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THE EFFECT OF POTENTIATED SULFONAMIDE ADMINISTRATION ON EQUINE THYROID FUNCTION

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# THE EFFECT OF POTENTIATED SULFONAMIDE ADMINISTRATION ON EQUINE THYROID FUNCTION

By

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ABSTRACT

THE EFFECT OF POTENTIATED SULFONAMIDE ADMINISTRATION ON EQUINE THYROID FUNCTION

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The project's specific aims were (1) to determine if a dose-response relationship existed between trimethoprim-sulfadiazine (TMP-SDZ) administration and thyroid gland function in euthyroid horses, and (2) to determine the reversibility of any such alterations.

Three groups of four horses each received either no treatment (group 1), oral TMP-SDZ at 15 mg/kg daily (group 2), or 30 mg/kg daily (group 3) for 8 weeks. Total and free thyroxine (TT4 & FT4), FT4 by equilibrium dialysis (FT4d), total and free triiodothyronine (TT3 & FT3), and thyrotropin (TSH) concentrations were measured weekly during an 8 week treatment period and an 8 week recovery period. Pituitary-thyroid axis function was assessed by performing thyrotropin-releasing hormone (TRH) stimulation tests prior to treatment and at 4 week intervals during treatment and recovery.

No significant differences between groups were observed in weekly basal serum concentrations of TT4, FT4, FT4d, TT3, FT3, and TSH. Within each group, subtle and physiologically insignificant differences over time were observed. All hormones increased after TRH administration, but response to TRH did not change from baseline values in any group, except for a greater increase in TSH 2 hours post-TRH at week 8 in group 3. The greater TSH peak after TRH stimulation implies that the pituitary gland may have become more sensitive to TRH with TMP-SDZ treatment at 30 mg/kg daily. Overall, however, treatment did not produce subclinical or clinical hypothyroidism.

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| | |
|---|---|
| ADR | adverse drug reaction |
| THF | tetrahydrofolate |
| PABA | *para*-aminobenzoic acid |
| DHF | dihydrofolate |
| TMP | trimethoprim |
| OMP | ormetoprim |
| PYR | pyrimethamine |
| SMZ | sulfamethoxazole |
| KCS | keratoconjunctivitis sicca |
| SDZ | sulfadiazine |
| HA | hydroxylamine |
| EPM | Equine Protozoal Myeloencephalitis |
| T4 | thyroxine |
| T3 | triiodothyronine |
| TSH | thyrotropin/thyroid stimulating hormone |
| TRH | thyrotropin releasing hormone |
| TPO | thyroid peroxidase |
| TG | thyroglobulin |
| FT4 | free T4 |
| FT3 | free T3 |
| TBPA | thyroid binding prealbumin |

| | |
|---|---|
| TBG | thyroid binding globulin |
| TT4 | total T4 |
| TH | thyroid hormone(s) |
| PTU | propylthiouracil |
| SDM | sulfadimethoxine |
| CHD | congenital hypothyroidism and dysmaturity |
| TH-MSD | thyroid hyperplasia-musculoskeletal deformity |
| NAT | N-acetyltransferase |
| NADP | nicotinamide adenine dinucleotide phosphate |
| FT4d | free T4 by equilibrium dialysis |
| TT3 | total T3 |
| RIA | radioimmunoassay |
| TFA | thyroid gland function assessment |
| BCS | body condition score(s) |

# CHAPTER 1

# LITERATURE REVIEW

## Adverse Drug Reactions

Recognition and understanding of adverse drug reactions (ADRs) are vital to patient care in both human and veterinary medicine. The phrase *adverse drug reaction* denotes an unintended or undesirable response to a medication that emerges when used at appropriate prophylactic, therapeutic, or diagnostic doses.[1] Drug toxicity is a more general term that further includes ailments seen with excessive doses. Adverse drug reactions can be divided into three categories: (1) pharmacologic toxicity, which is a reaction caused by the pharmacologically active parent drug and/or metabolite that may or may not involve the "therapeutic target;" (2) intrinsic toxicity, which typically has reproducible effects and is dependent on drug dose and chemistry; and (3) idiosyncratic toxicity, also termed drug hypersensitivity, which is usually not reproducible, but may still be dependent on drug dose and chemical traits.[1] This last category is further defined as an "interaction between drug and host variables including genetics, age, gender, disease, diet, other drugs, and other chemical exposures."[2] Authors distinguish intrinsic reactions from idiosyncratic ones by defining the latter as arising from an individual's unique physiologic response to the drug in question.

Adverse drug reactions, of which numerous syndromes have been reported in both human and animal species, occur with use of many medications. Expanding the medical community's knowledge of the etiopathogenesis of ADRs is clearly important. As this

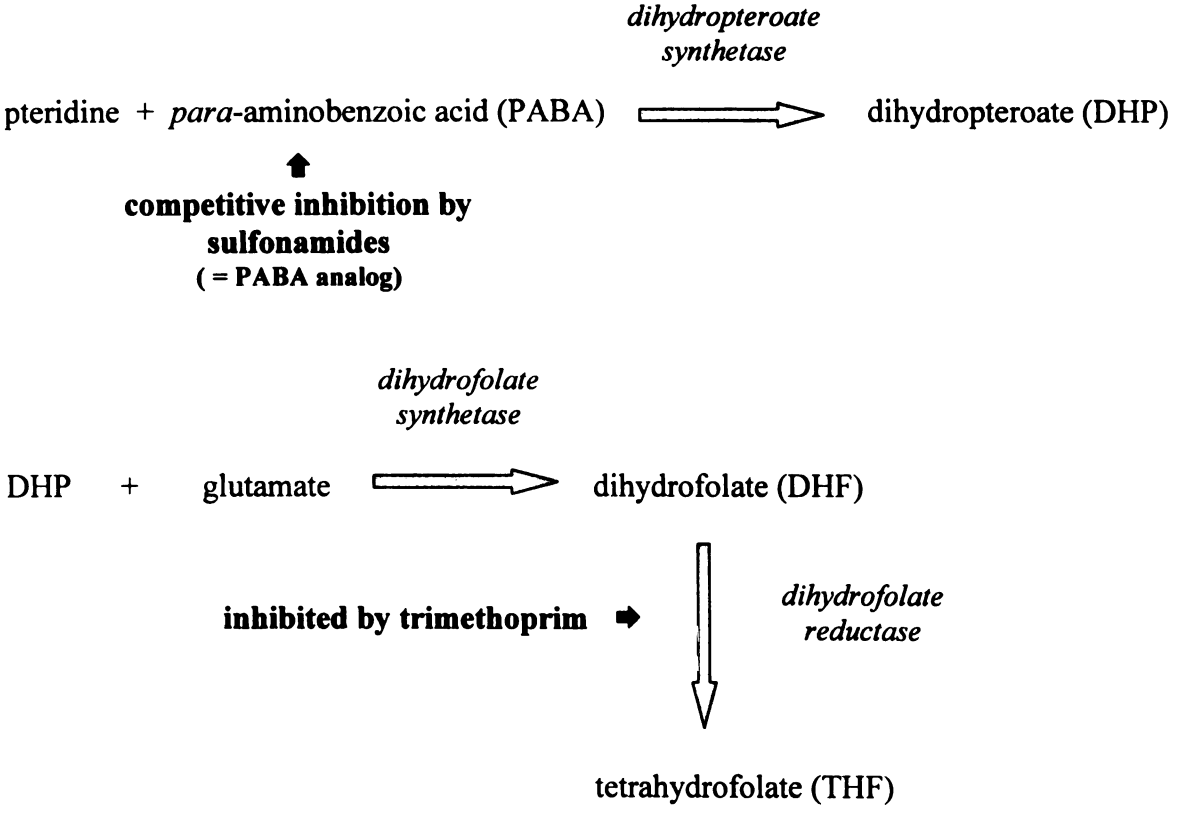
knowledge base improves, more informed therapeutic decisions can be made and more ADRs will potentially be avoided.

**Potentiated Sulfonamide Drugs**

Sulfonamide drugs were introduced in the early 1930s as effective and affordable agents for treatment of illnesses requiring broad-spectrum antimicrobial therapy.[1] For this review, the term "sulfonamide" will include all antimicrobial drugs derived from sulfanilamide (para-aminobenzenesulfonamide). Sulfonamides inhibit production of tetrahydrofolate (THF), the active form of folic acid (Figure 1). Specifically, they are analogues of *para*-aminobenzoic acid (PABA), a compound important in production of dihydrofolate (DHF). By competing with PABA, sulfonamides decrease DHF production by bacteria, and subsequent THF production. Potentiated sulfonamides were developed in the 1960s. These preparations combine a sulfonamide with a diaminopyrimidine compound, including trimethoprim (TMP), ormetoprim (OMP), or pyrimethamine (PYR). Pyrimidines directly inhibit dihydrofolate reductase (Figure 1), and thereby decrease conversion of DHF to THF, which is an important cofactor for DNA synthesis by bacteria.[1]

Potentiated sulfonamides exert their antimicrobial effects because bacterial cells must synthesize THF. Because mammalian cells do not synthesize folates and require a dietary source of folic acid, mammals are considered resistant to side effects of the primary action of sulfonamides. Nevertheless, because potentiated sulfonamides can also interfere with absorption and processing of folic acid to its active metabolites in mammals, prolonged administration can lead to signs of folic acid deficiency.[1]

**Figure 1.** Folate synthesis and sites of action of folate synthesis inhibitor drugs. (enzymes in *italics*)



## Adverse Reactions to Sulfonamides and Potentiated Sulfonamides

Despite regular reports of ADRs to sulfonamide and potentiated sulfonamide formulations over the past 70 years, they are still widely utilized in both human and veterinary medicine because of their low cost and continued efficacy. In fact, use of potentiated sulfonamides has increased dramatically with the increase in human *Pneumocystis carinii* infections, associated with the worldwide AIDS epidemic and the drug combination's efficacy against this organism.[1]

Recognition of a higher rate of ADRs associated with sulfonamide use in AIDS patients, compared to non-AIDS patients, has prompted further investigation of potential mechanisms of adverse effects.[1] However, the link between AIDS and the increase in ADR incidence remains unclear. Potential factors include genotypic and phenotypic variation, dose and length of drug therapy, drug formulation, and immunologic responses to parent drugs and their metabolites.

### *Pharmacologic Toxicities*

In a review of ADRs to sulfonamide drugs across several species, Cribb *et al.* (1996)[1] listed pharmacologic toxicities including nausea, vomiting, neurological signs, hematologic abnormalities, and renal tubular acidosis. While severe vomiting is rarely observed, nausea is more common (especially when the drugs are used at higher doses) and often leads to cessation of therapy. Sulfonamides are thought to act centrally, at the emetic center in the medulla, to produce these adverse effects. Other neurological signs, including headache, fine muscle tremors, anxiety, disorientation, and hallucinations, are adverse effects on the central nervous system that are infrequently observed.[1] The

mechanisms for these ADRs are not known, but abatement of clinical signs after withdrawal of the drugs supports that they are drug-induced effects.

In people, hematological abnormalities, including thrombocytopenia and neutropenia, have been described in patients being treated with potentiated sulfonamides. These ADRs are more commonly attributed to TMP and it is recognized that people with preexisting folic acid or vitamin $B_{12}$ deficiencies are at greater risk of developing these problems.[1] Hypoglycemia in patients receiving sulfanilamides was one of the earliest recognized adverse effects. In fact, recognition of this ADR served as motivation for development of related compounds for use as oral antidiabetic agents (e.g., sulfonylureas, such as tolbutamide, glipazide, and gliclazide). The mechanism of action appears to be a stimulatory effect on pancreatic beta cell secretion of insulin.[1] Currently, the more commonly prescribed sulfonamides rarely produce this effect.

Metabolic acidosis has also been recognized in patients receiving sulfanilamide or TMP-sulfamethoxazole (SMZ). These sulfanilamides can inhibit carbonic anhydrase activity and thereby limit renal tubular excretion of hydrogen ions. Retention of hydrogen ions leads to metabolic acidosis in the face of alkaline urine (type II or proximal renal tubular acidosis).[1, 3]

*Intrinsic Toxicities*

Intrinsic toxicities to potentiated sulfonamides include crystalluria and renal tubular necrosis, methemoglobinemia, and keratoconjunctivitis sicca (KCS).[1] Renal tubular necrosis may develop subsequent to the combined effects of crystalluria and direct tubular cell injury. Crystals are composed of either the parent sulfonamide or its

metabolites, and the degree of renal damage is inversely related to the sulfonamide solubility. For example, sulfadiazine (SDZ) is less soluble than SMZ and produces a higher incidence of crystalluria. Next, hydroxylamine (HA) metabolites of sulfonamides can gain entry to tubular cells and cause direct cell injury and necrosis. Both mechanisms can lead to obstruction of tubular flow, by aggregates of crystals or cellular debris, and progressive renal compromise. Fortunately, these effects are largely reversible with discontinuation of treatment.[1]

*Idiosyncratic Toxicities*

Finally, use of potentiated sulfonamides may produce idiosyncratic toxicities including dermatopathies (both urticarial and non-urticarial), immune-mediated disorders, blood dyscrasias, hepatic disease, and hypothyroidism. Idiosyncratic reactions can be due to the parent drugs or metabolites, but can also be non-drug related phenomena.[1] For example, unusual responses to the primary infection or reactions to inactive ingredients of a drug preparation (e.g., coloring or flavor) can also occur. Immune-mediated idiosyncratic reactions to sulfonamides may develop as drugs or metabolites bind to endogenous proteins and form "new antigens." Induction of an immune response to these "foreign" antigens can produce cellular damage and organ dysfunction. A wide variety of clinical signs could ensue depending on the tissue antigens involved.

Skin lesions are one of the more common complaints from patients on sulfonamide therapy. Documented abnormalities include papular dermatitis, urticarial

rashes, erythema multiforme, erythema nodosum, lupus erythematosus, toxic epidermal necrolysis, and photosensitization.[1]

In addition to the aforementioned blood dyscrasias related to folic acid deficiency, variable hematopoietic problems may develop with sulfonamide therapy, including eosinophilia, leukopenia and/or leukocytosis, macrocytosis, agranulocytosis, and aplastic anemia. Some of these disorders are associated with generalized, systemic illness. In horses, blood dyscrasias including anemia, neutropenia, and thrombocytopenia,[4] hemolytic anemia,[5] and a congenital syndrome in foals[6] have all been described. In this latter disorder, dams had been treated for equine protozoal myeloencephalitis (EPM) with a sulfonamide/pyrimidine combination and affected neonates had low folate concentrations, anemia, lymphoid aplasia, and bone marrow hypoplasia. Furthermore, some foals had epidermal necrosis and tubular nephrosis.

Although rare, idiosyncratic hepatocellular damage with potentiated sulfonamide administration has been recognized in human patients for over 50 years.[1] Liver enzyme activities are elevated and biopsy evaluation reveals hepatic necrosis and inflammatory infiltrates. Hepatitis may accompany ADRs with multiple organ involvement and go undetected without complete patient evaluation. Multiple sulfonamide formulations have been implicated; the mechanism is unknown. The problem has also been recognized as a reaction to TMP rather than the sulfonamide portion of the drug combination.

Other authors have described a variety of syndromes in dogs associated with sulfonamide therapy. Retinitis, myositis, anemia, fever, rashes, glomerulonephritis, polyarthritis, and hepatitis have all been reported in dogs receiving potentiated sulfonamides.[7, 8, 9, 10] Giger *et al.* (1985)[10] described polyarthritis as the most common

clinical problem in a series of dogs and proposed an immune-mediated process as the culprit. The author suggested that a genetic predisposition to ADRs might be occurring. A later review proposed that Doberman Pinschers had an inherent, poor ability to detoxify hydroxylamine-metabolites of sulfonamide drugs.[11]

Goitrogenic properties of potentiated sulfonamide drugs have been recognized for decades in multiple species.[1, 12-16] In people, subclinical hypothyroidism has been recognized as an idiosyncratic reaction to potentiated sulfonamide treatment.[17] Postulated mechanisms of decreased thyroid function include immune-mediated disease, thyroid gland enzyme inhibition by a drug metabolite, as well as altered metabolism and increased sensitivity in certain individuals to these drugs.[1, 2, 3, 17] Before continuing a more detailed discussion of the occurrence and mechanisms of hypothyroidism, a brief review of the hypothalamic-pituitary-thyroid axis and function of thyroid hormones is warranted.

