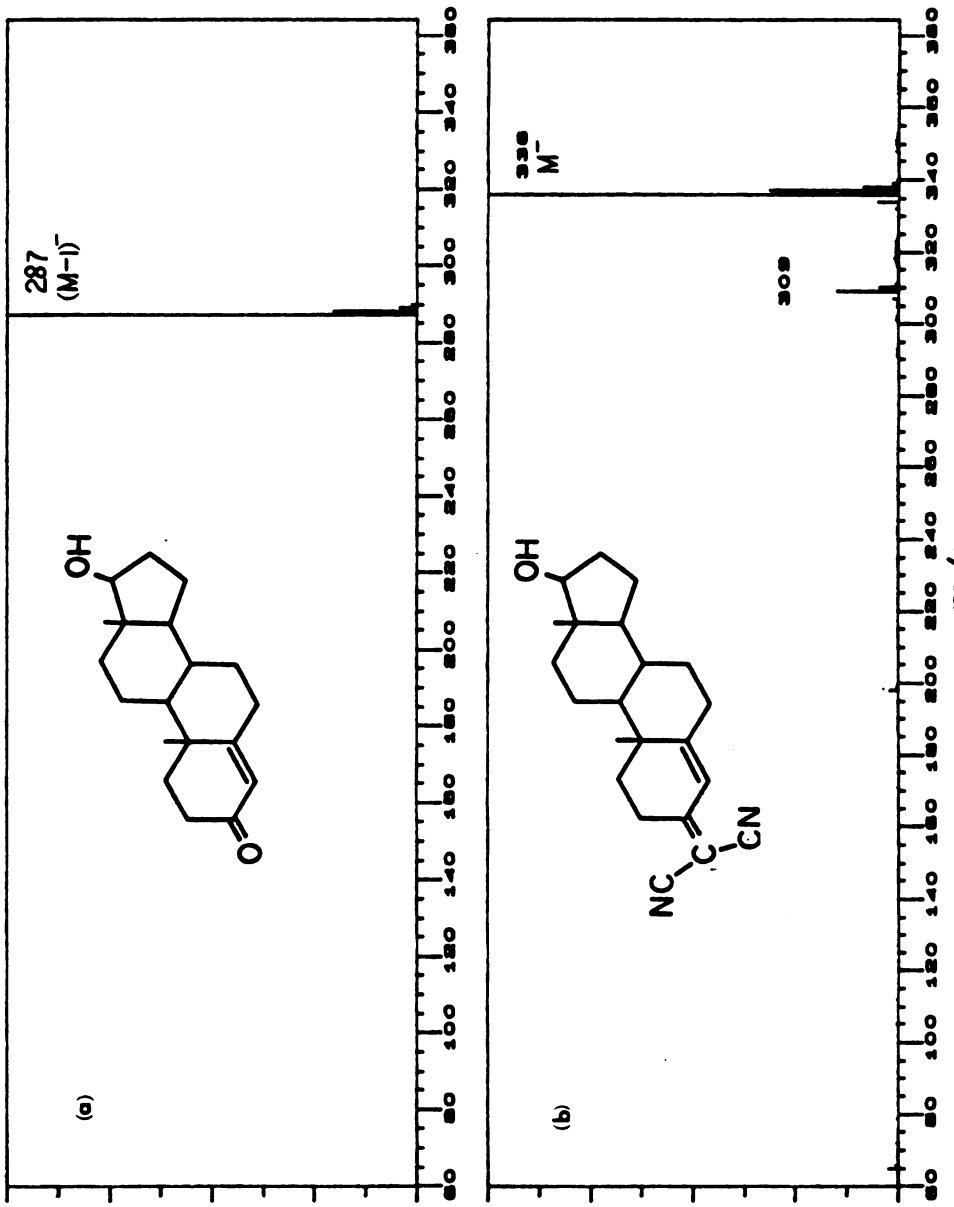


Figure 5: EC-NCI mass spectra of a) free testosterone
b) DCM of testosterone.

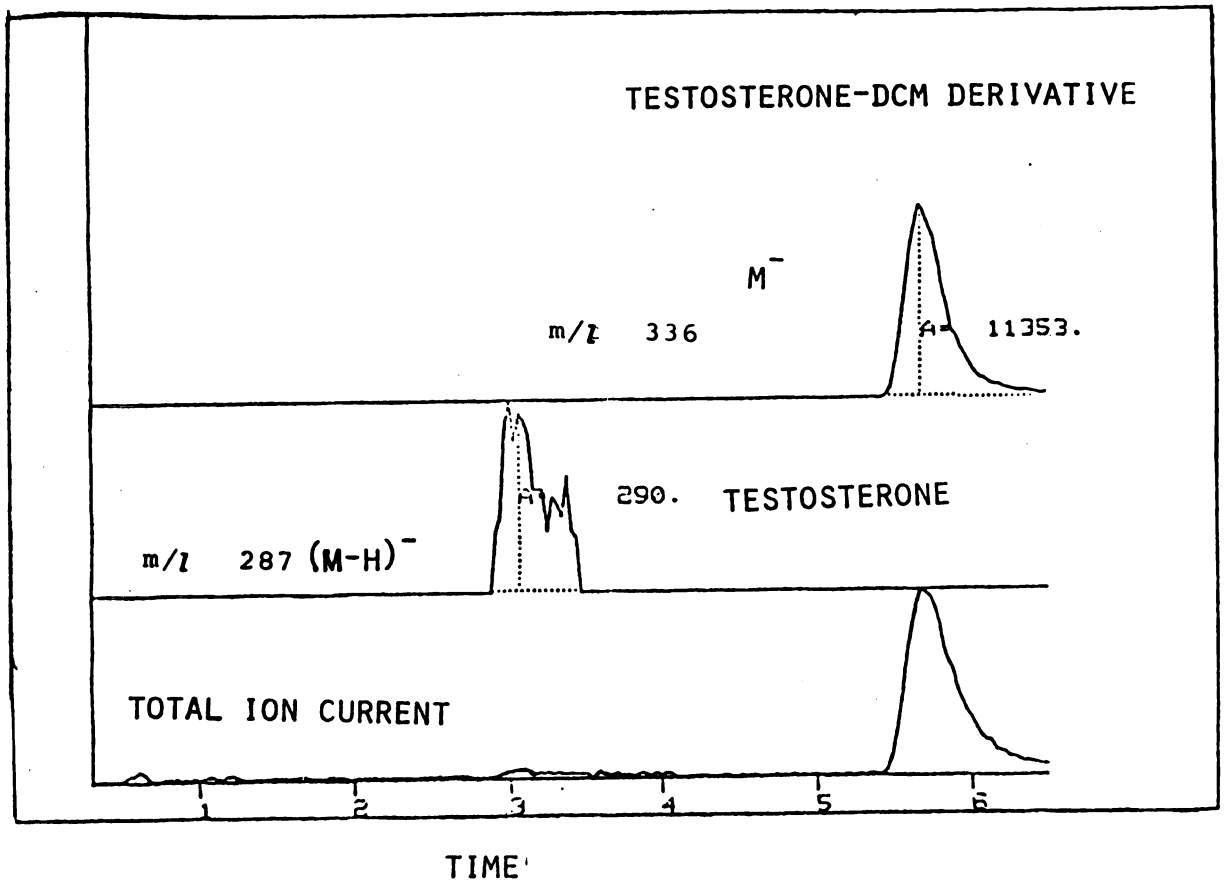


Figure 6: Reconstructed mass spectrum at m/z 336(M^- of DCM testosterone) and m/z 287($(M-1)^-$ of free testosterone) together with total ion current chromatogram resulting from injection of equal amount of free testosterone and DCM testosterone under EC-NCI conditions. The areas are indicated as follows: 290 area units for free testosterone and 11,353 units for DCM testosterone.

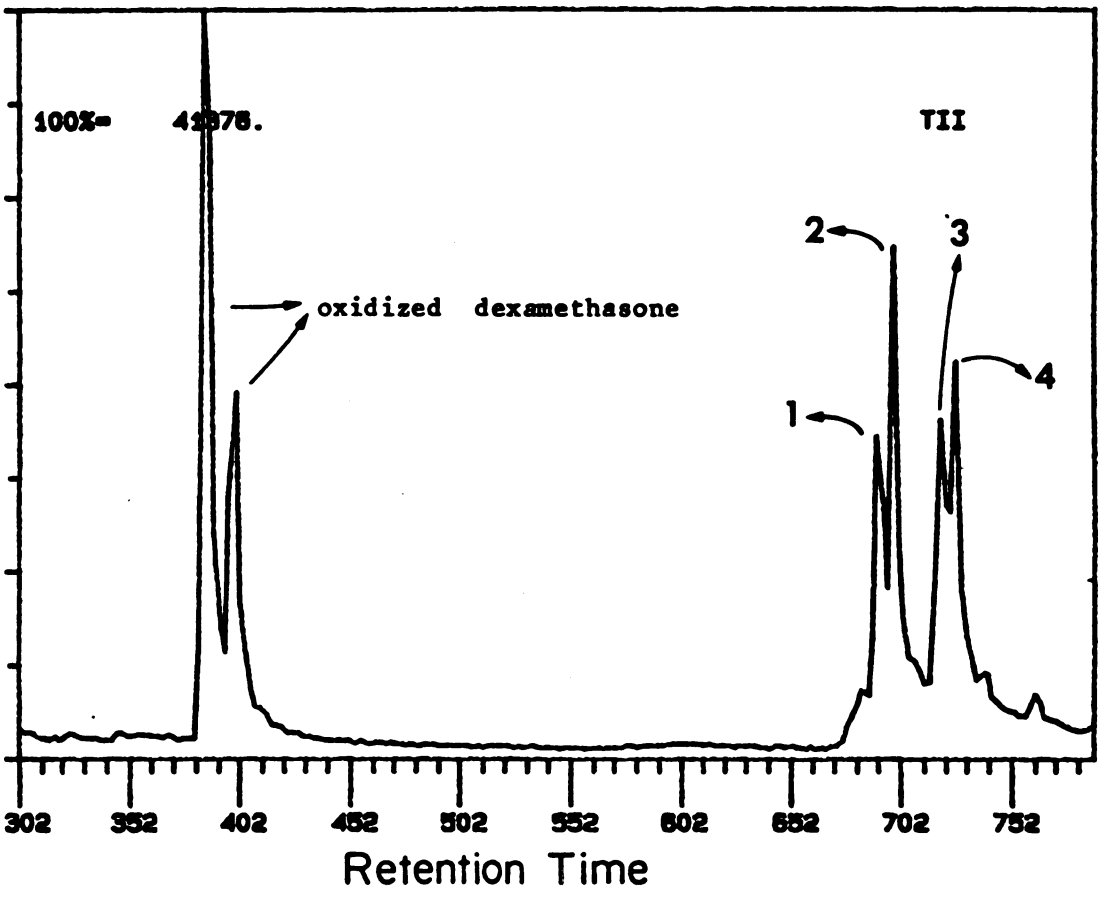


Figure 7: Reconstructed total ion current chromatogram resulting from coinjection of 100ng oxidized dexamethasone and 200ng PFA-TMS of dihydrotestosterone(four isomers labelled 1,2,3,4.)

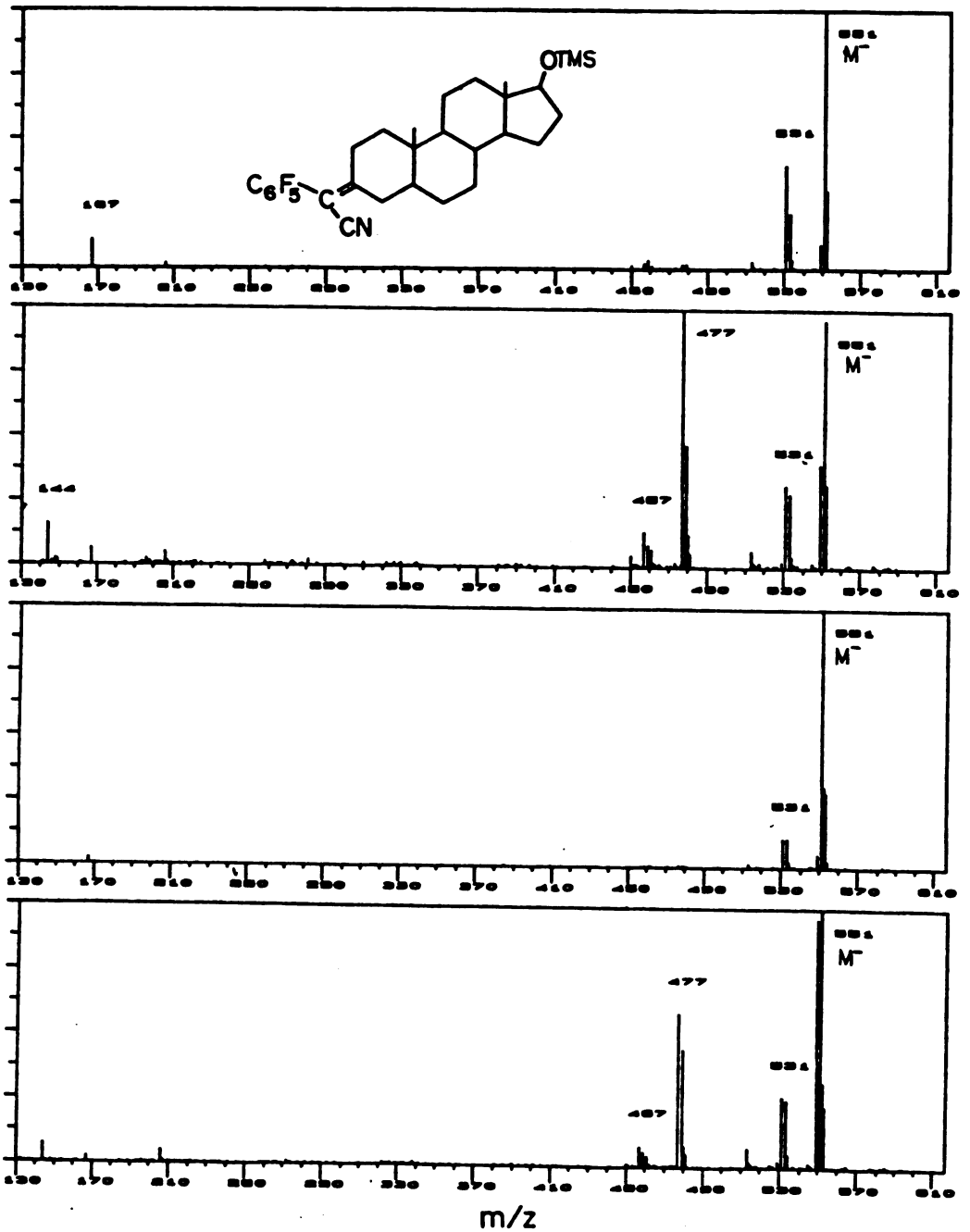


Figure 8: EC-NCI mass spectra of four isomers of PFA-TMS of dihydrotestosterone.

Chapter 3: An Oxidation Procedure for the Analysis of Corticosteroids.

Introduction:

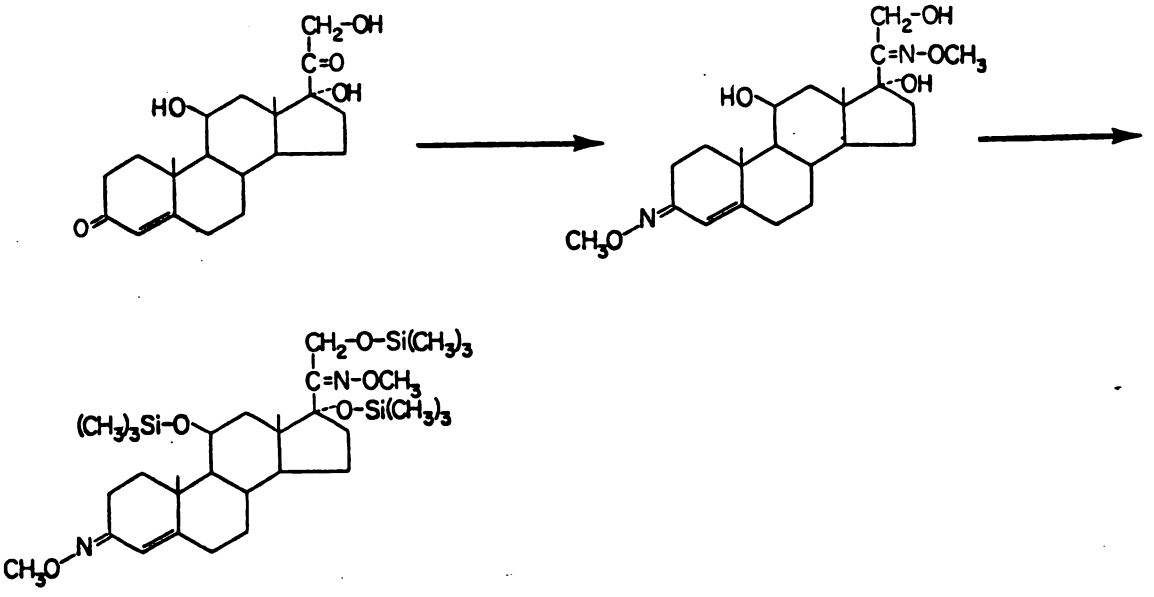
In 1949, Hench and co-workers reported a dramatic effect of cortisone in reducing inflammation in a patient with rheumatoid arthritis(53). This observation immediately evoked wide interest. The impact upon the medical world is demonstrated by the fact that, in the year following the first published report of the efficacy of cortisone in the treatment of rheumatoid arthritis, the Nobel prize in medicine was jointly awarded to Kendall and Reichatein, who were responsible for much of the basic chemical research that lead to the synthesis of the steroid, and to Hench, whose contribution has just been described. Since then adrenal corticosteroids and synthetic corticosteroids have become widely used in medicine. Although corticosteroids are best known for their antiinflammatory properties, they are also important in the areas of lymphoid tissue involution, anti-allergy effects, lysosomal stabilization effects and anti-stress effects. Because of the low dosage of synthetic corticosteroids due to their high potency, and their structural similarity to endogenous steroid hormones, a highly sensitive and selective assay is needed for the detection of these compounds in human beings and animals.

HPLC methods have been widely used in the quantitative analysis of natural and synthetic corticosteroids in biological fluids(54-60). The methods have been applied to bioavailability and pharmacokinetic studies of synthetic corticosteroids such as prednisolone, prednisone(58,59), and budesonide(60). The HPLC methods offer the advantage that the corticosteroids can be analyzed without derivatization. However, these methods lack the sensitivity required for the analysis of corticosteroids at low concentration(ng/ml).

Highly sensitive radioimmunoassays have been developed for a number of synthetic steroids(61-65). However, the specificity in radioimmunoassays remains questionable due to the cross-reactivity with other structurally similar compounds. The technique cannot unequivocally distinguish similar synthetic or endogenous corticosteroids. Thus false determinations are frequently encountered.

GC/MS has been widely used as a specific technique for the identification of drugs in biological fluids. The main problem in the use of GC/MS for corticosteroids analysis is that the reliable GC/MS determination of corticosteroids is possible only in the form of derivatives. Underivatized corticosteroids are thermally unstable under high GC oven temperature due to the presence of 17- and/or 21- hydroxy groups on the otherwise stable 20-ketopregnane side chain. The main decomposition route for steroids with a dihydroxyacetone side chain is the splitting of the 17-20 bond and the formation of the 17-ketone derivatives(66,67); there are other reactions(68) which must also be taken into account. The most generally used method for protection of

the corticosteroids side chain requires double derivatization: formation of the methoxime(MO) of the 20-keto group, followed by silyation(TMS) of the 17 and 21 hydroxy group(69,70) as shown in Scheme 4.



Scheme 4

So called MO-TMS derivatives have been used for the quantitative determination of prednisolone and prednisone in human plasma using GC/MS with chemical ionization(71), and in the detection of dexamethasone in horse urine by capillary column GC/MS with EC-NCI(72). In general, MO-TMS derivatives lack enough sensitivity when ionized by the EI or CI mode. Although one report(72) showed higher sensitivity in EC-NCI mode(250pg), the ions selected for SIM were not explained by fragmentation mechanisms.

It has been suggested that in order to produce stable silylated derivatives of corticosteroids, it is necessary to protect C-3 and C-20 ketones before the silylation step(69,70,73). One recent published work showed that it is not always true because the tetra-TMS derivative of dexamethasone was proved to be stable enough under a high GC oven temperature(74). Tetra-TMS dexamethasone showed better sensitivity than MO-TMS dexamethasone under both EI and CI conditions. Under CI conditions, it showed better sensitivity(100pg of sample produced a signal with S/N approximately 2.5) than previously reported GC/MS works.

Because of the thermal instability of corticosteroids, the appropriate derivative for the analysis of corticosteroids with ECD or EC-NCI needs not only to increase the electron affinity of the analyte but also to increase the thermal stability of the analyte. Despite the fact that ECD has been used for more than two decades, there is no high electron affinity derivative which has been proved to be suitable for the analysis of corticosteroids. Some high electron affinity derivatives which have been successfully used in C-19 steroids did not

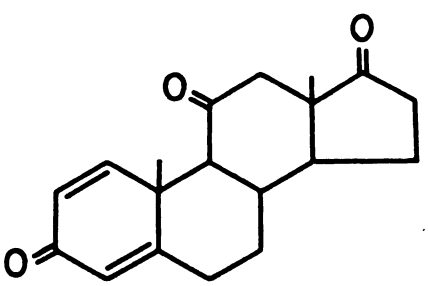
show promising results when they were used with corticosteroids. For example, pentafluorophenyl-dimethylsilyl(flophemesyl) proved to be a good derivative for the analysis of C-19 steroids by ECD(75-77). However, when it was used for the analysis of corticosteroids it failed to react with the sterically hindered C-17 hydroxy group(78). This could result in poor sensitivity because the C-17 hydroxy group is believed to be responsible for the thermal instability of corticosteroids. Because corticosteroids contain a dihydroxyacetone side chain which does not exist in most other types of steroids, a high electron affinity derivative which reacts only with this functional group would be the ideal ECD derivative for the analysis of corticosteroids by ECD. Boric acid has been used as a selective derivatization reagent because it selectively reacts with the dihydroxy acetone side chain of corticosteroids to form a cyclic derivative(79). Due to the selectivity of boric acid, Pool and coworkers proposed the use of high electron affinity boric acid derivatives(80-83) such as 3,5-bis(trifluoromethyl)-benzene boric acid, benzeneboric acid, and 4-bromobenzeneboric acid to not only selectively derivatize corticosteroids but also to increase the electron affinity of corticosteroids. However, it was found that they show multiple products or no products when used to make derivative of many bifunctional groups steroids such as cortisol, aldosterone, and 11-dehydrocorticosterone.

The dicyanomethylene(DCM) derivative mentioned in chapter 2 showed some promise for increasing the electron affinity of keto-steroids. However, it was found that in the case of corticosteroids, the steroids apparently degraded under the catalytic conditions(ammonium acetate/acetic acid) required for the most rapid production of the DCM derivatives.

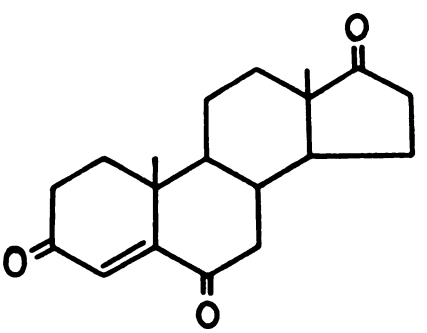
In general, there are two types of compounds which show high response under EC-NCI or ECD. One type is extensively halogenated compounds; the other is highly conjugated compounds. Because most steroids are neither heavily halogenated nor highly conjugated, they are expected to show poor response under EC-NCI or ECD. Studies of the structure-response relationship of steroids under ECD(83-85) indicates that despite the fact that most steroids do show a very poor response, a few steroids having specific structural characteristics show a high response to this detection technique.

In this laboratory, a similar study of the structure-response relationship of steroids under EC-NCI conditions produced results which do not completely agree with the reported ECD data (83). It was found that not all the steroids which reportedly have a high ECD response also have a high EC-NCI response. Two exceptions which show a high response under EC-NCI conditions Bare 1,4-androstadien-3,11,17-trione(IV) and 4-androsten-3,6,17-trione(V). Because it was found that some steroids, especially most synthetic corticosteroids can be chemically oxidized to these two high electron affinity steroid analogs, we reported here on the feasibility of converting a variety of steroids to variants of these

high electron affinity ketonic analogs as a basis for developing selective and sensitive methodology for the parent steroids.



(IV)

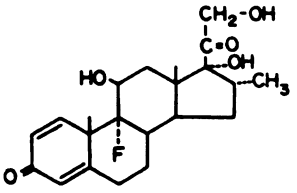


(V)

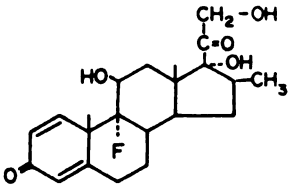
Experimental Section:

I. Chemicals

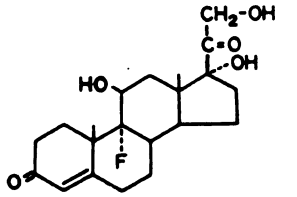
4-androsten-3,17-dione; 1,4-androstadien-3,17-dione; progesterone (4-pregnen-3,20-dione); testosterone (4-androsten-17β-ol-3-one); 6α-hydroxy testosterone; 4-pregnene-3,11,17-trione; 4-androsten-3,6,17-trione; prednisolone; 6α-methyl prednisolone; dexamethasone; 6β-hydroxy prednisolone; betamethasone; fludrocortisone; fluorometholone; cortisol; 6β-hydroxy cortisol were obtained from Steraloids, Inc. Flumethasone was a gift from Syntex Co. n-dodecane, chromium trioxide, pyridinium chlorochromate, sodium bismuthate were purchased from Aldrich, Inc.; Sephadex LH-20 from Sigma Chemical Company.; Methoxyamine hydrochloride and bis(trimethyl-silyl)-trifluoroacetamide(BSTFA) from Pierce Inc.Chrominum trioxide-pyridine complex was synthesized by slowly adding chromium trioxide in small portions to reagent grade pyridine.



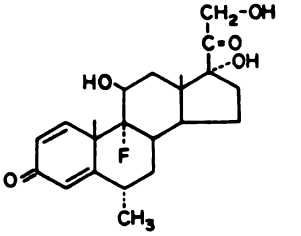
Dexamethasone



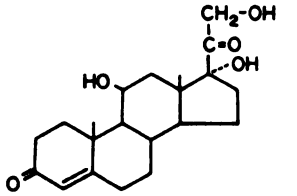
Betamethasone



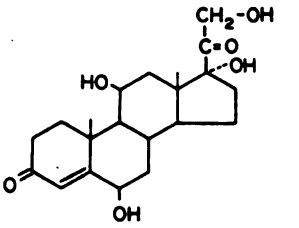
Fludrocortisone



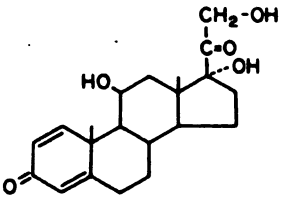
Fluorometholone



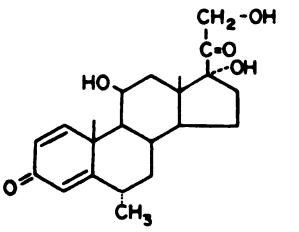
Cortisol



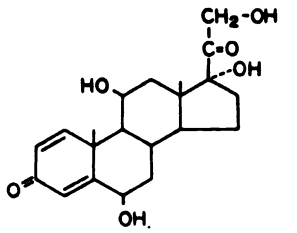
6β-OH-Cortisol



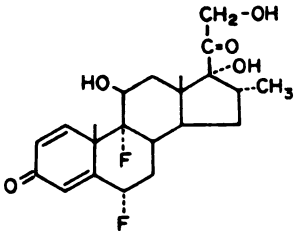
Prednisolone



6α-CH_3-Prednisolone



6β-OH-Prednisolone



Flumethasone

II. Instrumentation

The gas chromatography (GC) was performed on a Perkin-Elmer Sigma 3A gas chromatograph equipped with a flame ionization detector (FID) and an electron capture detector (ECD). The GC/MS conditions were described in chapter 2.

III. Oxidation of Steroids

For some corticosteroids, the same oxidation products could be produced by using different oxidation reagents. The criteria for the selection of oxidation reagent will be discussed in the Results and Discussion Section.

A. Sodium Bismuthate

11-keto-steroids(0.1 to 1 mg) were dissolved in 1 ml dichloromethane. 0.5 ml 15% acetic acid and 15 mg sodium bismuthate were added to the solution. The solution was allowed to react at room temperature for 1 hour (in darkness). The dichloromethane phase(bottom layer) was separated and the solvent was evaporated under a nitrogen gas stream. The residue was dissolved in 0.5 ml n-dodecane prior to GC/MS analysis. Since on-column injection is most effective when the inital oven starting temperature is below the boiling point of the solvent, the choice of n-dodecane allows use of a higher initial oven temperature and therefore, the chromatographic run time is reduced(86).

B. Chromium trioxide pyridine complex or pyridinium chlorochromate

Steroids (0.1-1mg) and oxidation reagent (15mg) were dissolved in 1ml solvent (pyridine for chrominum tri oxide-pyridine complex, dichloromethane for pyridinium chlorochromate). The solution was set at room temperature for 3-5 hours. Excess oxidation reagent was removed by filtration through a pasteur pipet packed with 3cm Sephadex LH-20. Ethyl acetate was used as eluent, the first 3ml of eluent were collected and the solvent was removed under N_2. The residue was dissolved in 0.5ml dodecane prior to GC-MS analysis.

