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A new robust technique for testing of glucocorticosteroids in dogs and horses

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A new robust technique for testing of glucocorticosteroids in dogs and horses

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DEDICATION

I want to dedicate this project to my wife, Jackie, and my children, Shauna, Luke and Jake for their patience and understanding without which this project would not have been possible.

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CHAPTER 1. INTRODUCTION

Glucocorticosteroids are a class of steroids that have been used to obtain beneficial and detrimental results in the horse and dog racing industry. Glucocorticosteroids, also known as glucocorticoids, are used to treat inflammation in horses and dogs that have been stressed or injured to allow the animals to return to the racing circuit more quickly than naturally. While these treatments, when used correctly, would benefit the animal just as anti-inflammatories benefit humans, they can also cause detrimental effects to the health of the animal.

Glucocorticoids are a key ingredient in maintaining homeostasis in the body. This homeostasis is affected by trauma, exercise and cold that increase production of the corticotropin releasing factor in the hypothalamus (Wilke *et al* 1982). The corticotropin releasing factor increases the production of glucocorticoids (Wilke *et al* 1982).

There are two reasons for interest in the ability to detect glucocorticoids in racing animals. First, glucocorticoid residuals in the animal are undesirable in the racing industry as the residuals can be evidence of dosing the animal. Since these chemicals decrease the amount of pain the animal experiences, the animal may increase trauma due to lack of feedback. The decreased pain may be a result of reduced pressure on the nerves due to less inflammation but the exact reason is unclear (Martindale, 2005). The second reason is that the treatment with glucocorticoids can affect performance that could affect the outcome of the pari-mutuel races. Pari-mutuel racing, dogs and horses, generated over $4 billion in revenues and receipts in the United States in 2002 (U.S. Census Bureau 2002).

This paper presents research to characterize and quantify glucocorticoids in equine urine and plasma and in canine urine. This paper also summarizes an elimination study of an under-examined glucocorticoid. The research incorporates a liquid\liquid extraction from a small aliquot of sample. This aliquot is analyzed for many glucocorticoids in a single liquid chromatography mass spectrometry run.

CHAPTER 2. LITERATURE REVIEW

Cortisol (hydrocortisone) is the principal glucocorticoid in horse and dog blood and also the most potent naturally produced corticosteroid (Gower, 1984). Cortisol is produced by the adrenal gland, decreases the body's response to stress, decreases inflammation, and can cause euphoria. One study states the normal range in horses to be 50-3500 nmol/L (Irvine *et al* 1988). Cortisol maintains homeostasis in the body. Introduction of compounds that mimic cortisol decreases cortisol production (Toutain *et al,* 1984).

Synthesis: "The adrenal cortex produces corticosteroids which are a group of C_{21} steroids" (Gallciano, 1985). "This group known as 4-en-3-ones contains a double bond at C-4 and an oxo group at C-3. These compounds also have a side chain at C-17 and may or may not contain a hydroxyl group. The 17- hydroxylated glucocorticoids are more powerful than the non-hydroxylated counterparts. There is hydroxyl at C-21 and an oxo group at C-20." "There also needs to be an oxygen function, hydroxyl or oxo group which may or may not be at C-11" (Gower, 1984). The steroid numbering system is illustrated using cortisol in figure 1.

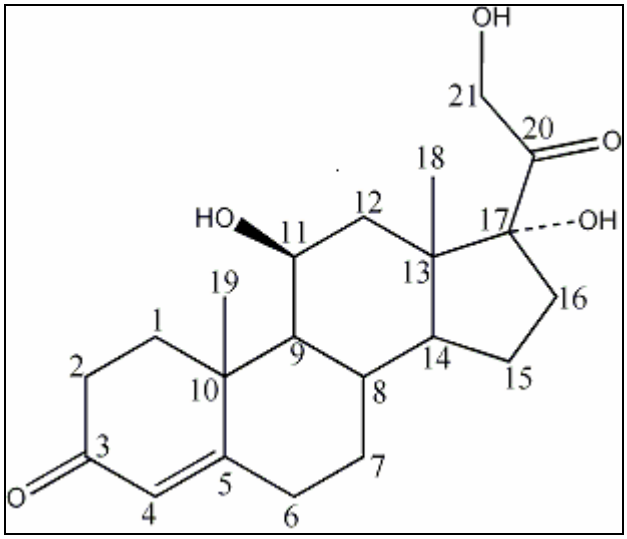

Figure 1. Chemical structure of cortisol with steroid numbering system (Wilke, 1982)