**Thyroid Gland Physiology and Hypothyroidism**

The thyroid gland produces hormones with numerous actions that are integral to homeostasis. In general, thyroid hormones are responsible for control of metabolic rate. The thyroid gland secretes two main hormones, thyroxine (T4) and triiodothyronine (T3), which act on cells throughout the body to increase basal metabolic rate. Production and secretion of T4 and T3 is controlled by thyroid stimulating hormone (TSH), or thyrotropin, which is produced and secreted by the anterior pituitary gland. The hypothalamic hormone, thyrotropin-releasing hormone (TRH), controls synthesis and release of TSH. Circulating concentrations of T4 and T3 regulate hypothalamic and

pituitary production and secretion of TRH and TSH, respectively. Following the classic feedback loop paradigm, low T3 concentrations lead to increased TSH synthesis and release, and vice versa. Normal T3 and T4 levels inhibit TSH production. In people, T3 and T4 are thought to *indirectly* affect TRH release; it is proposed that higher CNS centers dictate TRH production, in response to brain T3 concentrations.[18, 19]

Approximately 93% of hormone secreted by the thyroid gland is T4 and 7% is T3. At the tissue level, T4 is converted to T3, by 5′-deiodinase, and T3 serves as the more metabolically active of the two hormones. In addition, the thyroid gland also produces calcitonin, a hormone central to calcium metabolism.[18, 19]

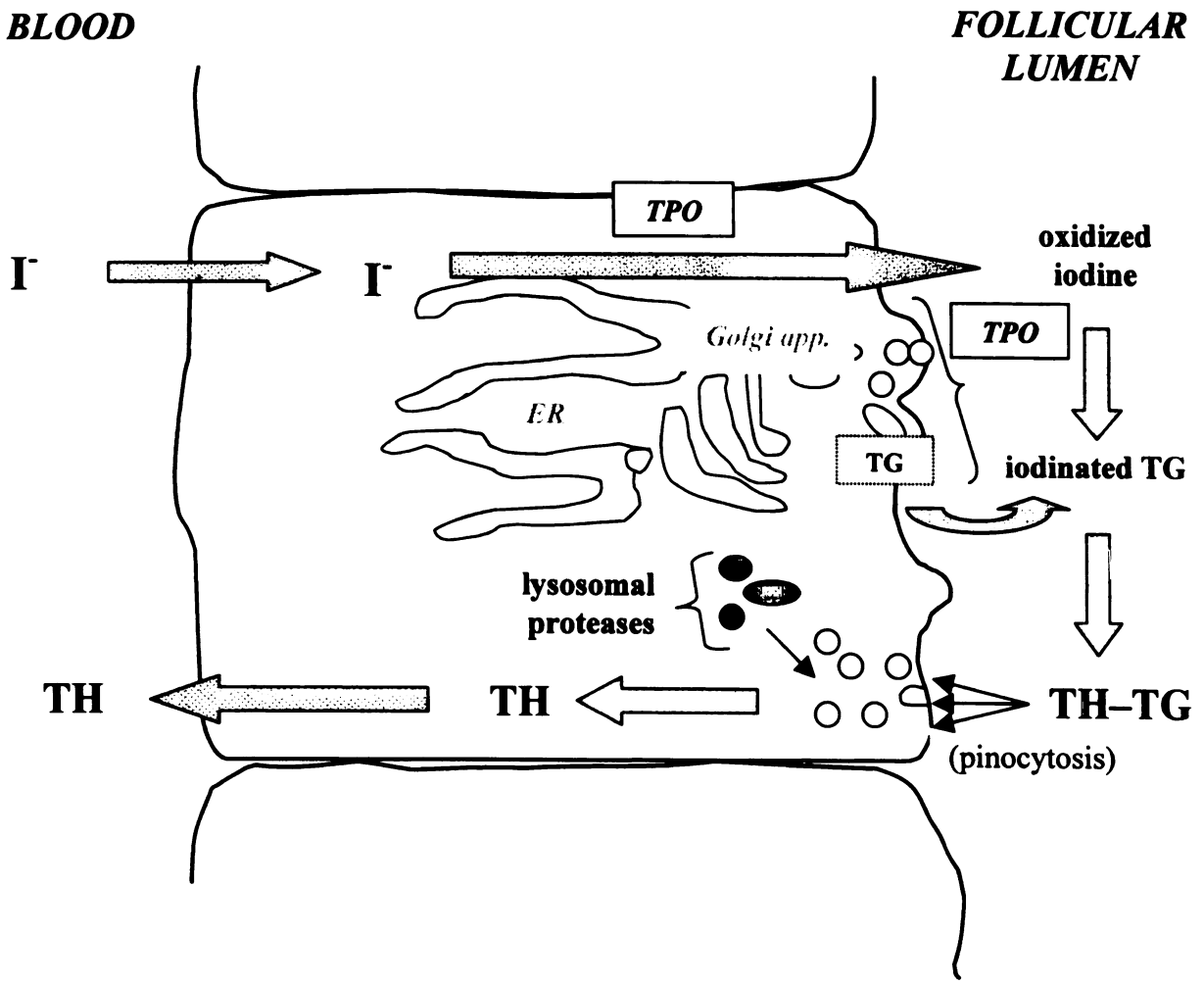
Synthesis of thyroid hormone requires iodine, which is actively taken up from blood as iodide via pumps located in the basal membrane of thyroid follicular cells. Activity of these pumps increases in response to increases in circulating TSH. Iodide ions are subsequently oxidized to iodine in a reaction catalyzed by thyroid peroxidase (TPO). The oxidation reaction occurs near the apical membrane of follicular cells, in close proximity to tyrosine-rich thyroglobulin (TG) molecules that are produced by the endoplasmic reticulum and Golgi apparatus of these glandular cells. In effect, as soon as TG is released into the cytosol, TPO catalyzes attachment of iodine to about one-sixth of the tyrosine residues on TG (a process called organification). In the cytosol and subsequently after secretion of TG across the apical membrane into the follicular space, mono- and diiodotyrosine residues gradually join to form T3 and T4 that remain attached to TG (Figure 2).[18, 19]

The thyroid gland can store, on average, a two to three month supply of thyroid hormones on TG as colloid in the follicular lumen. In response to TSH release, T4 and

T3 are cleaved from TG and released into circulation. Specifically, TSH release leads to pinocytosis of thyroid colloid through the apical membrane surface. Fusion of these pinocytosed vesicles with lysosomes results in release of T4 and T3 from TG. Free T4 (FT4) and T3 (FT3) then diffuse across the basal membrane and into the bloodstream. Upon entering the bloodstream, over 99% of secreted, FT4 and FT3 bind to plasma proteins, including albumin, thyroid binding prealbumin (TBPA) and, primarily, thyroid binding globulin (TBG). The protein-bound and free forms of each hormone comprise the total hormone concentration, i.e., bound T4 and FT4 make up the total T4 (TT4) concentration in serum.

Iodinated tyrosine residues that are not incorporated into thyroid hormones are deiodinated by action of a deiodinase enzyme and this iodine is recycled into the cytosolic iodine pool. This process allows unused iodine to remain inside the cell as a source for subsequent organification of TG.[18, 19]

**Figure 2.** Thyroid gland follicular cell: iodide/iodine transport and thyroid hormone synthesis. (TPO = thyroid peroxidase; TG = thyroglobulin; TH = thyroid hormone)



*Hypothyroidism*

Primary hypothyroidism is defined as a clinical syndrome accompanied by decreased circulating T4 and T3 concentrations and elevated TSH concentration.[18-20] The term subclinical hypothyroidism is used when circulating thyroid hormone concentrations are normal, but TSH concentration is elevated. Autoimmune disease and abnormal enzyme function, of both TPO and deiodinases, are two of the more prevalent causes of naturally occurring, primary hypothyroidism in people and animals.[18, 19]

Secondary hypothyroidism is a rare condition due to abnormal pituitary gland function. In this disorder, aberrant TSH production, or response to TRH, leads to decreased or absent stimulation of the thyroid gland. Disorders that cause decreased hypothalamic TRH synthesis, or poor TRH activity, have been reported and are termed tertiary hypothyroidism.[18, 19]

**Hypothyroidism Attributable to Potentiated Sulfonamides**

*Human Syndromes*

Although uncommon, reports of primary, subclinical and clinical hypothyroidism associated with sulfonamide therapy date back to the early use of these drugs.[1] In fact, in the early 20th century, these drugs were prescribed to treat hyperthyroidism.[1] At therapeutic doses, Cohen *et al.* (1980)[16] reported decreases in total T3 and T4 concentrations in people taking TMP-SMZ or TMP-sulfamoxole for only 10 days. In more recent years, delayed anti-thyroidal effects in patients taking sulfonamides have also been reported.[3]

*Rodent Syndromes*

Through a series of projects with rodents, researchers found an association between sulfonamide therapy, TSH hypersecretion, and goiter.[1, 12-15] An early study[12] evaluated effects of long-term TMP-SMZ, TMP, or SMZ therapy at various doses in rats. Monkeys served as a primate control group and received similar drug treatments. A dose-dependent increase in rat thyroid gland weight was reported after 13, 52 and 60 weeks of treatment with TMP-SMZ or SMZ alone. Histopathologic examination revealed glandular hyperplasia in all groups given SMZ. The extent of hyperplasia was less in the lower-dose groups. In addition, nodule formation was observed in some rats after 52-60 weeks of therapy at doses greater than 50 mg/kg of SMZ or TMP-SMZ. Vascular invasion and lung metastases of thyroid follicles were also evident in some rats that received greater than 150 mg/kg of SMZ or TMP-SMZ for 60 weeks. These changes and the associated decrease in body weight in affected rats resolved between 7-20 weeks after removal of SMZ from their diet.

Treatment with SMZ or TMP-SMZ did not produce goiter in any of the primate subjects included in this study. Monkeys tolerated long-term therapy at all doses although one death, attributed to anemia and nephropathy, was observed. Histopathologic evaluation of monkey thyroid glands did not reveal evidence of hyperplasia as was seen in rat thyroid glands. The goitrogenic effects of SMZ on rat thyroid glands could have been a consequence of elevated TSH production and activity on the thyroid gland or a direct hyperplastic effect on thyroid cells. Further, hyperplasia was reversible on cessation of treatment and these studies provided support for species

variation as monkeys appeared to be insensitive to anti-thyroidal effects of sulfonamide preparations.

In 1981, Cohen *et al.*[14] reported the effects of several formulations of potentiated sulfonamides (TMP-SMZ and TMP-sulfamoxole) on rat thyroid gland function, size and histopathology. This study had been motivated by a 1980 clinical report of decreased circulating thyroid hormone concentrations in men and women treated with these two drug combinations at pharmacologic doses for 10 days.[16] Thyroid gland function was evaluated by measurement of T3, T4, and TSH concentrations, and histopathologic examination of thyroid tissue was performed at the end of the study. Responses of rats treated with these two drug combinations were compared to those of negative (untreated) and positive (propylthiouracil [PTU]-treated) control rats. Propylthiouracil inhibits thyroid peroxidase and significantly decreases thyroid hormone production by the gland.[19] Thus, PTU-treatment provides a reliable positive control group. Three additional groups were treated with a single drug component; TMP, SMZ, or sulfamoxole. Doses of each drug were reported as pharmacologic equivalents of human doses based on body weight or as toxic doses (i.e., 20-fold greater than pharmacologic doses). Medications were given once daily orally for 10 days.

At pharmacologic doses, both TMP-SMZ and TMP-sulfamoxole decreased T4 concentrations (female rats), and increased TSH concentrations (males and females). Treatment with sulfamoxole alone resulted in decreased T3 and T4 concentrations in females, decreased T3 concentrations in males, and increased TSH concentrations in both sexes, with a greater TSH increase seen in females. Treatment with SMZ alone led to increased T4 concentrations in both sexes, increased TSH concentrations in males, and no

change in T3 concentrations. Treatment with TMP alone resulted in increased T4 concentrations, but no changes in T3 or TSH concentrations. With toxic doses, changes after 10 days were similar to those observed in PTU-treated controls. Both groups exhibited decreased T3 and T4 concentrations and increased TSH concentrations.

At the end of the study, an increase in thyroid gland weight was detected in rats treated with toxic doses of both drug combinations, pharmacologic doses of TMP-sulfamoxole, and PTU. The primary histopathologic change was hyperplastic goiter in rats receiving toxic and pharmacologic doses of TMP-sulfamoxole, a toxic dose of TMP-SMZ, and PTU. Other groups had normal histopathologic findings. The authors concluded that these two drug formulations retained goitrogenic properties at both therapeutic (pharmacologic) and toxic doses and that caution was still needed when prescribing these medications to people. Further, detection of increased TSH concentrations in all groups except rats treated with TMP alone provided support that goitrogenic effects were a consequence of elevated TSH production and activity on the thyroid gland.

*Canine Syndromes*

Prolonged treatment with sulfonamides is frequently prescribed for dogs with urinary tract infections, pyoderma, and respiratory disease. Only one potentiated sulfonamide formulation is approved for use in veterinary medicine, TMP-SDZ. However, generic formulations of TMP-SMZ are often used off-label due to the significantly lower cost. Over the past several decades, there have been several reports of canine hypothyroidism occurring in concert with long-term sulfonamide therapy. One

report described a case of clinical hypothyroidism in a dog with respiratory tract disease that had been treated with TMP-SDZ for at least 30 days on two separate occasions.[21] Clinical signs of hypothyroidism included exercise intolerance, alopecia, hyperpigmentation, and exudative skin lesions. Further assessment revealed low T4 and T3 concentrations and a high TSH concentration. Both clinical and laboratory abnormalities resolved with discontinuation of TMP-SDZ and initiation of thyroxine supplementation.

Next, in a series of 21 dogs,[22] the anti-thyroidal effects of TMP-SMZ, prescribed for treatment of pustular dermatitis, were evaluated. A dose of 30 mg/kg orally, twice daily, for 6 weeks was used to achieve effective drug levels in the skin. All subjects had T3 and T4 concentrations measured prior to and at the end of treatment. A limited number of dogs also had TSH-stimulation tests performed before, at the end of treatment, and 6-8 weeks after discontinuation of therapy to assess thyroid gland responsiveness. In addition, radionuclide imaging of the thyroid glands was performed in two dogs immediately after therapy was stopped to assess iodide uptake by the gland.

At the end of treatment, T4 concentrations were less than the values prior to therapy, while no difference was detected in T3 concentrations before and after treatment. The three dogs assessed with a TSH-stimulation test before and after treatment had reduced thyroid gland responsiveness (blunted increases in T4 and T3) at the end of the treatment period. Of two dogs that had a further TSH-stimulation test performed 6 to 8 weeks later, one had a normal response while the other remained hypothyroid. The two subjects that had nuclear imaging performed showed increased iodide uptake by the gland at the end of the treatment period. The investigators concluded that TMP-SMZ, at this

dose and duration of therapy, had suppressive effects on thyroid gland function in dogs. They proposed that the drug led to impaired organification and coupling, rather than impaired iodide uptake by the gland. They also implied that the toxic effects of SMZ-TMP affected thyroid hormone synthesis within the gland, but did not alter peripheral conversion of T4 to T3.

*Porcine Syndrome*

A study in swine evaluated the relationship between OMP-sulfadimethoxine (SDM) treatment and occurrence of poor viability of piglets, stillbirths, and congenital goiter.[23] The effects of two diets were also compared: one ration was similar to that used on a farm with a historical, congenital goiter problem; the other was a standard gestation diet. Three treatments consisted of the farm ration alone (ration 1, control), the farm ration plus 33.1 mg/kg OMP-SDM daily (ration 2); and the standard gestation ration plus 33.1 mg/kg OMP-SDM daily (ration 3). The feeding trial began 22 to 58 days prior to expected farrowing dates and continued until farrowing occurred.

Blood samples were collected from the pigs immediately prior to feeding twice weekly until farrowing, as well as within 24 hours of farrowing. After the final blood sample was collected, blood samples were also collected from the piglets, then all adults pigs and piglets were euthanatized. Three piglets from each litter were randomly selected and had multiple endocrine glands prepared for histopathologic evaluation.

All offspring from pigs fed rations 2 and 3 had congenital goiter (grossly enlarged thyroid glands) and moderate, diffuse thyroid hyperplasia. Mild to moderate thyroid hyperplasia was also found in adults fed rations 2 and 3, and one gilt fed ration 2 also had

a grossly enlarged thyroid gland. No significant differences were found in T4 concentrations in adults throughout the study or in offspring in any group. In addition, determination of SDM serum concentrations in piglets showed that appreciable amounts of the drug did not cross the placenta. This study was the first to suggest that sulfonamides may induce anti-thyroidal effects *in utero*.

## Equine Hypothyroidism

### *Naturally-Occurring Hypothyroidism*

Well-documented cases of naturally occurring equine hypothyroidism are scarce. Two case reports of suspected hypothyroidism, in a 2-year old colt[24] and a 2-year old gelding,[25] describe presenting complaints of multifocal to diffuse, non-pruritic alopecia. According to the respective owners, both patients had normal foal hair coats until approximately 5 months of age, when patchy hair loss began. These horses also exhibited small body size (compared to equivalent-aged horses from the respective farms). Hematologic and serum chemistry analyses in both cases were within normal limits. Bacterial, fungal, and ectoparasitic causes of hair loss were ruled out in both cases.