IV.The Isolation and Oxidation of Dexamethasone from Horse Urine

A single 5 mg dose of dexamethasone was administered intramuscularly to a cross-bred female horse. Urine samples were collected via a catheter during a peroid 2 hours to 46 hours at 4 hours intervals following administration of the drug. The urines were treated as shown in scheme 5. To a 20 ml aliquot of urine in a 300 ml flask, 5ug of internal standard (6α-methyl prednisolone in methanol) were added and vigorously stirred for 10 minutes. Then the solution was vigorously stirred with 100 ml CH_2Cl_2 for another 25 minutes. The dichloromethane extract was dried with anhydrous sodium sulfate and evaporated under nitrogen; the residue was dissolved in 200 µl pyridine and transferred to a 1 ml reacti-vial. Then the pyridine solution extract was oxidized with 15 mg of chromium trioxide-pyridine complex at room temperature for 3 hours. Following removal of pyridine by evaporation under nitrogen, 200 µl ethyl acetate was added which dissolved the oxidized steroids but

left a slurry of the excess oxidizing reagent. The suspended oxidation reagent was removed by filtering through a pasteur pipette packed with 5 cm Sephadex LH-20 using ethyl acetate as eluent. The first 3 ml of solution was collected and the solvent was evaporated under a nitrogen gas stream. The residue was dissolved in 100 µl n-dodecane and transferred to a 100 µl reacti-vial; 1 to 2 µl of the solution was injected into the GC-MS for analysis.

SCHEME FOR THE ANALYSIS OF DEXAMETHASONE IN HORSE URINE

HORSE URINE

Add internal standard (5 μg 6α-methyl-prednisolone to 20 ml horse urine)

EXTRACTION

CH_2Cl_2 (50ml)

1. Anhydrous Na_2SO_4
2. Evaporate to dryness
3. Dissolve in 200μl pyridine

OXIDATION

1. Approximately 15 mg $(py)_2CrO_3$
2. React at room temp. 3 hours
3. Evaporate pyridine
4. Dissolve in 200μl EtoAc

ISOLATE OXIDIZED PRODUCT

1. 5 cm column Sephadex LH-20 in EtoAc
2. Collect first 3 ml EtoAc
3. Evaporate EtoAc
4. Dissolve in 100μl n-dodecane

GC-MS

1. Inject 1% aliquot on column
2. EC-NCI with methane

Scheme 5.

V. The Isolation and Oxidation of 6β-hydroxy Cortisol from Human Urine

A C-18 Sep-pak cartridge was pre-washed with 2ml of methanol followed by 5ml of distilled water. An aliquot (10ml) of normal human urine was passed through the cartridge. Following a 5ml distilled water wash, steroids were eluted with 2ml of methanol. The methanol was dried under N_2 and dissolved in dichloromethane(only free steroids can be dissolved). Approximately 15mg of pyridinium chlorochromate was added to the solution. The solution was allowed to stand at room temperature for 5 hours and then treated as described in Section III of the Experimental. The residue was dissolved in 100µl dodecane prior to GC-MS analysis.

VI. The Formation of MO-TMS Derivative

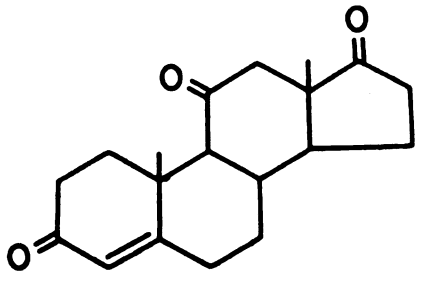
1 mg of corticosteroid is dissolved in 0.5 ml pyridine solution which contains 8% of methoxyamine hydrochloride. The solution is heated at 80°C for 3 hours. The solvent is removed under nitrogen and trimethylsilylimidazole(TMSI) was added; silylation is carried out at 80°C for 2 hours. Excess derivatization reagents are removed by filtration through a short column(2 cm) of Sephadex LH-20 slurry packed in a pasteur pipette using chloroform-hexane (1:1) as eluant. The steroid MO-TMS derivatives are eluted in the first 2 ml of eluent and the solvent is removed under nitrogen. The residue is dissolved in dodecane before the analysis.

Results and Discussion

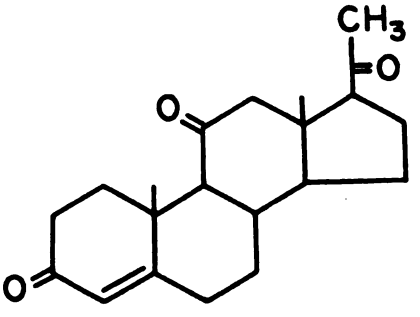
I. Structure-Response of Steroids

In ECD, it has been reported that the response of organic compounds are not always predicable. For example, benzo[a]pryene showed 15 times higher response than benzo[e]pyrene under ECD despite the fact that both compounds have same molecular weight, same number of double bonds, and similar electron affinity(87). Under EC-NCI conditions, similar results were reported in a recently published work(88). The response of different steroids under ECD has been reported(83-85). Several steroids showed unusually high ECD response. The relative response of many steroids under EC-NCI conditions was measured in this laboratory and listed in Table 1. Because of the similarity between EC-NCI and ECD, it is surprising that the EC-NCI data do not agree with the reported ECD data (83). For example, under ECD conditions, 4-androsten-3,11,17-trione(VI) and 4-pregnene-3,11,20-trione(VII) were reported to have a response about one half that of 1,4-androstadien-3,11,17-trione(IV). However, under EC-NCI conditions, 1,4-androstadien-3,11,17-trione(IV) gave a response much greater than that of the other two compounds. By examining the ECD data which were collected by using the ECD detector described in the experimental section, it was found that they showed a structure-response relationship similar to that of the EC-NCI data. Both 4-androsten-3,11,17-trione(VI) and 4-pregnene-3,11,20-trione(VII) showed a much poorer response compared to that of 1,4-androstadien-3,11,17-trione(IV). The reason for the different ECD data is not clear. One possible reason is that the

reported ECD data were collected with one of the very early designs of an ECD detector(1963); since then instrumental improvements have been made which might account for the difference in response.



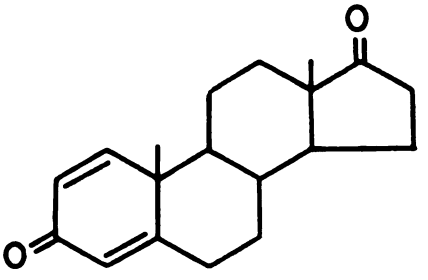
(VI)



(VII)

Two steroids which show a high response are 1,4-androstadien-3,11,17-trione(IV) and 4-androsten-3,6,17-trione(V)(Table I). Their responses are over two orders of magnitude higher than those of many other steroids. The reason for the high response of 4-androsten-3,6,17-trione(V) could possibly be due to its conjugated diketone system. However, the high response of 1,4-androstadien-3,11,17-trione(IV) cannot be explained simply by conjugation. By comparing the response of 1,4-androstadien-3,11,17-trione(IV) with that of 4-androsten-3,11,17-trione (VI) and 1,4-androstadien-3,17-dione(VIII),it appears that the functional groups (1-ene, 11-one,) are vital for the

high response of this compound. The response drops dramatically if either of these two groups is removed from the molecule.

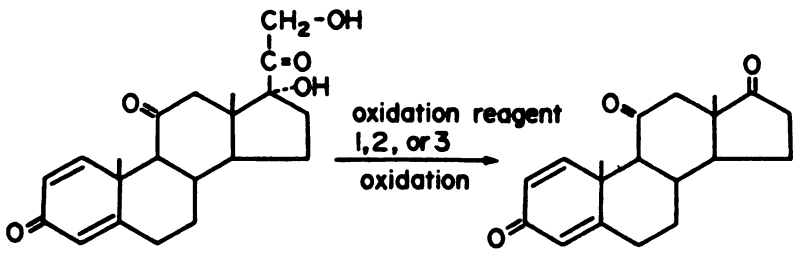


(VIII)

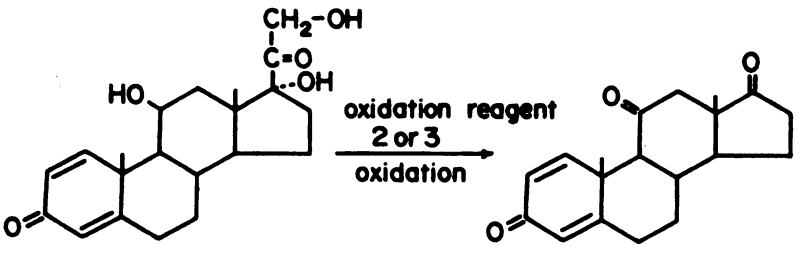
In 1965 Zlatkis and Lovelock suggested that the difficulty in predicting the response of conjugated electrophores was related to the fact that the structure one should consider is the resultant negative ion, not the parent molecule(89). In some instances it is now possible to elucidate the structure of negative ions formed under ECD conditions by using EC-NCI or atmospheric pressure ionization mass spectrometry(API-MS) because both techniques provide information about the mass of the resultant negative ions. In this case, since only the molecular anion is observed for 1,4-androstadien-3,11,17-trione(IV), the above explanation is not applicable unless the molecule rearranged to form a different structure with the same molecular weight.

II. Oxidation Reactions

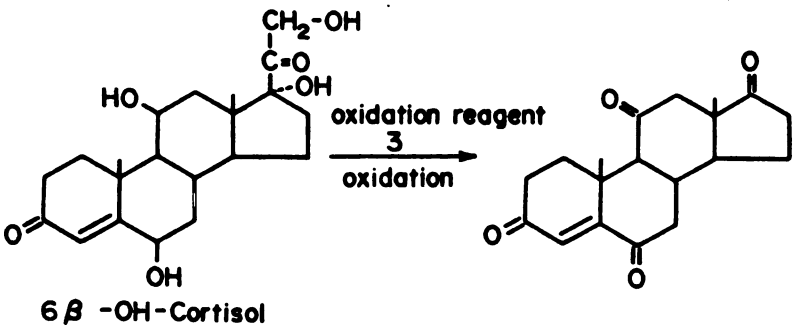
The high response of 1,4-androstadien-3,11,17-trione(IV) and 4-androsten-3,6,17-trione(V) provides an avenue for a sensitive and selective method for only those steroids that can be converted to these two structural analogs or substituted variants. By inspection of the structures of corticosteroids, it is clear that many have a structure which can be oxidized to one of these two high electron affinity structural analogs as shown in Scheme 6.



Prednisone



Prednisolone



6β -OH-Cortisol

1. Sodium bismuthate
2. Chromium trioxide-pyridine complex
3. Pyridinium chlorochromate

Scheme 6

Three different oxidation reagents, sodium bismuthate(90), chromium trioxide-pyridine complex(91) and pyridinium chlorochromate(92), are generally used. Sodium bithmuthate is able to oxidize the 17-OH but not 11-OH and 6-OH. The chromium trioxide-pyridine complex can oxidize 11-OH, 17-OH but cannot oxidize the 6-OH group effectively. However, pyridinium chlorochromate is able to oxidize all three hydroxy group(i.e., 6-OH, 11-OH and 17-OH). There are two important factors to be considered in choosing the oxidation reagent. First, the structure of the analyte must be considered. For example, prednisone(a 11-ketone corticosteroid) is one of the synthetic corticosteroids and, also, it is the major metabolite of prednisolone(a 11-OH corticosteroid). If the detection of only prednisone is desired, sodium bismuthate would be the proper oxidation reagent because sodium bismuthate is able to oxidize prednisone but not prednisolone to 1,4-dien-3,11,17-trione(IV). On the other hand, if the total concentration of prednisolone and prednisone is of interest, the chromium trioxide-pyridine complex would be the better choice of oxidizing reagent. The other important factor for choosing an oxidation reagent is that one should use the less potent one if two different oxidation reagents can produce the same oxidation product. In general, the stronger the oxidation reagent used the greater the chance of interference from the sample matrix.

In the preliminary study using a packed column, there was only one GC peak for oxidized dexamethasone, oxidized betamethasone, and oxidized flumethasone. However, two peaks showed up when the 25 m SE-54 widebore open tubular capillary column was used for the GC/MS analysis. Since these two peaks represent compounds having the same molecular weight and indistinguishable EI spectra, it is possible that they are configurational isomers. The possible reason for two isomeric products of the oxidation reaction is that the C-17 ketone enolizes under the oxidation conditions and, therefore, produces 16α-methyl as well as 16β-methyl oxidation products.

III. Structure-Response Relationship of Oxidized Corticosteroids

Ten important corticosteroids were oxidized according to scheme 6. The relative response of these oxidized corticosteroids were measured and listed in Table 2. All oxidized corticosteroids except oxidized cortisol show at least 2 orders of magnitude higher response than oxidized testosterone(the reference compound). By examining the structure-response of the oxidized corticosteroids, it appears that if the oxidized product has the basic structure of 1,4-androstadien-3,11,17-trione(IV) or 4-androsten-3,6,17-trione(V), the presence of extra functional groups does not significantly change their response. For example, oxidized dexamethasone and oxidized prednisolone have the same basic high electron affinity structure as 1,4-androstadien-3,11,17-trione(IV). As a result they show similar responses even though oxidized dexamethasone contains two extra functional groups (16-methyl and 9-fluorine) compared to oxidized

prednisolone. For oxidized steroids which do not contain the high electron affinity basic structure, the presence of extra functional groups may change their responses significantly. An example of this influence is provided by fludrocortisone. Although oxidized cortisol (4-androsten-3,11,17-trione) shows poor response under EC-NCI, the addition of a fluorine atom to the C-9 position (oxidized fludrocortisone) results in a sharp increase in its response.

IV. EC-NCI Mass Spectra of Oxidized Corticosteroids

The EC-NCI mass spectra of oxidized corticosteroids are generally very simple. The spectra of oxidized nonfluorinated corticosteroids each consist of only one peak representing the negative molecular ion, M^-. Spectra of oxidized fluorinated corticosteroids consist of M^-, $(M-HF)^-$ and/or $(M-HF-CH_3)^-$ for peaks. The only exception which has been found is oxidized cortisol which shows the $(M-2)^-$ ion. The EC-NCI mass spectra of oxidized corticosteroids which were obtained at source temperature of 150°C are shown in Table 3. As mentioned previously, corticosteroids with a 16-methyl group produced two oxidation products which had the same molcular weight and indistinguishable EI spectra. Under EC-NCI conditions, these two isomers produced the same ions but with different peak intensity ratios. For example, Figure 9 and Figure 10 show the total ion current profile and the negative ion mass spectra of oxidized dexamethasone. The latter showed that the ion currents of both isomers were carried by the molecular ion, M^-, and the fragment ions, $(M-HF)^-$. However, the peak intensity ratios of $M^-/(M-HF)^-$ are quite different for the two isomers. The major oxidation product

(retention time, 6.4 minutes) has an ion abundance ratio [$M^-/(M-HF)^-$] of 0.65 whereas the minor oxidation product (retention time, 6.7 minutes) has an ion abundance ratio of 0.2 at the same source temperature (150°C).

The EC-NCI mass spectra might change with the changing of source temperature. For example, the $(M-HF-CH_3)^-$ ion(m/z 295) did not appear in the mass spectrum which was taken at a source temperature of 100°C. However, in the mass spectrum which was taken at a source temperature of 300° C, it was the most intense peak.

V. Selecting Source Temperature

In ECD, the temperature of the detector is a very important operational parameter (93,94). A valid detection limit for a given compound using ECD cannot be determined without optimizing the detector temperature because the response of a compound could change dramatically with a change in detector temperature. For example, 10 pg of benzophenone gives an ECD response with a S/N of approximately 3 at a detector temperature of 150°C. However, at a detector temperature of 360°C, 1 ug of benzophenone is required to give the same response. Although ECD and EC-NCI are considered to be similar techniques, most published EC-NCI works do not mention whether the source temperature has any effect on the response of the analytes. For benzophenone, a similar temperature response phenomenon was observed in this laboratory under EC-NCI conditions. The response of benzophenone dropped to approximately 1/7 its initial response when the source temperature

increased from 110°C to 200°C. Oxidized corticosteroids generally showed slightly higher response at higher source temperatures. The response of oxidized prednisolone increased approximately 4 times when the source temperature increased from 120°C to 300°C. Under the same conditions, the response of oxidized dexamethasone increased approximately 2 times. From the results of temperature response study, it appears that it is better to use a high source temperature in the analysis of oxidized corticosteroids.

As mentioned previously, the appearence of the EC-NCI mass spectra could also change with the source temperature. Oxidized dexamethasone produces two major ions, M^- and $(M-HF)^-$, under EC-NCI conditions. Increasing the source temperature results in higher abundance of $(M-HF)^-$ fragment ions, but lower abundance of the molecular ion. The peak intensity ratio of $M^-/(M-HF)^-$ changes from .65 to .10 when the source temperature increases from 150°C to 200°C. The large difference in the intensity of M^- and $(M-HF)^-$ creates a situation which is unfavorable for selected ion monitoring (SIM). In general, it is preferable to monitor at least two prominent ions for each compound so that any ambiguity on one ion current profile can be clarified by comparison with the other ion current profile. The selection of two ions with a large difference in abundance will result in either poor sensitivity (which is established by the ion of low abundance) or poor selectivity(when identification of a compound is based on only one ion). For oxidized dexamethasone, because there is no significant increase in response with an increase in the source temperature, a low source temperature would be a better choice. For oxidized prednisolone, only the molecular ion is

observed in the EC-NCI spectrum. Because the higher source temperature results in higher response, a high source temperature would be a better choice.

VI. The Advantages of The Oxidation Method

A. The Sensitivity Advantage

In evaluating an analytical method, sensitivity and selectivity are two important criteria. The advantage of sensitivity in this oxidation method is demonstrated by a comparison of instrumental response to oxidized dexamethasone with that of the tetra-TMS and the MO-TMS derivatives of dexamethasone.

The EI spectrum of MO-TMS of dexamethasone shows heavy fragmentation; by selected ion monitoring of the most intense high mass ion(m/z=364), 5 ng of pure sample were required to produce a signal with a S/N of approximately 5 as shown in Figure 11. Under PCI conditions, by SIM of the protonated molecular ion and the $(M+H-CH_3OH)^+$ ion, 1 ng of the MO-TMS derivative of dexamethasone was needed to produce a signal with a S/N of approximately 4 as shown in Figure 12. Under the EC-NCI conditions, the MO-TMS derivative of dexamethasone produced some high mass ions. However, it is difficult to rationize a fragmentation pathway that leads to the formation of these ions; a published report on the EC-NCI of MO-TMS derivatives offers no suggestion(72). By SIM of the two most intense ions (m/z=489 and m/z=504), a 200 pg sample gives a peak with a S/N of approximately 2 (Figure 13).

The tetra-TMS dexamethasone has been reported to have better sensitivity than MO-TMS dexamethasone in both EI and PCI modes. In the conventional EI mode, by SIM of the molecular ion, it took 5 ng of pure sample to produce a signal with a S/N of approximately 2.5. In PCI mode, by SIM of the protonated molecule ion, 100 pg sample produced a signal with a S/N of approximately 2.5.

For oxidized dexamethasone under the EC-NCI conditions, by SIM of the M^- and $(M-HF)^-$ ions, 10 pg of sample can be detected with a S/N of approximately 2, as shown in Figure 14. By comparison all the detection limits mentioned above, it was showen that the sensitivity is improved by at least an order of magnitude by this oxidation procedure. Since most oxidized corticosteroids and oxidized dexamethasone have similar relative responses, their detection limits are expected in the 10 pg range.

B. The Selectivity Advantange

Hitherto, quantitative methodology for the analysis of a low electron afffinity compound by EC-NCI required the use of a general derivatization reaction to produce a derivative of higher electrophilicity. However, in many cases, not only the analyte is derivatized, but many other compounds which coexist with the analyte are also derivatized by the high electron affinity derivatization reagents. Sensitivity may be increased but there is no improvement in selectivity.

The oxidation method reported here shows very high potential in selectivity because only certain structural analogs show high response under EC-NCI. Most compounds which coexist in biological fluids with the analyte do not have a structure that can be oxidized to 1,4-androstadien-3,11,17-trione(IV) analogs, and therefore, do not interfere. For example, testosterone and progesterone are both present in human urine; under the oxidation conditions testosterone is oxidized to 4-androsten-3,17-dione and progesterone shows no reaction with the oxidation reagent. Both 4-androsten-3,17-dione and progesterone show much poorer response than do oxidized corticosteroids.

In earlier work by Matin and Amos (71), prednisolone was assayed in human plasma as the MO-TMS derivative and then analyzed by SIM, using chemical ionization. Since cortisol could interfere with prednisolone due to the overlapping between the molecular ion peak cluster of prednisolone and the molecular ion peak cluster of cortisol (overlap due to multiplicity of silicon isotope peaks), cortisol was selectively removed by derivatization with Girard T reagent prior to analysis by gas chromatography-mass spectrometry. If the oxidation method described here was used, this isolation procedure could be eliminated since oxidized prednisolone shows about 60 times higher response than oxidized cortisol.

C. The Advantage of Simple Modification Versus Derivative Formation

The steroids which undergo the oxidation reaction are converted to a ketonic steroid analog which is suitable for vapor phase analysis; other compounds such as organic acids not amenable to the oxidation reaction retain hydroxy or carboxylic groups and thus are not in a structural form suitable for vapor phase analysis. This serves as another degree of discrimination against potentially interfering compounds. On the other hand, the successive derivatization procedure for the formation of the common MO-TMS derivative often produces multiple products due to incomplete reactions which in turn complicate the analysis.

The usual strategy of preparing a volatile derivative often involves the formation of the trimethylsilyl derivative. Because of the presence of several trimethylsilyl groups in the MO-TMS and tetra-TMS derivatives of corticosteroids, the molecular ion is represented by a multiplet rather than as a single peak ion. In a recently published work (74), the tetra-TMS derivative was used for the quantitative analysis of dexamethasone. Because of the multiplicity of molecular ions [$^{13}C_6$,$^{2}H_3$], labelled dexamethasone was used as an internal standard so that the molecular ion peak cluster of labeled dexamethasone would not overlap with the molecular ion peak cluster of unlabelled dexamethasone. The synthetic work for this heavily labelled internal standard is considered to be fairly difficult (95). The amount of synthetic work could be reduced considerably if the oxidation method described here were used. Because no silicon atom is present in the

molecule, dexamethasone labelled with as few as three deuterium atoms should be sufficient to avoid the isotope peak overlap problem encountered with multiple TMS derivatives in quantitative analysis.

D. Distinguishing Dexamethasone and Betamethasone Isomers

Dexamethasone and betamethasone are two important synthetic corticosteroids which are difficult to distingish from each other. They have the same molecular weight and their MO-TMS derivatives give very similar EI spectra. The only difference between these two compounds is the different configuration of 16-methyl group. In order to distinguish these two isomers, methods that derivatize dexamethasone and betamethasone to a different extent have been reported.

Brooks and Lawson(96) proposed a method which derivatized dexamethasone to the 3,20-bis-MO-21-TMS derivative and betamethasone to the 3-MO-21-TMS derivative, followed by analysis by EI with an electron beam energy of 15eV. Houghton et al.(72) proposed another method of derivatization in which dexamethasone yielded mainly the bis-MO-tri-TMS derivative; betamethasone yielded a mixture of the mono-MO-tri-TMS and the bis-MO-tri-TMS derivative with a ratio of 3:1. Under EC-NCI conditions, mainly the bis-MO-tri-TMS was observed for dexamethasone and the mono-MO-tri-TMS was observed for betamethasone. Both methods are heavily based on chemical means. For instance, the reaction time for the formation of MO derivative has to be controlled precisely in order to form the mono-MO derivative for betamethasone and form the bis-MO for dexamethasone. With this oxidation procedure, it is possible to

distinguish between these two isomers, mainly by instrumental means. Both dexamethasone and betamethasone produce two oxidation products (one major, one minor) that can be easily separated by capillary GC. For dexamethasone, the major component has a shorter retention time than the minor component. However, for betamethasone, the major component has a longer retention time than the minor component. In addition to this difference, the EC-NCI spectra of these two oxidation products are quite distinguishable. For oxidized dexamethasone, the major product (with the shorter retention time) has a higher $M^-/(M-HF)^-$ ratio than the minor product (with the longer retention time). The opposite phenomenon is observed for oxidized betamethasone. Hence, by combining this oxidation procedure with capillary column EC-NCI-MS, a clear discrimination of these two isomers, as shown in Figure 15, is obtained by monitoring M^- and $(M-HF)^-$ ions.

Fluorometholone produces an oxidation product which has the same molecular weight and similar EI and EC-NCI spectra as oxidized dexamethasone and oxidized betamethasone. However, only one peak shows up in the capillary column chromatogram while both oxidized dexamethasone and oxidized betamethasone show two peaks in the capillary column gas chromatogram. In addition, it has a longer retention time than oxidized dexamethasone and oxidized betamethasone if the SE-54 capillary column is used in the analysis. Thus no confusion is expected.

E. The Advantages in Chemical Stability and Chromatographic Analysis Time

In addition to the advantages previously mentioned, the oxidation procedure also has advantages in terms of chemical stability and chromatographic analysis time. Because MO-TMS and tetra-TMS derivatives are moisture sensitive compounds, a special method is required to store the derivatized compounds. The most common method to store these moisture sensitive compounds is to dissolve them in a solvent which contains about 1% of TMS reagent (such as BSTFA). After the solution is transfered to a capillary tube, the tube is sealed over a bunsen burner. On the other hand, no special method is required for the storage of oxidized corticosteroids because they are neither moisture nor air sensitive. Oxidized corticosteroids can be stored in a reacti-vial for several months without noticeable change. This advantage is even more crucial when the concentration of the analyte is extremely low. A small amount of residual moisture is enough to destroy the TMS compound. The oxidized product, however, remains unchanged.

All the derivatives which have been used for corticosteroids, such as MO-TMS, tetra-TMS, and alkylboronate ester share a common feature in their gas chromatographic analysis, namely long retention times. In this study, even under the rigorous GC conditions used such as the use of a high boiling point solvent, high column head pressure, high oven heating rate, and ultra performance capillary column(thermally stable to 350°C), it still takes approximately 12.5 minutes for the MO-TMS derivative of dexamethasone to go through a 25 meter column. Under the

same conditions, the retention time of oxidized dexamethasone is only about 6.5 minutes. Almost one half of the chromatographic analysis time is saved. This advantage becomes more important as the number of analyses increases. For example, in quantitative applications, usually more than 10 analyses are necessary to construct a calibration curve. Therefore, the instrument time can be reduced significantly if this oxidation procedure is used.

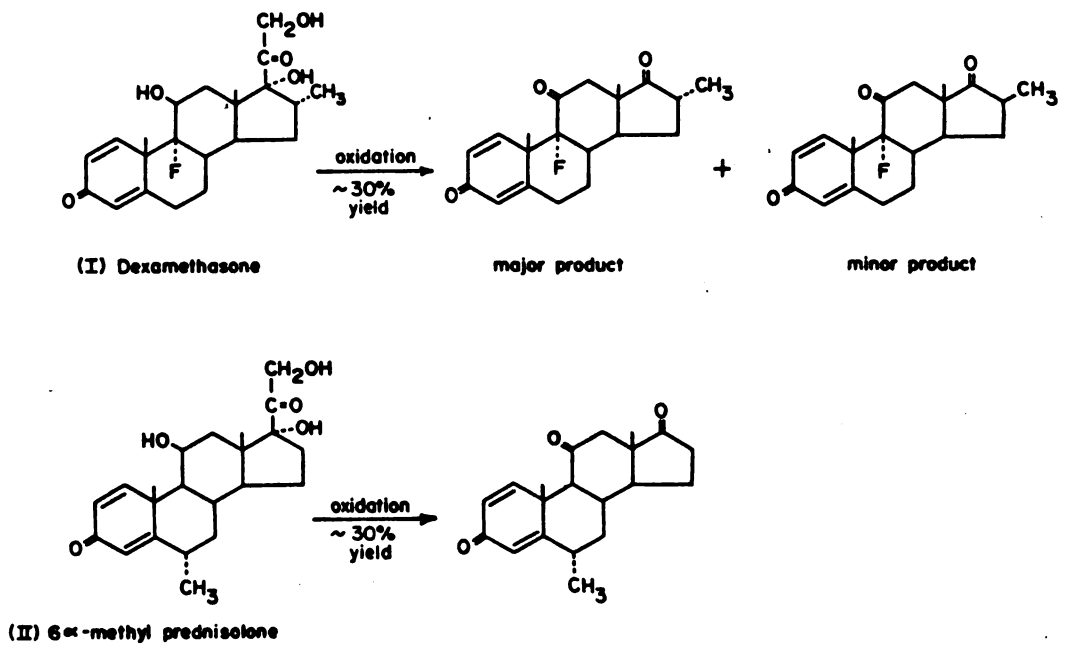
VII. The Feasibility of This Oxidation Procedure in The Analysis of Biological Fluids for Corticosteroids.

The feasibility of this procedure to analyze biological samples for corticosteroids is demonstrated by the quantitative analysis of horse urine for dexamethasone described in section (A) and the detection of 6β -hydroxy cortisol from human urine described in section (B).

A. Quantitative Analysis of Horse Urine for Dexamethasone

Dexamethasone is one of the most potent anti-inflammatory steroids. Its possible use to influence the performance of race horses and the relative ease with which one may obtain dosed horse urine prompted us to investigate the possibility of developing a quantitative method for this drug based on the proposed oxidation procedure. A structurally similar compound, 6α-methyl prednisolone, is used as the internal standard in the feasibility study because of the lack of general availability of an isotopically labeled internal standard. Both dexamethasone and the internal standard(6α -methyl prednisolone) were oxidized to

1,4-androstadien-3,11,17-trione analogs as shown in scheme 7.