Effects: Gower (1984) stated corticosteroids increase blood glucose level by promoting the hepatic conversion of amino acids to glucose. These compounds also oppose insulin in allowing glucose into adipose cells, which in effect mobilizes fat and releases free fatty acids to the liver. Corticosteroids cause a net reduction in protein synthesis in muscle and lymphatic tissue, which reduces antibody synthesis. This net protein loss is due to increased protein catabolism (Martindale, 2005). Deluca (1984) stated glucocorticoids also suppress intestinal calcium absorption therefore lowering plasma calcium levels. Lower plasma calcium levels can lead to bone decalcification.

Corticosteroids counteract the histamine and serotonin effects of increased capillary permeability and dilation which are part of the inflammatory response. This increased capillary resistance reduces the movement of leucocytes to the site of infection. Therefore, phagocytosis decreases (Gower, 1984). These chemicals can also cause adrenal gland atrophy (Ralston and Stenhouse, 1990).

Cholesterol is transformed by desmolase in the mitochondria to yield pregnenolone. "This reaction includes two hydoxylases and utilizes cytochrome P450" (Garrett and Grisham, 1999). The pregnenolone is transported to the endoplasmic reticulum and converted into progesterone, which involves a hydroxyl oxidation and the double bond being transferred to a different ring (Garrett and Grisham, 1999). The sequence is diagramed in figure 2 on page 4.

Metabolites: There are four major pathways for steroid degradation: (Gower, 1984).

1. "Reduction of the double bond at c-4 with accompanying reduction of the C-3 oxo group to a secondary alcohol group.
2. Reduction of the C-20 oxo group to a secondary alcohol group.
3. Oxidation of the 17B-hydroxyl group.
4. Further hydroxylation at various points in the steroid nucleus."

In addition, the corticosteroids can convert to carboxylic acid derivatives. The majority of the corticosteroids are excreted as sulfate compounds or as glucuronide compounds (Gower, 1984). Norman (2002) stated the principal conjugated product is

glucuronides. Volin (1994) stated corticosteroids are excreted as sulphuric and glucoronic acid compounds which are water-soluble.