Stanley *et al.*[24] (1982) described the 2-year old colt to be lethargic and cold intolerant. Both testicles were palpably small and had poor tone. Additionally, plasma testosterone concentration was extremely low (62.5 pg/ml; reference range, intact male, 200-980 pg/ml), suggestive of decreased testicular activity. Skin biopsies, performed on both affected horses, revealed many small hair follicles containing keratinized material and marked dermal collagenization.[24, 25]

Stanley *et al.*[24] diagnosed hypothyroidism based on a single measurement of decreased serum T3 concentration. The authors proposed that a "functional hypothyroidism" existed due to their documentation of concurrent, normal serum T4 and low T3 concentrations. In the 2-year-old gelding, Hillyer *et al.*[25] used a TSH stimulation test (bovine TSH, 5 IU given intramuscularly) to diagnose hypothyroidism. T3 and T4 were measured before and 3 and 6 hours after TSH administration. Compared to a group of clinically normal horses, the patient's T3 and T4 responses were blunted. Both cases showed significant clinical improvement with exogenous T3 supplementation of variable treatment courses (80 and 120 days, respectively).

In addition to these "juvenile" cases, multiple reports of equine goiter (visibly enlarged thyroid gland) in foals have been presented since the 1960s.[26-34] In some reports goiter was also detected in adult horses and in neonates goiter occurred both with and without concurrent developmental abnormalities, primarily affecting the musculoskeletal system. Goiter was determined to be a consequence of either excessive or deficient iodine intake in these reports (see below).

Excessive iodine intake has been shown to cause goiter with hypothyroidism in humans, rats, and mice.[19, 35] Based on knowledge from human medicine and data in rodents, the mechanism of thyroid gland dysfunction results from interference with iodine organification within follicular cells, termed the Wolff-Chaikoff effect.[19] This occurs via inhibition of thyroid peroxidase mRNA and protein synthesis. As a result, thyroglobulin molecules are not iodinated, and thyroid hormone production declines. This protects the individual from making excessive, and potentially harmful, amounts of thyroid hormones. This effect normally lasts only a few days in people. However, some

individuals, including human newborns and fetuses, may have a several week delay before normal thyroid function resumes.[19, 35] Perhaps this phenomenon occurs in equine neonates as well, and would explain why correction of the iodine imbalance, and thyroid hormone replacement, is beneficial for treatment until normal thyroid gland function can resume after several weeks.

Interestingly, low dietary iodine availability has been implicated in decreased thyroid gland function as well.[19] In this scenario, due to low follicular cell uptake and subsequent decreased thyroid hormone synthesis, the hypothalamus and pituitary gland are stimulated to produce increased amounts of TRH and TSH, respectively. This, in turn, leads to increased stimulation of the thyroid gland and eventual goiter formation, due to diffuse, follicular cell hyperplasia. With correction of iodine requirements, this condition responds well to therapy.

Baker *et al.*[26] (1968) reported enlarged thyroid glands, as well as elevated plasma inorganic iodide and thyroid hormone concentrations in lactating mares and foals on a breeding farm. The authors found dietary iodide intake to be greater than 48 mg iodine/mare/day, compared to 7 mg or less/mare/day on farms with no goiter complaints, due to inclusion of dried seaweed (kelp) in three affected farms' diets. Many of the goitrous foals in this report had contracted, forelimb flexor tendons as well. Thyroid histopathologic changes included distended to coalescing follicles of variable size, containing pale colloid. Also, flattened follicular epithelium was present with few luminal projections. Goiter and blood abnormalities were also observed in some yearlings and non-lactating mares. These changes resolved with correction of dietary iodine intake.

Drew *et al.*[28] (1975) reported goiter in four foals and a mare from a single stud. Two of the four foals died; the other two foals showed limb weakness. Histopathologic exam of one foal's thyroid gland showed marked follicular enlargement and signs of hyperplasia. One surviving foal was not treated and the other (last born) received supplemental potassium iodide based on a concern of iodine deficiency. This foal showed no improvement with therapy. Eventually, these occurrences of goiter were attributed to excessive iodine intake, from a proprietary compound added to the pregnant mares' daily ration for 2 months prior to the first observation of signs in the pregnant mare. Once this imbalance was identified, the ration was corrected. Protein bound iodine content in mare's serum was measured after the diet change, and the authors documented a gradual return to normal protein bound iodine values. Both surviving foals recovered fully.

Another syndrome of congenital hypothyroidism was first reported in 1981, synonymously termed congenital hypothyroidism and dysmaturity (CHD) syndrome or thyroid hyperplasia-musculoskeletal deformity (TH-MSD) syndrome.[36, 37] Although goiter was not a prominent feature of the syndrome, variable thyroid gland hyperplasia has accompanied forelimb flexural deformities (contracted tendons with frequent common digital extensor tendon rupture), mandibular prognathism, and delayed or abnormal ossification of cuboidal bones.[31, 34, 38-42] Variation in thyroid gland histopathologic changes is likely a consequence of differences in the severity of the insult and the time after birth at which thyroid glands were examined. In abortuses and neonates, typical pathologic findings were hyperplastic, follicular epithelium with cuboidal to columnar cells, with some stratified epithelia, as well as small amounts of

colloid.[36, 39, 43, 44] These changes were consistent with a primary, hypothyroid state and excessive TSH stimulation of a hypofunctioning gland. In weanlings with less severe musculoskeletal disease, thyroid gland pathology included variable sized follicles with colloid present, and low to flattened cuboidal epithelium. These changes were more consistent with a gland recovering from a hypothyroid state.

Various etiologies have been proposed for CHD or TH-MSD, including inherited thyroid hormone synthesis/release disorders, ingested thyrotoxins, and infectious agents. Heritability has largely been eliminated as a cause based on the sporadic nature of the syndrome and the variety of breeds affected.[39] Next, multiple necropsy exams of affected fetuses and foals have failed to identify evidence of an infectious agent. Proposed toxins that could be ingested by pregnant mares and lead to fetal iodine deficiency include excessive nitrate ingestion[36, 43] and glucosinolates.[45]

Nitrate and glucosinolate ingestion may be implicated in decreased thyroid gland function due to their effects on iodine metabolism. Nitrates can break the bond between iodine and the basal membrane transport protein integral to the active iodine pump in thyroid follicular cells.[46, 47] Glucosinolates are nonvolatile components of many plant species, including the mustard grasses, which are common weeds in spring and summer in some regions. The hydrolytic metabolites of glucosinolate are isothiocyanates, known antithyroid substances. Proposed mechanisms of action include inhibition of active iodide uptake by follicular cells and interference with thyroglobulin iodination and/or coupling of iodotyrosine residues within TG molecules. In addition, isothiocyanate derivatives have been shown to interfere with iodide oxidation to iodine.[19, 47]

A case report of a full-term, newborn foal with hypothyroidism and respiratory insufficiency has also been described.[48] This foal was unable to stand unassisted and had an abnormal suckle reflex immediately post-partum. Examination revealed hypothermia, dehydration, failure of passive transfer, bilaterally enlarged thyroid glands, and harsh lung sounds. Respiratory acidosis and compensatory metabolic alkalosis were also present. A TRH stimulation test was performed and revealed a decreased response compared to an age-matched, normal foal (results were obtained after the foal's death). The affected foal had an initial favorable response to treatment, but died after 8 days of hospitalization. Post-mortem examination found variably-sized thyroid follicles filled with colloid and severe, diffuse, pulmonary atelectasis. The authors proposed that a hypothyroid condition *in utero* led to abnormal surfactant production and subsequent respiratory insufficiency. No gestational or dietary factor that may have altered iodine availability was discovered during further investigation of the history or the owner's farm.

While a common cause of these syndromes of congenital hypothyroidism has not been established, several possibilities exist. Further, the cause is likely multifactorial in many instances. Nevertheless, all current theories emphasize the importance of proper iodine intake by pregnant mares for normal fetal development.

*Experimentally-Induced Hypothyroidism*

In 1970, surgical thyroidectomy was first performed as a disease model for equine hypothyroidism. Lowe *et al.* described effects of thyroidectomy on thyroid gland responsiveness[49] as well as on growth and metabolism.[50] The studies reported

development of lethargy, hypothermia and cold sensitivity, growth retardation, dull and slow-to-shed hair coats, edema of the face and distal, hind limbs, elevated cholesterol concentrations, non-detectable T4 concentrations, decreased feed consumption and weight gain, and delayed epiphyseal growth plate closure and incisor eruption. The authors also showed that iodinated casein supplementation ameliorated or even reversed these signs of hypothyroidism. Next, after recognizing the CHD syndrome in western Canada, a group of researchers in Saskatoon performed partial thyroidectomies in late-term equine fetuses and successfully reproduced the abnormalities seen in foals with naturally occurring CHD.[51]

More recently, Vischer *et al.*[52] (1999) examined hemodynamic effects of thyroidectomy in sedentary horses. Negative effects of the hypothyroid state on hemodynamic function in people, rodents, and dogs motivated this study. Thyroidectomy produced expected changes including decreases in heart rate, respiratory rate, body temperature, cardiac output, and responsiveness to β-adrenergic stimulation. In addition, blood volume, plasma volume, and electrocardiographic PQ and QT intervals increased. These alterations were partly reversible with oral thyroid hormone supplementation.

In 2002, Breuhaus *et al.*[53] published data from hypothyroid horses, with disease induced by administration with PTU. In this study, within 4 weeks of the start of PTU treatment, horses showed decreased gland responsiveness to TRH stimulation as well as increased basal TSH concentrations. However, development of clinical signs of hypothyroidism was not apparent in any of the subjects.

In a second study evaluating drug-induced hypothyroidism, Johnson *et al.*[54] (2003) described effects of PTU and bromocryptine on thyroid function in mares. Their

results supported the use of PTU treatment as a model of primary hypothyroidism, with elevated TSH and lowered TT4 and TT3 concentrations after 28 days of therapy with PTU. The authors hypothesized that bromocryptine would lead to secondary hypothyroidism due to the drug's dopaminergic, suppressive effects on pituitary hormone release. Specifically, the study tested whether the drug would lower TSH concentrations and subsequently lead to a hypothyroid state. They found no evidence in support of this latter hypothesis.

## Pathophysiology of Sulfonamide-Associated Hypothyroidism

### *Sulfonamide Metabolism*

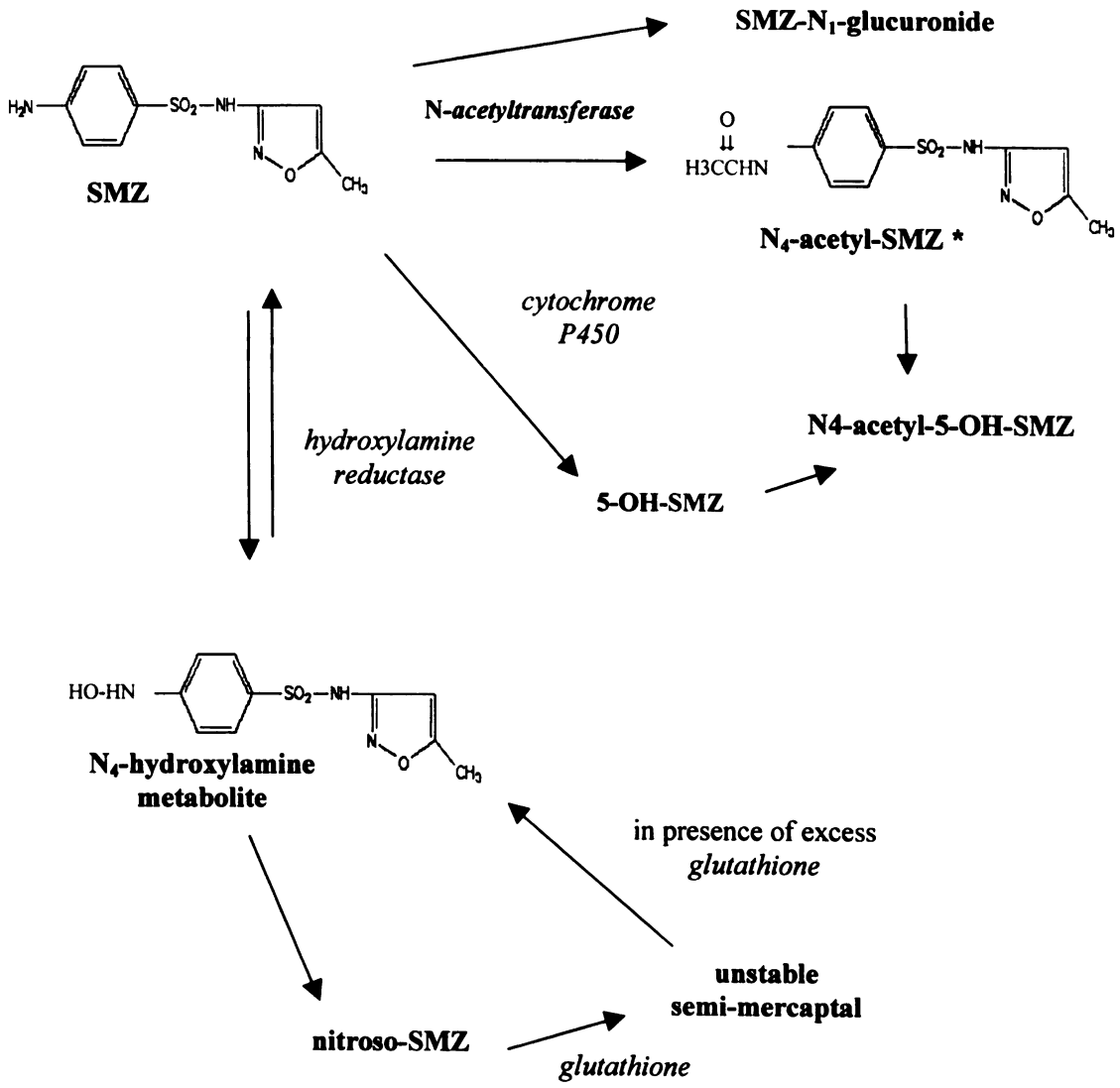
It has been proposed that adverse reactions to sulfonamide drugs are linked to the effects of sulfonamide metabolites. The metabolic pathways for SMZ are shown in Figure 3. The parent drug, SMZ, is acetylated or hydroxylated (i.e., oxidized) at the $N_4$ position on the aromatic ring. The majority of the parent drug is acetylated *in vivo*. In addition, hydroxylation can occur at the $C_5$-methyl group and glucuronidation may occur at the $N_1$ position.[1]

N-acetyltransferase (NAT) is the primary enzyme responsible for acetylation. Acetylated SMZ is predominantly excreted via renal tubular excretion. The proportion eliminated through this route can vary with pH and degree of plasma protein binding of drug metabolites. Two forms of N-acetyltransferase, NAT1 and NAT2, play important roles in eliminating sulfonamides. Polymorphisms in these enzymes likely result in variability to metabolize these drugs in different species. NAT1, with highest activity in the liver, has the greater role in sulfonamide breakdown; NAT2, found in mononuclear

leukocytes, has provided a means of studying sulfonamide metabolite production *in vitro*.[1, 2, 17]

The polymorphisms in NAT affect the rate of drug acetylation. Slow and rapid acetylator phenotypes have been described in people, rodents and dogs.[2] This trait is thought to be recessive, i.e. homozygous recessive individuals are "slow" acetylators, whereas heterozygous and homozygous dominant subjects are classified as "rapid" acetylators. Research in this field suggests that certain human ethnic groups (Asian) and dog breeds (Doberman Pinscher) are more likely to display specific phenotypes.[2, 11] The possibility exists that additional species or breeds within species also exhibit this phenotype.

**Figure 3.** Sulfonamide metabolism – e.g. sulfamethoxazole (SMZ).
Enzymes in *italics*; * = actively excreted by renal tubules.



*Mechanisms of Toxicity*

The clinical significance of genetic polymorphisms and resultant phenotypic variation is an indirect one. "Slow" acetylator individuals may produce more hydroxylamine metabolites of sulfonamide preparations, because more parent drug is available for oxidation.[11] Consequently, greater concentrations of the oxidized metabolites are produced. Hydroxylamine metabolites (SMZ-HA) are readily formed by action of cytochrome P450 enzymes (specific names vary by species) at the $C_5$ position. In addition, SMZ-HA can spontaneously be converted to even more reactive, nitroso-SMZ metabolites. These hydroxylamine compounds can also be modified into other toxic metabolites by NAT action (acetoxy-SMZ). In addition, SMZ-HA may be converted back to the parent sulfonamide compound. Researchers have theorized that these metabolites are central to development of idiosyncratic reactions. Initially, two main pathways were proposed: (1) direct cytotoxicity by reactive metabolites; (2) metabolite–host antigen binding and subsequent induction of host immunologic responses.[1-3, 55]

More recent studies suggest that sulfonamide metabolites can also affect enzyme activity, which then leads to organ dysfunction. Sulfamethazine has been shown to reversibly inhibit TPO and thereby decrease iodine organification and coupling by the thyroid gland.[17] As detailed above, these two processes are central to thyroid hormone synthesis. By inhibiting thyroid hormone production, TRH and TSH secretion increase, consistent with primary hypothyroidism. These results are consistent with previous findings in rats and mice regarding the goitrogenic effects of long-term sulfonamide therapy that was attributed to prolonged TSH stimulation.[12-14] However, a potential

mechanistic basis for the changes in hormone concentrations, inhibition of TPO activity, was now established.