Scheme 7

Under EC-NCI, the oxidized 6α-methyl prednisolone produced only the molecular ion (m/z = 312). A calibration curve was prepared from control horse urine containing a standard amount of pure dexamethasone mixed in proportion with the internal standard prior to solvent extraction and oxidation. The variance between different preparations of samples with the same concentration was greater than the variance between repeated injections of the same sample into the GC-MS (RSD=8%

vs. RSD=4%); in other words, the variance in the preparation of sample is greater than the variance in the mass spectrometric determination. Three separate preparations for each standard concentration of dexamethasone in control urine were made in order to enhance the accuracy of the calibation curve. A standard calibration curve approximating a straight line from 0 to 35 ng/ml is shown in Figure 16. The relative standard deviation of each point is on the order of 8%. The correlation coefficient of the curve is 0.998.

The selected ion current profiles of the horse urine extracts following oxidation at 14 hours and 38 hours are shown in Figure 17. The M^- and the $(M-HF)^-$ ions of the oxidized dexamethasone were chosen along with the molecular ion of the oxidized internal standard (m/z = 312) for selected ion monitoring. The concentrations of dexamethasone at 14 hours and at 38 hours urine were 24 $\pm$ 1.9 ng/ml and 3.1 $\pm$ 0.2 ng/ml, respectively. Because no purification step other than solvent extraction is used in the sample processing, the low degree of interference that can be seen in Figure 17b demonstrates the high selectivity inherent in this methodology. In fact, the only major background peak in Figure 17b(with a retention time of 7.3 minutes) was proven to be a minor oxidation product (approximately 4% of the total oxidation yield) of the interal standard (6α-methyl prednisolone)(Figure 18).

B. The Detection of 6β-hydroxy Cortisol from Human Urine

Many methods for determination of the activity of drug metabolizing enzyme systems have been developed using animal subjects, but few of them are applicable to man(97). Besides evaluation of the pharmacokinetics of several test substances which are metabolized by mixed function oxidases, determination of the excretion rate of certain endogenous compounds has often been used to reflect the activity of the drug hydroxylating enzyme systems. 6β-hydroxy cortisol is a polar, unconjugated metabolite of cortisol(98). It is formed by the mixed function oxidases in the liver and is excreted by the kidney. Thus, the urinary excretion rate of 6β-hydroxy cortisol can be used as a non-invasive tool to indicate enzyme induction or inhibition in man.

When pyridinium chlorochromate was used as the oxidation reagent, one oxidation product, 4-androsten-3,6,11,17-tetraone, was produced. Only one ion can be selected for SIM because only the molecular ion was produced under the EC-NCI conditions. The problem with choosing only one ion for SIM was described in Section IV of the Results and Discussion. In an attempt to make a derivative which would produce at least two ions for the SIM, chromium trioxide-pyridine complex was tried as the oxidation reagent. It was hoped that after the major oxidation product, 4-androsten-6-ol-3,11,17-trione, was converted to its 6-TMS derivative, it would produce more than one ion under EC-NCI conditions. Unfortunately, only the $(M-TMSOH)^-$ ion was found under EC-NCI.

In another experiment in which the 4-androsten-6-ol-3,11,17-trione oxidation product was converted to its 6-trifluoro acetate(TFA) derivative before EC-NCI analysis, the derivatized compound was found to be thermally unstable under high GC oven temperature. Since none of the other derivatives tried provided more than one ion under EC-NCI conditions, pyridinium chlorochromate was chosen as the oxidation reagent in the detection of 6β-hydroxy cortisol in human urine. Steroids are extracted by C-18 Sep-pak and then oxidized by pyridinium chlorochromate. The final solution is injected into a capillary GC-MS with EC-NCI analysis. The reconstructed gas chromatogram shows a major peak which has the same retention time and the same EC-NCI mass spectrum as oxidized 6β-hydroxy cortisol (mw=314) (Figure 19).

VIII. The Disadvantage of the Oxidation Procedure

A. Different Corticosteroids Might Produce The Identical Oxidation Product

There is some possibility that different corticosteroids or a corticosteroid and its metabolites could form the same oxidation product thereby complicating the analysis. Of all the corticosteroids which have been studied, only prednisolone and prednisone are able to produce the same oxidation product when chromium trioxide-pyridine complex is used as the oxidation reagent. This problem can be solved by selection of different oxidation reagents as mentioned in Section II of the Results and Discussion.

Corticosteroids are metabolized principally by reduction in ring A, reduction of the ketone at C-20, and cleavage of the side chain. The possibility of producing the same oxidation product from a synthetic corticosteroid and its metabolites is not easy to evaluate mainly because of the lack of adequate information about the metabolites of most synthetic corticosteroids. The excretion and metabolism of radioactively labelled dexamethasone have been studied by several investigators. Hague et al(99) reported three unconjugated polar metabolites in urine after dexamethasone adminstration, but did not report their identity. The metabolites of dexamethasone in horse urine have been reported(100); four metabolites(11-dehydrodexamethasone, 11-ketone dexamethasone, 20-hydroxy dexamethasone, and 6-hydroxy dexamethasone) were suggested but they lack clear identification and quantitation. In addition, because the authors treated the urine sample with mixed glucuronidase and sulphatase before the separation of these metabolites, it lacked the information about whether these metabolites were excreted as a conjugated form or as a free form. In most cases, only the free form could possibly result in interference because only solvent extraction is used in the isolation of dexamethasone from horse urine and it will not extract conjugated metabolites from the the urine. Recently, the major unconjugated metabolite of dexamethasone in horse urine was identified by EC-NCI mass spectrum as 1,4-androstadien-9-fluoro-16α -methyl-6,11,16-triol-3,17-dione(101). Because of the extra functional groups, 6- and 16-hydroxy, it is unlikely that this compound will produce the same oxidation product as the analyte dexamethasone. Therefore, no major interference is expected.

IX. Future Improvements of This Oxidation Method

A. The Use of Isotopically Labelled Standards to Replace the Structurally Similar Internal Standards

In quantitative analysis of a organic compound, a stable isotopically labelled internal standard is often the best choice of internal standard simply because it has almost identical physical and chemical properties as the analyte. The use of an isotopically labelled internal standard is even more important in this project as is explained in the following paragraph.

It is generally believed that the source pressure and source temperature play an important role in the production of EC-NCI mass spectra as well as in determining the response of an analyte. In quantitative analysis, if a structurally similar compound is used as the internal standard, the analyte and the internal standard may experience slightly different source conditions because they normally do not elute from the column at the same time. The difficulty in controlling the source conditions can be demonstrated by the facts that a). the $M/(M-HF)^-$ ratio of the oxidized dexamethasone often changed after the source was cleaned and b). the construction of the calibration curve and the quantitation of dexamethasone from horse urine need to be done in the same day when 6*methyl prednisolone is used as the internal standard. If an isotopically labelled internal standard is used, both internal standard and the analyte should experience the same source conditions because they have the same retention time. The precision

could be improved if both the analyte and the internal standard experience the same source conditions.

B. Increase Oxidation Reaction Yield

Currently, the oxidation reaction yield for most corticosteroids is approximately 30% and the oxidation yield for 6β-hydroxy cortisol is in the 10% range. Development of a higher yield method would certainly improve the overall sensitivity of this oxidation method. In an experiment in which the dexamethasone was first oxidized with sodium bismuthate and then oxidized with chromium trioxide-pyridine complex, about 45% yield was observed. However, it required a two step oxidation reaction.

Table 1: Structure vs response of representative steroids under EC-NCI

steroids	relative response under EC-NCI	relative response under ECD (83)
1,4-androstadien-3,17-dione	1	0.45
progesterone(4-pregnen-3,20-dione)	1	2.2
4-androsten-3,11,17-trione	6	24.8
4-pregnen-3,11,20-trione	4	25.0
1,4-androstadien-3,11,17-trione	350	53.5
4-androsten-3,6,17-trione	350	---

Table 2. Response of oxidized corticosteroids relative to oxidized testosterone (4-androsten-3,17-dione) under EC-NCI

Analyte	Major Oxidation Product	Relative Response Under EC-NCI
testosterone	4-androsten-3,17-dione	1
dexamethasone	9-fluoro-16α-methyl-1,4-androstadien-3,11,17-trione	700
betamethasone	9-fluoro-16β-methyl-1,4-androstadien-3,11,17-trione	700
fludrocortisone	9-fluoro-4-androsten-3,11,17-trione	700
fluorometholone	6α-methyl-9-fluoro-1,4-androstadien-3,11,17-trione	700
cortisol	4-androsten-3,11,17-trione	6
6β-hydroxyl-cortisol	4-androsten-3,6,11,17-tetraone	525
prednisolone	1,4-androstadien-3,11,17-trione	350
6α-methyl-prednisolone	6α-methyl-1,4-androstadien-3,11,17-trione	350
6β-hydroxy-prednisolone	1,4-androstadien-3,6,11,17-tetraone	1050
flumethasone	6α,9-difluoro-16α-methyl-1,4-androstadien-3,11,17-trione	700

Table 3. Tabular EC-NCI mass spectra of oxidized product of indicated corticosteroids under EC-NCI conditions

Analyte (parent steroid)	Molecular Weight of oxidized product	Ion/Relative Intensity
dexamethasone (oxidized)	330	M^-/65 $(M-HF)^-$/100 $(M-HF-CH_3)^-$/4
betamethasone (oxidized)	330	M^-/26 $(M-HF)^-$/100 $(M-HF-CH_3)^-$/2
fludrocortisone (oxidized)	318	M^-/56 $(M-HF)^-$/100
fluorometholone (oxidized)	330	M^-/48 $(M-HF)^-$/100 $(M-HF-CH_3)^-$/3
cortisol (oxidized)	300	$(M-2)^-$/100
6β-hydroxyl cortisol (oxidized)	314	M^-/100
prednisolone (oxidized)	298	M^-/100
6α-methyl prednisolone (oxidized)	312	M^-/100
6β-hydroxyl prednisolone (oxidized)	312	M^-/100
flumethasone (oxidized)	348	M^-/36 $(M-HF)^-$/100 $(M-2HF)^-$/7

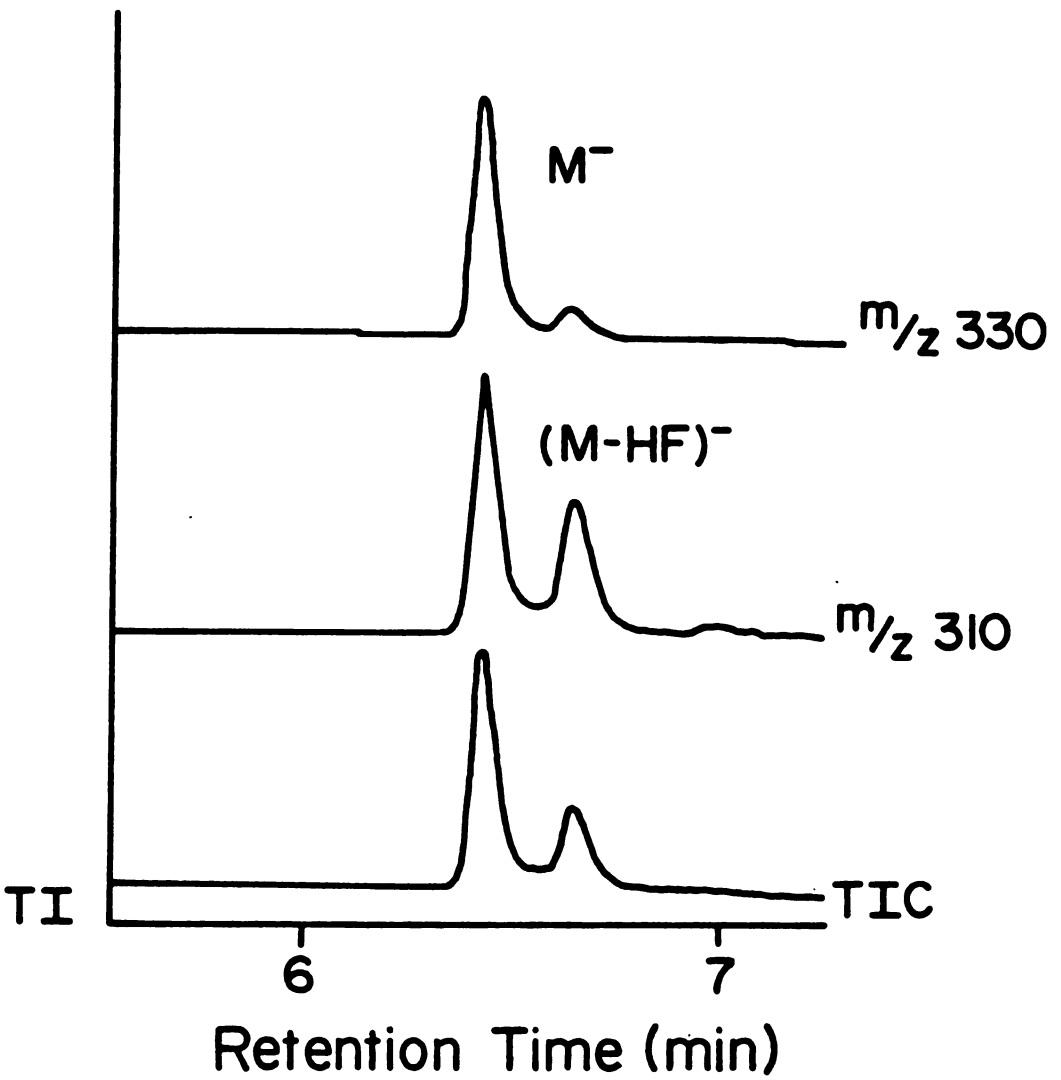


Figure 9. Total ion and selected ion chromatograms of oxidized dexamethasone.

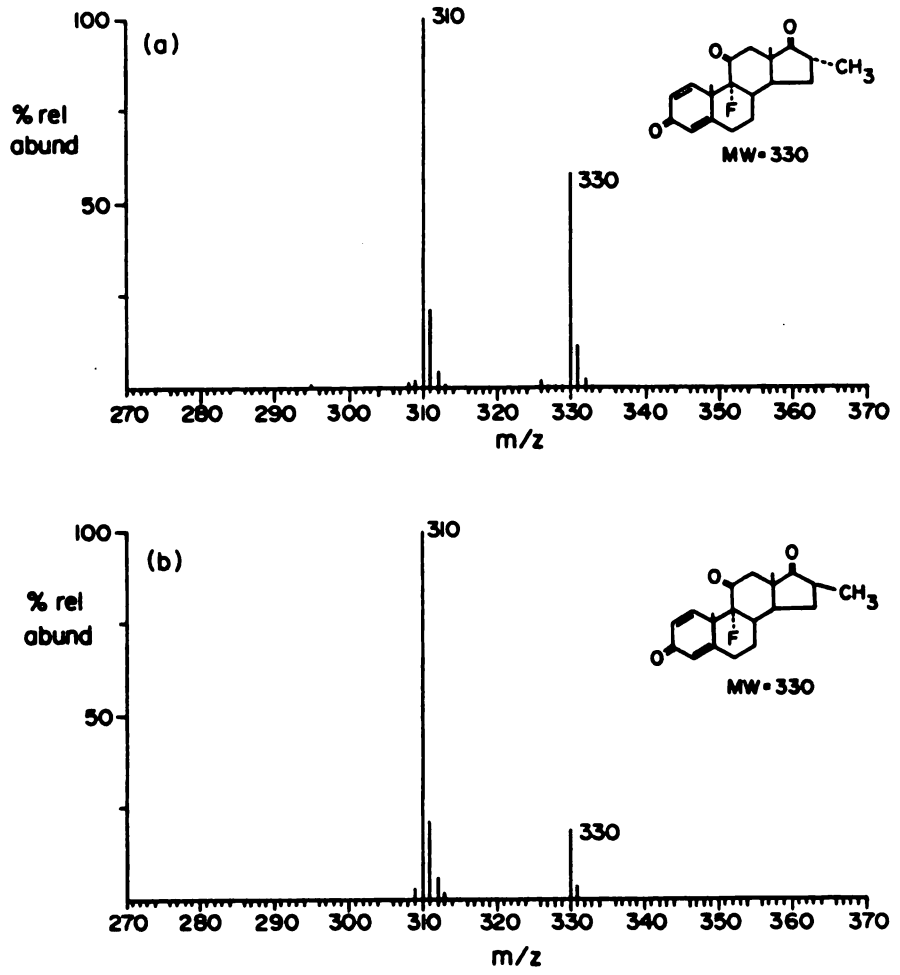


Figure 10. EC-NCI mass spectra of oxidized dexamethasone: a) the major oxidation product, b) the minor oxidation product.

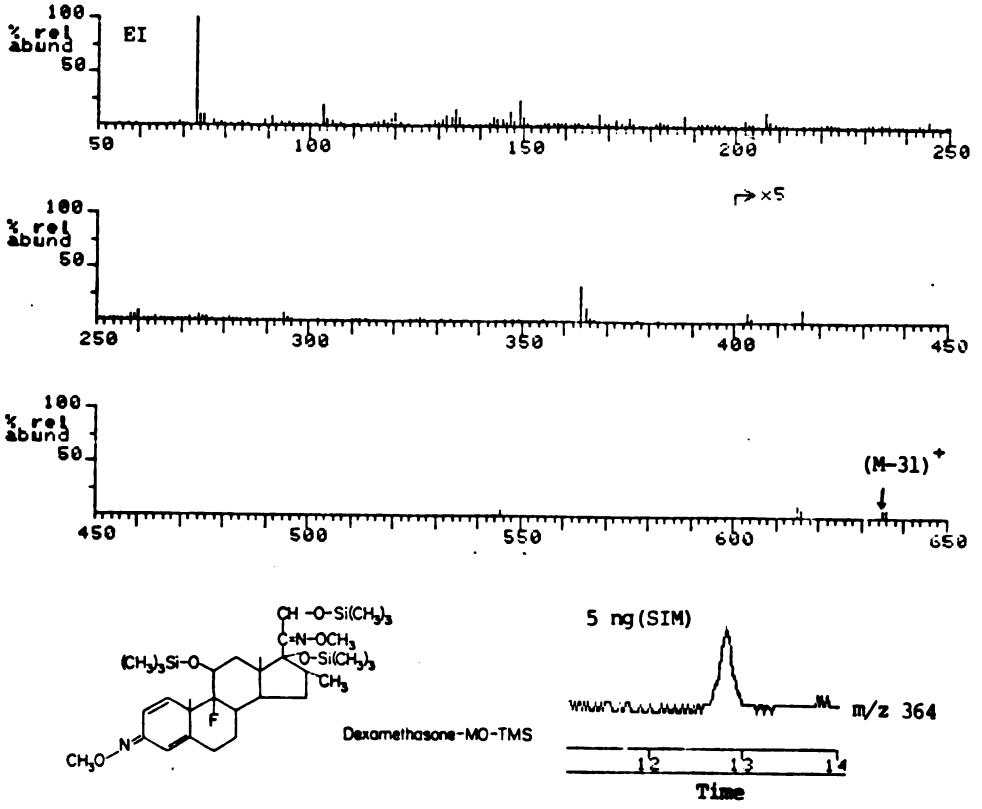
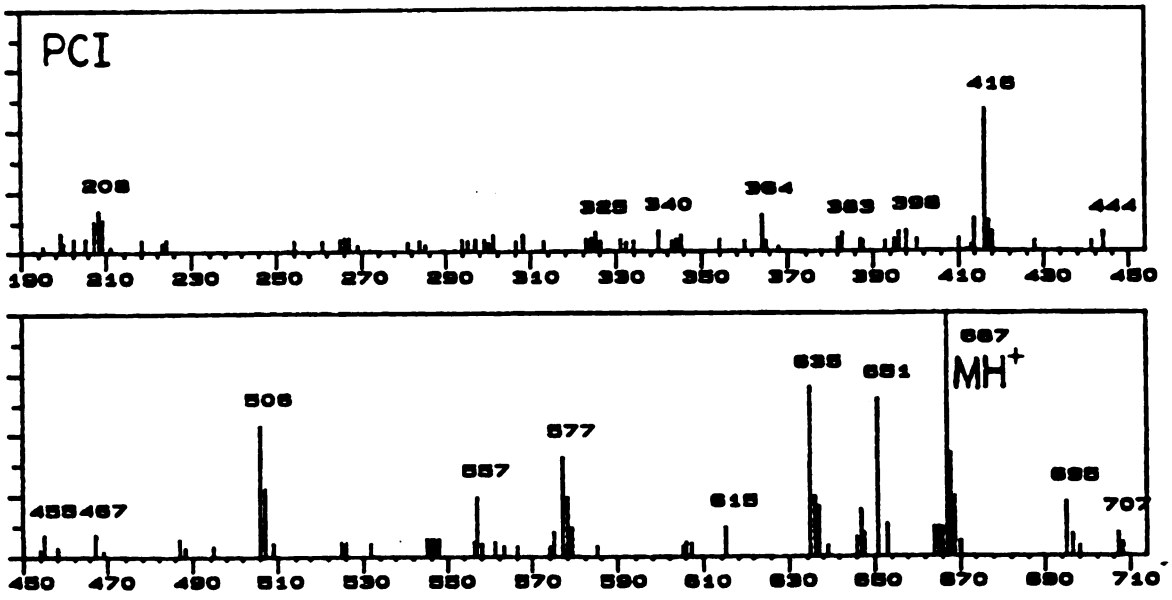


Figure 11. EI mass spectrum of MO-TMS of dexamethasone and the detection of low level of MO-TMS of dexamethasone by selected ion monitoring of m/z 364 under EI conditions.



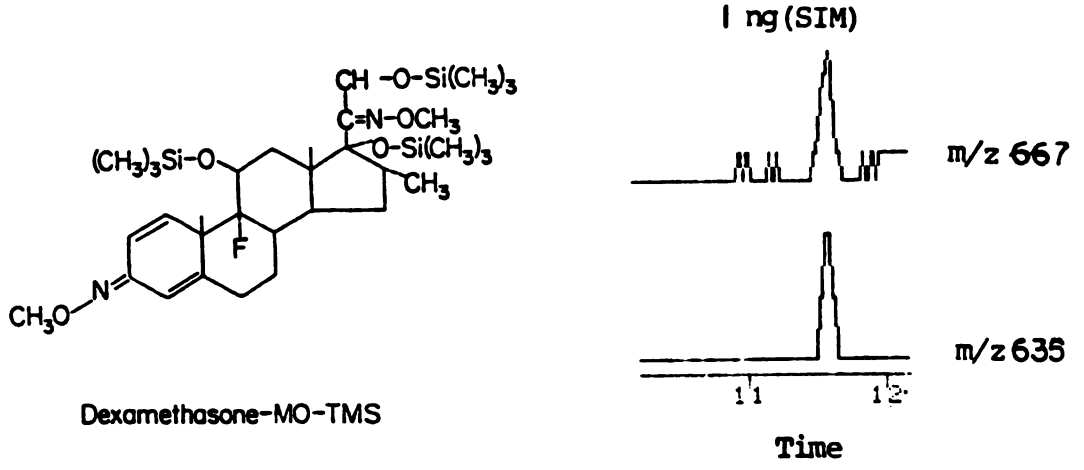


Figure 12. PCI mass spectrum of MO-TMS of dexamethasone and the detection of low level of MO-TMS of dexamethasone by selected ion monitoring of MH^+ and $(MH-CH_3OH)^+$ ions under methane PCI conditions.

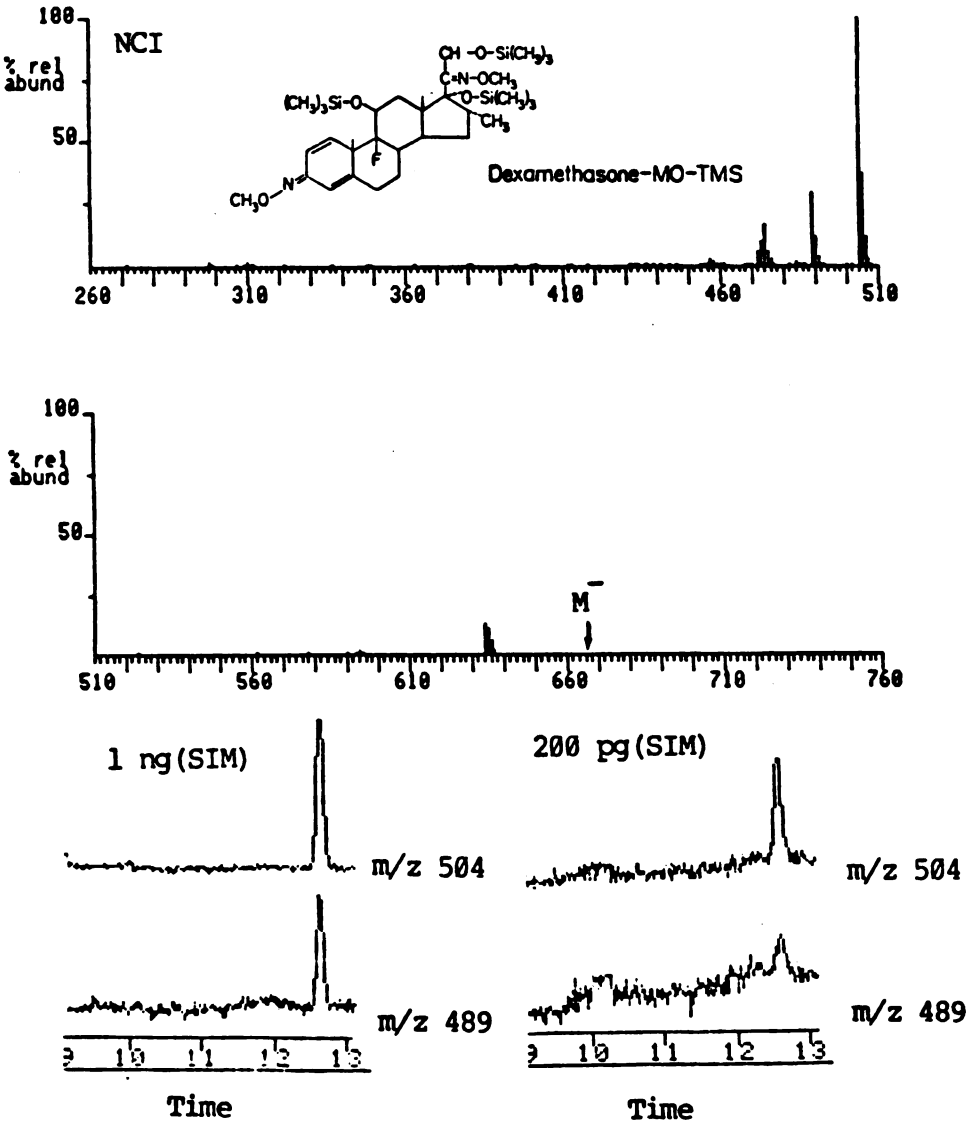


Figure 13. EC-NCI mass spectrum of MO-TMS of dexamethasone and the detection of low levels of MO-TMS of dexamethasone by selected ion monitoring of m/z 504 and m/z 489 ions under EC-NCI conditions.

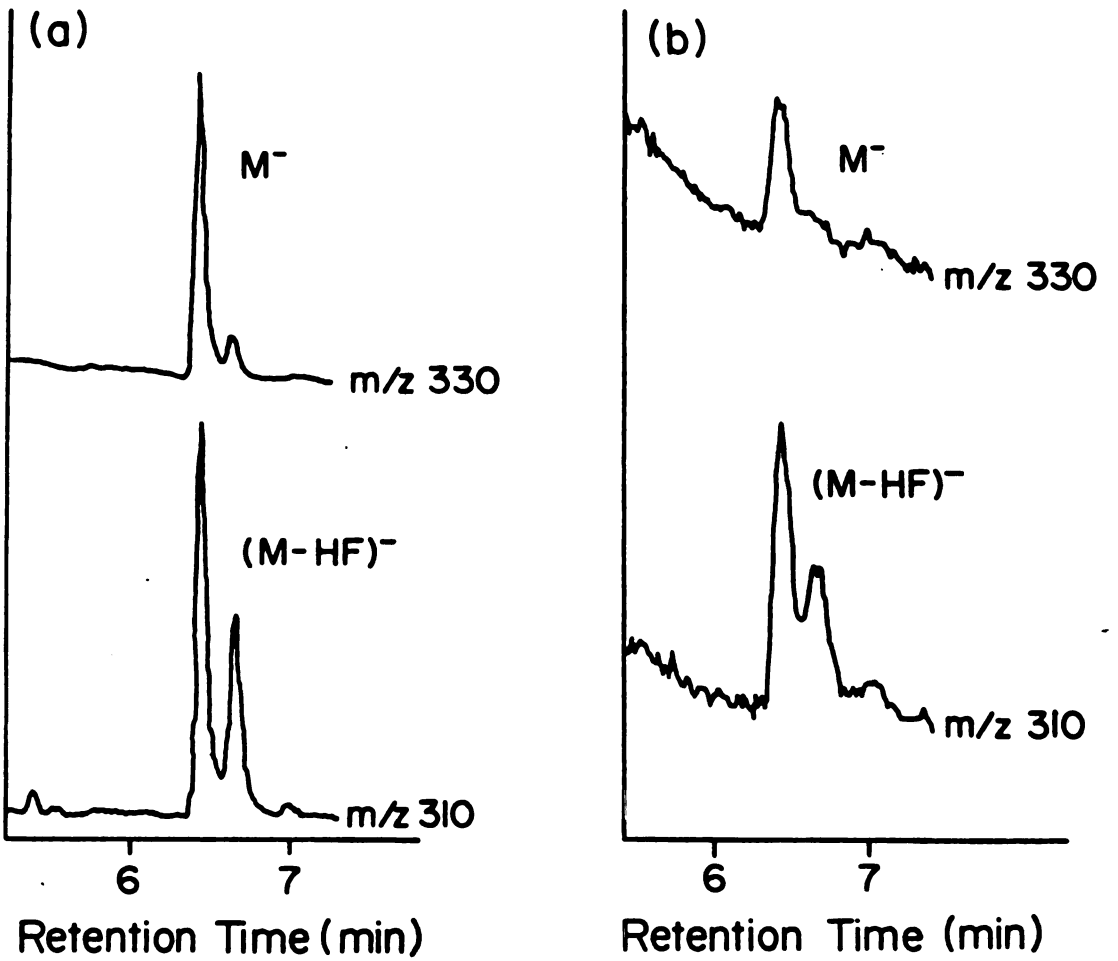


Figure 14. Detection of low levels of oxidized dexamethasone by selected ion monitoring of M^- and $(M-HF)^-$ ions under EC-NCI conditions: a) 200pg injected on-column, b) 10pg injected on-column.