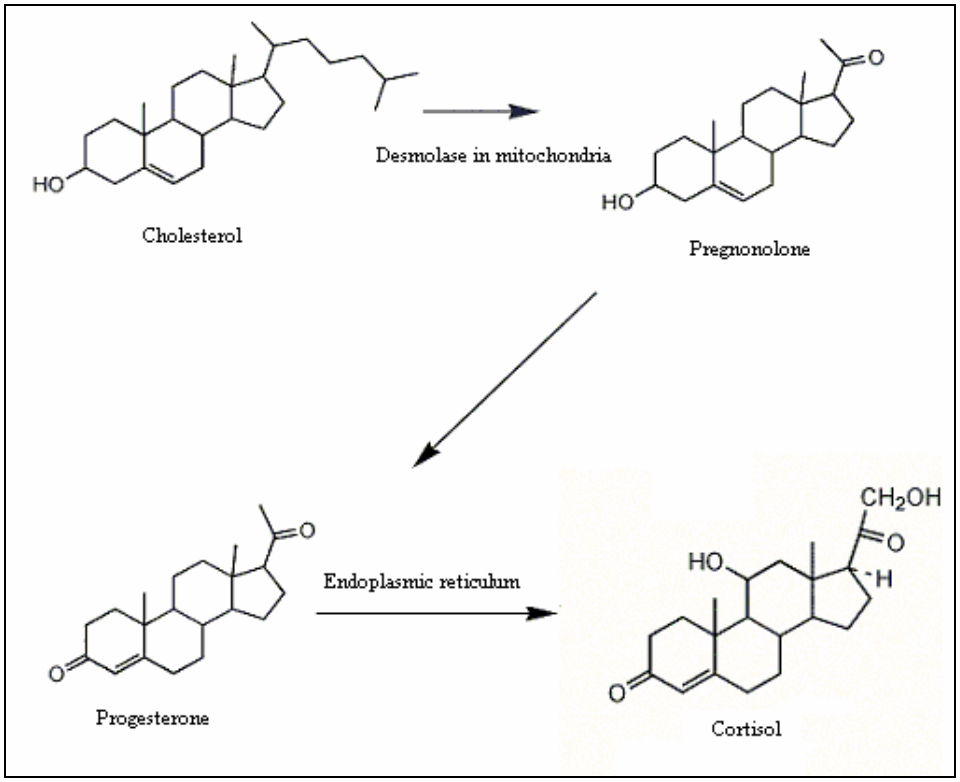

Figure 2. Route from cholesterol to cortisol (Garrett and Grisham, 1999)

Some glucocorticoid metabolites in equine urine are described below. Dexamethasone's major metabolite is 9-fluoro-16 alpha-methyl-6 beta,11- beta, 16- beta trihydroxy-1, 4- andostadiene-3,17-dione, M.W.=364 (Maylin and Skrabal, 1982). Betamethasone's main excretionary products in horses are betamethasone and 6-hydroxy betamethasone (Rodchenkov, 1988). Methylprednisolone's main metabolites are methylprednisone, 20-dihydromethylprednisolone, 20-dihydromethylprednisone (Ralston and Stenhouse, 1990), 17,21-dihydroxy-6alpha-methyl-1,4 pregnadiene-3,11,20-trione, 6alfa-methyl-17,20 beta,21-trihydroxy-1-4 pregnadiene-3,11 dione and 6 alpha –methyl-11b,17,20 beta,21-tetrahydroxy-1,4 pregnadien-3-one (Gallciano *et al*,1985). Cortisol's main metabolites are 20-beta-dihydrocortisol (Popot *et al*,1996), (Sams,1996), 11-beta-

hydroxyandrosterone (Popot *et al*,1994), 11-beta-hydroxyetiochlanolone (Popot *et al,* 1994), 20- beta dihydrocortisone (Popot *et al,* 1996) and cortisone (Popot *et al,* 1996). Some of these compounds are illustrated in Figure 3 on page 6.

Excretion: Tracking the excretion pharmacokinetics of steroids presents challenges. The pH of horse urine varies widely and the pH affects excretion rates (Sams, 1996). The pH of urine of a rested horse is about 8.4 while the plasma pH is about 7.4 (Sams, 1996). The pH of equine urine can decrease to as low as 5.4 after exercise (Sams, 1996). Irvine *et al* (1988) examined post race urine samples from 69 thoroughbred and 41 standard bred horses and found a pH range of 5.3 to 9.6. Alkaline pH levels of urine, such as those in a resting horse, can greatly increase excretion of the conjugated corticosteroids compared to the excretion rates when the pH of the urine is more acidic than that of the blood (Sams, 1996).

Factors affecting glucocorticoid concentration: Many glucocorticoids are endogenous, produced naturally by the body (Lewis, 1998). Therefore, an animal will constantly have concentrations of these endogenous compounds in its system. This concentration level of endogenous glucocorticoids is the baseline concentration level for that animal if the animal is resting, not under stress and has not recently engaged in exercise.

Exogenous compounds are produced from outside the subject (Lewis, 1998). These compounds can be those, which could be found naturally in the body, or they could be synthesized compounds. Examples of endogenous and exogenous corticosteroids are in table 1 on page 8.

Detection of doping with endogenous glucocorticoids is challenging due to individual differences in baseline levels in the subjects. These parameters make litigation against the abusers more difficult since the glucocorticoid levels must be proven to be greater than normal baseline levels for that particular animal. This situation is complicated further because levels of these substances can increase up to 50% in response to exercise (McKenna, 1994).