Other researchers have suggested that susceptible individuals have an inherent defect in detoxification of HA-sulfonamides. Several studies have been completed addressing glutathione function and its role in drug metabolism.[2, 17] However, results have been equivocal. It has been proposed by some authors that HA-sulfonamide metabolites play a role in hematologic toxicities. As HA-metabolites are detoxified via oxidation reactions, hemoglobin is also oxidized to methemoglobinemia leading to hemolytic anemia. Sulfasalazine, and its HA-metabolite, sulfapyridine, have most commonly been linked to methemoglobinemia. Fortunately, this sulfonamide drug is rarely administered systemically in human medicine today. Another hematologic disease, glucose-6-phosphate deficiency, can be exacerbated by treatment with sulfonamides. This deficiency results in an inability of red blood cells to reduce nicotinamide adenine dinucleotide phosphate (NADP) molecules. Consequently, glutathione stores are quickly depleted and reactive, oxidized sulfonamide metabolites accumulate. These metabolites are proposed to increase cell membrane fragility and lead to hemolysis.[1]

All in all, research to date has documented several mechanisms by which potentiated sulfonamide drugs may be toxic to the patients receiving them. In addition to potential direct toxicity that could affect all people and animals receiving the drug combinations (e.g., inhibition of TPO activity), it has also become clear that a number of patient factors, including metabolism of the drugs and increased susceptibility to adverse effects of particular drugs and/or their metabolites, places certain species and individuals within each species at increased risk. Finally, both the dose and duration of drug therapy

are additional factors that influence the potential for ADRs to potentiated sulfonamides in each individual patient.

**Potentiated Sulfonamide Treatment in the Horse**

Sulfonamides in combination with trimethoprim are one of the most commonly prescribed antibiotics by equine veterinarians. Sulfonamide drugs are an effective and affordable antibiotic choice for many diseases and injuries, including respiratory, urinary tract, and minor bacterial infections. Furthermore, because they can be administered orally, equine practitioners consistently dispense potentiated sulfonamides for administration to horses by their owners.

In addition, sulfonamides are used in the treatment of horses suspected to have EPM. Long-term SDZ and PYR combinations have been recommended for treatment of EPM based on their efficacy in the treatment of coccidial diseases in human medicine.[4] The dramatic increase in awareness and diagnosis of EPM during the 1990s led to a substantial increase in the number of horses receiving long-term (3-12 months) treatment with potentiated sulfonamides. While other medications to treat EPM infections are being marketed, combinations of TMP and/or PYR with a sulfonamide remain a common therapeutic choice. Debates still exist regarding dosage, as evidenced by the wide range of published doses, from 15-60 mg/kg once or twice daily.[56]

Some practitioners empirically use human TMP-SMZ formulations in combination with PYR to treat EPM. The equine veterinary community has not fully addressed the interaction and potential additive risk of ADRs that are potentially created by using two pyrimidine drugs simultaneously. In fact, in human medicine, this practice

is not recommended because of the significant alterations in folic acid production associated with use of two pyrimidines.[56] Nonetheless, this practice is common in equine medicine and no in-depth investigations into ADRs or long-term side effects due to potentiated sulfonamide therapy in equids have been pursued to date.

With the growing understanding of potentiated sulfonamide action and metabolism in equids and other species, the potential for ADRs to these drugs cannot be ignored or underestimated. Thus, it is imperative that further studies of potential ADRs to these drug combinations be pursued in the species in which they are commonly used. To that end, the research presented in this thesis focuses on potential anti-thyroidal effects of long-term (8 weeks) administration of PYR-SDZ to healthy horses at doses that are commonly prescribed by equine practitioners.

# CHAPTER 2

## THE EFFECT OF POTENTIATED SULFONAMIDE ADMINISTRATION ON EQUINE THYROID FUNCTION


### Abstract

*Objectives*

The first objective was to determine the dose-response relationship between trimethoprim-sulfadiazine (TMP-SDZ) administration and thyroid function in euthyroid horses by measuring basal serum concentrations of total and free thyroxine (TT4 and FT4), FT4 by equilibrium dialysis (FT4d), total and free triiodothyronine (TT3 and FT3), and thyrotropin (TSH), as well as thyrotropin releasing hormone (TRH)-stimulated serum concentrations of TT4, FT4, FT4d, TT3, FT3, and TSH. The second goal was to determine the reversibility of any TMP-SDZ-induced alterations in thyroid gland function established in the first objective.

*Subjects*

Twelve, adult, euthyroid horses of various breeds were divided into three treatment groups.

*Design*

The study included three groups of horses that were studied for a 16 week period: during 8 weeks of drug administration and 8 weeks of recovery. Group 1 (n=4) received no treatment and served as a control group (to assess random effects of time), group 2 (n=4) received TMP-SDZ at the lower recommended dose (15 mg/kg, PO, q 24 h) for 8 weeks, and group 3 (n=4) received the higher recommended dose (30 mg/kg, PO, q 24 h)

for 8 weeks. Medication was administered with 2 ounces of water and 4 ounces of sweet feed between 1 P.M. and 3 P.M. Each group consisted of three mares and one gelding and all were housed in adjacent paddocks and fed grass/alfalfa hay *ad libitum*.

Basal (resting) concentrations of TT4, FT4, FT4d, TT3, FT3, and TSH were determined weekly by radioimmunoassay (RIA). Details regarding RIAs for TT4, FT4, FT4d, FT3, and TSH appear in Appendices B and C. The TSH radioimmunoassay was performed as previously described with minor modifications. Thyroid gland function was further ascertained by use of the TRH stimulation test, according to published methodology. At the start of the study (week 0), thyroid hormone and TSH concentrations were assayed on baseline samples prior to TRH administration, and then 2 and 4 hours later, i.e. thyroid function assessment (TFA).

TMP-SDZ treatment continued for 8 weeks. TFA was repeated in all horses after 4 and 8 weeks of treatment, as well as 4 and 8 weeks following cessation of treatment. In addition, the same individual recorded body condition scores (BCS) every 4 weeks during the study period.

Data were analyzed by one and two-factor, repeated measures analysis of variance for main effects of treatment (dose level) and/or time. If F-ratios were significant ($p<0.05$), a Student Newman-Keuls or Dunnett's post-hoc test was performed to detect specific differences.

*Results*

All 12 horses remained healthy during the study period and no adverse clinical effects of 8 weeks of treatment with TMP-SDZ at either dose were observed. Body

condition scores ranged from 4 to 6.5 (out of 9) and, although BCS increased ($p<0.01$) from 5.1 ± 0.2 to 5.5 ± 0.2 (mean ± standard deviation) for all subjects over the 16-week study period, differences between treatments were not observed.

No significant differences between treatment groups were observed in weekly basal serum concentrations of TT4, FT4, FT4d, TT3, and FT3. Similarly, differences between treatment groups were not observed in weekly basal serum concentrations of TSH except at week 16 when mean TSH concentration for group 1 was greater ($p<0.05$) than the mean values for group 2 and group 3. Within each treatment group, differences were observed over time. In group 1 (controls), mean FT4 values at weeks 1, 2, 7, and 9 through 16 were lower ($p<0.05$) than baseline concentration (week 0) and mean FT3 concentration at week 1 was higher ($p<0.05$) than baseline. In group 2 (15 mg/kg), mean FT4 concentrations at week 1 and weeks 7 through 16 were lower ($p<0.05$) than baseline concentration, as were mean FT3 concentrations at weeks 8 and 16. In Group 3 (30 mg/kg), mean FT4 concentrations at weeks 1, 7, and 9 through 16 were lower ($p<0.05$) than baseline concentration and mean FT3 concentrations at weeks 4, 5, 7, and 8 were lower ($p<0.05$) than baseline concentration. Differences in weekly mean TSH concentrations were not observed except for a decrease ($p<0.05$) from baseline concentration at weeks 3, 7, and 10 in group 3 (30 mg/kg). No other significant differences in hormone concentrations were found between treatment groups within any weekly time period.

Concentrations of all thyroid hormones and TSH increased after TRH administration, yet responsiveness to TRH did not change from baseline values (week 0) in any group, except in group 3. After 8 weeks of treatment, mean TSH concentration in

group 3 was significantly greater ($p<0.05$) 2 hours after TRH administration compared to the baseline (week 0) 2-hour post-TRH sample concentration and the equivalent samples from groups 1 and 2. This difference in group 3 was not found after 4 weeks of treatment and did not persist at 4 or 8 weeks after cessation of TMP-SDZ administration.

*Conclusion and Clinical Significance*

Administration of TMP-SDZ for 8 weeks, at doses commonly recommended for treatment of equine infections, did not produce clinical signs of hypothyroidism. However, an exaggerated TSH response after TRH administration to horses treated with the highest dose for 8 weeks suggests that potentiated sulfonamides may affect pituitary responsiveness.

While only two doses of TMP-SDZ were evaluated in this study, the findings imply that most healthy horses should tolerate long-term therapy with this drug formulation without concern of development of thyroid dysfunction. Further research is necessary to investigate if higher doses or longer durations of therapy may induce primary hypothyroidism. In addition, future studies should include histologic evaluation of the thyroid gland.

## Introduction

Sulfonamides in combination with trimethoprim, or potentiated sulfonamides, are one of the antimicrobial agents most commonly prescribed by equine veterinarians. These drug combinations continue to be an effective and affordable antibiotic choice for many diseases and injuries, including respiratory, urinary tract, and minor wound

infections. Furthermore, because they can be administered orally, equine practitioners routinely dispense potentiated sulfonamides for administration by their clients.

In addition, sulfonamides in combination with pyrimethamine are used in the treatment of horses suspected to have EPM.[4] The dramatic increase in awareness of EPM over the past decade has led to a substantial increase in the number of horses receiving long-term (3-12 months) treatment with potentiated sulfonamides. While other medications to treat EPM infections are being marketed, potentiated sulfonamide combinations remain a popular choice.

*Sulfonamide Treatment and Hypothyroidism*

For several decades, the medical community has recognized sulfonamide-associated adverse drug reactions in multiple species, including toxic effects on the thyroid gland.[1, 12-14, 21, 22] Historically, due to this ability to inhibit thyroid gland function, sulfonamides were actually used to treat hyperthyroidism in human patients.[16] The mechanisms of sulfonamide toxicity involve blocking organic binding of iodine to thyroglobulin and coupling of iodothyronines to form T4 and T3.[17, 55] Thyroid peroxidase (TPO) catalyzes both of these metabolic reactions. Sulfonamide-induced inhibition of TPO, reversible with discontinuation of sulfonamide treatment, has been demonstrated as an important mechanism of hypothyroidism associated with sulfonamide treatment in rats.[17]

Subclinical and clinical hypothyroidism induced by sulfonamide therapy, prescribed for ailments other than hyperthyroidism, has been described in humans[3, 16], rodents[12], and dogs.[21, 22] A "hypersensitivity reaction" to sulfonamides was considered

the cause of decreased thyroid hormone concentrations in human patients.[3] This hypersensitivity was thought to be limited to patients with an unidentified, inherited defect in detoxification of reactive metabolites of sulfonamides. Specifically, authors proposed that the HA-metabolite of sulfamethoxazole, formed by action of TPO on the parent sulfamethoxazole, was cytotoxic to thyroid cells *in vitro* and was able to produce hypothyroidism *in vivo* in patients that are unable to detoxify the HA-metabolite.[3] An alternate theory suggested that covalent binding of the HA-metabolite to several macromolecules in thyroid cells, including TPO, may lead to formation of antibodies against these new "antigens" and thus induce autoimmune hypothyroidism.[3]

*Assessment of Thyroid Gland Function*

The variety of methods used to assess thyroid gland function has hampered comparison of results from prior studies. For example, some authors have reported resting thyroid hormone concentrations, while others have evaluated the response of the thyroid gland during stimulation tests.[3, 12-14, 21, 22, 63] In human medicine, assay of serum TSH concentration is the accepted clinical screening test for evaluation of hypothyroidism. This single sample test is both practical and economical and, when combined with concurrent T4 measurement, allows categorization of patients as hypothyroid (low T4 and high TSH) or as subclinically hypothyroid (normal T4 and high TSH).[20] In studies of sulfonamide-induced thyroid gland dysfunction, it is noteworthy that a consistent experimental finding has been increased basal serum concentrations of TSH during sulfonamide treatment.[12, 14, 17, 21, 22] Recently, a TSH assay has been

developed for use on equine serum samples[53, 54, 60] and provides an important new research tool for assessing thyroid gland function in horses.

In the multiple species studied, sulfonamide-associated hypothyroidism appears to be dependent on dose and duration of sulfonamide treatment, as well as the sulfonamide formulation administered. For example, when a trimethoprim/sulfadiazine combination was administered (30 mg/kg, PO, q 24 h) to healthy dogs for 4 weeks, decreases in T3 and T4 concentrations were not found and response to exogenous TSH was normal.[63] However, endogenous TSH concentrations were not measured in this study. For this reason, it is possible that states of subclinical hypothyroidism were missed. When an ormetoprim/sulfadimethoxine combination was administered (27.5 mg/kg, PO, q 24 h) to dogs for 8 weeks, impaired thyroid gland function and increased thyroid gland weight was produced (Primor® package insert, SmithKline Beecham, Exton, PA).

In the latter investigation, enlarged basophilic cells (thyrotroph cells that produce TSH) were found in the pituitary glands of treated dogs, providing support for a primary hypothyroid condition with a secondary increase in TSH release from the pituitary gland. A similar observation of altered pituitary thyrotroph cells was made in rats administered high doses (2 g/kg) of sulfonamides for 4 weeks.[13] Furthermore, additional studies in rats described similar pituitary thyrotroph hypertrophy and increased thyroid gland at higher doses weights,[12, 14] consistent with the expected, compensatory increase in TSH production.

In dogs with pyoderma treated with a trimethoprim/sulfamethoxazole combination (60 mg/kg, PO, q 24 h), serum TT4 and FT4 concentrations were decreased and response to exogenous TSH was diminished after 6 weeks of therapy. When

assessed, return to apparently normal thyroid function required 8 to 12 weeks after cessation of sulfonamide treatment.[22]

*Goals of the Thesis*

Because of the widespread use of potentiated sulfonamide medications in equine practice, investigating a possible connection between use of these drugs and equine thyroid gland function is clearly important. In addition, with the prevalent use of thyroid hormone supplementation in horses that may in fact be euthyroid, research into a potential cause of thyroid dysfunction is critical for the equine veterinary community. The results should provide important information about thyroid gland physiology in horses and may help clarify some of the current confusion about hypothyroidism in horses. In people, thyroid hormone therapy in euthyroid patients has been shown to have negative effects.[64, 65] If more accurate thyroid function assessment is obtainable, perhaps fewer equids will be unnecessarily treated with thyroid hormone supplement.

Thus, we tested the hypothesis that oral administration of a TMP-SDZ combination changes hypothalamic-pituitary-thyroid axis function in horses. Furthermore, we hypothesized that this effect was dependent on both dose and duration of treatment and was reversible following cessation of treatment. Although this proposal did not address potential differences between sulfonamide formulations, we investigated sulfadiazine because this drug is the only sulfonamide approved for use in the horse and is the most commonly used sulfonamide for long-term treatment of EPM.

## Materials and Methods

*Detailed Methodology*

Twelve, healthy, adult horses, including nine mares and three geldings, age 5-15 years, were housed in outdoor grass paddocks and fed timothy/alfalfa mix hay *ad libitum*. Following at least two weeks of being managed and fed consistently, subjects' baseline thyroid gland function was evaluated by use of the TRH stimulation test: measurement of concentrations of thyroid hormones and TSH before and 2 and 4 hours after administration of 1 mg of TRH[a] intravenously.[61] All blood sampling started at 9 A.M.

Uniprim®[b] powder was the product selected for this investigation in which the horses were separated into three groups, with three mares and one gelding in each group. Group 1 (n=4) received no treatment and served as a control group (to assess random effects of time), group 2 (n=4) received Uniprim® at the lower recommended dose (15 mg/kg, PO, q 24 h) for 8 weeks, and group 3 (n=4) received the higher recommended dose (30 mg/kg, PO, q 24 h) for 8 weeks. All treatments were administered between 1 P.M. and 3 P.M. and the study was performed from March through July of 2002.