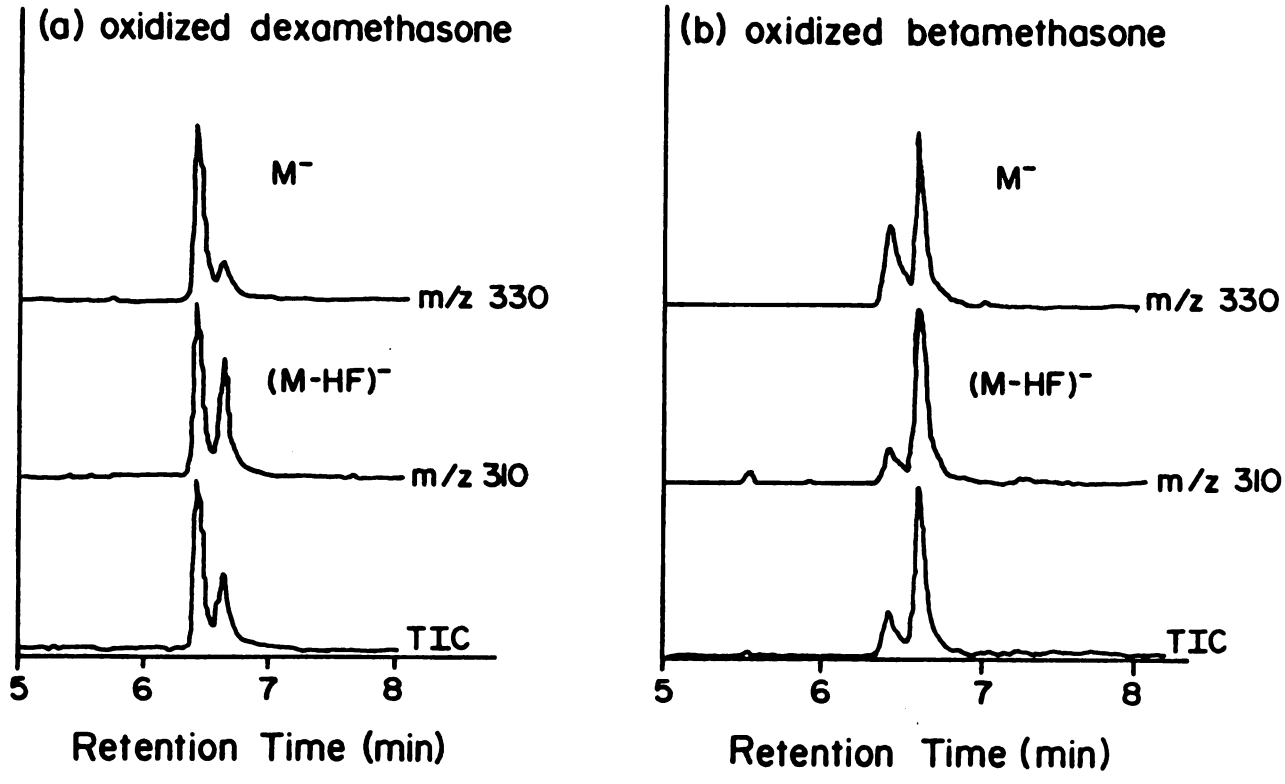


Figure 15. a) Reconstructed total ion current(TIC) and reconstructed ion currents of M^- and $(M-HF)^-$ of oxidized dexamethasone. b) Reconstructed TIC and reconstructed ion currents of M^- and $(M-HF)^-$ of oxidized betamethasone.

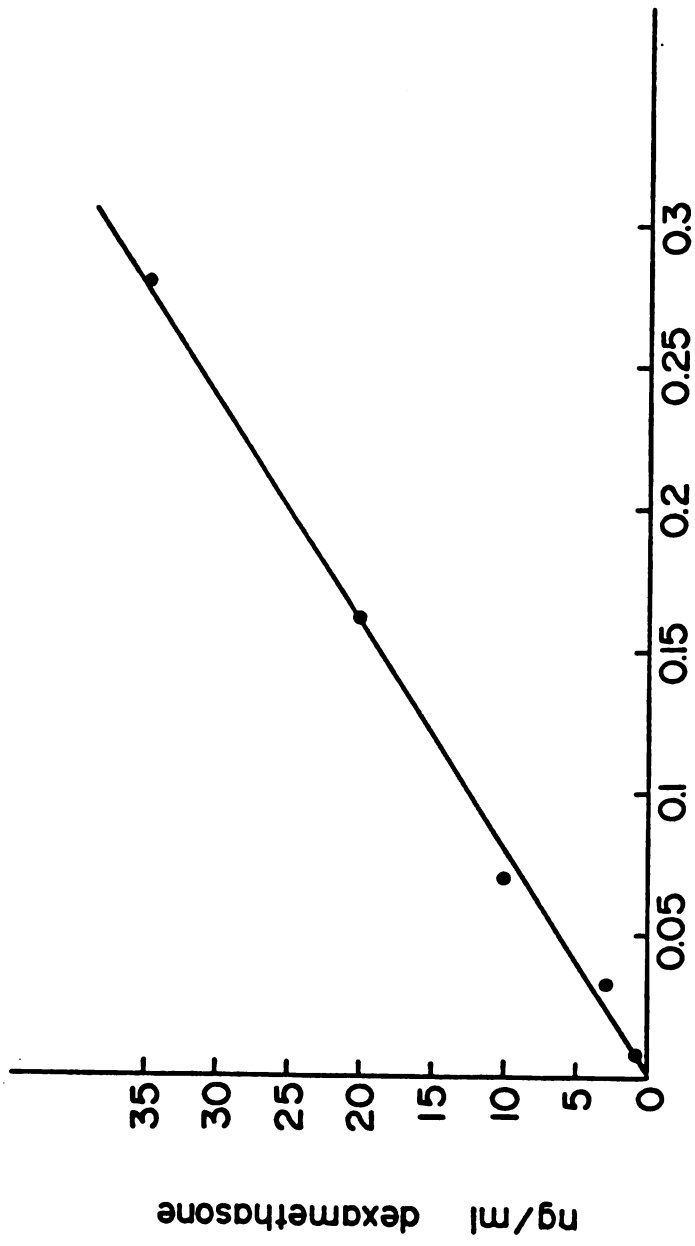
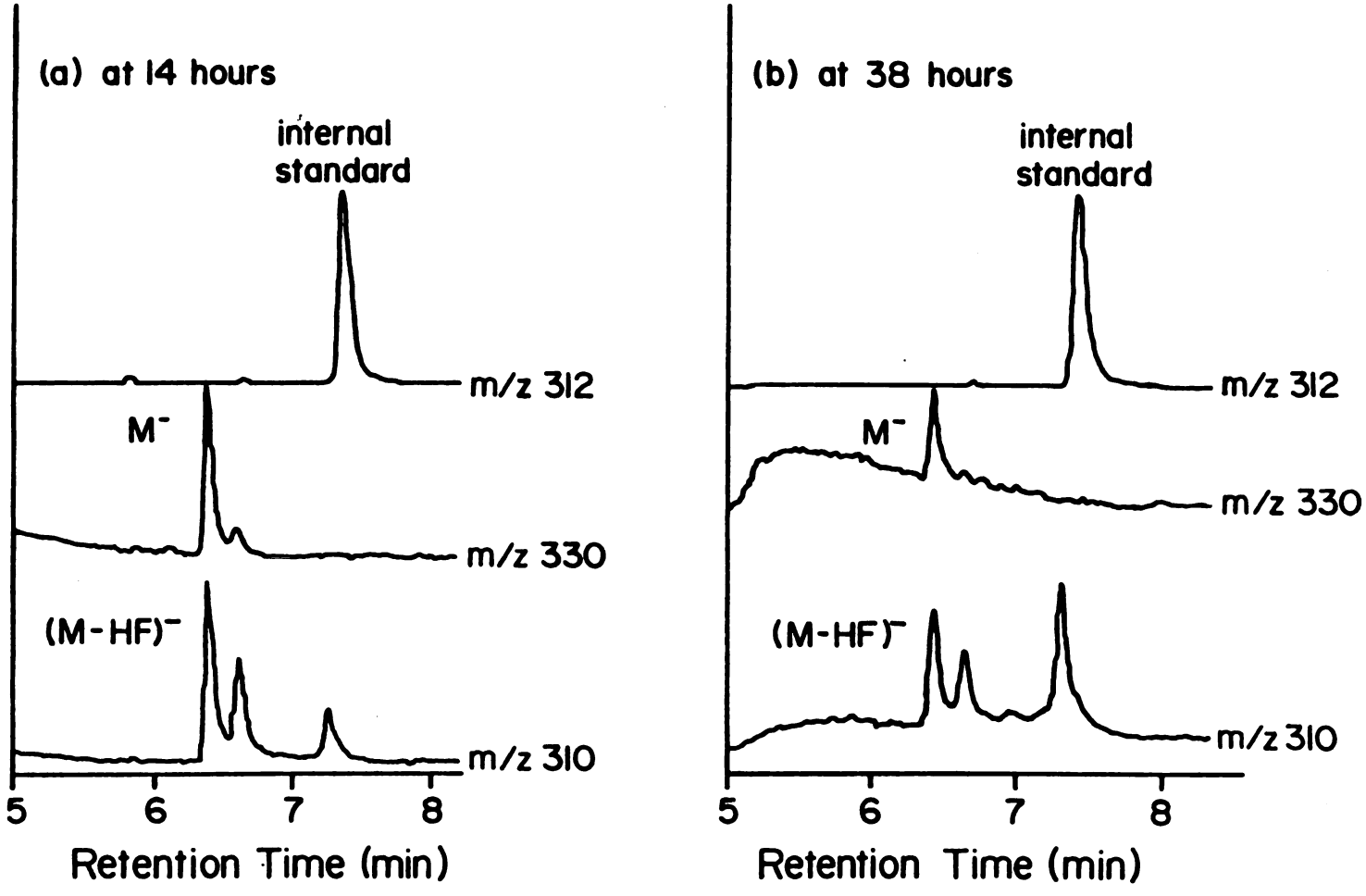


Figure 16. Standard curve of dexamethasone spiked into control horse urine using selected ion monitoring focusing on major ions of oxidized dexamethasone(m/z 330 and m/z 310) and oxidized internal standard(m/z 312).

Figure 17. Use of selected ion monitoring to quantitatively determine the oxidized products of dexamethasone extracted from urine samples obtained at a) 14 hours and b) 38 hours after adminstration of 5mg drug to a horse.


(a) at 14 hours
internal standard
m/z 312
M⁻
m/z 330
(M-HF)⁻
m/z 310
5
6
7
8
Retention Time (min)
(b) at 38 hours
internal standard
m/z 312
M⁻
m/z 330
(M-HF)⁻
m/z 310
5
6
7
8
Retention Time (min)

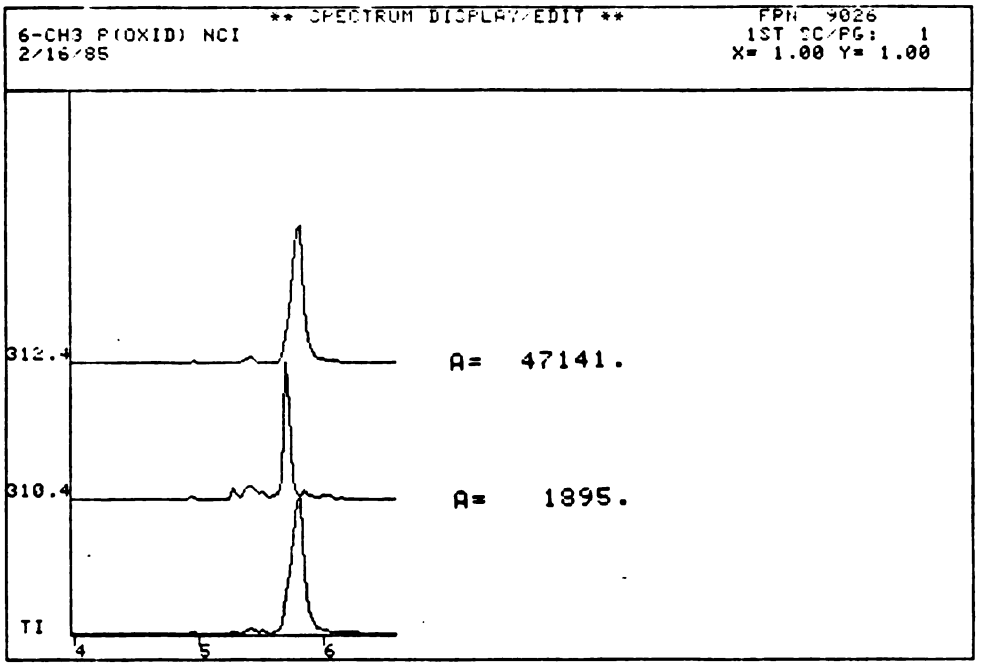


Figure 18. Reconstructed total ion current and reconstructed ion currents of m/z 312 and m/z 310. The m/z 310 is the M^- of the by-product of 6α-methyl prednisolone of the oxidation reaction.

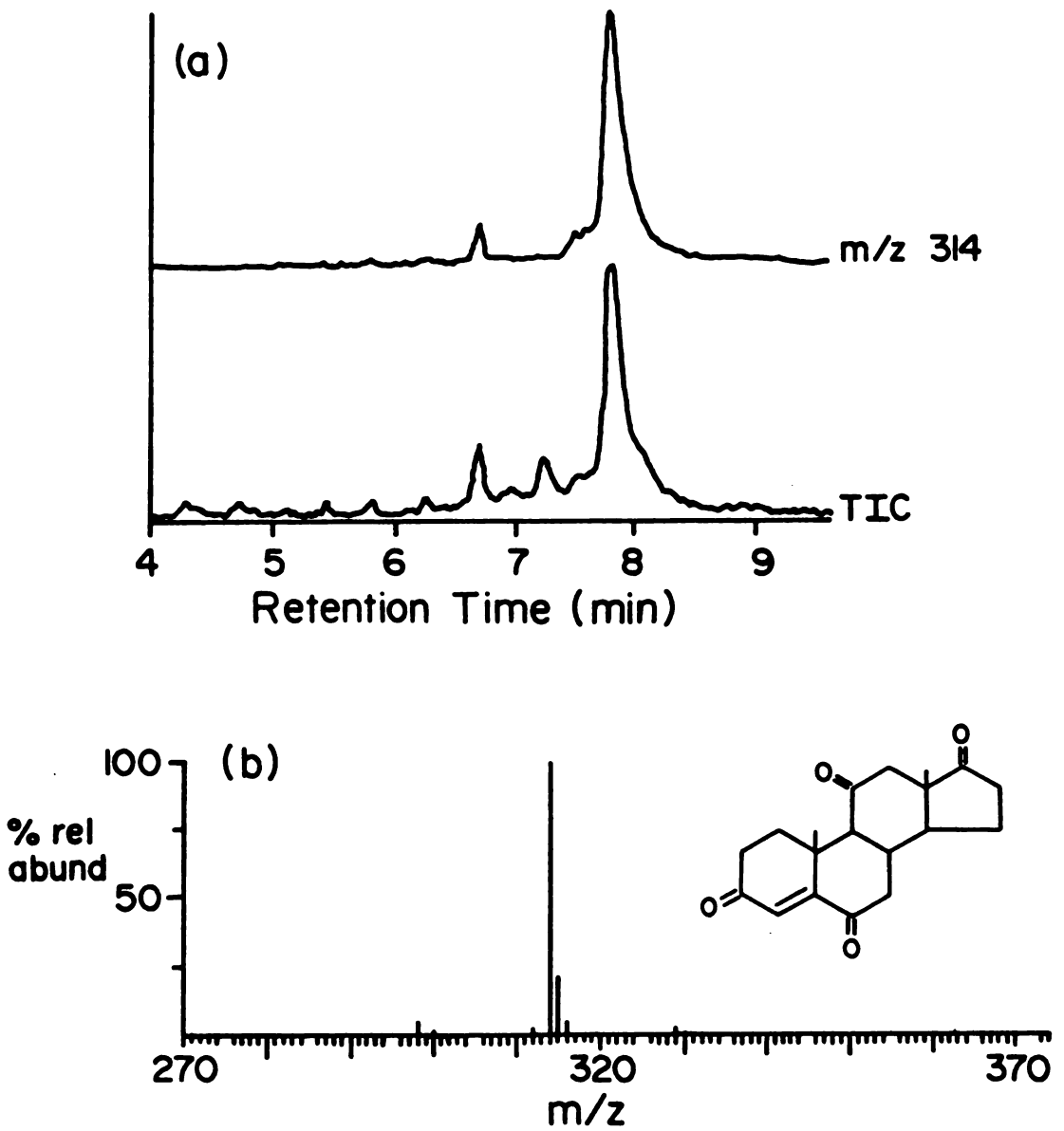


Figure 19. a) Reconstructed total ion current of human urine extract and reconstructed ion current of m/z 314(M^- of oxidized 6β-hydroxy cortisol). b) EC-NCI mass spectrum of the major peak in a) with retention time of 7.8 minutes.

Chapter 4. The Pentafluorobenzyl Derivative for Estrogens

Introduction:

Estradiol and other estrogen metabolites are present in the urine at level 100 to 1000 times lower than those of the androgen and corticosteroid metabolites. Because the concentration of estrogens is low, a highly sensitive and highly selective method is needed to measure the concentration of estrogens in urine. The pentafluorobenzyl(PFB) derivative is probably one of the most widely used derivatives in the analysis of organic compounds by EC-NCI. It has been used in the analysis of carboxylic acids(102) and prostaglandins(103,104). The major reasons for its wide use are: a). the total ion current under EC-NCI conditions often increased dramatically for PFB derivatives and b). the negative charge often stays with the analyte and not with the pentafluorobenzyl moiety when the single bond connecting the analyte and PFB moiety breaks during EC-NCI. Therefore, it produces an intense $(M\text{-}PFB)^-$ ion which is suitable for selected ion monitoring.

The PFB derivative can be formed only with compounds that have an acidic hydrogen. Because all the estrogens have a phenolic hydrogen in the A ring, the possibility of using a PFB derivative in the analysis of estrogens was studied.

Experimental Section:

1. Materials

Pentafluorobenzyl bromide, BSTFA were purchased from Pierce Inc.; estrone, estradiol from Steroids Inc.; tetrabutylammonium(TBA) hydrogen sulfate, sodium hydroxide from Aldrich Inc.

2. Synthesis of PFB derivatives

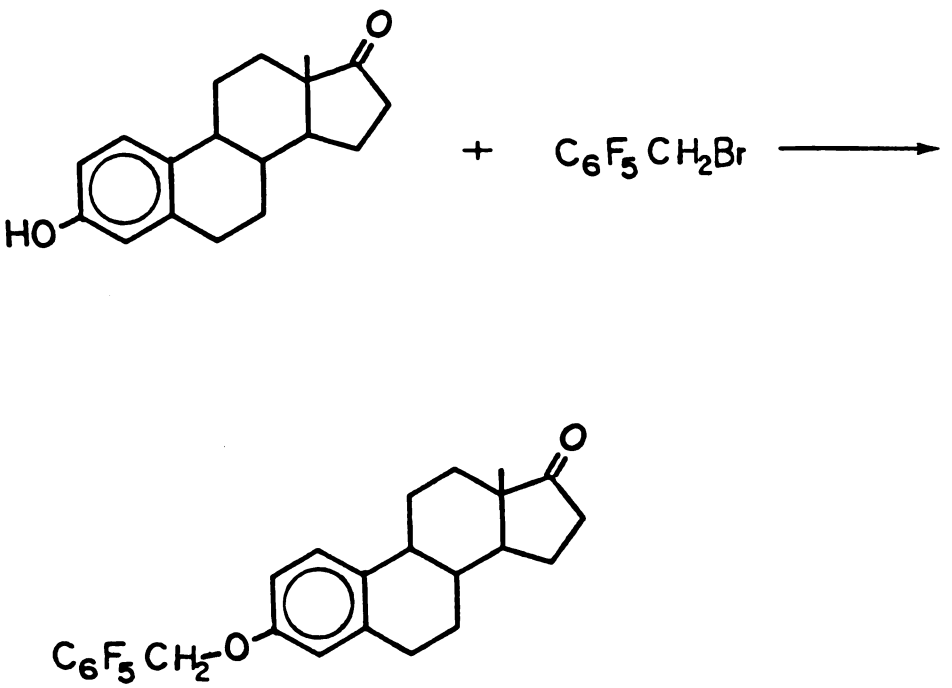
To 1 ml of dichloromethane containing 0.2 mg sample is added 1 ml of an aqueous solution which is 0.1 M in TBA hydrogen sulfate and 0.2 M in sodium hydroxide. 20 µl of PFB bromide is added and the reaction mixture which is stored in the dark for 30 minutes. The dichloromethane layer is isolated and the solvent is evaporated with a nitrogen stream. The residue is dissolved in 1 ml hexane before the analysis.

3. Synthesis of TMS derivative

Estrogens which have nonacidic hydroxy groups such as the 17-hydroxy in estradiol have to be treated with a TMS reagent in order to improve their chromatographic peak shape. After the PFB reaction is completed, the compound is dissolved in 100 µl of BSTFA. The solution is set at room temperature for 1 hour. Following the removal of excess BSTFA with nitrogen, the residue is dissolved in hexane before the analysis.

Results and Discussion:

The PFB derivative is formed as shown in Scheme 8.



Scheme 8

1. The EC-NCI Mass Spectra

The EC-NCI mass spectra of PFB-estrone and PFB-TMS-estradiol are shown in Figure 20. For both PFB-estrone and PFB-TMS-estradiol, over 95% of the total ion current is carried by the $(M-PFB)^-$ ion(m/z=269 for PFB-estrone, m/z=343 for PFB-TMS-estradiol).

2. The Response Under EC-NCI

The response of PFB-TMS-estradiol was measured relative to the oxidized dexamethasone as shown in Figure 21. 100 ng of oxidized dexamethasone and 80 ng of PFB-TMS-estradiol were coinjected to capillary GC for EC-NCI detection. Results show that the PFB-TMS-estradiol had a slightly higher response than did the oxidized dexamethasone. Since the detection limit of the oxidized dexamethasone has been measured to be approximately 10 pg, the sensitivity of PFB-TMS-estradiol is expected in the same range.

3. The Feasibility of the PFB Derivative in the Analysis of Estradiol in Urine

The use of the PFB derivative in the analysis of estradiol in urine is being studied by Mr. J. Leary of this laboratory. Preliminary results showed that high background interference is the major problem. The interference may be caused by many endogeneous compounds such as organic acids and fatty acids in the human urine which also contain acidic hydrogens that react with PFB bromide. Therefore, a more extensive sample clean up procedure for the urine will be necessary.

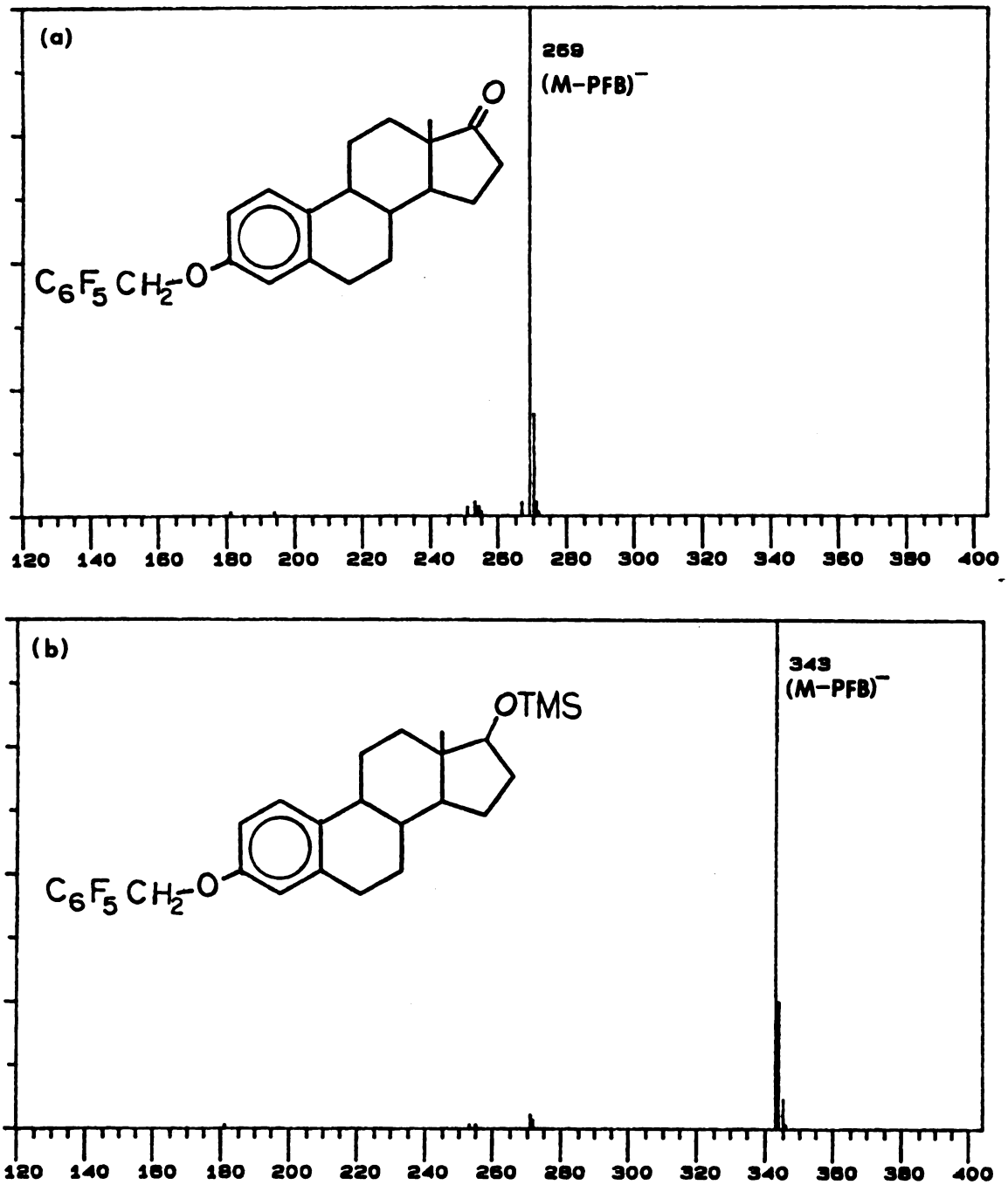


Figure 20. a) EC-NCI mass spectrum of PFB-estrone.
b) EC-NCI mass spectrum of PFB-TMS-estradiol.

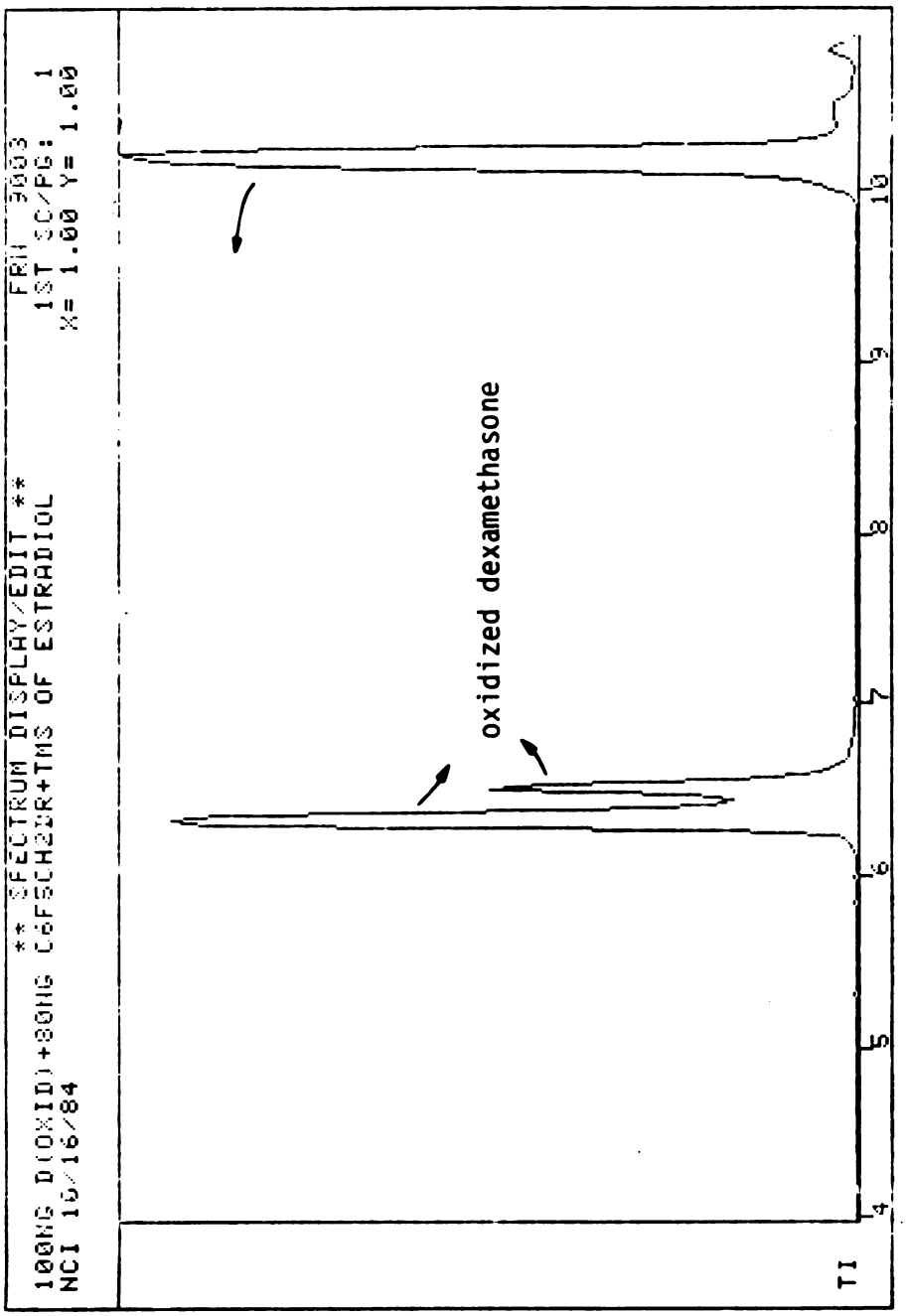


Figure 21. Reconstructed total ion current chromatogram resulting from coinjection of 100ng oxidized dexamethasone and 80ng PFB-TMS-estradiol.