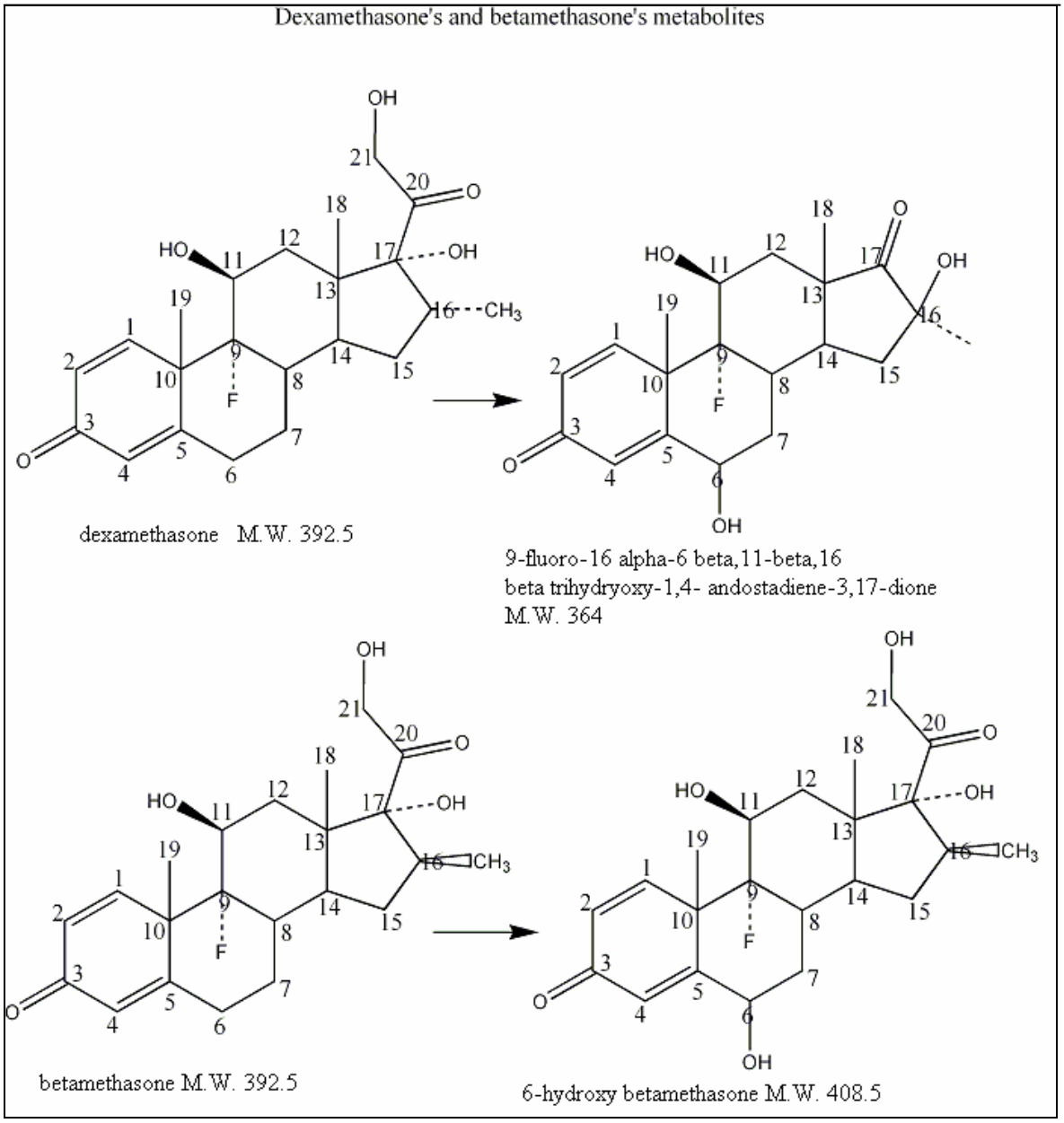

Figure 3. Dexamethasone and betamethasone with major metabolites

(Rodchenkov, 1988, Skrabalak, 1982)