Basal (resting) concentrations of TT4, FT4, FT4 by equilibrium dialysis (FT4d), TT3, FT3, and TSH were determined weekly by radioimmunoassay (RIA). A TRH stimulation test was repeated in all subjects after 4 and 8 weeks of treatment. To assess reversibility of these effects, the TRH stimulation test was again repeated 4 and 8 weeks after discontinuation of treatment. As with the baseline sampling, all sampling started at 9 A.M. Overall, thyroid function in response to TRH administration was assessed five times in each horse.

After each blood sample collection and adequate clot formation, samples were centrifuged at 3000 rpm at 4°C for 10 minutes. Serum was harvested and frozen at –20°C until hormone assays were performed. Concentrations of TT4, FT4, FT4d, TT3, FT3, and TSH were determined by radioimmunoassay (RIA).[57-59] Details regarding RIAs for TT4, FT4, FT4d, FT3, and TSH appear in Appendices B and C. The TSH radioimmunoassay was performed as previously described[53, 54, 60] with some modifications.

*Statistical Analysis*

Data were analyzed by a one-way or two-factor, repeated measures analysis of variance for main effects of treatment (dose level) and/or time using a commercially available software program.[c] When F-ratios were significant ($p<0.05$), a Student Newman-Keuls or Dunnett's post-hoc test was performed to detect specific differences. A sample size of 4 horses per group was chosen on the basis of being able to detect a 25% difference in the increase in thyroid hormone concentrations after administration of TRH.

**Results**

All 12 horses remained healthy during the study period and no adverse clinical effects of 8 weeks of treatment with TMP-SDZ at either dose were observed. Body condition scores ranged from 4 to 6.5 and, although BCS increased ($p<0.01$) from 5.1 ± 0.2 to 5.5 ± 0.2 (mean ± standard deviation) for all subjects over the 16-week study period,

differences between treatments were not observed. The only additional change observed was the anticipated shedding of hair coat from spring to summer.

No significant differences between treatment groups were observed in weekly basal serum concentrations of TT4, FT4, FT4d, TT3, and FT3. Similarly, differences between treatment groups were not observed in weekly basal serum concentrations of TSH except at week 16 when mean TSH concentration for group 1 was greater ($p<0.05$) than the mean values for group 2 and group 3. Within each treatment group, differences were observed over time (Table 1). In group 1 (controls), mean FT4 values at weeks 1, 2, 7, and 9 through 16 were lower ($p<0.05$) than baseline concentration (week 0) and mean FT3 concentration at week 1 was higher ($p<0.05$) than baseline. In group 2 (15 mg/kg), mean FT4 concentrations at week 1 and weeks 7 through 16 were lower ($p<0.05$) than baseline concentration, as were mean FT3 concentrations at weeks 8 and 16. In Group 3 (30 mg/kg), mean FT4 concentrations at weeks 1, 7, and 9 through 16 were lower ($p<0.05$) than baseline concentration and mean FT3 concentrations at weeks 4, 5, 7, and 8 were lower ($p<0.05$) than baseline concentration. Differences in weekly mean TSH concentrations were not observed except for a decrease ($p<0.05$) from baseline concentration at weeks 3, 7, and 10 in group 3 (30 mg/kg). Although an increasing trend in TSH appears to be present over the 16-week study period in group 1, this was not a statistically significant finding.

Concentrations of all thyroid hormones and TSH increased after TRH administration (Tables 2-6), yet responsiveness to TRH did not change from baseline values (week 0) in any group (Figures 4-8), except in group 3. After 8 weeks of treatment, mean TSH concentration in group 3 was significantly greater ($p<0.05$) 2 hours

after TRH administration compared to the baseline (week 0) 2-hour post-TRH sample concentration and the equivalent samples from groups 1 and 2 (Figures 4 and 6). This difference in group 3 was not found after 4 weeks of treatment and did not persist at 4 or 8 weeks after cessation of TMP-SDZ administration (Figures 5, 7, and 8).

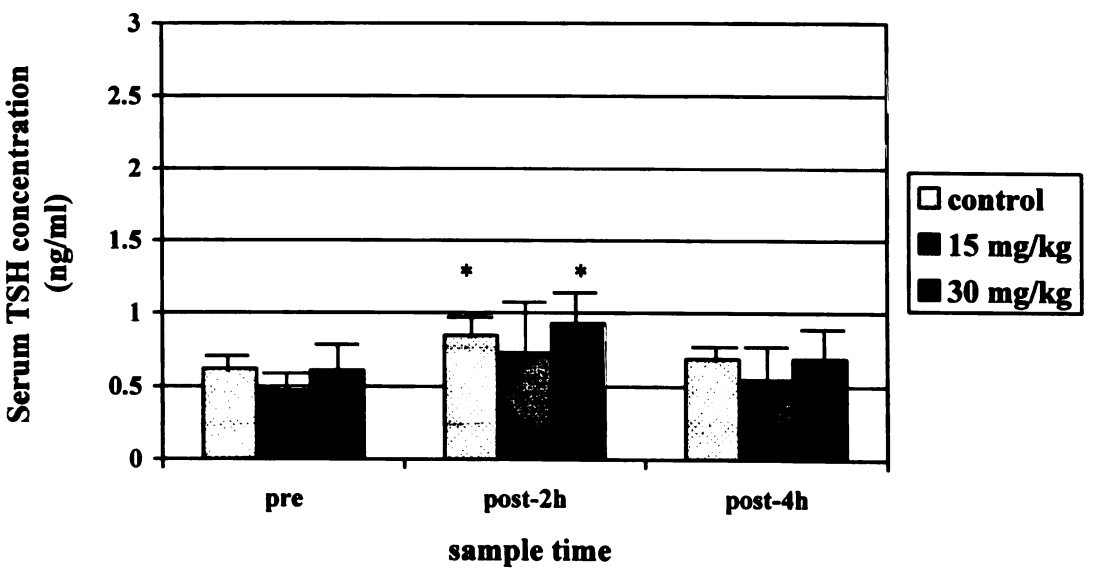



**Figure 4.** TRH Stimulation test results for serum TSH (mean + standard deviation) prior to treatment (week 0).
* = difference within group from pre-TRH value; $P < 0.05$.

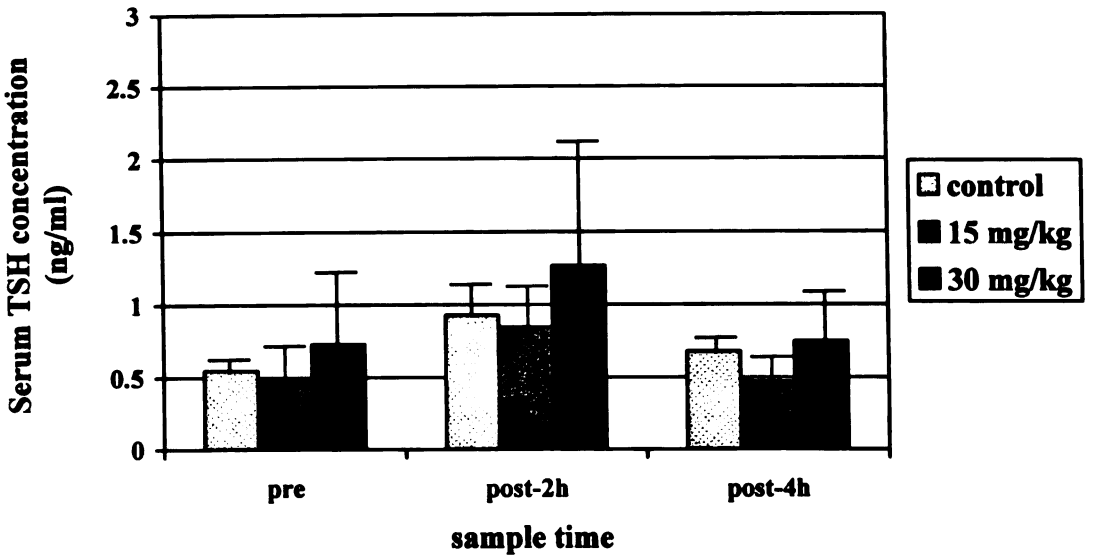



**Figure 5.** TRH stimulation test results for serum TSH (mean + standard deviation) after 4 weeks of treatment (week 4).
* = difference within group from pre-TRH value; # = difference between groups at same sampling time; $P < 0.05$.

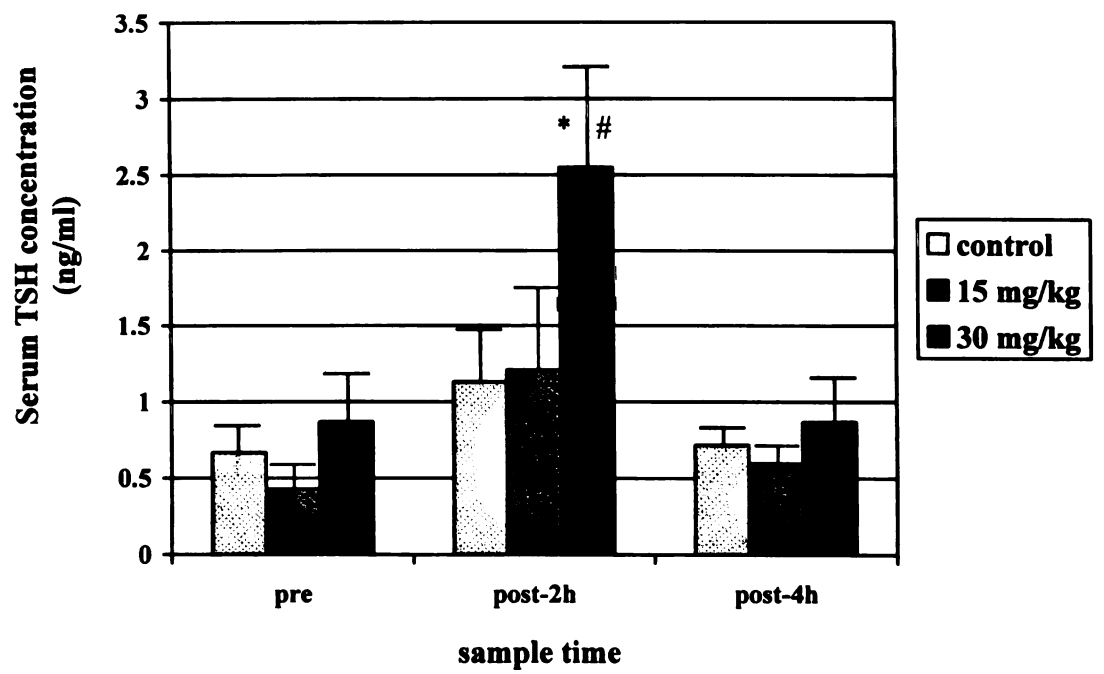



**Figure 6.** TRH Stimulation test results for serum TSH (mean + standard deviation) after 8 weeks of treatment (week 8).
* = difference within group from pre-TRH value; # = difference between groups at same sampling time; $P < 0.05$.

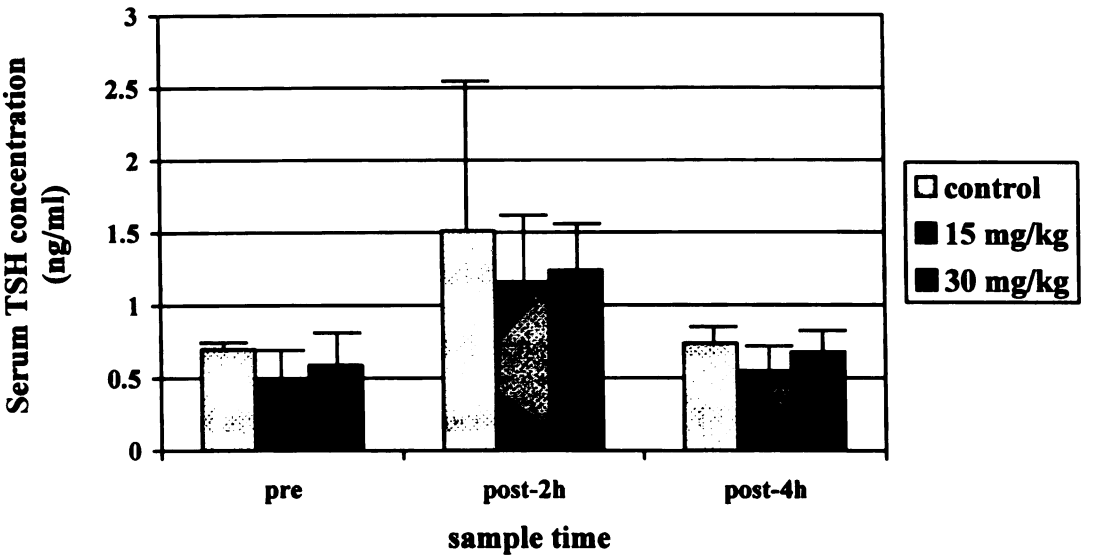



**Figure 7.** TRH stimulation test results for serum TSH (mean + standard deviation) 4 weeks after cessation of treatment (week 12).
* = difference within group from pre-TRH value; $P < 0.05$.

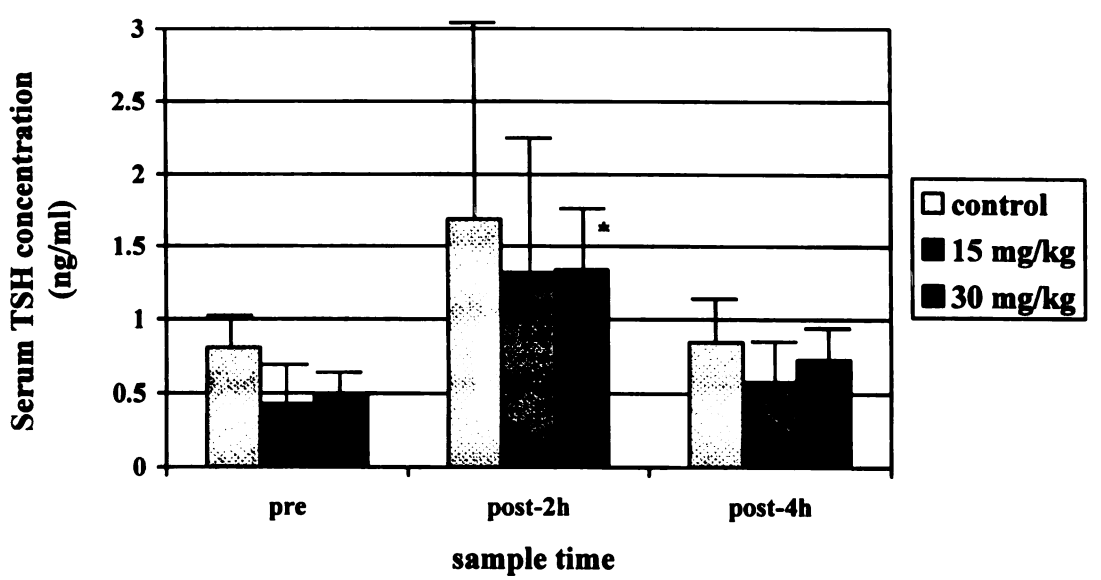



**Figure 8.** TRH Stimulation test results for serum TSH (mean + standard deviation) 8 weeks after cessation of treatment (week 16).
* = difference within group from pre-TRH value; $P < 0.05$.

**Discussion**

Long-term TMP-SDZ therapy at 15 mg/kg, q 24 h and 30 mg/kg, q 24 h did not produce clinical signs of hypothyroidism or appear to have substantial adverse effects on thyroid function in healthy horses. These findings in horses were not completely unexpected in light of prior research and clinical observations in other species. In the early stages of decreased thyroid gland function, an elevated TSH concentration may be the only indicator of altered thyroid function. Over time, TT4 and TT3 concentrations may subsequently decrease, while a normal FT3 concentration, the most physiologically active form of thyroid hormone, is typically maintained. This progression has been attributed to a greater shift of T4 and T3 from protein-bound to their unbound (free) forms. In addition, greater conversion of T4 to T3 by 5'-deiodinase occurs at the tissue level. As disease progresses, FT4 and FT3 would be the last forms of thyroid hormones that would be expected to have their concentrations fall below the lower limit of the reference range.[19]

The decrease in FT4 concentrations in weeks 7 through 16, in the face of minimal change in concentrations of other hormones, does not fit the typical profile of a drug-induced hypothyroid state. Next, the time relationship of these decreased FT4 concentrations contrasts with patterns observed in rats and dogs. Specifically, in those studies, laboratory findings supportive of hypothyroidism were present by the end of the treatment regimen, and hormone concentrations returned to normal ranges within 8 weeks.[12, 14, 22] More importantly, this decrease was found in all three groups further eliminating treatment with TMP-SDZ as a causative factor.