Chapter 5: The Modification of an HP 5985A GC/MS for Negative Ion Detection

Introduction

Negative ions produced in the source of a magnetic sector mass spectrometer are easily detected by a standard electron multiplier, because the kinetic energy of the negative ions (approximately 8kV) in a magnetic sector mass spectrometer is high enough to bombard the cathode (approximately -2kV) of the electron multiplier with sufficient energy to expel electrons at the first stage(105). Unfortunately, negative ions in a quadrupole mass spectrometer have very low kinetic energy (approximately 10-20V). Thus, negative ions produced in a quadrupole mass spectrometer will not impact on the cathode of a standard electron multiplier with sufficient energy to be detected.

In a continuous dynode electron multiplier, although there are no discrete stages, the overall operation can be described as if the electrons were passing from "stage" to "stage" as they "bounce" along the surface of the active element of the continuous dynode. In the normal positive mode, positive ions impact on the cathode (first "stage" of continuous dynode electron multiplier, potential approximately -2kV), thereby generating an equivalent number of electrons. Amplification is accomplished through a "cascading effect" of expelled electrons because the positive gradient potential, "stage" after "stage", results in the number of electrons increasing from "stage" to "stage". In the last "stage" (anode), the potential is about 2kV higher than that in the

first "stage" (cathode); in other words, the anode is at ground potential.

One approach to the detection of negative ions on a quadrupole mass spectrometer is to apply a high positive potential (approximately +2kV) on the cathode (Figure 22) so that the cathode is also at a sufficiently positive potential for efficient collection of negative ions(106). Because the electron multiplier has to be operated under a positive gradient potential, the voltage on the anode is normally operated at approximately +4kV (floated at +4kV). Several disadvantages have been reported concerning this approach with a floating electron multiplier. First, complex preamplifier circuity is required to reference the signal to ground potential. Second, a special high voltage floating coaxial signal feedthrough is required on the vaccum envelope to prevent microphonic noise and charge leakage. Third, the noise due to stray electrons was reported to be 30 times larger than that observed in a conventional electron multiplier(107).

A novel approach which was developed by Stafford and coworkers(108) is to convert the negative ions to secondary positive ions, which are subequently detected by a conventional positive ion electron multiplier. Negative to positive ion conversion can be accomplished by impacting the primary negative ions on a conversion dynode held at a high positive potential (approximately +3kV) as shown in Figure 23. This methodology eliminates the high voltage problem associated with floating the anode of an electron multiplier at a high positive potential. A further advantage of this negative ion detector is that the noise level from

stray electrons is significantly reduced because electrons do not produce positive ions when they impact on the conversion dynode. Thus, they are not detected by the electron multiplier.

A general trend and important observation has been reported by Stafford(108). That is, with the conversion dynode in operation, the gain of the detection system is mass dependent, the gain of the multiplier increases with the mass of the detected ion. This relationship supports the assumption that positive ion fragments are produced by the impact of negative ions on the dynode surface. It is possible that the larger negative ions yield more fragments on impact and could increase the overall gain of the system. Because the conventional positive ion multiplier exhibits a reduction in gain with higher mass and because the higher mass ions are often more interesting, the increased gain at high mass in the conversion dynode multiplier is considered as a major advantage.

Because of the advantages of the conversion dynode that were mentioned previously, one of the Hewlett Packard 5985A Gas chromatograph/Mass spectrometers in the MSU/NIH regional mass spectrometry facility was modified for negative ion detection based on the conversion dynode concept.

The diagram of normal positive ion detection of the HP 5985A GC/MS is shown in Figure 24.

1. The Ion Source

A negative ion circuit board was built so that all the ion source voltages were converted from the positive ion mode to the negative ion mode. The circuit diagram which was used to convert draw out voltage from positive ion mode to negative ion mode is shown in Figure 25. Similar circuits were used to convert ion focusing, entrance lens, and x-ray plate voltages. A CI volume which was designed for negative ions was purchased from Hewlett Packard Inc.

2. The Detector

The Galileo 4770 electron multiplier was replacted by a Galileo 4770B electron multiplier. Unlike the 4770 multiplier, the ion entrance aperture of the electron multiplier and the ion deflector shield (x-ray plate) are separated from each other in the 4770B electron multiplier. In the negative mode, the voltage on the ion deflector plate was set to +2.5KV (which was suppilied by a Bertan 602B high voltage power supply) so that the negative ions can be converted to positive ions and then detected by the electron multiplier. The voltage on the ion entrance aperture was set between 0 and +10V. In the positive mode, the same voltage (0-+255V) was applied to both the ion entrance aperture and ion deflector plate.

In order to apply a high voltage to the conversion dynode (ion deflector plate), the ion deflector low voltage feedthrough which was mounted on the detector flange was replaced by a MHV high vaccum high voltage feedthrough (Cermeseal Inc.). An additional high vaccum feed through was mounted so that a voltage could be applied to the ion entrance plate.

The diagram of the modified negative ion detection system of the HP 5985A GC/MS is shown in Figure 26.

3. The Rods

After the modification of the detector and the reversing of all source voltages, negative ions can now be easily detected by this modified instrument. However, the negative ion peak shape was not as good as its positive ion conterpart. Quite often, it showed unresolved peak shapes and incorrect isotopic aboundances(as shown in Figure 27). Bruins(109) reported that it is necessary to reverse the d.c. polarity of the rods when the operation is changed from positive to negative ions and vice versa. However, no sigificant improvement in peak shape was observed by switching the polarity of the rods in the modified HP 5985A GC/MS.

In an experiment in which the ion source, the voltage supply, the "negative ion " circuit board, and the detector were all the same as before, instead of using the old rods, rods which had been verified to be spherically symmetrical by the manufacturer were used. The result was quite satisifactory. Well resolved peaks were observed without changing the polarity of the rods. This experiment proved that replacment of the old rods with symmetrical rods was necessary. After the symmetrical quadruple rods were purchased and mounted, the peak shape was improved significantly. A tuning file which was recorded about one year after installation of the symmetrical rods is shown in Figure 28.

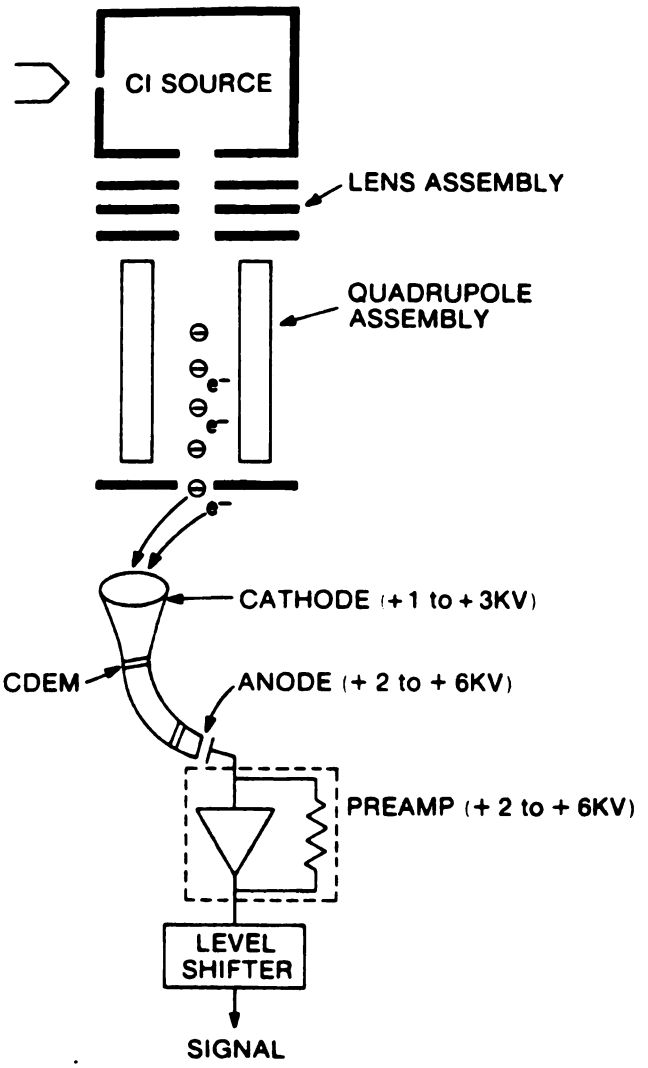


Figure 22. Off-ground negative ion continuous dynode electron multiplier.

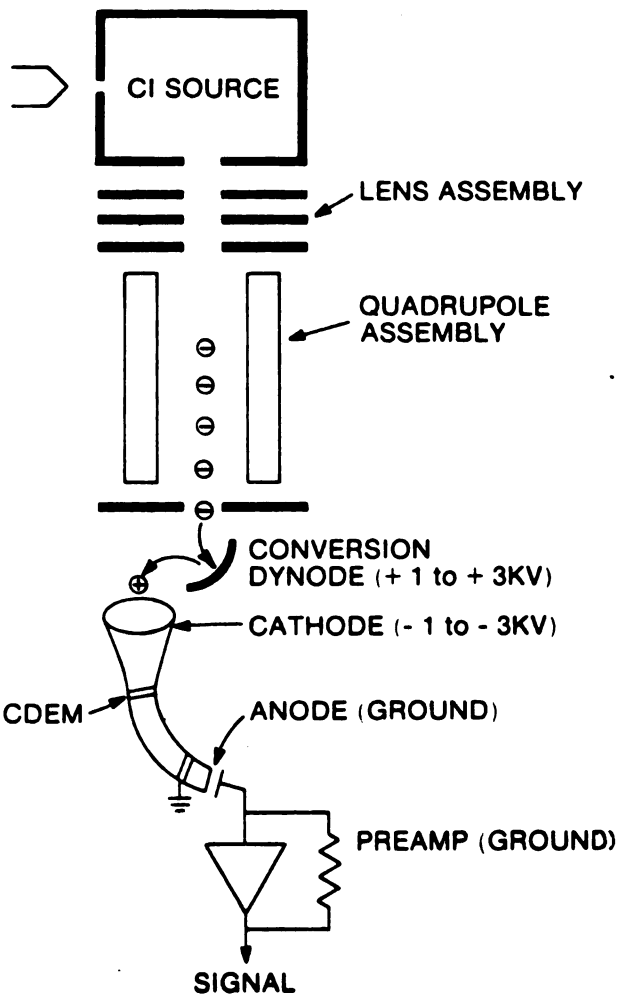


Figure 23. Conversion dynode negative ion detector.

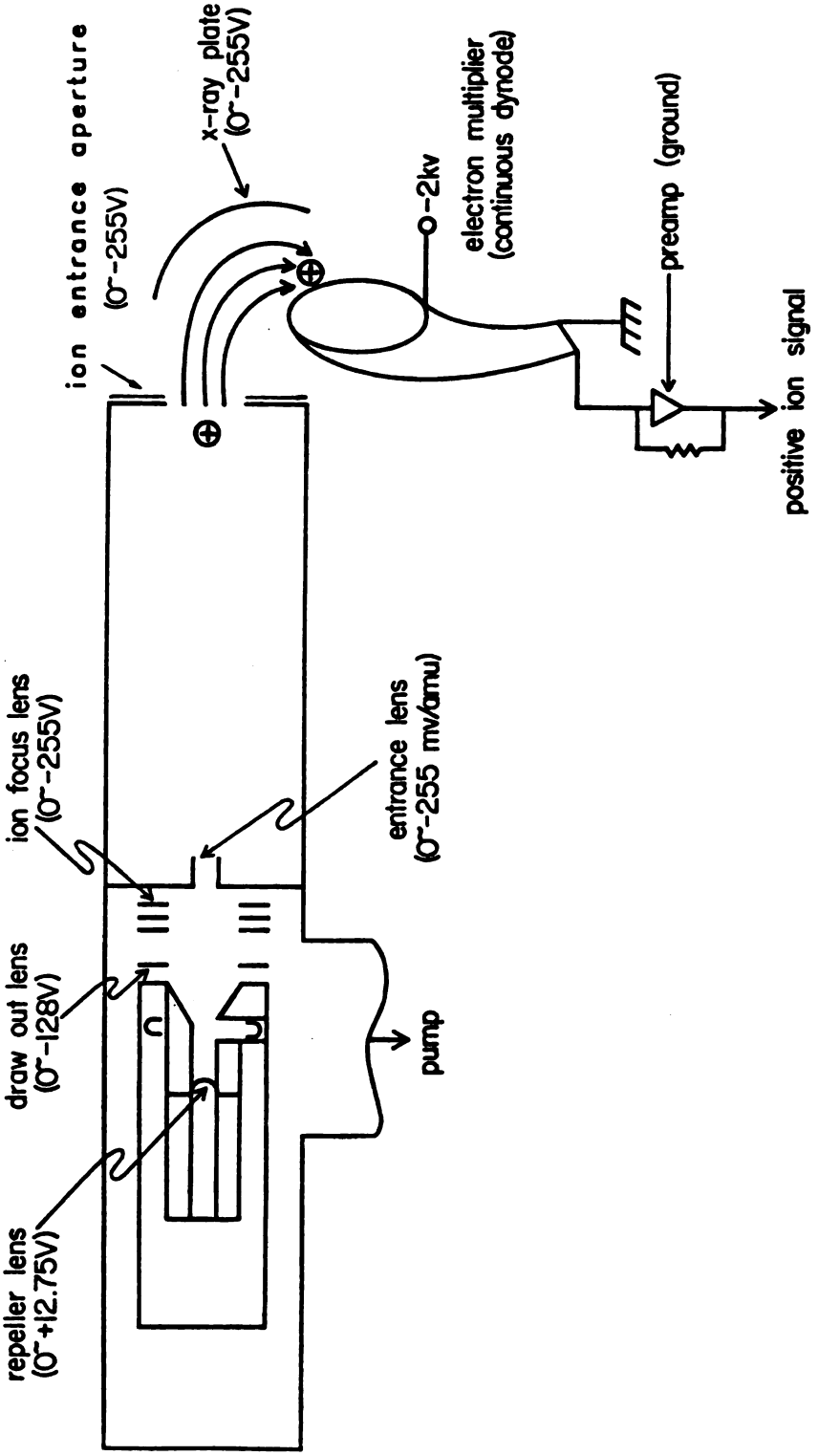


Figure 24. HP 5985A GC/MS: Schematic diagram for positive ion detection(normal).

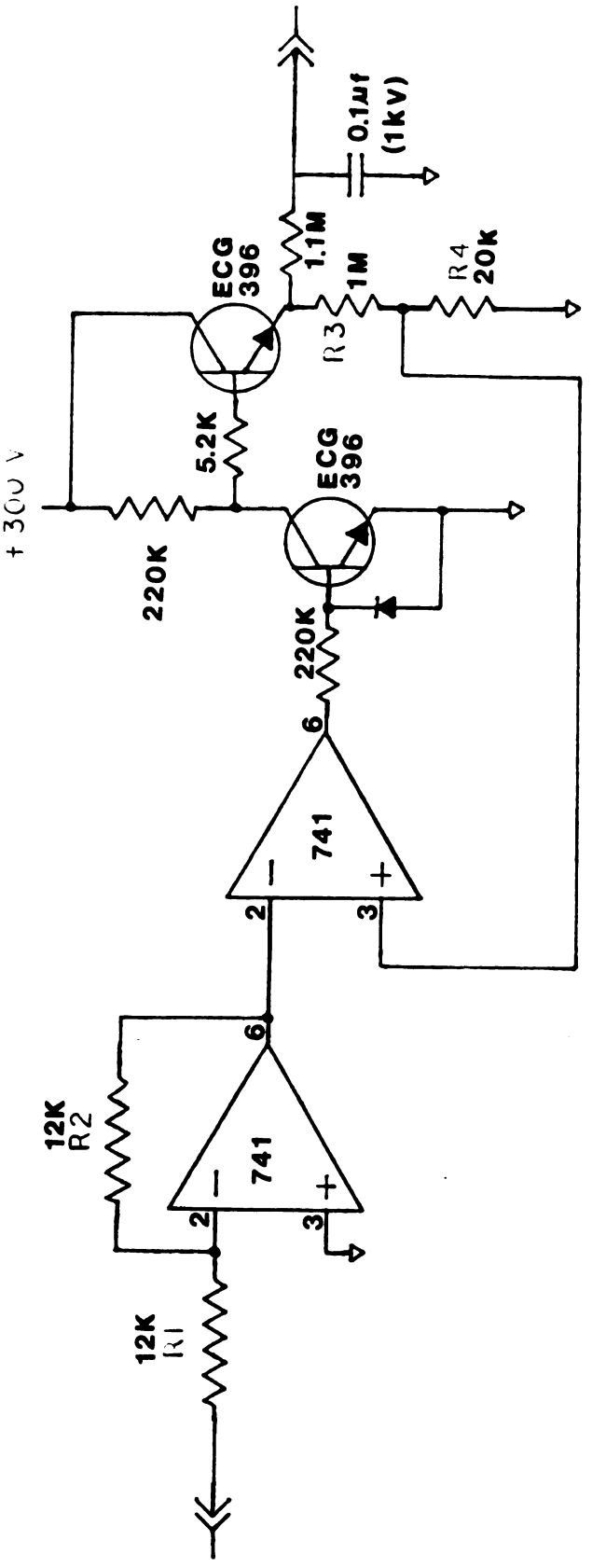


Figure 25. Circuit diagram for the conversion of draw out voltage.

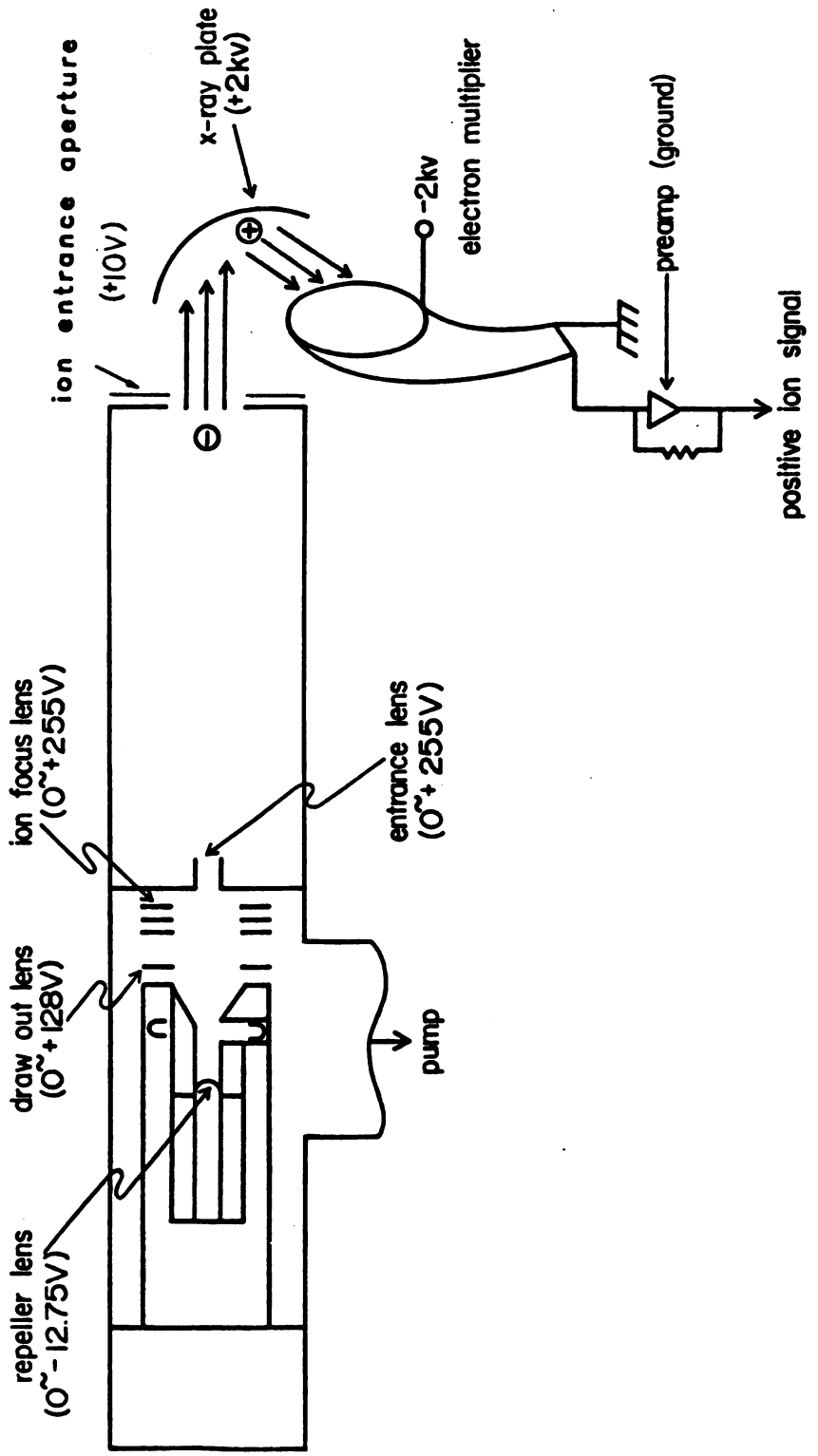
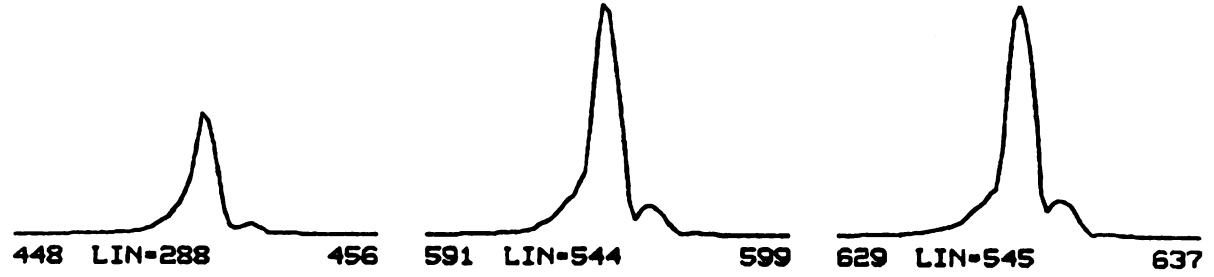


Figure 26. HP 5985A GC/MS: Modified electrically for negative ion detection.

```
* CURRENT PARAMETERS *
L05985-10041H.I

DATE (FPN 1002 ): 3/2/83
REPELLER(V)=10.8          EM VOLTAGE(V)=2600
DRAWOUT(V)=76.5           AMU GAIN=190
ION FOCUS(V)=170          AMU OFFSET=97
ENT LENS(MV/AMU)=124      MASS AXIS GAIN=.998489
X-RAY(V)=56               MASS AXIS OFFSET=-.538086
EMISSION(UA)=300          IONS: POSITIVE
ELECT. ENERGY(EV)=200     ACTUAL SOURCE TEMP =152
LOG AMP OFFSET=1
```



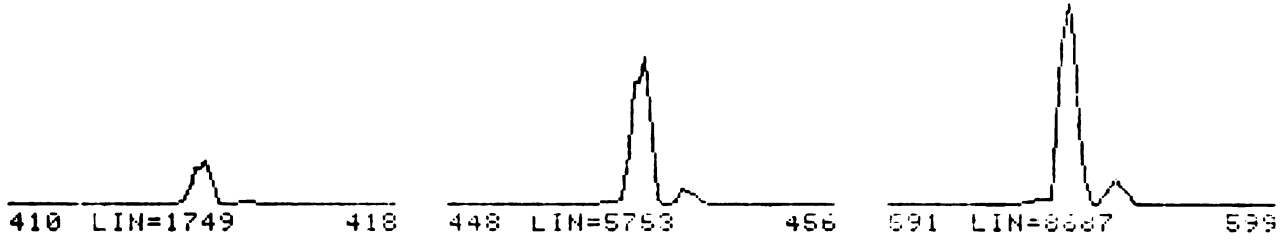
ACTUAL MASS	ABS ABUND	REL ABUND	FWHM	ACTUAL ISO MS	ISO ABUND	ACTUAL ISO RATIO	EFF WIDTH
452.21	287	100	.61	453.21	25	8.71	.622
594.97	543	189.19	.68	595.93	75	13.81	.66
633.06	544	189.54	.7	634.02	86	15.8	.683

```
OPTION?
```

Figure 27. A negative ion tuning file with the old rods.

```
                    * CURRENT PARAMETERS *
                       [05985-10041H]

DATE (FRN 1001 ): 12/27/84
REPELLER(V)=3.89              EM VOLTAGE(V)=1400
DRAWOUT(V)=43.5               AMU GAIN=180
ION FOCUS(V)=70               AMU OFFSET=102
ENT LENS(MV/AMU)=80           MASS AXIS GAIN=.998897
X-RAY(V)=99                   MASS AXIS OFFSET=-.277212
EMISSION(UA)=300              IONS: POSITIVE
ELECT. ENERGY(EV)=175         ACTUAL SOURCE TEMP =188
LOG AMP OFFSET=1
```



ACTUAL MASS	ABS ABUND	REL ABUND	FWHM	ACTUAL ISO MS	ISO ABUND	ACTUAL ISO RATIO	EFF WIDTH
414.06	1748	100	.41	415.07	145	8.29	.532
452.04	5752	329.06	.42	453.02	645	11.21	.54
594.82	8686	496.91	.41	595.83	1049	12.07	.474

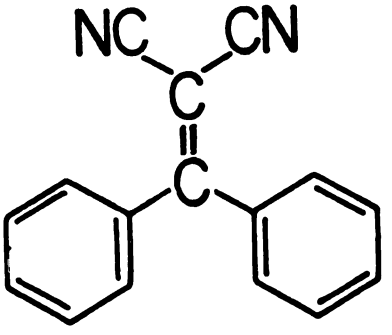
```
OPTION?
```

Figure 28. A negative ion tuning file with the new rods.

Chapter 6. Electron Impact Mass Spectra of Dicyanomethylene Derivatives of Benzophenone Analogs

Introduction

The DCM derivative has been studied for features that may enhance the response of ketonic compounds under electron capture negative chemical ionization conditions. In the course of characterizing the dicyanomethylene (DCM) derivative of benzophenone(IX) by electron impact(EI) mass spectrometry, it was observed that the EI mass spectrum was characterized by a base peak at m/z=165 corresponding to $(M-65)^+$. This ion cannot be explained by a simple fragmentation mechanism.



(IX)

The EI mass spectrum of the DCM derivative of benzophenone has been reported by Reichert(110) but with no explanation for the major peak corresponding to $(M-65)^+$. In this chapter, a fragmentation mechanism in which the double bond connecting the parent structure and the DCM moiety is saturated by the hydrogen atoms from the ring system before its cleavage is proposed to rationalize the production of the $(M-65)^+$ ion. The evidence which comes from the EI mass spectra of several model compounds(including stable-isotope labelled analogs) as well as from the MS/MS study are presented to support the proposed fragmentation mechanism.

Experimental

1. Chemicals

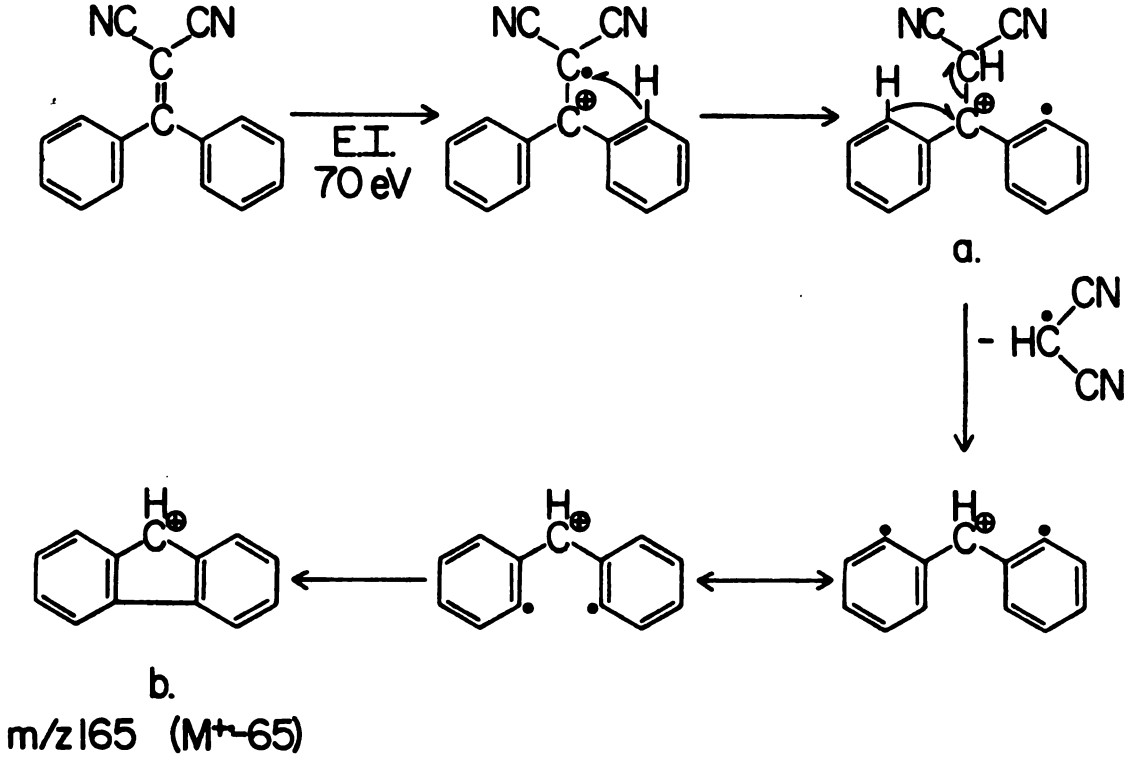
Most of the DCM derivatives were prepared by condensing benzophenone analogs which are commerically available with malononitrile in the presence of a buffer consisting of ammonium acetate and acetic acid in ethyl acetate. This mixture was then refluxed for approximately four hours. Yields varied from 10 to 90%. Frequently, the reaction product would be accompanied by dark colored by-products (polymer of malononitrile ?) which were readily separated by TLC. Pentadeutero-benzophenone was synthesized from hexadeutero-benzene and benzoylchloride by conventional methods. The 2,4-dimethylbenzophenone was prepared from bromobenzene and 2,4-dimethylbenzonitrile. 9-Dicyanomethyl fluorene (XI) was prepared by reduction of compound X (condensation product of 9-fluorenone and malononitrile) by $LiAlH_4$.