Exercise and breed of horse affect glucocorticoid levels. A study of trotting horses and standardbred horses gave an arithmetic mean post race cortisol level in serum of 71 ng/ml with a range of 39-141 ng/ml. The mean cortisol levels in these same horses at rest were 31 +/- 2.1 ng/ml in the trotting horses. The range in the resting trotting horses was

24.3-37.8 ng/ml. The mean cortisol level in the resting standardbred horses was 19 +/- 2.6 ng/ml, with a range of 7.1- 41.3 ng/ml (Shultz *et al,* 1994).

Prescribed veterinary treatment of injuries can cause lingering drug effects. For example, using 25 mg of prednisolone injected intratendinously can relieve pain (measured as lameness) for 6 days. Prednisolone can be detected 16 days after treatment using reversed phase High Pressure Liquid Chromatography (HPLC) and off-line detection with immunoassay (Shultz, 1994).

Endogenous steroid suppression is the phenomenon of endogenous steroid production decreasing due to the presence of an administered corticosteroid. Toutain *et al* (1984) demonstrated this phenomenon by injecting different horses with various synthetic glucocorticoids. This study demonstrated that after injection with dexamethasone the cortisol concentrations in plasma were depressed for approximately four days. A prednisolone injection depressed cortisol plasma concentrations for a period of nearly 21 days (Toutain *et al,* 1984).

Toutain *et al* (1983) demonstrated this effect in canine subjects. In an experiment using five mixed breed dogs of both sexes, dexamethasone was given intravenously (IV) at a dose of 1 mg/kg body weight or intramuscularly (IM) in either 0.1 or 1 mg/kg dose by body weight. The compounds administered were dexamethasone alcohol and dexamethasone 21-isonictinate solution and dexamethasone 21-isonictinate as a suspension. The solutions, whether administered IV or IM, initially depressed cortisol levels. These cortisol levels returned to baseline within 48 hours for the solutions. The suspension depressed cortisol levels for 10 days in the 0.1 mg/kg test and one month in the 1 mg/kg test.

Some glucocorticoid concentration levels are affected by the route of administration. Methylprednisolone can be used with intra-articular (inside joint cavity) administration for relief of acute and long-term joint ailments. Methylprednisolone is excreted more quickly when given intra-articularly than intra-muscularly (Ralston and Stenhouse, 1990). Dexamethasone showed no difference in excretion rates due to route of

administration (Dumsia, 1976). The horses in the various route of injecting dexamethasone study were dosed intramuscularly or with an IV of dexamethasone ranging in concentration from 40-85 ug/kg (Dumsia, 1976). Several glucocorticoids are listed in table 1.

Table 1. List of various endogenous and exogenous glucocorticoids.

Chemical name	M.W.	Chemical formula	Exogenous
amcinonide	502.6	$C_{28}H_{35}FO_7$	y
betamethasone	392.5	$C_{22}H_{29}FO_5$	y
clobetasol propionate	467.0	$C_{25}H_{32}ClFO_5$	y
clocortolone pivalate	495.0	$C_{27}H_{36}ClFO_5$	y
cloprednol	392.9	$C_{21}H_{25}ClO_5$	y
cortisone	360.5	$C_{21}H_{28}O_5$	n
cortivazol	530.7	$C_{32}H_{38}N_2O_5$	y
desoximethasone	376.5	$C_{22}H_{29}FO_4$	y
dexamethasone	392.5	$C_{22}H_{29}FO_5$	y
diflorasone diacetate	494.5	$C_{26}H_{32}F_2O_7$	y
diflucortolone valerate	478.6	$C_{27}H_{26}F_2O_5$	y
flucloronide	487.4	$C_{24}H_{29}ClFO_5$	y
flumethasone pivalate	494.6	$C_{27}H_{36}F_2O_6$	y
fluocinolone acetonide	452.5	$C_{24}H_{30}F_2O_6$	y
fluocortolone	376.5	$C_{22}H_{29}FO_4$	y
fluorometholone	376.5	$C_{22}H_{29}FO_4$	y
fluprednisolone	378.4	$C_{21}H_{27}FO_5$	y
flurandrenolide	436.5	$C_{24}H_{33}FO_6$	y
formocortal	569.1	$C_{29}H_{38}ClFO_8$	y
hydrocortamate hcl	512.1	$C_{27}H_{41}NO_6$ -HCL	y
hydrocortisone	362.5	$C_{21}H_{30}O_5$	n
medrysone	344.5	$C_{22}H_{32}O_5$	y
meprednisone	372.5	$C_{22}H_{28}O_5$	y
prednisolone	360.4	$C_{21}H_{28}O_5$	y
prednisone	358.4	$C_{21}H_{26}O_5$	y
triamcinolone	394.4	$C_{21}H_{27}FO_6$	y