An alternative explanation for the decrease in FT4 during the latter half of the study period could include change in season and daily turnover of thyroid hormones. Similarly, although not suspected in our study, nonthyroidal illness and sample handling errors can significantly alter measured FT4 concentrations.[19, 57] Before this decrease in FT4 may be interpreted erroneously, it should also be emphasized that the baseline values (week 0) were the highest values measured and that all FT4 values measured remained within the laboratory reference range (6-24 pmol/L) throughout the study period. Finally, a similar decrease in FT4d was not observed. Because determination of FT4d is now considered a superior method for assessment of functional, non-protein bound thyroxine, there may be little to no functional significance of the apparent decline in FT4. Although beyond the work of this study, further investigation of differences in FT4 and FT4d in horses undergoing various manipulations of the hypothalamic-pituitary-thyroid axis will be necessary to elucidate the significance of these differences in concentrations between FT4 and FT4d.

Next, the finding of decreased FT3 concentrations in group 2 (15 mg/kg) and group 3 (30 mg/kg) horses during the latter half of the treatment phase of the project (week 8 in group 2 and weeks 4, 5, 7, and 8 in group 3), in light of normal TT4 and TT3 concentrations, was unexpected. While these decreases would be consistent with a sulfonamide-induced effect that is dependent on both dose and duration, drug-induced hypothyroidism was not further supported by an increase in TSH concentration at the same sampling times. Although the rapid return of FT3 to a baseline-equivalent value by week 9 could also suggest elimination of a drug effect, it is again important to emphasize that mean FT3 concentrations remained within the reference range (1.7-5.2 pmol/L) at all

times. Thus, other factors that may transiently affect thyroid function (e.g., changes in ambient temperature from week to week) could also have played a role in the changes in FT3 concentrations that were found.

As stated, the significantly lower TSH concentrations found in group 3 (30 mg/kg) at weeks 3, 7, and 10 were opposite to our hypothesis, i.e. that TSH should increase with treatment dose and duration. Once again, random variation between horses and other factors that may have influenced thyroid function over the course of the 16-week study period may likely explain these observations. While increases in TSH concentration, supportive of hypothyroidism, were not induced by treatment with TMP-SDZ, an exaggerated TSH response to TRH stimulation was observed in group 3 (30 mg/kg) after 8 weeks of drug administration. This finding suggests that an increase in pituitary gland sensitivity to TRH may develop in horses receiving long-term, higher doses of TMP-SDZ.

Studies in human and animal subjects have clearly demonstrated that hypothyroid effects of potentiated sulfonamides are both dose and duration dependent and are observed more commonly after prolonged use of high dosages.[14, 16, 22] Thus, it is possible that the dosages and treatment duration selected may have been insufficient to induce obvious adverse effects of sulfonamides. For this study, we selected two drug dosages (15 and 30 mg/kg) based on both recommended therapeutic dosages and clinical experience. Although once daily dosing may not be the most widely used treatment regimen (the drug combinations are frequently administered twice daily in practice), it was selected on the basis of the manufacturer's recommended dose and on pharmacokinetic data for Uniprim®. Next, a treatment course of 8 weeks was selected

because studies in other species report suppression of thyroid function within 10 days to 8 weeks.[12-14, 16, 21-23] Further, the treatment course for most bacterial infections in horses would likely be 8 weeks or less. However, these drug combinations are sometimes used clinically at even higher dosages (60 mg/kg, q 24 h, or 30 mg/kg, q 12 h) and for longer durations (6-12 months) for treatment and prophylaxis of EPM. Thus, the findings of this study do not exclude the possibility that potentiated sulfonamide therapy could have detrimental effects on the function of the hypothalamic-pituitary-thyroid axis when horses are treated with higher dosages for longer periods.

Evaluating potential adverse effects of 8 weeks of treatment with common doses of TMP-SDZ on the hypothalamic-pituitary-thyroid axis was informative, even if no biologically significant changes were observed or substantiated. Thus, the project provided useful information with respect to a typical treatment regime. Most importantly, this research supports that treatment with TMP-SDZ at these dosages for 8 weeks should be safe, with regard to thyroid function, in the majority of horses. Nonetheless, future work may need to investigate effects of longer treatment courses at higher dosages to conclusively determine the safety of these drug combinations in horses. Another concern that was not addressed in this study is the potential that sick horses may be at greater risk than healthy horses for adverse effects of potentiated sulfonamides on the hypothalamic-pituitary-thyroid axis. The euthyroid sick syndrome is well recognized in people and some other species of veterinary interest and could clearly change patient susceptibility to potential adverse drug reactions.

Concurrent treatment with other drugs can alter the results of thyroid gland function tests. Although thyroid gland function may be completely normal, some

medications can affect measured concentrations of thyroid hormones drastically. For example, protein-bound drugs, like phenylbutazone, cause marked decreases in bound thyroid hormone concentrations, and thus lower serum concentrations of total T4 and T3. This is due to displacement of thyroid hormones from plasma proteins, such as albumin, TBPA, and TBG.[66, 67] In our study, this was not a factor, as none of the subjects were being treated with phenylbutazone or other non-steroidal anti-inflammatory medications. Subjects were infrequently sedated with alpha-2 agonist medication (xylazine at 0.4-0.6 mg/kg IV) or acepromazine (0.04 mg/kg IV) for use in various teaching procedures. Xylazine has no reported negative effects on thyroid hormone binding.[68] Acepromazine, while largely protein-bound in plasma, has no reported effects on thyroid hormone binding.[69]

One important issue when administering oral potentiated sulfonamide drugs is adequate gastrointestinal absorption. It has been suggested that these drugs are best absorbed when the stomach is empty,[4] so as to limit drug binding to ingested proteins and competition for mucosal absorption. However, in one study comparing absorption and other pharmacokinetic data for oral TMP-SDZ paste (Tribrissen®, Kenilworth, NJ) between fed and unfed horses, fasting had no effect on either drug's serum concentrations.[70] In contrast, in another study evaluating the effect of feeding on absorption of phenylbutazone, TMP, and SDZ, a significant decrease in peak plasma concentrations of TMP, but not SDZ, was found in the fed state.[71]

In this project, horses were maintained on grass pasture continuously. However, pasture was limited such that supplemental hay was provided daily in the mornings between 8-10 A.M. To avoid administering the TMP-SDZ combination to recently fed

subjects, we chose a daily treatment time between 1 and 3 P.M. Unfortunately, this management program did not allow complete restriction of food prior to drug therapy. Although the possible effect is not known, the drug was purposely administered in only a small quantity of 12% sweet feed to limit any negative effects on drug absorption. It would have been ideal to document drug absorption by measuring serum concentrations of TMP and SDZ at weekly or monthly intervals but such analyses were beyond the scope of the budget for the project. Again, it warrants emphasis that the dosage protocol selected followed recommendations by the drug manufacturer.

Gender differences in hypothyroid effects of sulfonamides have been reported in experimental studies in rats.[14, 72] Cohen *et al.*[14] reported a greater decrease in TT4 in female rats, compared to male rats, at pharmacologic doses of TMP-SMZ, TMP-sulfamoxole, and sulfamoxole alone. The investigators did not propose any explanations for a gender difference. Fullerton *et al.*[72] evaluated sulfamethazine treatment alone in rats for 12, 18, and 24 months. Results supported the development of primary hypothyroidism at high doses (significant decrease in TT4 and increase in gland weights). Of interest, female rats had a more marked and earlier decrease in TT4 concentrations than male rats. Similar observations of gender differences have not been experimentally documented or anecdotally noted in other species. Clearly, this study was not designed to assess gender differences in horses.

Environmental factors can also exert a substantial influence on both function of the hypothalamic-pituitary-thyroid axis and tissue utilization of thyroid hormones. This project began in early spring and was completed in the summer months. With increased ambient temperature and humidity, one would expect thyroid hormone concentrations to

decrease, as has been observed in other mammalian species including cattle, sheep, and people.[58, 73] This physiologic response could falsely lead to diagnoses of hypothyroidism, either clinical or subclinical. However, our results did not show an overall downward trend in thyroid hormone concentrations. Thus, it appears as though a seasonal effect of lower thyroid gland output either did not occur or random variation in the thyroid hormone concentrations obscured such a trend.

Multiple tests have been described to assess thyroid gland function. As in human medicine, the current "gold standard" screening test for clinical hypothyroidism in small animal veterinary practice is documentation of increased baseline TSH concentration. In addition, some clinicians recommend measurement of baseline total and free T4 and T3 concentrations or the same thyroid hormones following TRH administration. While not required to make a diagnosis of hypothyroidism, TSH response to TRH stimulation can also be useful in evaluating the ability of the gland to respond to this stimulus.[18, 19] Prior work has shown that TSH concentrations in horses peak within 45-90 minutes following TRH administration.[53] While this study did not collect samples during before 2 hours after TRH administration, a significant rise in TSH was observed at 2 and 4 hours post-TRH administration in all treatment groups, supporting the conclusion that both the pituitary and thyroid glands remained responsive to TRH and TSH, respectively, in the horses in this study. Further, the apparently exaggerated response of TSH 2 hours after TRH administration after 8 weeks of treatment in group 3 horses (30 mg/kg) was both an unexpected and interesting finding. The exaggerated TSH response suggests increased responsiveness of the pituitary gland to TRH and it could be argued that this finding is supportive of early or mild, subclinical hypothyroidism. Specifically, the pituitary gland

could become more sensitive to TRH if it were primed to increase TSH output, as in primary hypothyroidism. This would be consistent with studies in rats that described pituitary thyrotroph hypertrophy and increased thyroid gland weights after treatment with sulfonamides.[12, 14]

Lastly, considerable species variation has been demonstrated in the severity of ADRs to sulfonamide drugs. Thus, it is possible that equine hypothalamic-pituitary-thyroid axis and thyroid gland function is not affected by sulfonamide drugs or their metabolites, as has been reported in chickens, monkeys, and guinea pigs.[55] Specifically, horses may not exhibit the "slow acetylator" phenotype described in people and dogs.[11, 17] As a result, drug metabolites may be less likely to accumulate and lead to alterations in thyroid gland follicular cell function. However, a final point worthy of consideration is that it may be more important to focus on the individual patient, rather than the species population as a whole, when investigating potential ADRs to potentiated sulfonamides. Both the presence of disease and individual risk factors for idiosyncratic ADRs could make individual horses susceptible to drug-induced hypothyroidism. Thus, the clinician should remain astute to clinical signs and investigation of suspected drug-induced dysfunction of the hypothalamic-pituitary-thyroid axis should be pursued in patients that may develop clinical signs consistent with hypothyroidism while being treated with potentiated sulfonamides.

# CONCLUSION

Extended therapy with sulfonamide drugs has long been associated with a variety of adverse drug reactions. Hypothyroidism associated with sulfonamide drug therapy has been well documented in humans, dogs, and rodents, and is suspected to occur in swine.[1, 12-16, 21-23] Evaluating potential adverse effects of potentiated sulfonamides on equine thyroid gland function was the focus of this research study. This is an important issue in veterinary medicine because of the widespread use of trimethoprim/sulfonamide combinations in equine practice, as well as the widely held misconception regarding the occurrence of equine hypothyroidism.

Today, equine hypothyroidism is routinely diagnosed based on non-specific clinical signs and low, basal serum thyroid hormone concentrations. It is likely that this approach results in many false positive diagnoses and perpetuates the belief in the veterinary community that equine hypothyroidism is a common disorder.

Hypothyroidism is a poorly documented disease entity in the equine species. Congenital cases in neonates have been associated with suspected increased dietary nitrate content or decreased iodine content during gestation.[26, 36-38] Practitioners have suggested that primary hypothyroid states occur in many horses, ponies, and miniature horses with a variety of vague clinical signs, such as obesity/abnormal fat deposition, dull hair coat, and poor fertility. While endocrine disease may exist in some patients, another underlying disease process is more likely than hypothyroidism in a majority of these patients.

The findings of this study suggest that typical potentiated sulfonamide treatment regimens are not associated with decreased function of the thyroid gland. While the results can help practitioners in their antimicrobial treatment choices, we still must consider the risks of adverse reaction occurrence with higher dosages and longer durations of treatment, as reported in other animal species.

## APPENDIX A

**Footnotes**

a TRH, P2162, chemical grade, Sigma-Aldrich Inc., St. Louis, MO, USA.

b Uniprim®, Macleod Pharmaceuticals, Inc., Fort Collins, CO, USA.

c SigmaStat®, Jandel Scientific, St. Paul, MN, USA.

d Clinical Assays Gammacoat™ M Total T4 $^{125}$I RIA Kit, DiaSorin Inc., Stillwater, MN, USA.

e Clinical Assays Gammacoat™ M Free T4 $^{125}$I RIA Kit, DiaSorin Inc., Stillwater, MN, USA.

f Free T4 by equilibrium dialysis, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA.

g Clinical Assays Gammacoat™ M Free T3 $^{125}$I RIA Kit, DiaSorin Inc., Stillwater, MN, USA.

h Dr. A.F. Parlow, National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases, Harbor-UCLA Medical Center, Torrance, CA, USA.

i Normal Rabbit Serum, S20-100ML, Chemicon International, Inc., Temecula, CA, USA.

j Rabbit IgG Immunoprecipitation Reagent, R8633, Sigma-Aldrich, Inc., St. Louis, MO, USA.

# APPENDIX B

## Thyroid Hormone Assays – Details and Modifications

*Total Thyroxine (TT4)*

Total T4 was measured in duplicate with a commercially available solid-phase radioimmunoassay (RIA) kit.[d] Antibody-coated polypropylene tubes (12x75 mm), $^{125}$I-T4, buffer solutions, and standards were included in the kit. Specificity data from the manufacturer revealed 92% cross-reactivity with D-thyroxine, 2.1% cross-reactivity with D- and L-triiodothyronine, and less than 0.1% cross-reactivity with other iodothyronines. Modifications were made to the assay protocol to enhance the analytical sensitivity of the assay. Specifically, the volume of sample or standard added was increased from 10 to 25μl. Next, the standard curve was shifted to a lower range by mixing equal volumes of 0 and 13 nmol/L standards and by discarding the highest standard (257 nmol/L). Thus, the standard curve ranged from 6.5-156 nmol/L. The sensitivity limit of the assay, defined as the concentration of TT4 at 90% specific binding, was 3 nmol/L (data from 10 assays). When L-thyroxine was added to aliquots of pooled equine serum to create increases of 26, 52, and 78 nmol/L, 106, 104, and 95% of added TT4 was measured in the assay. A pool of equine serum with a high concentration of TT4, 67 nmol/L, was diluted 50% and 25% in zero standard. Assay of these diluted samples yielded recovery rates of 96% and 113%, respectively, after correction for dilution. Repeatability was determined in three pools of equine serum chosen to have low (8 nmol/L), middle range (22 nmol/L), and high (67 nmol/L) TT4 concentrations. The intraassay coefficients of variation (CV) for 10 replicates of each pool were 0.072, 0.042, and 0.030, respectively. In 10 assays,

the interassay CV for equine serum pools with TT4 concentrations of 6, 18, and 35 nmol/L were 0.237, 0.069, and 0.073, respectively.

*Free Thyroxine (FT4)*

Free T4 was measured using a commercially available solid-phase RIA kit.[c] Assays were performed in the Endocrinology Laboratory at the Diagnostic Center for Population and Animal Health at Michigan State University. This assay has not yet been validated for use in horses.

*Free Thyroxine by Equilibrium Dialysis (FT4d)*

A commercially available RIA kit[f] was modified for FT4d determination, in duplicate. The procedures for equilibrium dialysis and RIA of FT4 in dialysate were done as per the manufacturer's protocol. The manufacturer reported less than 0.044% cross-reactivity with other iodothyronines. The sensitivity of the assay, defined as the concentration of FT4 at 90% specific binding, was 1.8 pmol/L (mean of 10 assays). Estimates of dilutional parallelism and recovery were made using dialysates of equine serum. When samples of a serum pool of dialysate with a FT4concentration of 25 pmol/L were diluted with dialysate buffer to 50%, 25%, and 12.5% of the original concentration, 88, 96 and 96% of expected amounts of FT4 were recovered in the assay, respectively. When aliquots of T4 equivalent to 4, 11, 31 and 68 pmol/L were added to the same pool of equine dialysate, 104, 137, 109, and 105% of the respective added T4 was measured in the assay. Repeatability was determined with equine serum pools with concentrations of FT4 of 13 and 24 pmol/L. For 10 replicates of each pool, the respective

intraassay CVs were 0.075 and 0.078. In 10 different assays, the respective interassay CVs were 0.166 and 0.074.