2. Instrumentation

Electron impact ionization mass spectra were obtained either with a Hewlett-Packard 5985A GC-MS-DS or with a Finnigan 4000 GC-MS-DS. Samples were introduced through a 3% OV-1 or 3% SP-2100 GLC column (2m x 2mm) at temperatures ranging from 150 to 200°C. Exact mass measurements were performed by peak matching on a Varian-MAT CH-5 double-focussing instrument with sample introduction by direct probe. Collisionally-activated dissociation (CAD) mass spectra were obtained with an Extranuclear Laboratories triple quadrupole mass spectrometer with sample introduction by direct probe. In all cases, the ionizing voltage was 70eV and the ion source temperature was 200°C. The CAD spectra were obtained with a pressure of 1×10^{-4} torr of Ar in the collision cell (second quadrupole).

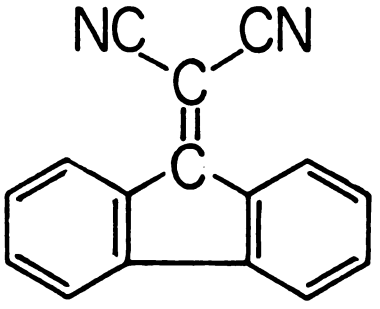
Results and Discussion

The mass spectrum of the DCM derivative of benzophenone is presented in Figure 29. The base peak at m/z 165 $(M-65)^+$ corresponds to the loss of $^{\cdot}CH(CN)_2$ from the molecular ion (confirmed by exact mass measurement at 165.0696 compared to calculated value of 165.0704 for $C_{13}H_9$); this elimination requires cleavage of a double bond. The following mechanism for this elimination is proposed:(Scheme 9)

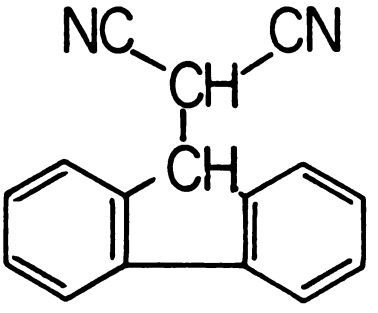


Scheme 9

This mechanism suggests that the molecular ion of the DCM derivative of benzophenone which undergoes this elimination rearranges to a fluorene-like structure upon electron impact. To assess this possibility, authentic 9-dicyanomethylfluorene (XI) was prepared by reduction of 9-dicyanomethylene-fluorene (X) according to the method of Campaigne et. al. (111).



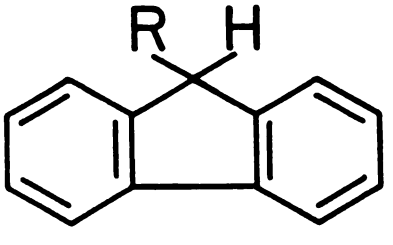
(X)



(XI)

The mass spectrum of 9-dicyanomethyl-fluorene (XI) is presented in Figure 30; note that it, too, is characterized by a base peak at m/z 165 corresponding to loss of $\cdot CH(CN)_2$ from the molecular ion (confirmed by exact mass measurement of 165.0699 compared to 165.0704 calculated for $C_{13}H_9$).

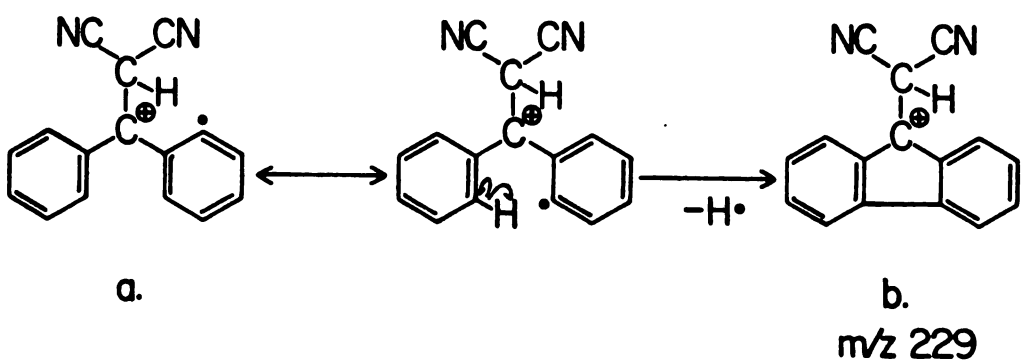
To obtain direct evidence that the ion of mass 165 in the mass spectra of the DCM derivative of benzophenone, 9-dicyanomethyl-fluorene (XI), fluorene (XII R=H), and 9-bromo-fluorene (XII R=Br) have comparable structural features, this ion from each compound during EI was subjected to collisionally-activated dissociation (CAD). The relative importance of this ion of mass 165 in the EI mass spectrum of each compound can be seen in Table 4. The results of CAD analysis of m/z 165 using a triple quadrupole mass spectrometer are shown in graphical form in Figure 31. The most prominent daughter ions appear to result from loss of hydrogen, elements of acetylene, and 1,3-butadiene from the parent ion (m/z 165) to produce peaks at m/z 164, 139, and 115, respectively. The data are normalized to the ion current at m/z 164, the most abundant daughter ion. The intensity of the parent ion peak(m/z 165) ranged from 2 to 3 times greater than that of m/z 164 during replicate CAD analyses of m/z 165 from these four compounds. The daughter ion mass spectra of mass 165 from these four compounds are identical within experimental error. These CAD data provide convincing evidence that the ion of mass 165, produced by fragmentation of any one of these four compounds, has a fluorene-like structure.



(XII)

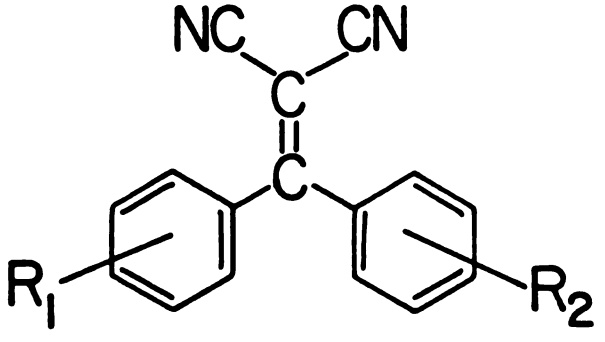
The mass spectrum of the DCM derivative of 9-fluorenone (X) indicates no peak corresponding to loss of $\cdot CH(CN)_2$ from $M^{+\cdot}$. This result is consistent with the theme represented in Scheme 9; following electron impact ionization, compound X would have no driving force to undergo this rearrangement because X already has the fluorene-like stable structure. Otherwise, the mass spectrum of X is characterized by a base peak representing $M^{+\cdot}$(m/z 228) and another peak, one tenth as intense, representing M-HCN; no other significant peaks appear in the mass spectrum.

The mass spectrum of the DCM derivative of benzophenone (Figure 29) also exhibits an intense peak corresponding to loss of a hydrogen from the molecular ion. Although other mechanisms exist, this elimination also could be rationalized through a molecular rearrangement leading to a substituted fluorene-like species as illustrated in Scheme 10 (which assumes the molecular ion in the form of ion a. in Scheme 9):



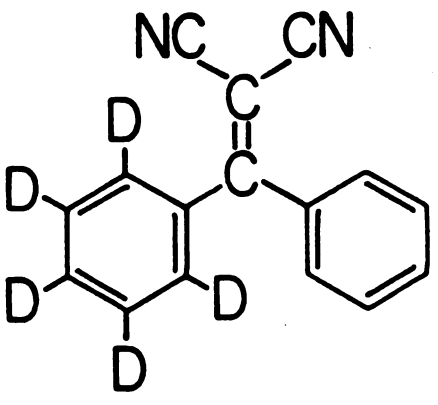
Scheme 10

Additional evidence for the rearrangement in Scheme 9 is provided in Table 5 which is a summary of mass spectra of the DCM derivative of benzophenone analogs of the general structure XIII. In nearly each case, a significant peak was observed for an ion corresponding to M^+-65. As expected, the loss of HCN from $M^{+\cdot}$ was frequently observed (110,113) and in cases of the chlorinated analogs, the loss of $^{\cdot}Cl$ from $M^{+\cdot}$ was a dominant process. Fragmentation of the bond between the dicyanoethylene and (substituted) phenyl moieties was found very rarely; apparently only a small fraction of the molecular ions retain the benzophenone-like structure which would permit them to generate an ion representing the (substituted) phenyl moiety. The mass spectrum of benzophenone (and underivatized analogs) often exhibit a significant peak at m/z 77 as well as at a m/z value corresponding to loss of the phenyl moiety from the molecular ion. That is, the C-C bond between the carbonyl and phenyl moieties of the (underivatized) benzophenones can be easily cleaved (112).

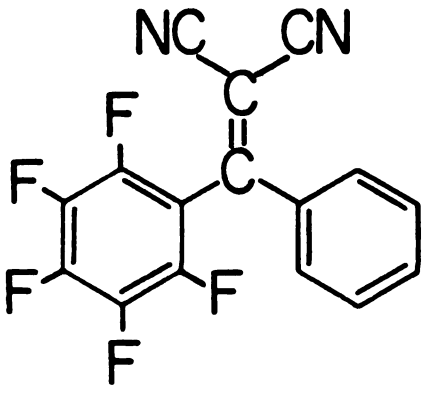


(XIII)

Other corroborative evidence for the characteristic proposed rearrangement as illustrated by Scheme 9 is provided by the mass spectra of the DCM derivative of compounds XIV and XV. The spectrum of the pentadeutero compound exhibits approximately equal ion abundance for the loss of $\cdot CD(CN)_2$ and $\cdot CH(CN)_2$ which is consistent with the symmetry of available hydrogens in Scheme 10. Incidentally, the spectrum of VI also indicates approximately equal ion abundance for $(M-H)^+$ and $(M-D)^+$. The experiment involving compound XV was designed to preclude the pathway illustrated in Scheme 9 because compound XV does not contain a hydrogen to donate from one of the phenyl groups. However, the mass spectrum of XV exhibits some ion current corresponding to the elimination represented in Scheme 10; the fact that both hydrogen and fluorine participate in this rearrangment may reflect the similar bond strengths of C-F and C-H. The essence of the mass spectrum of compound XV is as follows: M^+(100%), $(M-F)^+$ (26%), $(M-HF)^+$ (36%), $(M-HCN)^+$ (16%), $(M-CH(CN)_2)^+$ (3%). The loss of HF from XV is consistent with the tendency of these compounds to form the fluorene-like structure in Scheme 9 and Scheme 10.



(XIV)



(XV)

Williams et. al. (113) and Reichert (110) have reported that the mass spectra of benzolmalononitriles exhibit a peak representing the elements of the corresponding benzonitriles as formed via intramolecular rearrangement. In the mass spectra of the DCM derivative of the benzophenone analogs, there are no such peaks. The absence of benzonitrile fragments in these spectra is consistent with the proposed rearrangement (Scheme 9) of these molecules to form a more rigid structure (fluorene-like species) upon electron impact ionization.

This fragmentation study of model compounds suggests a fundamental mechanism by which to rationalize the principal bond cleavages observed during the mass spectrometry of the DCM derivatives of benzophenone analogs. The driving force for this mechanism, involving cleavage of a double bond, is apparently provided by the formation of a stable fragment ion having a putative fluorene-like structure. Collisionally-activated dissociation (CAD) mass spectra of this fragment ion generated from several fluorene analogs and DCM-benzophenone derivatives during EI indicate nearly identical fragmentation patterns. The CAD data together with conventional mass spectra provide evidence for the formation of a common ion of fluorene-like structure. These results should facilitate interpretation of the mass spectra of similar derivatives of other benzophenone analogs.

Table 4

Abbreviated Tabular EI Mass Spectra of Fluorene Derivatives

Compound Name	Relative Abundance	
	M	m/z 165
9-dicyano-methyl-fluorene	m/z 230 (13%)	100% $(M-CH(CN)_2)^+$
fluorene	m/z 166 (100%)	90% $(M-H)^+$
9-Bromo fluorene	m/z 244 (7%)	100% $(M-Br)^+$

Table 5*

Abbreviated Tabular Mass Spectra of DCM-Benzophenone Analogs Represented by Structure XIII

*) In this table the m/z value is reported together with the relative abundance of the ion (in parenthesis)

NAME \ ION	M^+	$(M-HCN)^+$	$(M-CH(CN)_2)^+$	$(M-R_1)^+$	$(M-(R_1+R_2))^+$
DCM-2,4-dimethyl-benzophenone $2R_1 = CH_3$, $R_2 = H$	258 (100)	231 (57.3)	193 (8.6)	243 (15.2)	
DCM-4,4'-dimethoxy-benzophenone $R_1 = R_2 = OCH_3$	290 (100)	263 (0.9)	225 (7.2)	259 (8.7)	228 (1.1)
DCM-4,4'-dimethyl-benzophenone $R_1 = R_2 = CH_3$	258 (100)	231 (7.6)	193 (20.3)	243 (86.9)	228 (15.7)
DCM-4,4'-dichloro-benzophenone $R_1 = R_2 = Cl$	298 (73.8)	271 (2.7)	233 (10.1)	263 (86.8)	228 (100)
DCM-4-chloro-benzophenone $R_1 = Cl$, $R_2 = H$	264 (68)	237 (4.0)	199 (11)	229 (100)	
DCM-4,4'-bis (N,N-dimethyamine) benzophenone $R_1 = R_2 = N(CH_3)_2$	316 (100)	289 (0.6)	251 (2.0)	272 (3.3)	228 (4.4)
DCM-3,4-dichloro-benzophenone $2R_1 = Cl$, $R_2 = H$	298 (53.9)	271 (6.1)	233 (14.7)	263 (100)	228 (70)

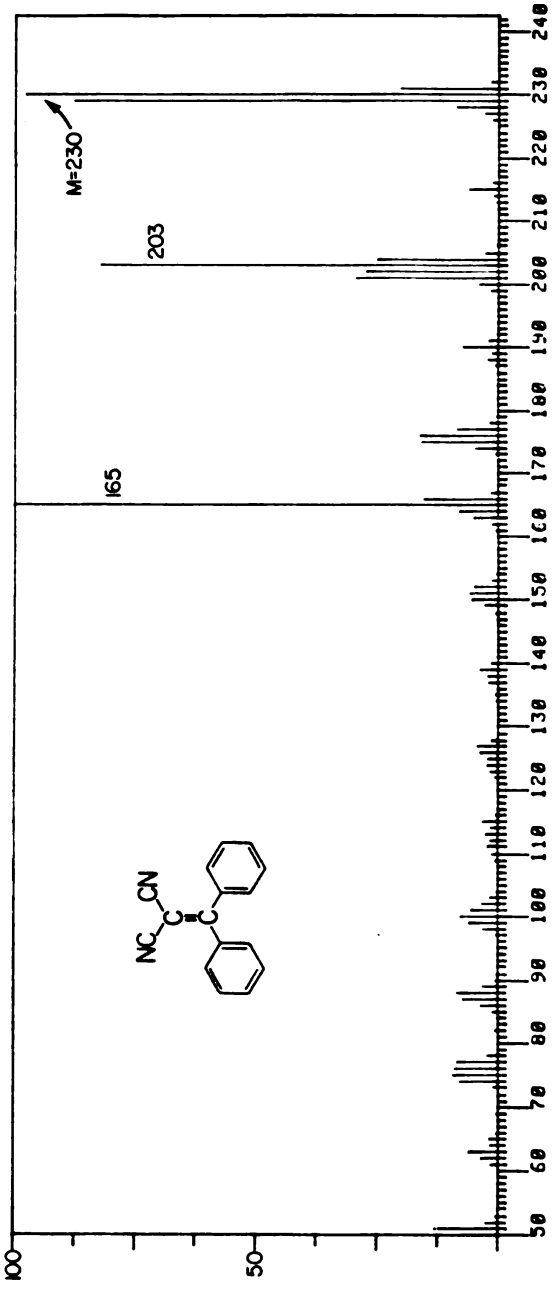


Figure 29. EI mass spectrum of dicyanomethylene derivative of benzophenone(IX).

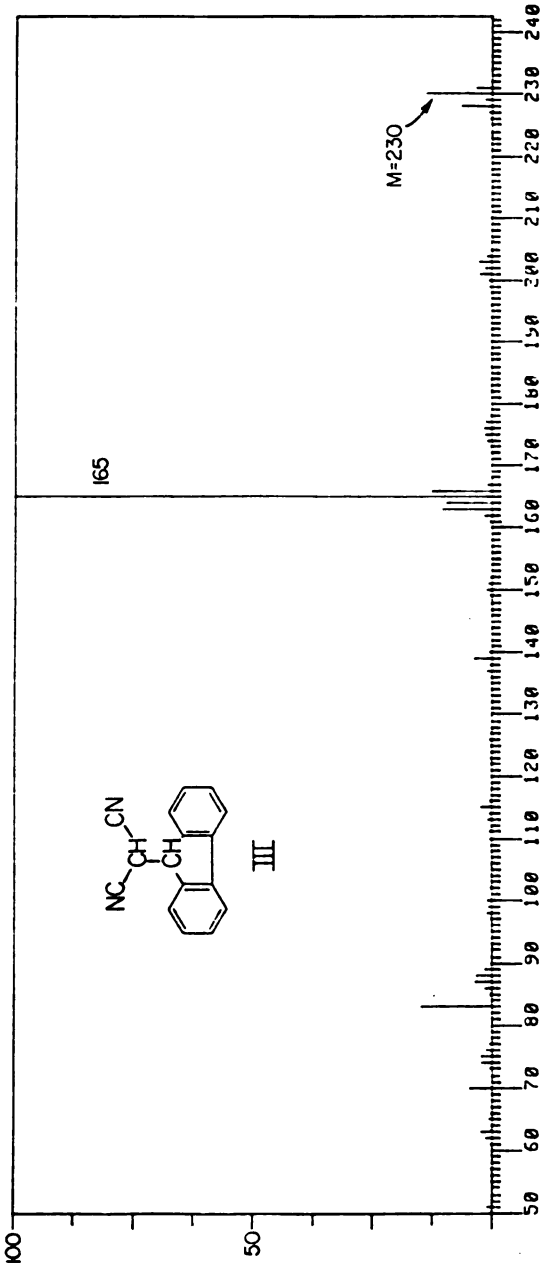


Figure 30. EI mass spectrum of 9-dicyanomethyl-fluorene(XI).

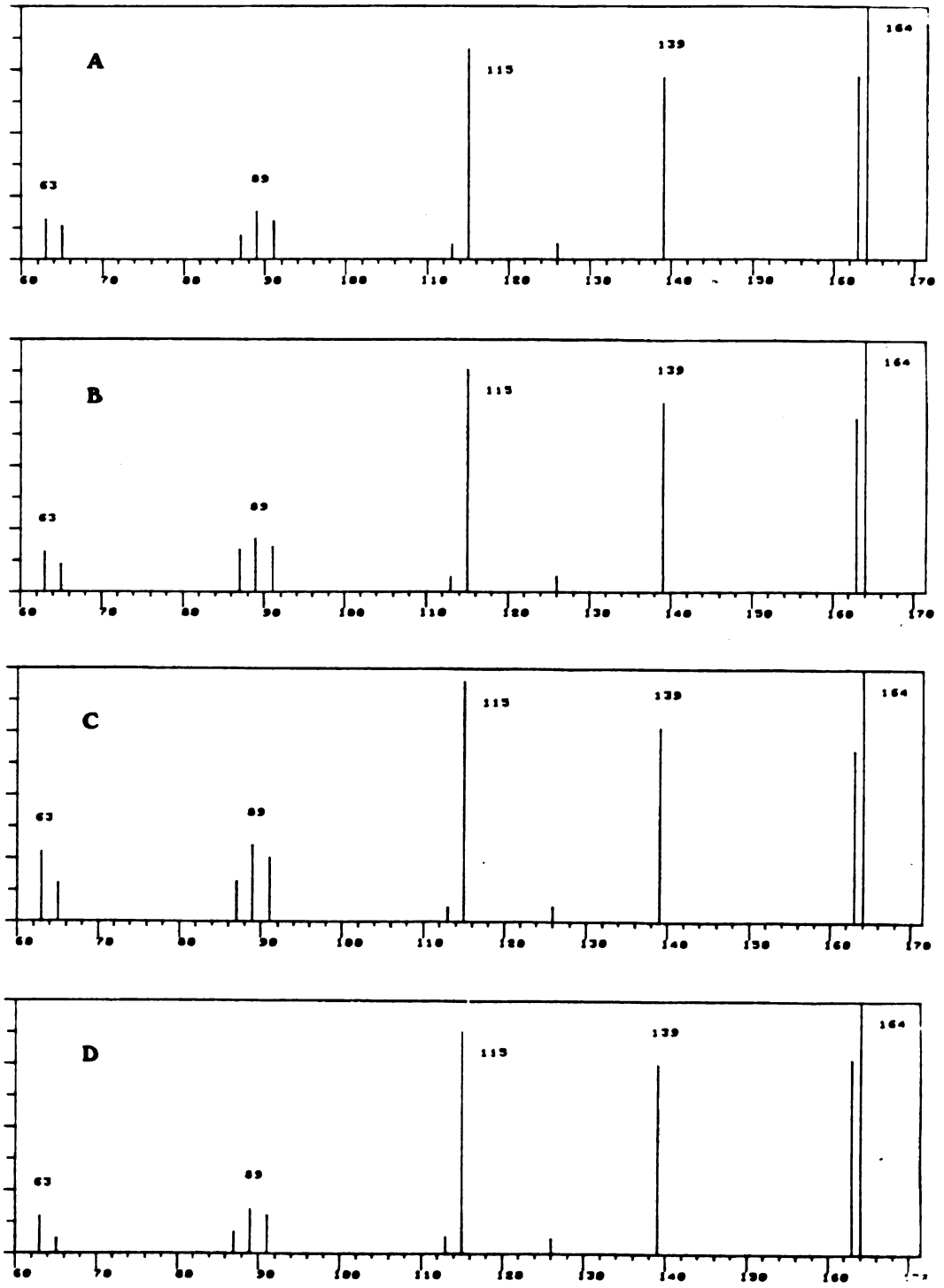


Figure 31. Daughter ion (CAD) mass spectra of the ion of mass 165 produced upon EI for each of the indicated compounds: A, DCM derivative of benzophenone; B, 9-dicyanomethyl fluorene; C, 9-bromo-fluorene; D, fluorene.

Chapter 7. The Negative Ion Mass Spectra of Dicyanomethylene Derivatives of 9-Fluorenone and Benzophenone

Introduction

In negative ion chemical ionization (NCI) mass spectrometry, if a gas such as methane or isobutane is used as the auxiliary gas, no negative reagent ions of the kind found in positive CI are produced in the ion source. The major purpose of the auxilliary gas is to slow down the high energy primary electron beam in order to produce low energy secondary electrons which can be captured by compounds which have high electron affinities and large electron capture cross sections. Because negatively charged reagent ions are not produced in large quantity in the CI source, ion / molecule reactions between anions and analyte molecules are not expected to occur. Therefore, unlike in positive CI, methane or isobutane NCI does not favor production of negative ions of greater mass than that of the molecular ion of the analyte. However, several compounds have been reported to produce negative ions with masses higher than the analyte (115-121) when either methane or isobutane is used as the reagent gas. There have been several explanations for this phenomenon, including ion / molecule interactions (115), and fast radical / molecule reactions followed by electron capture (116-121).

In the course of exploring new high electron affinity derivatives for ketones, dicyanomethylene(DCM) derivatives produced from the reaction of malonitrile with ketone moieties were investigated. The negative ion mass spectra of DCM 9-fluorenone and benzophenone analogs are often characterized by intense fragment ions which cannot be explained as the fragment ions of the molecular anions. A possible mechanism for the formation of these unusual fragment ions was investigated as presented in this chapter.

Experimental Section

1. Materials

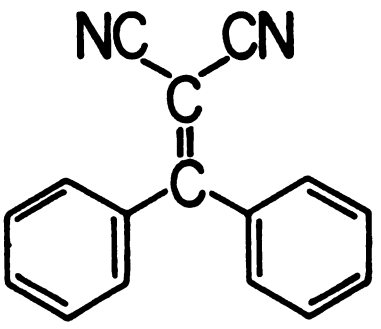
All the DCM derivatives were synthesized as described in the experimental section of chapter 6. The d_4-9-fluorenone was synthesized through cyclization of the d_5- benzophenone using palladium acetate as the catalyst(122).

2. Instrumentation

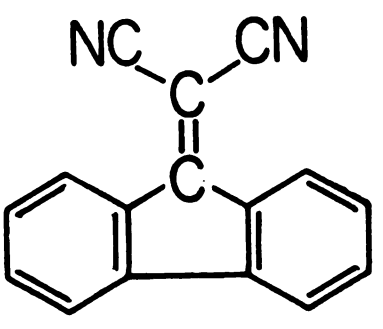
Negative ion mass spectra were obtained with a Hewlett Packard 5985A GC/MS. Samples were introduced through a 25 meter SE-54 widebore capillary column with temperature programming from 150°C to 250°C at 15°C/minute. Collisionally-activated dissociation(CAD) experiments were done with an Extranuclear Laboratories triple quadrupole mass spectrometer with sample introduction by direct probe.

Results and Discussion

The methane NCI mass spectra of the DCM derivatives of benzophenone (IX) and 9-fluorenone (X) are shown in Figure 32. The negative ion mass spectra of these two compounds are characterized by three major ions: M^-, $(M-25)^-$, and $(65)^-$. Similar ions are also predominant in the negative ion mass spectra of several other benzophenone analogs. Upon cursory inspection, one might suggest possible elemental formulas for the two fragment ions such as $(M-C_2H)^-$ for the $(M-25)^-$ ion and $(C_5H_5)^-$ for the $(65)^-$ ion. It is very difficult, however, to draw straightforward mechanisms that produce fragments of these formulas from unimolecular decomposition of the negative molecular ion. Another set of plausible formulas for the two fragments are $(M+H-CN)^-$ and $(CH(CN)_2)^-$. $(M+H-CN)^-$ requires the existence of an $(M+H)^-$ ion, for which there is no strong evidence in the mass spectra shown in Figure 32. $(CH(CN)_2)^-$ requires the existence of an $(M+H)^-$ ion or the rearrangement of an hydrogen atom from an aromatic ring to the carbon connecting the dicyano groups.

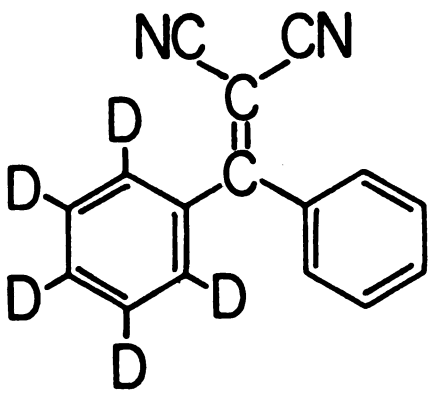


(IX)

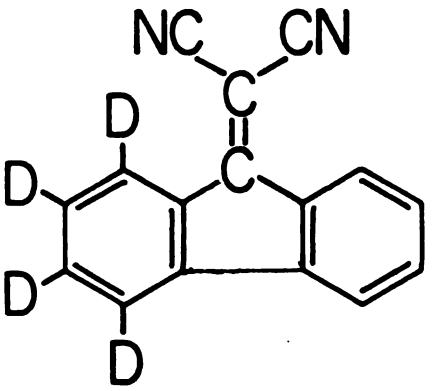


(X)

In an attempt to elucidate the structures of these unusual fragment ions, the following deuterated compounds were synthesized: the DCM derivative of d_5- benzophenone (XIV) and the DCM derivative of d_4-9-fluorenone (XVI).



(XIV)



(XVI)

The deuterated compounds were analyzed by methane negative ion mass spectrometry and the spectra are shown in Figure 33. Surprisingly, they are also characterized by the same three major ions as their non-deuterated analogs: M^-, $(M-25)^-$, and $(65)^-$. It is obvious from these results that neither the fragment ion of mass 65 nor the "$(M-25)^-$" fragment ion contains any hydrogen atoms that are rearranged from the original molecules. Any auxilliary hydrogen atoms in these two species must come from some other species in the ion source. Since the fragment ion of mass 65 and the "$(M-25)^-$" fragment ion surely do contain some auxiliary or rearranged hydrogen, the following experiments were performed to determine the source of that hydrogen.