*Total Triiodothyronine (TT3)*

Total T3 was measured in duplicate by charcoal separation RIA. The methods and validation for use of this assay in the horse have been described previously.[23]

*Free Triiodothyronine (FT3)*

Free T3 was measured in duplicate using a commercially available solid-phase RIA based on competition of endogenous FT3 with a $^{125}$I-T3 derivative.[g] The kit protocol described 100% antibody cross-reactivity with L-T3 and less than 0.2% cross-reactivity with other iodothyronines. The assay procedure was modified by prolonging the duration of incubation from 90 min to 3 h in a 37°C water bath. This change was done to assure equilibration of maximal binding for assay runs that consisted of a standard curve and 53 samples. The sensitivity of the assay, defined as the concentration of FT3 at 90% specific binding, was 1.2 pmol/L (based on data from 10 assays). In analog-based RIA for FT3, there are multiple binding interactions between the endogenous hormone, the T3-derivative, assay antibody, and endogenous binding proteins. Under these conditions, assessment of dilutional parallelism and recovery is not possible. For equine serum pools with concentrations of 1.7 and 5.6 pmol/L (10 replicates), the intraassay CVs were 0.099 and 0.034, respectively. The interassay CVs for equine serum pools with FT3concentrations of 1.4, 4.3 and 7.7 pmol/L were 0.140, 0.095, and 0.082, respectively (10 assays).

# APPENDIX C

## Equine Thyrotropin Assay – Details and Modifications

Assay reagents were supplied by Dr. A. F. Parlow[h] and the methodology was based on published validation.[53, 54, 60] These publications describe using a two-antibody RIA. Lyophilized, highly purified equine TSH[h] (eTSH, AFP-5144B) was solubilized in phosphate-buffered saline (PBS) to 121µg/ml. Following iodination of purified eTSH by the chloramine-T method, eight serial dilutions of the non-iodinated eTSH were prepared, as well as duplicate buffer-only (zero standard), non-specific binding, and serum sample (from two euthyroid horses) tubes. All tubes contained 200µl aliquots of standard, solution, or sample. The TSH antiserum[h] (anti-ovine developed in rabbit, AFP-C33815), shipped frozen and then thawed to room temperature, was diluted 1:20,000 and 1:35,000 in 0.01M PBS containing 0.05M EDTA and 2.5% normal rabbit serum.[i] These two dilutions of the primary antibody were added to the aforementioned tubes in duplicate. Tubes were then vortexed and incubated for 48 h at 4°C. Then, 200µl of the initial radiolabelled TSH (48,703 counts/200µl) was added and the tubes were again mixed and incubated for 48 h at 4°C.

The secondary, precipitating antibody[j] was added undiluted to each tube at two volumes, 150µl and 200µl, and the tubes were vortexed and incubated for 48 h. Unfortunately, specific binding was poor for all of the four possible combinations of the different dilutions of primary and secondary antibodies. An attempt to improve binding was made by using less dilute primary antibody solutions (1:1,000, 1:5,000, and 1:20,000) and larger volumes of secondary antibody (200µl and 400µl). The highest

specific binding (14.7%) was obtained with a 1:1,000 dilution of the first antibody and 400 µl of the second antibody. At that point, a second column separation was performed (see *Equine Thyrotropin Iodination* section).

Following this second column separation, fractions associated with the two highest count peaks were diluted in PBS/bovine serum albumin (BSA) solution to achieve radioactivity of approximately 30,000 counts/200µl. The assay protocol was followed with duplicate tubes for total counts, non-specific binding, and primary antibody dilution of 1:10,000. In addition, reagent combinations with 500µl and 1000µl of secondary antibody were prepared. To assess the effectiveness of the precipitating antibody, gamma scintillation counting was done after centrifugation without washing with PBS, as well as after the described double-centrifugation with PBS wash. The highest specific binding of TSH was achieved with fraction 4 using 1000µl of second antibody. After a single centrifugation, specific binding was 25.2%; after PBS washing and a second centrifugation, binding decreased minimally to 23.6%.

*Standard Curve and Assay*

To establish a TSH standard curve, eight serial dilutions of TSH (ranging from 0.156 ng/ml to 20 ng/ml), along with non-specific binding tubes and a zero standard, were assayed in duplicate. Tracer radioactivity was measured at 30,686 counts/200µl prior to use, using 5µl of iodinated TSH/ml of 1% BSA in PBS. The best specific binding was achieved using 200µl of primary antibody (1:22,500 dilution), 200µl of standard or sample, and 1000µl of secondary antibody. All samples were assayed in a single batch. Repeatability was determined with equine serum pools with concentrations

of TSH of 0.516 and 0.632 ng/ml. For 5 replicates of each pool, the respective intraassay CVs were 0.315 and 0.119.

*Equine Thyrotropin Iodination*

The highly purified equine TSH was radioiodinated by the chloramine-T method. To prepare TSH aliquots, the frozen, lyophylized protein was solubilized in 0.5M PBS, pH 7.5, at 100μg/ml. This volume was then divided into 5μg and 2μg aliquots and frozen. For use in the column, an elution buffer of 0.03M PBS, 0.02% sodium azide, 0.15M sodium chloride (pH 7.4), and 0.2% bovine serum albumin (BSA) was also prepared. The BSA-elution buffer (30ml) was run through the G-25 column approximately 24 hours prior to iodination. A transfer solution for use in transferring the iodinated TSH to the G-25 column was prepared as well. It consisted of 1g potassium iodide and 16g sucrose diluted to 100ml with double-distilled water, frozen in 1ml aliquots.

Just prior to iodination, the chloramine-T solution was made at a concentration of 1mg chloramine-T/ml of 0.03M PBS. At that time, a metabisulfite solution was made at a concentration of 3mg metabisulfite/ml of 0.03M PBS. For iodination, a 5μg TSH aliquot was thawed and transferred to a vial of 1mCi of iodine-125 ($^{125}I$). This combination was then transferred back to the original TSH vial. Twenty-five (25) μl of the chloramine-T solution were then added to this vial and incubated for 45 seconds (5μg chloramine-T/μg TSH). Then, 25 μl of the metabisulfite solution were added and incubated for an additional 45 seconds (15μg metabisulfite/μg TSH). One hundred (100) μl of transfer solution was then added and the final solution was pipetted on top of a PD-

10 column containing 10ml of G-25 (Amersham Inc.). The radioiodinated TSH was separated from unincorporated $^{125}I$ by running the solution through the gel column with the elution buffer and collecting 15, 1ml fractions. These fractions were vortexed and 20µl aliquots of each fraction were counted by a gamma-counter. Gamma scintigraphy showed two significant raw count peaks corresponding to fractions 4 and 9. Fraction 4 was used to prepare the radioiodinated tracer.

Very poor specific binding of the labeled TSH was achieved in two attempts at setting up a TSH standard curve. At that point, a second protein separation of the previously iodinated TSH was performed (approximately 60 days after the initial iodination). In this procedure, a G-50 column was made by putting ~10ml of G-50 (Amersham Inc.) in a 10ml glass pipet. A 1% BSA in PBS solution was run through the column. The iodinated TSH solution was pipetted onto the G-50 column and 16 fractions of 1ml each were collected. These tubes were vortexed, and then 10µl aliquots were counted on the gamma counter. Again, two distinct peaks were observed and the fraction associated with the first peak (fraction 4) was used to prepare the radiolabeled tracer.

## APPENDIX D

**Table 1.** Weekly serum concentrations (mean ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and TSH for horses administered a trimethoprim/sulfadiazine combination at 0, 15 mg/kg, or 30 mg/kg, PO, q 24 h for 8 weeks. Week 0 = baseline values; weeks 1-8 = treatment; weeks 9-16 = recovery.

| Hormone | Group | Week 0 | Week 1 | Week 2 |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 26.0 ± 4.8 | 33.0 ± 7.0 | 24.8 ± 2.8 |
| | 15 mg/kg | 21.5 ± 4.4 | 25.3 ± 7.1 | 17.8 ± 1.0 |
| | 30 mg/kg | 30.3 ± 10.3 | 35.0 ± 9.3 | 24.3 ± 6.2 |
| FT4 (pmol/l) | control | 21.5 ± 1.3 | 16.5 ± 1.0* | 17.0 ± 3.6* |
| | 15 mg/kg | 21.0 ± 2.9 | 14.8 ± 2.6* | 19.3 ± 0.5 |
| | 30 mg/kg | 20.5 ± 2.9 | 15.8 ± 2.2* | 19.3 ± 2.1 |
| FT4d (pmol/l) | control | 20.5 ± 3.7 | 23.8 ± 3.8 | 21.8 ± 1.0 |
| | 15 mg/kg | 21.5 ± 3.9 | 21.5 ± 4.5 | 20.5 ± 3.1 |
| | 30 mg/kg | 21.3 ± 3.6 | 20.5 ± 2.5 | 21.8 ± 3.3 |
| TT3 (nmol/l) | control | 0.9 ± 0.2 | 1.0 ± 0.2 | 1.0 ± 0.4 |
| | 15 mg/kg | 1.0 ± 0.4 | 0.9 ± 0.1 | 1.0 ± 0.3 |
| | 30 mg/kg | 0.8 ± 0.3 | 1.0 ± 0.2 | 0.9 ± 0.1 |
| FT3 (pmol/l) | control | 2.8 ± 0.6 | 4.5 ± 1.0* | 2.5 ± 0.5 |
| | 15 mg/kg | 3.3 ± 0.5 | 4.3 ± 1.0 | 2.9 ± 0.6 |
| | 30 mg/kg | 3.3 ± 0.6 | 4.4 ± 0.2 | 2.2 ± 0.2 |
| TSH (ng/ml) | control | 0.62 ± 0.17 | 0.59 ± 0.16 | 0.59 ± 0.11 |
| | 15 mg/kg | 0.47 ± 0.18 | 0.33 ± 0.17 | 0.43 ± 0.26 |
| | 30 mg/kg | 0.61 ± 0.2 | 0.50 ± 0.21 | 0.49 ± 0.21 |

* = significant difference within the same group from baseline value (Week 0), $p<0.05$.
# = significant difference between control and both treated groups during same week, $p<0.05$.

**Table 1 (cont'd).**

| Hormone | Group | Week 3 | Week 4 | Week 5 |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 25.8 ± 6.2 | 21.8 ± 5.0 | 27.0 ± 11.3 |
| | 15 mg/kg | 20.8 ± 5.7 | 19.3 ± 5.4 | 21.5 ± 6.2 |
| | 30 mg/kg | 28.8 ± 7.8 | 25.0 ± 10.2 | 24.0 ± 9.0 |
| FT4 (pmol/l) | control | 19.3 ± 1.0 | 17.8 ± 1.3 | 20.5 ± 3.7 |
| | 15 mg/kg | 19.5 ± 3.1 | 20.8 ± 1.5 | 21.5 ± 3.9 |
| | 30 mg/kg | 19.0 ± 2.2 | 17.8 ± 3.6 | 19.3 ± 3.3 |
| FT4d (pmol/l) | control | 21.8 ± 2.8 | 19.0 ± 2.4 | 29.3 ± 9.2 |
| | 15 mg/kg | 23.5 ± 4.0 | 20.5 ± 1.0 | 27.8 ± 9.1 |
| | 30 mg/kg | 22.8 ± 4.6 | 17.8 ± 5.1 | 23.0 ± 5.5 |
| TT3 (nmol/l) | control | 1.1 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.2 |
| | 15 mg/kg | 1.0 ± 0.4 | 1.3 ± 0.3 | 1.1 ± 0.3 |
| | 30 mg/kg | 0.9 ± 0.2 | 1.1 ± 0.3 | 0.9 ± 0.3 |
| FT3 (pmol/l) | control | 2.3 ± 0.6 | 1.7 ± 0.4 | 2.4 ± 0.5 |
| | 15 mg/kg | 2.0 ± 0.6 | 2.3 ± 0.7 | 2.6 ± 0.6 |
| | 30 mg/kg | 2.0 ± 0.2 | 1.8 ± 0.3* | 1.7 ± 0.7* |
| TSH (ng/ml) | control | 0.54 ± 0.11 | 0.55 ± 0.11 | 0.64 ± 0.10 |
| | 15 mg/kg | 0.45 ± 0.22 | 0.49 ± 0.31 | 0.46 ± 0.24 |
| | 30 mg/kg | 0.42 ± 0.13* | 0.73 ± 0.46 | 0.48 ± 0.16 |

* = significant difference within the same group from baseline value (Week 0), $p<0.05$.
# = significant difference between control and both treated groups during same week, $p<0.05$.

**Table 1 (cont'd).**

| Hormone | Group | Week 6 | Week 7 | Week 8 |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 20.5 ± 7.3 | 18.5 ± 8.4 | 22.8 ± 11.6 |
| | 15 mg/kg | 18.3 ± 5.5 | 15.0 ± 6.2 | 16.8 ± 7.4 |
| | 30 mg/kg | 21.5 ± 5.7 | 20.5 ± 7.6 | 21.0 ± 11.9 |
| FT4 (pmol/l) | control | 18.3 ± 2.9 | 14.5 ± 1.9* | 17.8 ± 5.3 |
| | 15 mg/kg | 19.8 ± 4.0 | 13.0 ± 1.8* | 15.0 ± 3.6* |
| | 30 mg/kg | 17.3 ± 1.0 | 14.3 ± 5.7* | 17.8 ± 6.0 |
| FT4d (pmol/l) | control | 22.0 ± 4.4 | 21.0 ± 7.0 | 16.8 ± 4.6 |
| | 15 mg/kg | 22.3 ± 7.9 | 23.8 ± 7.8 | 17.0 ± 5.4 |
| | 30 mg/kg | 18.0 ± 2.4 | 22.5 ± 7.9 | 13.5 ± 7.9 |
| TT3 (nmol/l) | control | 1.2 ± 0.5 | 1.0 ± 0.2 | 1.1 ± 0.3 |
| | 15 mg/kg | 1.4 ± 0.2 | 0.9 ± 0.2 | 0.9 ± 0.4 |
| | 30 mg/kg | 1.2 ± 0.2 | 0.9 ± 0.1 | 1.0 ± 0.4 |
| FT3 (pmol/l) | control | 2.5 ± 1.0 | 2.3 ± 0.6 | 2.3 ± 0.7 |
| | 15 mg/kg | 2.8 ± 0.5 | 2.1 ± 0.5 | 1.7 ± 0.5* |
| | 30 mg/kg | 2.1 ± 0.4 | 1.9 ± 0.2* | 1.9 ± 0.4* |
| TSH (ng/ml) | control | 0.65 ± 0.05 | 0.66 ± 0.12 | 0.67 ± 0.21 |
| | 15 mg/kg | 0.4 ± 0.17 | 0.44 ± 0.22 | 0.43 ± 0.19 |
| | 30 mg/kg | 0.5 ± 0.16 | 0.41 ± 0.18* | 0.87 ± 0.38 |

* = significant difference within the same group from baseline value (Week 0), $p<0.05$.
# = significant difference between control and both treated groups during same week, $p<0.05$.

**Table 1 (cont'd).**

| Hormone | Group | Week 9 | Week 10 | Week 11 | Week 12 |
|---|---|---|---|---|---|
| TT4 (nmol/l) | control | 22.8 ± 9.9 | 17.3 ± 4.6 | 19.5 ± 9.8 | 20.3 ± 8.3 |
| | 15 mg/kg | 22.8 ± 6.6 | 15.0 ± 5.0 | 18.8 ± 5.6 | 18.0 ± 5.7 |
| | 30 mg/kg | 26.3 ± 10.7 | 21.8 ± 10.1 | 25.5 ± 11.0 | 25.3 ± 12.0 |
| FT4 (pmol/l) | control | 14.0 ± 3.6* | 10.0 ± 2.9* | 11.5 ± 2.4* | 12.5 ± 1.3* |
| | 15 mg/kg | 14.0 ± 1.4* | 11.5 ± 1.3* | 12.8 ± 1.9* | 12.3 ± 1.5* |
| | 30 mg/kg | 13.5 ± 3.1* | 13.0 ± 3.8* | 13.3 ± 3.7* | 13.8 ± 4.6* |
| FT4d (pmol/l) | control | 26.5 ± 8.2 | 19.3 ± 8.7 | 24.5 ± 6.9 | 15.5 ± 5.7 |
| | 15 mg/kg | 30.8 ± 6.7 | 22.3 ± 2.8 | 25.3 ± 7.7 | 14.5 ± 4.7 |
| | 30 mg/kg | 28.3 ± 11.0 | 24.5 ± 10.7 | 26.3 ± 12.8 | 17.3 ± 12.4 |
| TT3 (nmol/l) | control | 1.0 ± 0.2 | 0.9 ± 0.1 | 1.0 ± 0.2 | 1.4 ± 0.4 |
| | 15 mg/kg | 1.0 ± 0.4 | 0.8 ± 0.3 | 0.9 ± 0.3 | 0.9 ± 0.6 |
| | 30 mg/kg | 1.1 ± 0.2 | 0.8 ± 0.2 | 1.3 ± 0.6 | 1.1 ± 0.2 |
| FT3 (pmol/l) | control | 2.7 ± 0.5 | 2.6 ± 0.3 | 2.8 ± 0.6 | 2.9 ± 0.7 |
| | 15 mg/kg | 2.8 ± 0.4 | 2.5 ± 0.7 | 2.5 ± 0.4 | 2.3 ± 1.3 |
| | 30 mg/kg | 3.0 ± 0.8 | 2.8 ± 0.4 | 3.6 ± 1.1 | 2.4 ± 0.6 |
| TSH (ng/ml) | control | 0.60 ± 0.05 | 0.60 ± 0.11 | 0.69 ± 0.15 | 0.70 ± 0.05 |
| | 15 mg/kg | 0.40 ± 0.13 | 0.53 ± 0.22 | 0.47 ± 0.31 | 0.48 ± 0.23 |
| | 30 mg/kg | 0.47 ± 0.18 | 0.37 ± 0.11* | 0.49 ± 0.10 | 0.59 ± 0.26 |

* = significant difference within the same group from baseline value (Week 0), $p<0.05$.
# = significant difference between control and both treated groups during same week, $p<0.05$.

**Table 1 (cont'd).**

| Hormone | Group | Week 13 | Week 14 | Week 15 | Week 16 |
|---|---|---|---|---|---|
| TT4 (nmol/l) | control | 20.5 ± 9.1 | 24.8 ± 10.3 | 20.8 ± 5.5 | 21.8 ± 6.9 |
| | 15 mg/kg | 21.8 ± 4.6 | 17.0 ± 5.8 | 25.3 ± 5.7 | 19.0 ± 5.0 |
| | 30 mg/kg | 23.0 ± 11.0 | 28.3 ± 16.6 | 36.8 ± 19.9 | 26.3 ± 20.1 |
| FT4 (pmol/l) | control | 12.3 ± 3.1* | 12.5 ± 1.7* | 12.8 ± 1.0* | 12.3 ± 1.3* |
| | 15 mg/kg | 12.8 ± 1.7* | 11.3 ± 1.9* | 14.3 ± 1.5* | 11.5 ± 1.9* |
| | 30 mg/kg | 12.3 ± 2.6* | 13.3 ± 4.4* | 15.3 ± 2.9* | 10.8 ± 4.6* |
| FT4d (pmol/l) | control | 21.3 ± 6.4 | 25.5 ± 8.9 | 21.5 ± 1.3 | 16.8 ± 5.8 |
| | 15 mg/kg | 24.5 ± 4.5 | 18.0 ± 4.7 | 24.3 ± 5.0 | 18.5 ± 2.9 |
| | 30 mg/kg | 19.8 ± 6.2 | 24.3 ± 12.8 | 30.0 ± 11.0 | 19.8 ± 11.2 |
| TT3 (nmol/l) | control | 1.2 ± 0.4 | 1.3 ± 0.5 | 0.9 ± 0.1 | 0.9 ± 0.3 |
| | 15 mg/kg | 1.1 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.3 | 0.7 ± 0.2 |
| | 30 mg/kg | 1.1 ± 0.2 | 1.1 ± 0.4 | 0.8 ± 0.3 | 1.0 ± 0.4 |
| FT3 (pmol/l) | control | 2.7 ± 0.9 | 3.0 ± 1.2 | 1.8 ± 0.5 | 1.9 ± 0.6 |
| | 15 mg/kg | 2.5 ± 0.2 | 2.3 ± 0.7 | 2.8 ± 1.2 | 1.7 ± 0.5* |
| | 30 mg/kg | 2.4 ± 0.2 | 2.5 ± 0.6 | 2.4 ± 1.0 | 2.2 ± 0.8 |
| TSH (ng/ml) | control | 0.66 ± 0.16 | 0.71 ± 0.15 | 0.71 ± 0.18 | 0.81 ± 0.21# |
| | 15 mg/kg | 0.46 ± 0.23 | 0.46 ± 0.29 | 0.45 ± 0.2 | 0.43 ± 0.28 |
| | 30 mg/kg | 0.45 ± 0.14 | 0.46 ± 0.19 | 0.46 ± 0.13 | 0.49 ± 0.17 |

* = significant difference within the same group from baseline value (Week 0), $p<0.05$.
# = significant difference between control and both treated groups during same week, $p<0.05$.

## APPENDIX E

**Table 2.** Baseline (week 0) serum concentrations (mean concentrations ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and TSH in samples collected before and 2 and 4 hours after administration of 1 mg of TRH to treated and control group subjects.

| Hormone | Group | Pre | 2 hours | 4 hours |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 26 ± 4.83 | 35.5 ± 4.36 | 43.3 ± 7.27 |
| | 15 mg/kg | 21.5 ± 4.43 | 51 ± 27.5 | 48 ± 10.1 |
| | 30 mg/kg | 30.3 ± 10.3 | 36 ± 13.9 | 43.8 ± 15.2 |
| FT4 (pmol/l) | control | 21.5 ± 1.29 | 24.25 ± 1.26 | 27.0 ± 2.16 |
| | 15 mg/kg | 21 ± 2.94 | 29.25 ± 2.36 | 32 ± 3.16 |
| | 30 mg/kg | 20.5 ± 2.89 | 24.25 ± 3.1 | 26 ± 2.71 |
| FT4d (pmol/l) | control | 20.5 ± 3.7 | 28.75 ± 4.27 | 34.25 ± 4.35 |
| | 15 mg/kg | 21.5 ± 3.87 | 38.33 ± 10.79 | 48.5 ± 13.48 |
| | 30 mg/kg | 21.25 ± 3.59 | 25 ± 5.29 | 30.5 ± 7.85 |
| TT3 (nmol/l) | control | 0.93 ± 0.22 | 2.1 ± 0.52 | 1.85 ± 0.45 |
| | 15 mg/kg | 1.03 ± 0.39 | 3.45 ± 1.23 | 2.25 ± 0.77 |
| | 30 mg/kg | 0.83 ± 0.29 | 1.38 ± 0.61 | 1.60 ± 0.37 |
| FT3 (pmol/l) | control | 2.80 ± 0.6 | 5.05 ± 1.45 | 4.83 ± 1.54 |
| | 15 mg/kg | 3.28 ± 0.49 | 10.13 ± 4.29 | 7.23 ± 2.72 |
| | 30 mg/kg | 3.25 ± 0.62 | 4.93 ± 1.16 | 4.80 ± 0.43 |
| TSH (ng/ml) | control | 0.62 ± 0.17 | 0.85 ± 0.13 | 0.69 ± 0.12 |
| | 15 mg/kg | 0.47 ± 0.18 | 0.73 ± 0.38 | 0.55 ± 0.26 |
| | 30 mg/kg | 0.61 ± 0.2 | 0.93 ± 0.28 | 0.69 ± 0.22 |

**Table 3.** Serum concentrations (mean concentrations ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and TSH in samples collected before and 2 and 4 hours after administration of 1 mg of TRH to treated and control group subjects after 4 weeks of treatment.

| Hormone | Group | Pre | 2 hours | 4 hours |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 21.75 ± 4.99 | 28.5 ± 5.07 | 34.75 ± 5.31 |
| | 15 mg/kg | 19.25 ± 5.37 | 35 ± 9.31 | 43.25 ± 6.99 |
| | 30 mg/kg | 25 ± 10.23 | 30.75 ± 10.81 | 37.5 ± 12.4 |
| FT4 (pmol/l) | control | 17.75 ± 1.26 | 21.25 ± 0.96 | 23.75 ± 1.5 |
| | 15 mg/kg | 20.75 ± 1.5 | 25.75 ± 2.5 | 29.5 ± 3 |
| | 30 mg/kg | 17.75 ± 3.59 | 20.0 ± 2.58 | 23.5 ± 1.91 |
| FT4d (pmol/l) | control | 19 ± 2.44 | 25 ± 4.24 | 28.5 ± 5.07 |
| | 15 mg/kg | 20.5 ± 1 | 38.0 ± 7.61 | 47.5 ± 9.29 |
| | 30 mg/kg | 17.75 ± 5.06 | 25.5 ± 5.51 | 29.25 ± 6.13 |
| TT3 (nmol/l) | control | 1.15 ± 0.19 | 1.73 ± 0.61 | 1.55 ± 0.42 |
| | 15 mg/kg | 1.25 ± 0.3 | 3.13 ± 1.14 | 2.25 ± 0.93 |
| | 30 mg/kg | 1.05 ± 0.26 | 1.78 ± 0.13 | 1.45 ± 0.23 |
| FT3 (pmol/l) | control | 1.7 ± 0.39 | 2.7 ± 0.91 | 2.33 ± 0.79 |
| | 15 mg/kg | 2.3 ± 0.69 | 6.4 ± 3.79 | 4.05 ± 1.23 |
| | 30 mg/kg | 1.78 ± 0.26 | 3.2 ± 0.55 | 2.63 ± 0.35 |
| TSH (ng/ml) | control | 0.55 ± 0.12 | 0.93 ± 0.28 | 0.68 ± 0.12 |
| | 15 mg/kg | 0.49 ± 0.31 | 0.85 ± 0.36 | 0.48 ± 0.22 |
| | 30 mg/kg | 0.73 ± 0.46 | 1.27 ± 0.83 | 0.75 ±0.27 |

**Table 4.** Serum concentrations (mean concentrations ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and TSH in samples collected before and 2 and 4 hours after administration of 1 mg of TRH to treated and control group subjects after 8 weeks of treatment.

| Hormone | Group | Pre | 2 hours | 4 hours |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 22.75 ± 11.62 | 33.25 ± 11.62 | 42.25 ± 16.09 |
| | 15 mg/kg | 16.75 ± 7.37 | 36 ± 8.37 | 43 ± 8.91 |
| | 30 mg/kg | 21 ± 11.86 | 30.5 ± 11.36 | 41.5 ± 8.81 |
| FT4 (pmol/l) | control | 17.75 ± 5.32 | 23 ± 4.97 | 27.25 ± 6.29 |
| | 15 mg/kg | 15 ± 3.56 | 23.75 ± 6.75 | 23.75 ± 3.86 |
| | 30 mg/kg | 17.75 ± 6.02 | 22.5 ± 4.8 | 25.25 ± 3.3 |
| FT4d (pmol/l) | control | 16.75 ± 4.57 | 30.75 ± 9.25 | 39.0 ± 11.52 |
| | 15 mg/kg | 17.0 ± 5.42 | 39.0 ± 4.83 | 45 ± 5.29 |
| | 30 mg/kg | 13.5 ± 7.85 | 22.75 ± 8.1 | 35.25 ± 10.34 |
| TT3 (nmol/l) | control | 1.1 ± 0.28 | 2.73 ± 0.79 | 2.1 ± 0.64 |
| | 15 mg/kg | 0.9 ± 0.37 | 3.45 ± 0.66 | 1.98 ± 0.41 |
| | 30 mg/kg | 0.98 ± 0.35 | 2.45 ± 0.26 | 2.3 ± 0.54 |
| FT3 (pmol/l) | control | 2.28 ± 0.67 | 6.0 ± 2.60 | 4.6 ± 1.73 |
| | 15 mg/kg | 1.73 ± 0.54 | 8.63 ± 1.45 | 4.43 ± 0.95 |
| | 30 mg/kg | 1.88 ± 0.43 | 4.75 ± 0.66 | 4.63 ± 0.5 |
| TSH (ng/ml) | control | 0.67 ± 0.21 | 1.13 ± 0.39 | 0.72 ± 0.11 |
| | 15 mg/kg | 0.43 ± 0.19 | 1.21 ± 0.6 | 0.6 ± 0.13 |
| | 30 mg/kg | 0.87 ± 0.38 | 2.55 ± 1.05 | 0.87 ± 0.28 |

**Table 5.** Serum concentrations (mean concentrations ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and TSH in samples collected before and 2 and 4 hours after administration of 1 mg of TRH to treated and control group subjects 4 weeks after cessation of treatment.

| Hormone | Group | Pre | 2 hours | 4 hours |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 20.25 ± 8.3 | 32 ± 10.03 | 39.5 ± 12.87 |
| | 15 mg/kg | 18.0 ± 5.72 | 38 ± 4.97 | 51.75 ± 12.69 |
| | 30 mg/kg | 25.25 ± 11.95 | 47.25 ± 13.07 | 53.75 ± 9.6 |
| FT4 (pmol/l) | control | 12.5 ± 1.29 | 17.5 ± 2.08 | 19.75 ± 1.89 |
| | 15 mg/kg | 12.25 ± 1.5 | 19.5 ± 1 | 22.0 ± 2.16 |
| | 30 mg/kg | 13.75 ± 4.57 | 20 ± 3.16 | 22 ± 1.63 |
| FT4d (pmol/l) | control | 15.5 ± 5.74 | 25.75 ± 8.61 | 30.25 ± 13.3 |
| | 15 mg/kg | 14.5 ± 4.65 | 30.75 ± 12.15 | 48 ± 13.59 |
| | 30 mg/kg | 15.67 ± 12.37 | 23.33 ± 13.23 | 27.33 ± 11.47 |
| TT3 (nmol/l) | control | 1.35 ± 0.37 | 2.55 ± 1 | 1.75 ± 0.26 |
| | 15 mg/kg | 0.93 ± 0.55 | 3.13 ± 0.62 | 2.15 ± 0.26 |
| | 30 mg/kg | 1.05 ± 0.24 | 2.85 ± 0.57 | 2.38 ± 0.42 |
| FT3 (pmol/l) | control | 2.85 ± 0.72 | 6.58 ± 2.41 | 3.83 ± 0.81 |
| | 15 mg/kg | 2.3 ± 1.34 | 8.8 ± 2.36 | 5.45 ± 0.94 |
| | 30 mg/kg | 2.4 ± 0.59 | 7.98 ± 1.78 | 6.23 ± 0.97 |
| TSH (ng/ml) | control | 0.7 ± 0.05 | 1.51 ± 1.03 | 0.74 ± 0.15 |
| | 15 mg/kg | 0.48 ± 0.23 | 1.16 ± 0.64 | 0.55 ± 0.2 |
| | 30 mg/kg | 0.59 ± 0.26 | 1.24 ± 0.28 | 0.68 ± 0.16 |

**Table 6.** Serum concentrations (mean concentrations ± standard deviation) of TT4, FT4, FT4d, TT3, FT3, and (TSH) in samples collected before and 2 and 4 hours after administration of 1 mg of TRH to treated and control group subjects 8 weeks after cessation of treatment.

| Hormone | Group | Pre | 2 hours | 4 hours |
|---|---|---|---|---|
| TT4 (nmol/l) | control | 21.75 ± 6.90 | 42.67 ± 11.5 | 49.5 ± 11.85 |
| | 15 mg/kg | 19 ± 4.97 | 44 ± 9.83 | 61 ± 9.90 |
| | 30 mg/kg | 26.25 ± 20.14 | 51.67 ± 17.10 | 56.5 ± 20.86 |
| FT4 (pmol/l) | control | 12.25 ± 1.26 | 18 ± 2 | 19 ± 2.45 |
| | 15 mg/kg | 11.5 ± 1.91 | 17.75 ± 1.71 | 22.0 ± 1.41 |
| | 30 mg/kg | 10.75 ± 4.57 | 16.33 ± 3.79 | 19.5 ± 3.70 |
| FT4d (pmol/l) | control | 16.75 ± 5.80 | 31.0 ± 12.12 | 40.5 ± 13.40 |
| | 15 mg/kg | 18.5 ± 2.89 | 41.0 ± 6.58 | 56.5 ± 0.71 |
| | 30 mg/kg | 19.75 ± 11.18 | 35.33 ± 15.50 | 43.75 ± 11.44 |
| TT3 (nmol/l) | control | 0.88 ± 0.30 | 3.16 ± 1.18 | 2.08 ± 0.75 |
| | 15 mg/kg | 0.65 ± 0.17 | 3.38 ± 0.64 | 2.2 ± 0.59 |
| | 30 mg/kg | 1.0 ± 0.36 | 3.06 ± 0.61 | 2.28 ± 0.69 |
| FT3 (pmol/l) | control | 1.85 ± 0.58 | 9.73 ± 6.93 | 5.33 ± 1.92 |
| | 15 mg/kg | 1.7 ± 0.45 | 10.03 ± 2.64 | 7.15 ± 2.89 |
| | 30 mg/kg | 2.15 ± 0.79 | 8.3 ± 2.52 | 6.18 ± 1.72 |
| TSH (ng/ml) | control | 0.81 ± 0.21 | 1.69 ± 1.35 | 0.85 ± 0.32 |
| | 15 mg/kg | 0.43 ± 0.28 | 1.32 ± 0.93 | 0.58 ± 0.28 |
| | 30 mg/kg | 0.49 ± 0.17 | 1.34 ± 0.38 | 0.73 ± 0.22 |

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