When nitrogen is used instead of methane as the NCI gas, only negative molecular ions, i.e., no fragment ions, are found in the mass spectra of compounds (IX) and (X). When hydrogen-containing gases such as hydrogen or ammonia are used in NCI instead of methane, the following ions are produced from compounds (IX) and (X): M^-, $(M-25)^-$, and $(65)^-$. When D_2 is used as the NCI gas instead of methane, the following high abundance ions are produced from compounds (IX) and (X): M^-, $(M-24)^-$, $(M-25)^-$, $(65)^-$, and $(66)^-$(Figure 34). In summary, NCI forms $(M+H-CN)^-$ and $(CH(CN)_2)^-$ from compounds (IX) and (X) when hydrogen atoms are present in the NCI gas. NCI forms $(M+D-CN)^-$, $(M+H-CN)^-$, $(CH(CN)_2)^-$, and $(CD(CN)_2)^-$ when deuterium atoms are present in the NCI gas. NCI forms no fragments of these compounds when hydrogen isotopes are not present in the NCI gas. The only logical explanation for these results is that hydrogen isotopes in the NCI gas are involved in the production of the fragment ions that are represented in the NCI mass spectra of compounds (IX) and (X). It is very possible that the MH^- ions are formed and then fragment to produce $(M-25)^-$ and $(65)^-$ ions.

In a CAD experiment in which the molecular anion of compound(X)(m/z 228) was chosen as the parent ion, no daughter ion was observed when Ar was used as the collision gas. This observation could mean that the $(M-25)^-$ and $(65)^-$ ions are not the daughter ions of the M- ion or it could mean that increasing the internal energy during the collision process is high enough to expel an electron from the negative ion thereby annihilating it. This could be a manifestation of the intrinsic differences in stability of negative ions and positive ions. Neutralization of a positive ion requires the capture of an electron and

involves a collision process. However, a negative ion can merely eject an electron to form a neutral molecule.

Three possible mechanisms that account for the formation of the MH^- ions are shown in equations I-III.

I. Ion / molecule reaction

a). $M + e \longrightarrow M^- + CH_4 \longrightarrow MH^- + CH_3^{\cdot}$

b). $M + H^- \longrightarrow MH^-$

II. Capture of two electrons by $(M+H)^+$ ion

$MH^+ + e \longrightarrow MH + e \longrightarrow MH^-$

III. Molecule / radical reaction prior to electron capture

$M + H^{\cdot} \longrightarrow MH^{\cdot} + e \longrightarrow MH^-$

All three mechanisms postulate the presence of an $(M+H)^-$ ion for which no significant peak is observed in the NCI spectra of any of the compounds studied in this chapter. In all three cases the $(M+H)^-$ species must be thought of as either a transition state in the production of the fragment ions or as an unstable intermediate species with a very short half-life. Nevertheless, the three mechanisms account

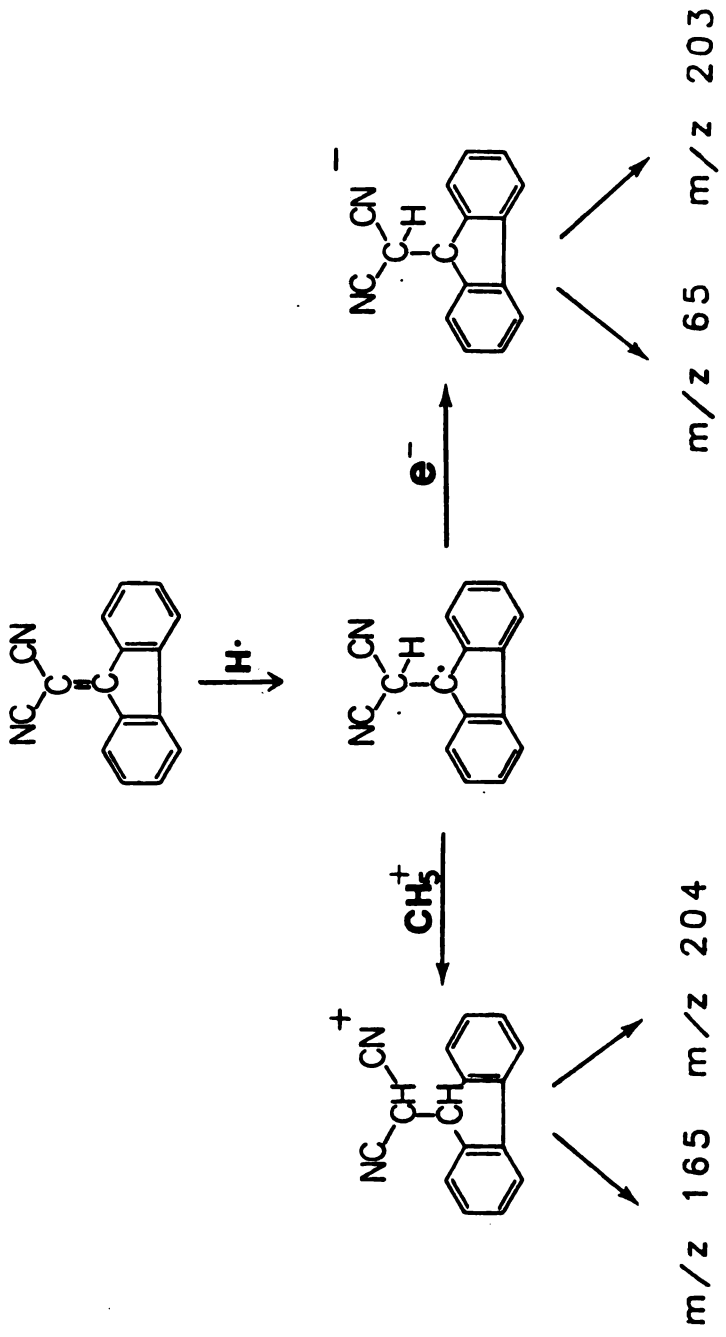
for the production of the $(M+H)^-$ species in very different ways.

Although the instrument which is used for the experiment(a quadrupole instrument) cannot identify whether the H^- ion is produced in the ion source, it is generally believed that the H^- ion is not produced in large quantity under methane NCI. In addition, because H^- is such a strong base, it should be able to extract a proton from most organic compounds to form the $(M-1)^-$ ion. However, no significant abundance is observed for $(M-1)^-$ ion in this experiment. Thus, it is unlikely that equation Ib represents the pathway for the production of the MH^- ion. A triple quadrupole mass spectrometer (TQMS) was utilized to determine whether an ion / molecule reaction produces the $(M-25)^-$ and $(65)^-$ ions. In the TOMS study, the M^- ion of DCM 9-fluorenone (compound X) was chosen as the parent ion by using the first quadrupole as a mass filter. Methane was introduced into the second quadrupole operated in the RF-only mode. Ion / molecule reactions have been shown to occur in the second quadrupole region of the TQMS if a reactive collision gas is used (123). In the case of compound (X), however, no ion / molecule reaction products were formed when methane was used as the collision gas. This experiment also reduces the possibility that ion / molecule reactions account for the $(M-25)^-$ and $(65)^-$ ions in this case even though Hass et al(115) postulated such reactions to explain the high abundance of $(M+H)^-$ ions in the methane NCI mass spectra of some organonitriles.

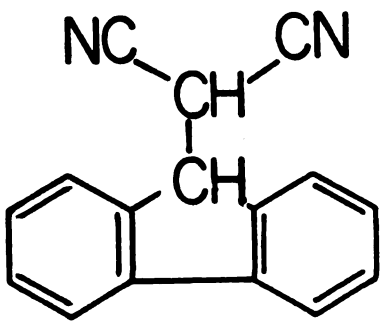
Equation II postulates that a positive protonated molecule $(M+H)^+$ is formed and that it is required to capture two electrons to form the $(M+H)^-$ ion that subsequently decomposes into the $(M-25)^-$ and $(65)^-$ fragment ions. Capturing two electrons in this fashion is not expected to happen faster than the capture of one electron by the highly conjugated parent molecule, so one would not expect any ions formed in this fashion to have abundances comparable to those of the molecular anion; however, the $(M-25)^-$ and $(65)^-$ ions are two of the most intense peaks in the spectrum and often contain more than 50% of the total ion current. Therefore, equation II seems unlikely to account for the observed ion abundances in the NCI mass spectrum of DCM 9-fluorenone.

A molecule / radical reaction prior to ionization (equation III) was proposed by McEwen and Rudat (116,117) to explain the negative CI (methane) mass spectrum of tetracyanoquinodimethane (TCNQ) in which a series of major ions was observed that corresponded to the addition of H, CH_3, and C_2H_5 to TCNQ with or without the loss of one or more cyano groups. The positive CI (methane) mass spectrum of TCNQ was similar to the negative ion mass spectrum in that the major ions corresponded to the addition of H, CH_3, and C_2H_5 with or without the loss of one or more cyano groups. Primarily because of the symmetry of the results in the positive and negative ion spectra, McEwen and Rudat proposed that a molecule / radical reaction followed by either electron capture or protonation was the most reasonable mechanism.

For DCM 9-fluorenone(X) and DCM benzophenone(IX), a similar phenomenon has been observed. Figure 35 shows the positive CI (methane) mass spectrum of both DCM benzophenone and DCM 9-fluorenone. Both compounds produce two major fragment ions: $(M-24)^+$ and $(M-63)^+$. These ions cannot be explained from the fragmentation of $(M+H)^+$ ion and have been proved by TQMS data not to be the daughter ions of the $(M+H)^+$. However, these two ions can be explained by a molecule / radical reaction prior to protonation. If the molecule / hydrogen radical product $(M+H)^\cdot$ were formed, the $(M+H)^\cdot$ compound could either capture an electron and then fragment to produce $(M+H-CN)^-$ = $(M-25)^-$ and $(CH(CN)_2)^-$ = $(65)^-$ ions or it could react with CH_5^+ to form a $(M+H_2)^+$ ion, and then fragment to form $(M+H_2-CN)^+$ = $(M-24)^+$ and $(M+H_2-CH(CN)_2)^+$ = $(M-64)^+$ ions as shown in Scheme 11. Therefore, the most reasonable mechanism for our data is equation III in which a molecule / hydrogen radical reaction occurs. In the ion source, the $(M+H)^\cdot$ product could capture an electron to form $(M+H)^-$ which in turn could decompose to fragment ions or it could capture a proton from CH_5^+ to form $(M+H_2)^+$ which then could decompose to form fragment ions.


NC
CN
C
H
H·
e⁻
CH₅⁺
m/z 203
m/z 65
m/z 204
m/z 165

One explanation for why the postulated $(M+H)^-$ ion is not readily observed in the negative ion mass spectra of compound IX and compound X could be that the resultant $(M+H)^-$ ion is very unstable if the hydrogen radical is added to the carbon-carbon double bond between the cyano groups and the aromatic rings. In order to demonstrate the instability of the $(M+H)^-$ ion, dicyanomethylene 9-fluorene (compound X) was reduced to 9-dicyanomethylfluorene (compound XVII) and then studied by negative CI (methane) mass spectrometry. The negative ion mass spectrum(Figure 36) of compound (XVII) showed only two peaks, each corresponding to a fragment ion:$(65)^-$ and $(M-1)^-$. More than 80% of the total ion current is carried by the $(65)^-$ ion. This phenomenon proves that compound (XVII) forms a very unstable molecular anion and suggests that reduction or addition of $H^\cdot$ to the double bond of the DCM moiety is responsible for the instability of the charged molecule. The structures of compound (XVII) and the molecule-hydrogen radical compound are considered to be similar. In both cases, the carbon-carbon double bond connecting the cyano groups to the fluorene rings is reduced to a single bond. Therefore, it is believed that the molecule-hydrogen radical product also forms a very unstable $(M+H)^-$ anion and ultimately fragments to form $(M-25)^-$ and $(65)^-$ anions.



(XVII)

When isobutane was used as the auxiliary gas instead of methane, two more ions(m/z 121,m/z 285) were observed in the negative ion mass spectra of compound (X) as shown in Figure 37. These two ions could also be explained by the molecule radical reaction as proposed in equation III. If a $C_4H_9^{\cdot}$ radical is added to the carbon-carbon double bond between the cyano groups and the fluorene ring of the neutral molecule, the radical addition product could capture an electron to form the ion of mass 285 or capture an electron and then fragment to produce an ion of mass 121 with the following formula: $(C_4H_9C(CN)_2)^-$. The intensity of the peak at m/z 285 is less than the intensity of the peak at m/z 121 because the resultant $(M+C_4H_9)^-$ ion is unstable as explained previously. This explanation is supported by the isobutane NCI spectrum of DCM d_4-9-fluorenone. In that spectrum, the peak at m/z 121 does not show a mass shift due to deuterium isotopes. This finding indicates strongly that any hydrogens in the ion of mass 121 come from the isobutane gas rather than from the isotopically-labeled fluorenone rings.

In all previous NCI studies, if a compound was found to ionize by dissociative electron capture, the intensity of fragment ions increased with increasing ion source temperature. The $(M-25)^-$ and $(65)^-$ anions that were produced by NCI of compounds (IX) and (X) did not follow this behavior pattern. The abundance of these fragments decreased with increasing ion source temperature(Figure 38). This unusual phenomenon may be due to a decrease in the hydrogen radical population at higher temperatures, or to an increasingly fast reverse molecule radical reaction at higher temperature.

The abundance of fragment ions also depends on the source pressure. The abundance of fragment ions shows a maximum with increasing of isobutane pressure as shown in Figure 39.

Ocassionally, ions of mass 180 and mass 182 are observed in the NCI mass spectra of compound (IX) and compound (X) respectively. These ions cannot be explained by the molecule/radical reactions or by the unimolecular decomposition. The NCI mass spectra of deuterated compounds show that all the deuterium atoms which are labelled to the molecule still remain on this ion (Figure 37). Furthermore, when D_2 is used as the auxiliary gas, these ions do not change their mass. One explanation for these observations is that the compounds could react with an oxygen-containing species to form the original molecules(benzophenone and 9-fluorenone). An exact mass measurement of these two ions(m/z 180, m/z 182) should help to elucidate their structure. Unfortunately, no instrument in the Mass Spectrometry Facility can perform the exact mass measurement of a negative ion.

Unlike the reports of McEwen(116,117) and Budzikiewicz(119), this chapter presents a number of unusual NCI mass spectra in which no clear evidence for the formation of ions with mass higher than the molecular weight of the analyte are presented. The results of this study will help provide guidelines for the interpretation of negative ion mass spectra which indicate fragment ions that cannot be rationized by straightforward decomposition of the molecular anion. Therefore, for NCI it seems better to use N_2 or Ar rather than hydrogen containing gases if the only purpose is the production of low energy electrons.

Otherwise, these molecule/radical reactions can significantly complicate the usually simple mass spectra obtained from electron capture NCI.

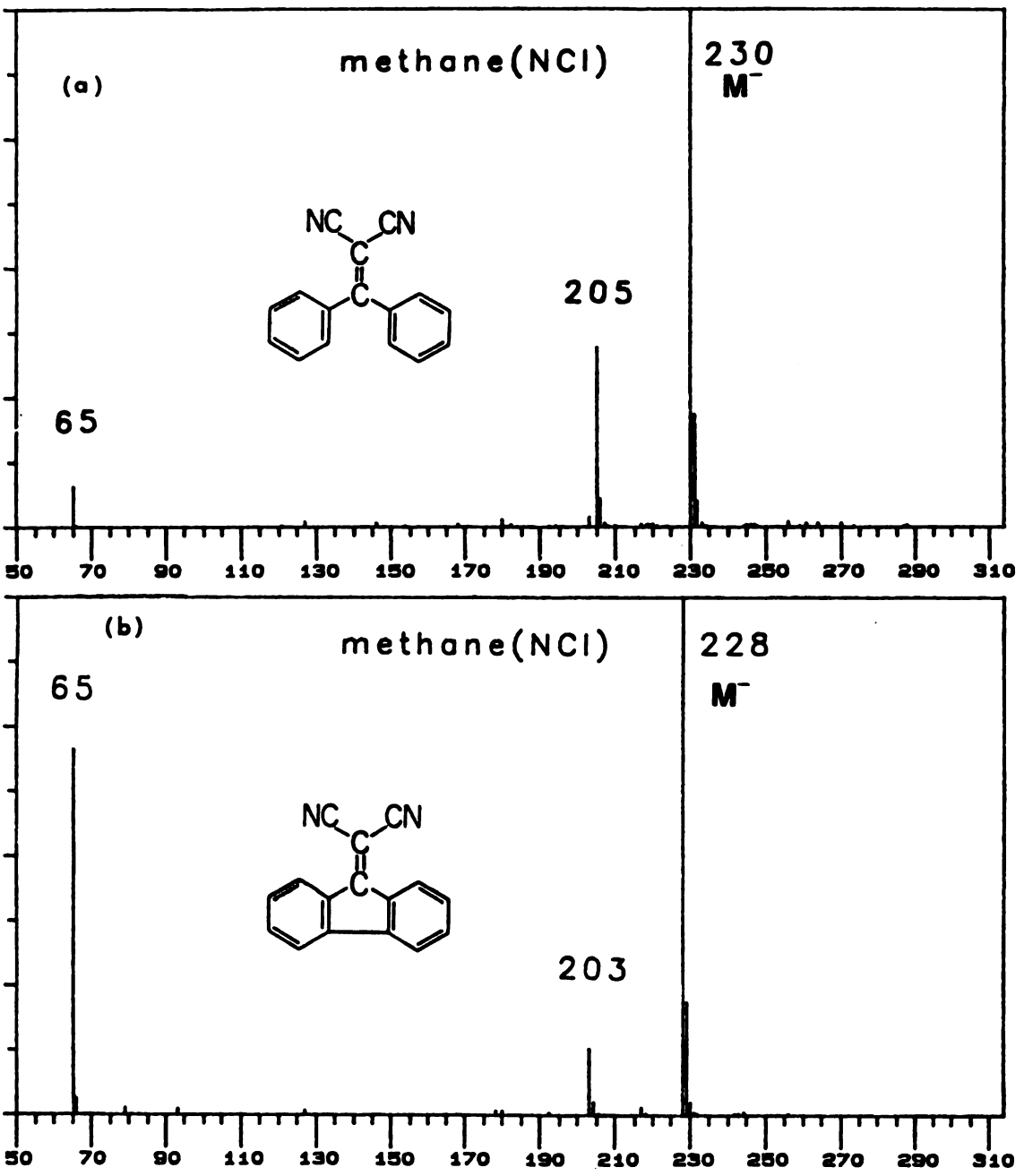


Figure 32. Methane NCI mass spectra of a) DCM benzophenone, b) DCM 9-fluorenone.

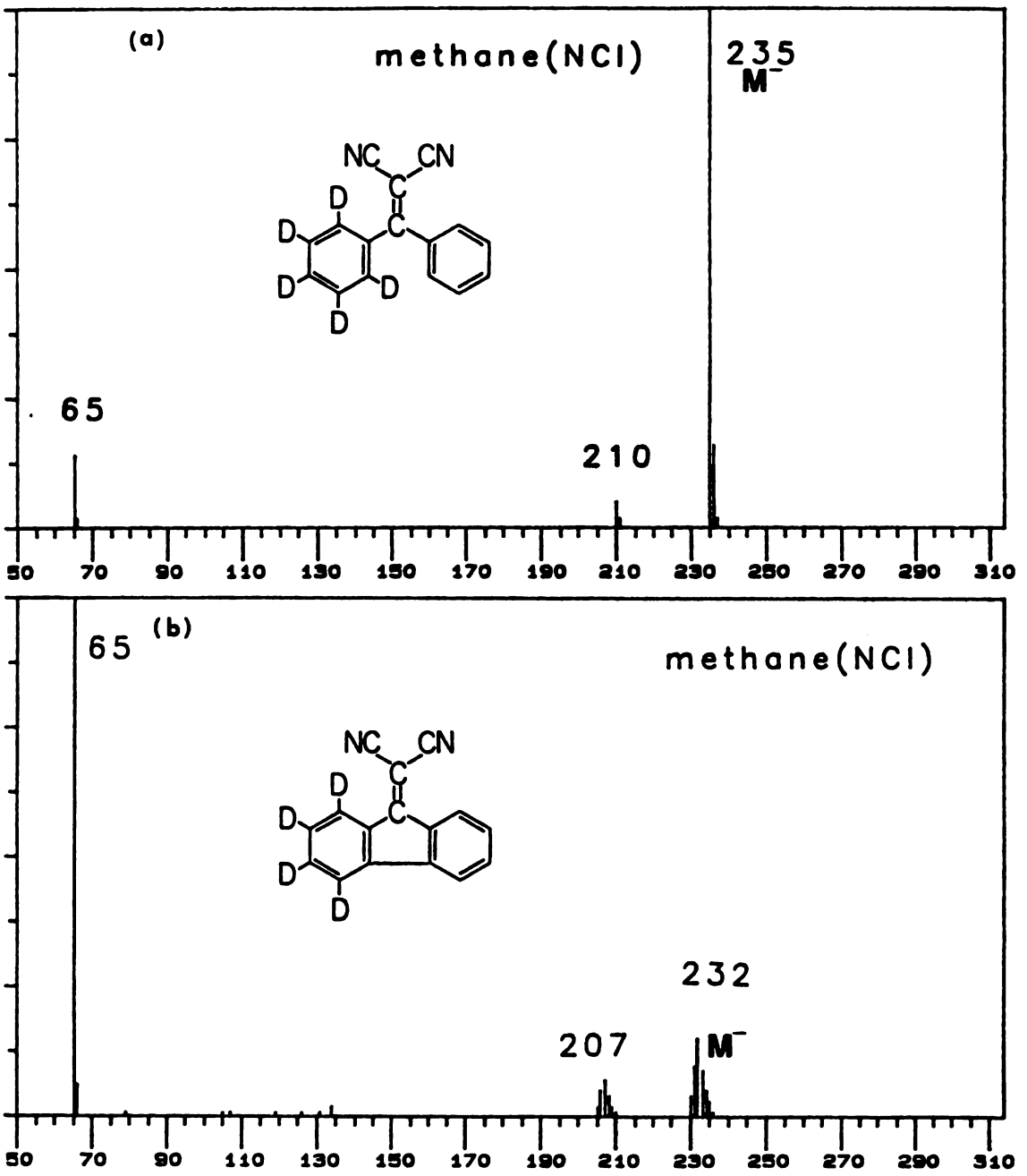


Figure 33. Methane NCI mass spectra of a) DCM d_5-benzophenone, b) DCM d_4-9-fluorenone.

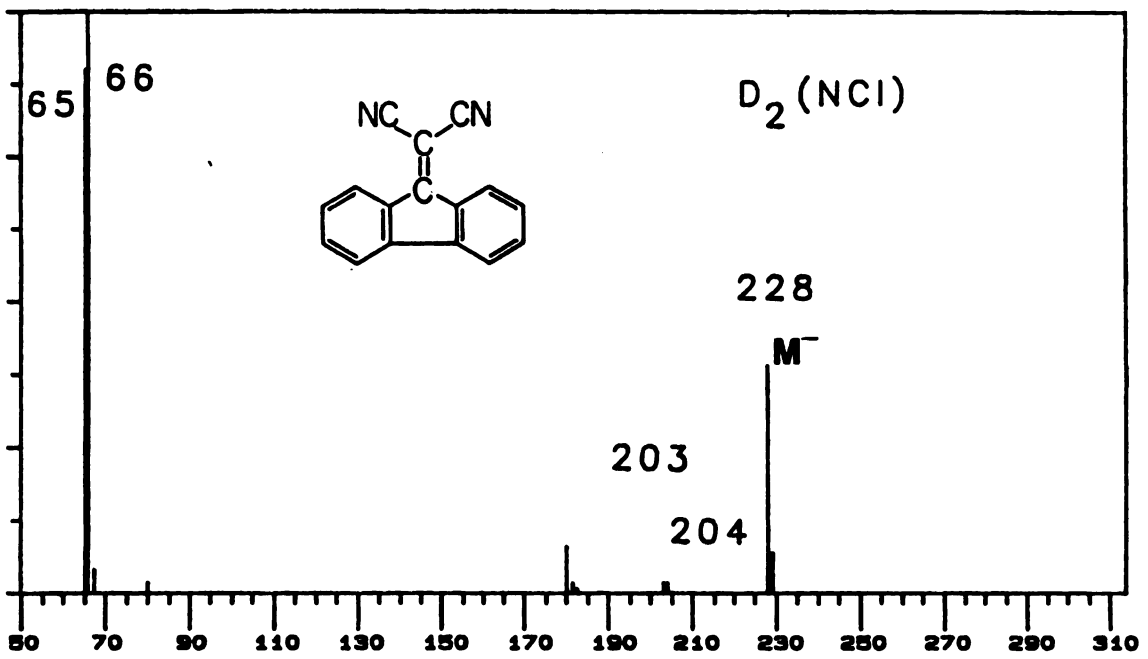


Figure 34. Deuterium NCI mass spectrum of DCM 9-fluorenone.

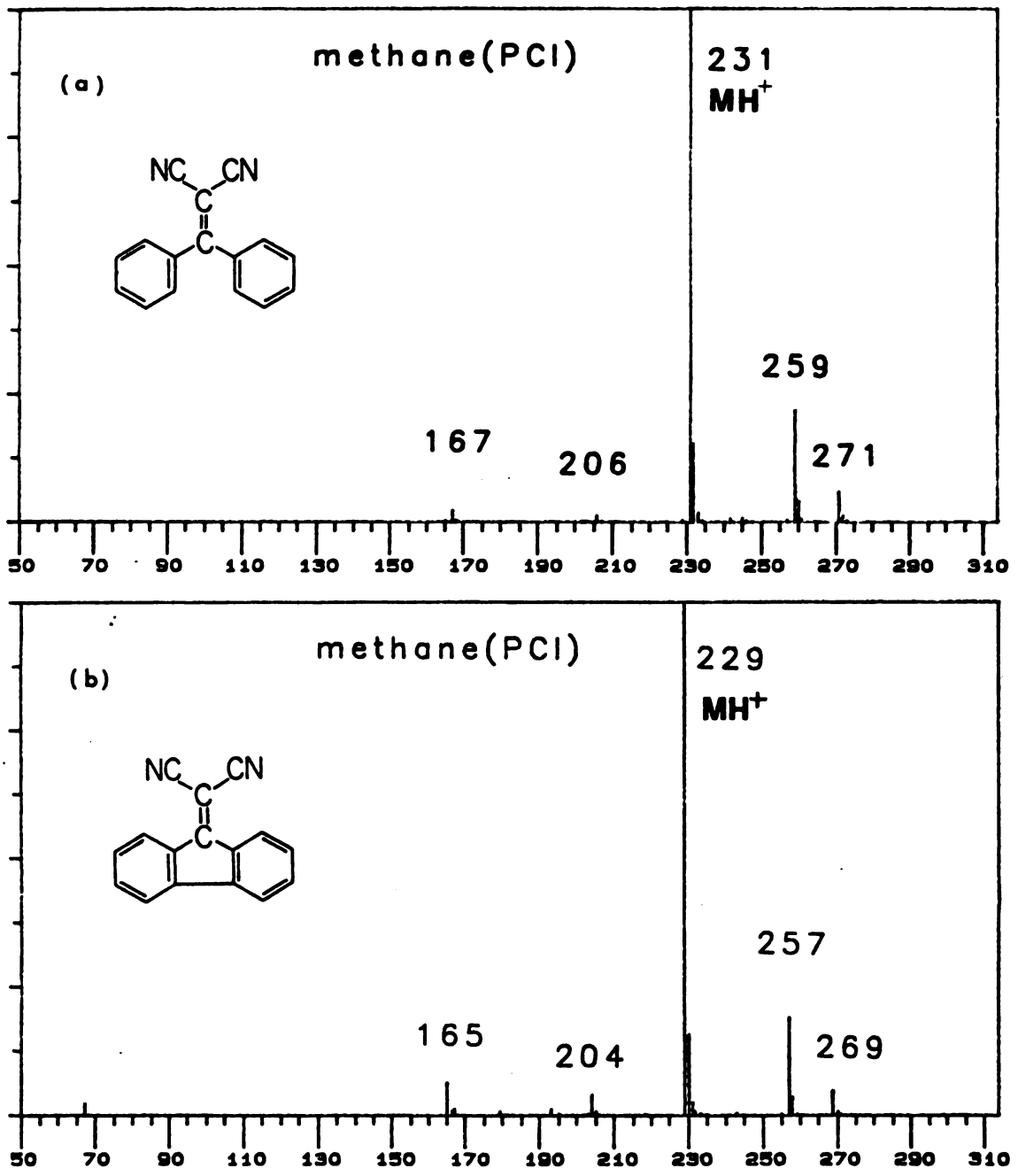


Figure 35. Methane PCI mass spectra of a) DCM benzophenone, b) DCM 9-fluorenone.

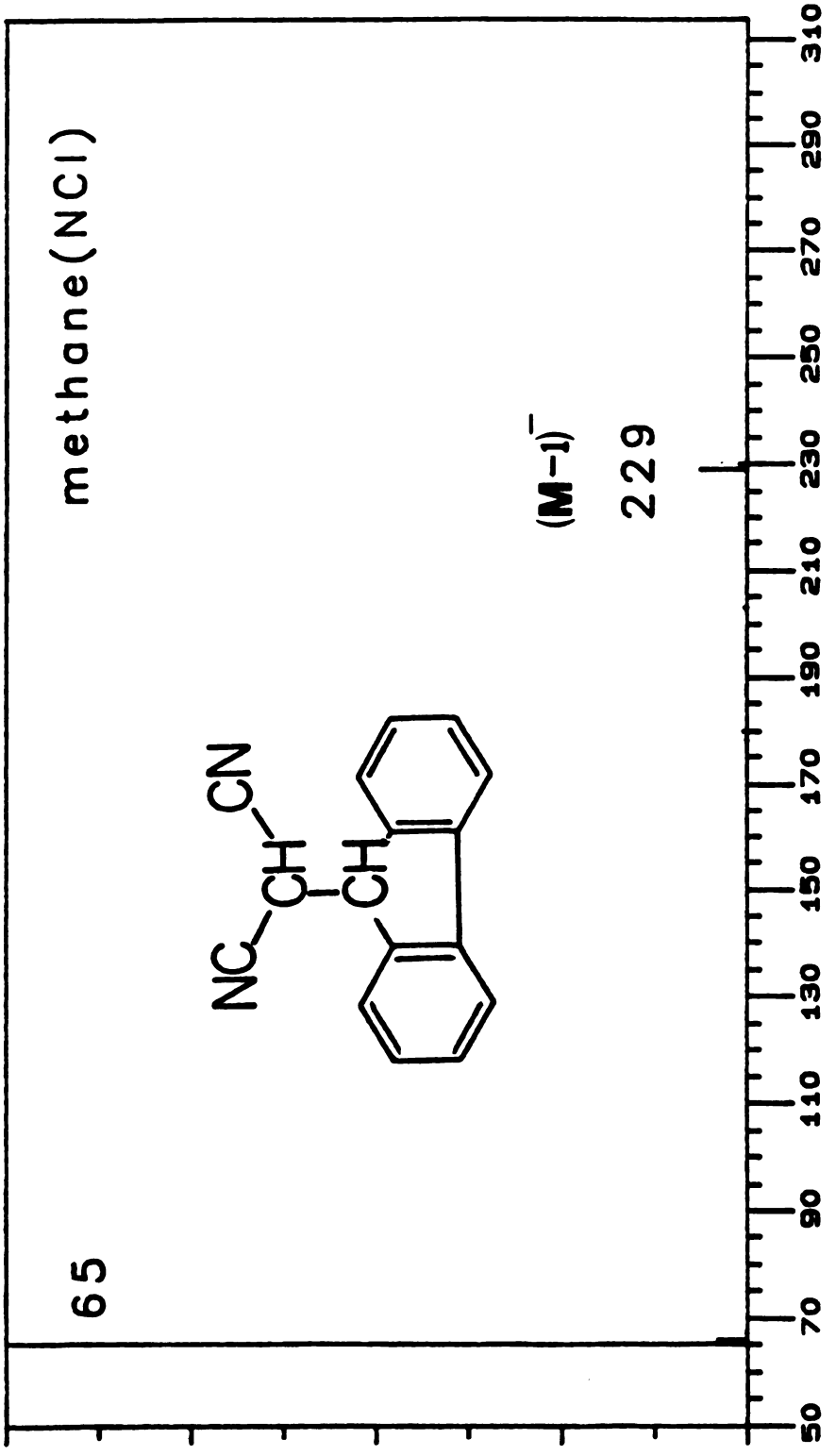


Figure 36. Methane NCI mass spectrum of 9-dicyanomethyl-fluorene.

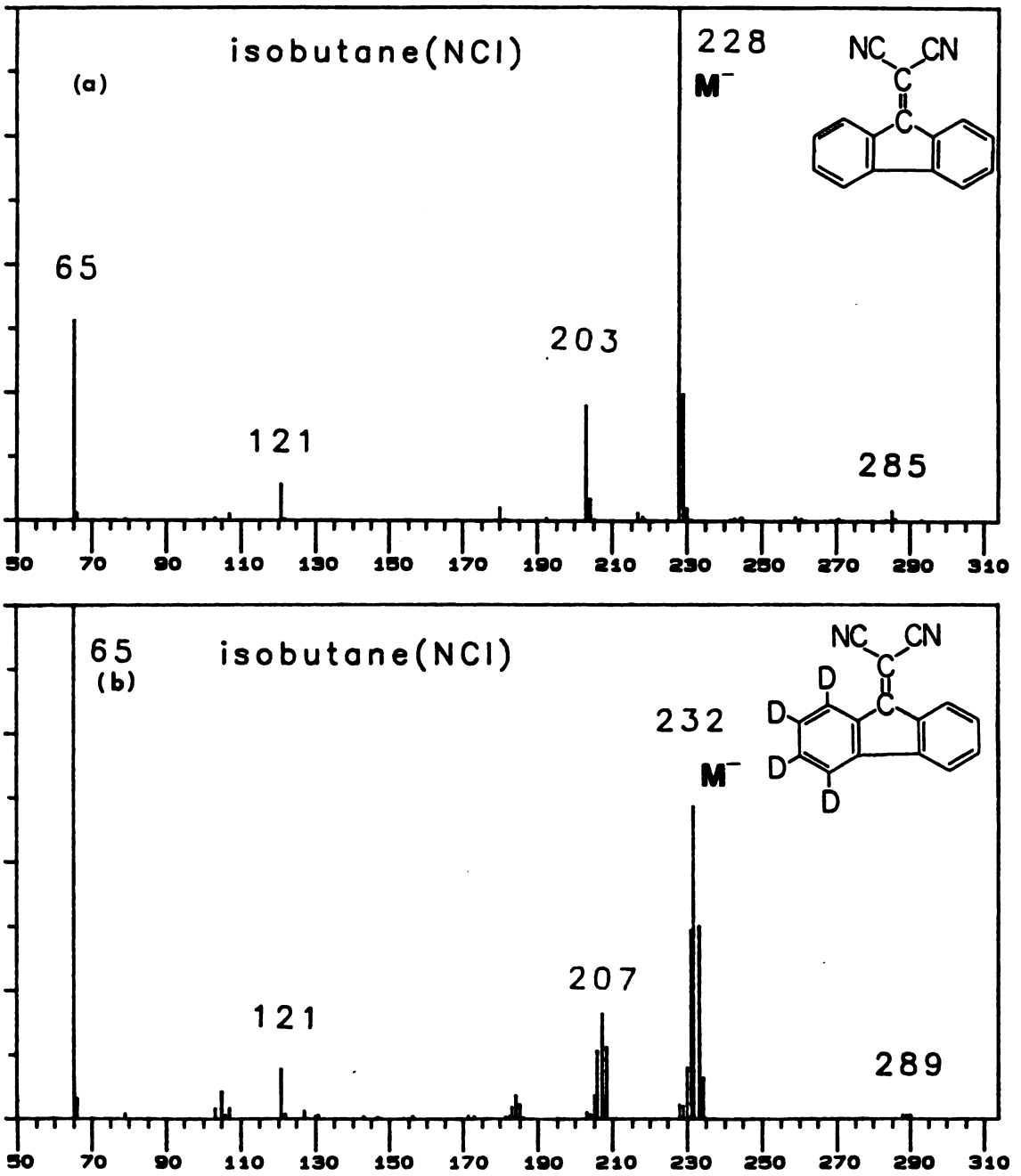


Figure 37. Isobutane NCI mass spectra of a) DCM 9-fluorenone, b) DCM d_4-9-fluorenone.

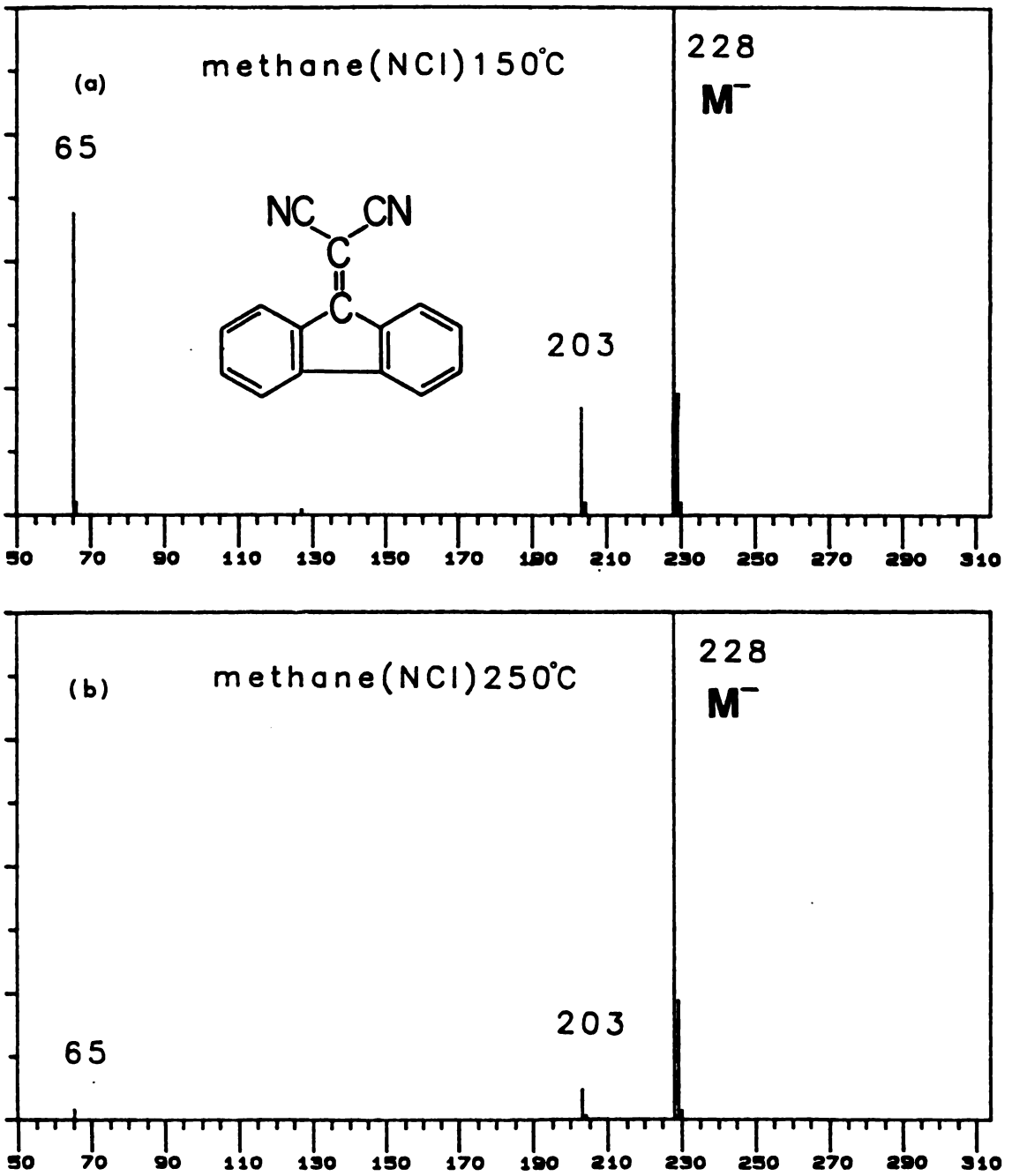


Figure 38. Methane NCI mass spectrum of DCM 9-fluorenone at a) 150°C, b) 250°C.

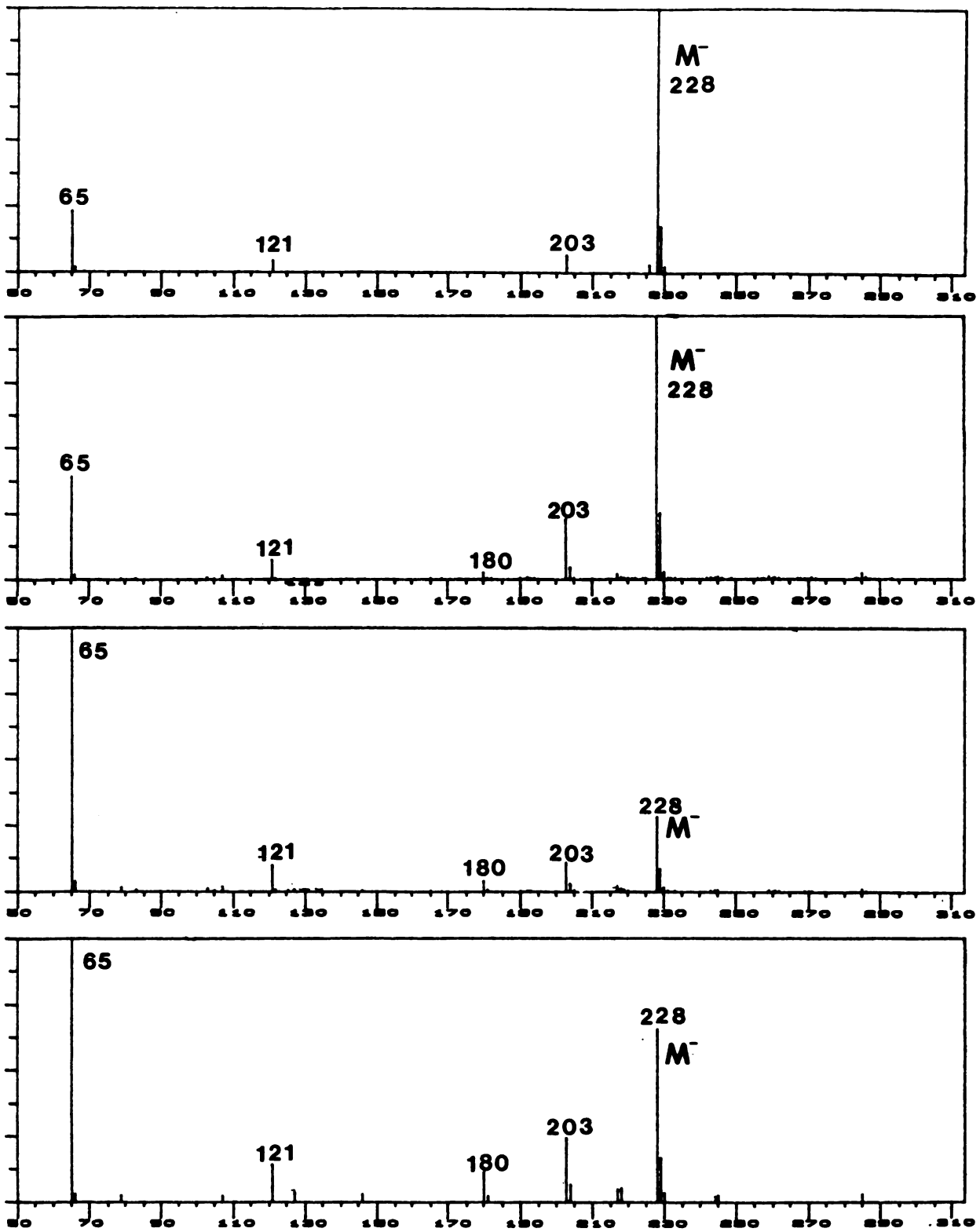


Figure 39. Isobutane NCI mass spectrum of DCM 9-fluorenone at a) $1*10^{-4}$, b) $2*10^{-4}$, c) $3*10^{-4}$, d) $4*10^{-4}$.

LIST OF REFERENCES

LIST OF REFERENCES

1. C. E. Melton, " Principles of mass spectrometry and negative ions "; M. Dekker: New York, 1970 p 190.

2. R. C. Dougherty, C. R. Weisberger, J. Am. Chem. Soc. 90, 6570 (1968)

3. R. C. Dougherty, J. Chem. Phys. 50, 1896 (1969)

4. D. F. Hunt, G. C. Stafford, F. W. Crow, J. W. Russell, Anal. Chem. 48, 2098 (1976)

5. J. G. Dillard, Chem. Review. 73, 589 (1973)

6. J. H. Bowie, B. D. Williams, " MTP International Review of Science, Physical Chemistry, Series Two ", Vol. 5, A. Maccoll, Ed., Butter-worth, London, 1975 pp 89-127

7. F. H. Field, " MTP INternational Review of Science, Physical Chemistry, Series one ", Vol. 5, A. Maccoll, Ed. BUtterworth, London, 1972, Chapter 5.

8. K. R. Jennings, " Mass Spectrometry ", Vol. 4, R. A. W. Johnstone, Ed., The Chemical Society, Burlington House, London, 1977, Chapter 9.

9. A. Hadjiantoniou, L. G. Christophorou, J. G. Carter, J. Chem. Soc. Faraday II, 69, 1691 (1973)

10. A. hadjiantoniou, L. G. Christophorou, J. G. Carter, J. Chem. Soc. Faraday II. 69, 1704 (1973)

11. L. G. Christophorou, A. Hadjiantoniou, J. G. Carter, J. Chem. Soc. Faradady, 69, 1713 (1973)

12. J. P. Johnson, D. L. McCorkle, L. G. Christophorou, J. G. Carter, J. Chem. Soc. Faraday II, 71, 1742 (1975)

13. K. R. Jennings, " High Performance Mass Spectrometry: Chemical Application " M. L. Gross, Ed., American Chemical Society, Washington, D. C., Chapter 9.

14. R. T. Alpin, H. Budzikiewicz, C. Djerassi, J. Am Chem. Soc. 87, 3180 (1965)

15. S. R. Robinson, C. H. J. Wells, R. B. Turner, J. F. Todd, J. Chem. Soc. Perkin Trans. II, 1363 (1976)

16. J. F. J. Todd, R. B. Turner, B. C. Webb, C. H. J. Wells, J. Chem. Soc. Perkin Trans. II, 1167 (1973)

17. D. R. Dakernieks, I. W. Fraser, J. L. Garnett, I. K. Gregor, Talanta. 23, 701 (1976)

18. C. L. Brown, K. P. Gross, T. Onak, J. Am. Chem. Soc. 94, 8055 (1972)

19. E. M. Chait. W. B. Askew, C. B. Matthews, Org. Mass Spectrom. 2, 1135 (1969)

20. I. K. Gregor, K. R. Jennings, D. K. Sensharma, Org. Mass spectrom. 12, 93 (1977)

21. J. H. Bowie, B. D. Williams, " MTP International Review of Science, Physical Chemistry, Series Two " Vol. 5, A. Maccoll, Ed., Butterworth, London, 1975, pp 89-127

22. M. VonArdenne, K. Steinfelder, R. Tummler, " Elektronenanlagerungs-Massenspektrographic Organischer Substanzen ", Springer-Verlag, New York, 1971

23. H. D. Beckey, F. J. Comes, "Topics in Organic Mass Spectrometry", A. L. Burlingame, Ed., Wiley-INterscience, New York, 1970, p 65.

24. F. H. Field, Acc. Chem. Res. 1, 42 (1968)

25. C. E. Klotz, " Fundamental Processes in Radiation Chemistry " P. Ausloos, Ed., John Wiely and sons, New York, 1968, p 40.

26. H. P. Tannenbaum, J. D. Roberts, R. C. Dougherty, Anal. Chem. 47, 49 (1975)

27. I. Dzidic, D. I. Carroll, R. N. Stillwell, E. C. Horning, Anal. Chem. 47, 1308 (1975)

28. A. P. Brunis, A. J. Ferrer-Correia, A. G. Harrison, K. R. Jennings, R. K. Mitchum, Adv. in Mass Spectrom. 7, 355 (1978)

29. A. L. C. Smith, F. H. Field, J. Am. Chem. Soc. 99, 6471 (1977)

30. E. A. Berger, R. C. Dougherty, Biomed. Mass Spectrom. 8, 238 (1981)

31. S. G. Lias, P. Ausloos, " Ion Molecule Reactions " American Chemical Society, Washington. D. C., 1975, Chapter 4.

32. L. G. Christophorou, Chem. Rev. 76, 409 (1976)

33. D. F. Hunt, F. W. Crow, Anal. Chem. 50, 1781 (1978)

34. R. C. Dougherty, Anal. Chem. 53, 625A (1981)

35. M. Deinzer, D. Griffin, T. Miller, J. Lamberton, P. Freeman, V.

Jonas, Biomed. Mass Spectrom. 9, 85 (1982)

36. M. A. Saleh, J. Agric. Food Chem. 31, 748 (1983)

37. E. A. Stemmler, R. A. Hites, Anal. Chem. 57, 684 (1985)

38. J. E. Lovelock, P. G. Simmonds, W. J. Vandenheuvel, Nature. 247, 197 (1963)

39. K. J. Koshy, J. Chromatogr. 126, 641 (1976)

40. C. F. Pool, E. D. Morgan, J. Chromatogr. 115, 587 (1975)

41. C. F. Pool, A. Zlatkis, Anal. Chem. 52, 1003A, (1980)

42. E. D. Morgan, C. F. Pool, J. Chromatogr. 89, 225 (1974)

43. E. D. Morgan, C. F. Pool, J. Chromatogr. 104, 351 (1975)

44. C. F. Pool, E. D. Morgan, Org. Mass Spectrom. 10, 537 (1975)

45. J. Attal, S. M. Hendeles, K. B. Eik-Nes, Anal. Biochem. 20, 394 (1967)

46. R. A. Mead, C. C. Hattmeyer, K. B. Eik-Nes, J. Chromatogr. Sci. 7, 394 (1969)

47. K. T. Koshy, D. J. Kaiser, A. L. VanDer Silk, J. Chromatogr. Sci. 13, 97 (1975)

48. D. G. Petterson, J. S. Holler, M. A. Baird, Paper Present at 30th ASMS Meeting. Honolulu 1982 pp 36

49. S. J. Gaskell, " Analysis of Steroids in Methods of Biological Analysis ", Vol. 29, D. Glick, Ed., Wiely (Interscience) New York, P. 385-434 (1983)

50. A. C. Cope, K. E. Hoyle, J. Amer. Chem. Soc. 63, 735 (1941)

51. G. H. Alt, G. A. Gallegos, J. Org. Chem. 36, 1000 (1971)

52. F. S. Prout, J. Org. Chem. 18, 928 (1953)

53. P. S. Hench, E. C. Kendall, C. H. Slocumb, and H. F. Polley Proc. Staff Meet. Mayo Clin. 24, 181 (1949)

54. F. A. Fltzpatrick, Adv. Chromat. 16, 16 (1978)

55. M. L. Rocci, and W. J. Jusko, J. Chromat. 224, 221 (1981)

56. C. Lee, B. Amos, S. B. Matin, J. Pharm. Sci. 70, 669 (1981)

57. C. P. Devries, M. Lomecky-Janousek, and C. Popp-Snijders, J. Chromat. 183, 87 (1980)

58. J. Q. Rose, A.M. Yruchak, and W. J. Jusko, J. Pharmacokinet. Biopharm. 9, 389 (1981)

59. J.Q. Rose, A. M. Yruchak, and W. J. Jusko, Biopharm, Drug Dispos. 1, 247 (1980)

60. A. Ryrfeldt, M. Tonnesson, E. Nelson, and A. Wibby, J. Steroid Biochem. 9, 389 (1981)

61. A.W. Meikle, L. G. Lagerquist, and F. H. Tyler, Steroids. 32, 202 (1973)

62. D. I. Chapman, M. S. Moss, and J. Whiteside, Vet. Rec. 100, 447 (1977)

63. T. Kano, A. Mizuchi, and Y. Miyachi, Nippon Naibumpi Gakkai Zasshi. 54, 654 (1978)

64. M. Hichens and A. F. Hogans, Clin. Chem. 20, 266 (1974)

65. S. Miyabo, T. Nakamura, S. Kuwajima, and S. Kishida, Eur, J. Clin. Pharmacol. 20, 277 (1981)

66. E. Bailey, J. Endocrinol. 28,131 (1964)

67. H. Gohfried, Steroids. 5, 385 (1965)

68. R. S. Rosenfeld, M. C. Lebau, R. D. Jandorek, and T. Salumaa J. Chromat. 8, 355 (1962)

69. W. L. Gardiner, E. C. Horning, Biochim. Biophys. Acta. 155, 524 (1966)

70. S. Hara, T. Watabe, and Y. Ike, Chem. Pharm. Bull. 14, 1311 (1966)

71. S. B. Matin and B. Amos, J. Pharm. Sci. 67,923 (1978)

72. E. Houghton, P. Teale, M. C. Dumasia, and J. K. Wellby, Biomed. Mass Spectrum. 9, 459 (1982)

73. W. J. A. Vandenheuvel, J. L. Patterson, and K.L. K. Braly, Biochim. Biophys. Acta. 144, 691 (1969)

74. Y. Kasuya, J. R. Althaus, J. P. Freeman, R. k. Mitchum, and J. P. Skelly, J. Pharm. Sci. 446, 733 (1984)

75. E. D. Morgan and C. F. Poole, J. Chromatogr. 89, 225 (1974)

76. E. D. Morgan and C. F. Poole, J. Chromatogr. 104, 351 (1975)

77. C. F. Poole and E. D. Morgan, Org. Mass Spectrom. 10, 537 (1975)

78. C. F. Poole, A. Zlatkis, W. F. Sye, S. Sighawangcha, and E. D. Morgan, Lipid. 15, 734 (1980)

79. C. F. Poole, A. Zlatkis, J. Chromatogr. 184, 99 (1980)

80. C. F. Poole, S. Singhawangcha, A. Zlatkis, J. Chromatogr. 186, 307 (1979)

81. C. F. Poole, S. Singhawangcha and A. Zlatkis, Analyst. 104, 82 (1979)

82. C. F. Poole, S. Singhawangcha and A. Zlatkis, J. Chromatogr. 158, 33 (1978)

83. J. E. Lovelock, P. G. Simmonds, W. J. A. Vandenheuvel, Nature. 197, 249 (1963)

84. K. J. Kosky, J. Chromatogr. 126,641 (1976)

85. C. F. Poole and E.D. Morgan, J. Chromatogr. 115, 587 (1975)

86. E. Houghton, P. Teale, Biomed. Mass Spectrom. 8, 358 (1981)

87. Eric. P. Grimsrud, Electron Capture Theroy and Practice In Chromatography. A. Zlatkis and C. F. Poole, Ed., Nerterlands, 1981, p 110.

88. L. R. Hilpert, G. D. Byrd, and C. R. Vogt, Anal. Chem. 56, 1842 (1984)

89. A. Zlatkis and J. E. Lovelock, Clin. Chem. 11, 259 (1965)

90. A. Bowers, P. G. Holton, E. Necoechea, and F. A. Kinel, J. Chem Soc. 7, 405 (1961)

91. G. I. Poos, G. E. Arth, R. E. Beyler, and L. H. Sareh, J. Am. Chem. Soc. 75, 422 (1953)

92. E. J. Corey and J. W. Suggs, Tetrahedron Lett. 31, 2047 (1975)

93. E. D. Pellizzar, J. Chromatogr. 98, 348 (1974)

94. C. F. Poole, Chromatogr. 118, 280 (1976)

95. R. K. Mitchum, Personal Communication.

96. C. J. W. Brooks, A. M. Lawson, Excerpta Medica Int. Congress Series No. 219, Proc. 3rd Int. Congress Hormonal Steroids. 238, 1970 (1972)

97. A. H. Conney, Pharmacol Rev. 19, 317 (1967)

98. P. Saenger, Enid Forster, J. Kream, J. Clin. Endocrinol. Metab. 52, 381 (1981)

99. N. Haque, K. Thrasher, E. E. Werk, H. Ct Knovles, and L. J. Sholiton, J. Clin. Endocrinol. Metab. 34,44 (1972)

100. Minooc. Dumasia, Marian W. Horner, Edward Houghton, Michael S. Moss, Biochem. Soc. Trans. 4, 119 (1976)

101. D. S. Skrabalak, T. R. corvey, and J. D. Henion, J. Chromatogr. 315, 359 (1984)

102. R. Strife, R. Murphy. J. Chromatogr. 305, 3 (1984)

103. K. A. Waddell, S. E. Barrow, C. Robinson, M. A. Orchard, C. T. Dollery, and I. A. Blair, Biomed. Mass spectrom. 11,68 (1984)

104. S. E. Barrow, K. A. Waddell, M. Ennis, C. T. Dollery, and I. A. Blair, J. Chromatogr. 239, 71 (1982)

105. E. Larsen, K. Kjelgaard, Int. J. Mass Spectrom. Ion Phys. 11, 149 (1973)

106. J. M. Goodings, J. M. Jones, D. A. Parkes, Int. J. Mass Spectrom. Ion Phys. 9, 417 (1972)

107. A. L. C. Smit, M. A. J. Rosetto, F. H. Field, Anal. Chem. 48, 2042 (1976)

108. G. Stafford, J. Reeher, R. Smith, M. Story, in Dynamic Mass Spectrometry, Vol. 5, Ed. by D. Price and J. F. J. Todd, p. 55. Heyden, London (1977)

109. A. P. Bruins, Biomed. Mass Spectrom. 10, 46 (1983)

110. C. Reichert. "Structure-Activity Relationships", ed. H.L. Holmes. Defense Research Establishment, Suffield Ralston, Alberta, Canada, p.319, (1975).

111. E. Campaigne, D. Mais, and E.M. Yokley: Synthetic Comm. 4, 379 (1974).

112. M.M. Bursey and F.W. McLafferty: J. Amer. Chem. Soc. 88, 4484 (1966).

113. D.H. Williams, R.G. Cooks, J.H. Bowie, D. Madsen, G. Schroll, and S.O. Lawesson, Tetrahedron. 23, 3173 (1967).

114. D.J. Collins and J.J. Hobbs: Aust. J. Chem. 27, 1731 (1974).

115. K. L. Bush, C. E. Parker, D. J. Harvan, M. M. Bursey and J. R. Hass, Appl. Spectrosc. 35, 85 (1981)

116. C. N. McEwen and M. A. Rudat, J. Am. Chem. Soc. 101, 6470 (1979)

117. C. N. McEwen and M. A. Rudat, J. Am. Chem. Soc. 103, 4343 (1981)

118. N. B. H. Henis, K. L. Busch and M. M. Bursey, Chim. Acta. 53, L31 (1981)

119. D. Stockel and H. Budzikiewicz. Org. Mass Spectrom. 17, 376 (1982)

120. E. A. Stemmler and R. A. Hites, Anal. Chem. 57, 684 (1984)

121. G. W. Dillow and I. K. Gregor, Org. Mass Spectrom. 20, 317 (1985)

122. B. Ackerman, L. Eberson, E. Jonsson and E. Pettersson, J. Org. Chem. 40, 1365 (1975)

123. D. Fetterolf and R. Yost, Int. J. Mass spectrom. Ion Proc. 62, 33 (1984)