Performance effects: In 1988, a study of 87 horses was conducted to determine the influence of glucocorticoids on performance. The horses had been timed several times on the same 5/8 mile track without corticosteroid injections. The results were averaged and standard deviation calculated for each horse (Watrin, 1988). Varied amounts of

dexamethasone (10-40 mg), flumethasone (5-10 mg) and prednisolone (75mg) were given to test horses 2-8 hours prior to the race. They raced on the same track in the medicated state as in the non-medicated trials. The route of administration was not mentioned in the study. A measurable effect was defined as a difference of 1/5 of a second from the subject's average time. Of these 87 horses, 43% showed no effect to the drugs, 38% showed an improvement in times and the remainders were measurably slower (Watrin, 1988). Unfortunately, the Watrin study did not state whether these horses were assessed for pain prior to the races, which could account for these scattered results.

Soma *et al* (1998) compared equine responses to exercise after administration of dexamethasone (0.05mg/kg), flunixin (1.1mg/kg) or saline (10 ml). The compounds were administered intravenously. Dexamethasone was administered 12 hours prior to the tests. The other compounds were administered two hours prior to exercise.

Heart rates were similar in all three tests groups after exercise on a treadmill. Oxygen consumption and CO_2 production were lower in the flunixin subjects than in the other groups (Soma *et al,* 1988). Glucose levels increased in the dexamethasone group compared to the other groups after exercise. Cortisol levels decreased in the dexamethasone group and did not increase during exercise. This was not true with flunixin or saline. Soma *et al* (1998) stated that the preliminary evidence showed that dexamethasone and flunixin might influence the horse's performance.

Chemical analysis: There are several methods for detecting corticosteroids in equine and canine fluids. Immunoassay, **G**as **C**hromatography/**M**ass **S**pectrometry (GC/MS), **T**hin **L**ayer **C**hromatography (TLC) and **H**igh **P**erformance **L**iquid **C**hromatography / **M**ass **S**pectrometry (HPLC/MS) appear to be the most common in the literature. These techniques are used after the extraction\concentration process has been completed.

The extraction\concentration process reduces the volume of matrix to near zero. This reduction of matrix volume concentrates the analytes of interest making detection and quantification easier. This process also reduces or eliminates interfering compounds from

the aliquot which will be analyzed. The extraction process was usually a liquid \liquid extraction.

A liquid\liquid extraction for the purpose of extracting glucocorticoids from bodily fluids involves adding an organic solvent, or extractant, to an aliquot of sample and mixing the liquids together in a test tube. The organic solvent, during the mixing process, extracts the organic components from the inorganic phase, which is mostly water and inorganic salts.

The mixed sample is placed in a centrifuge to stratify the two phases, organic and inorganic. The organic phase is carefully removed from the test tube with a Pasteur pipette and placed into another clean test tube which is placed in an evaporator. The evaporator contains a heated water bath and dispenses a stream of inert gas, usually nitrogen or helium, into the tube to speed evaporation and prevent reactions with oxygen. After the organic phase has evaporated, the test tube is removed from the evaporator. A small amount of organic solvent, 100 ul or less is added to the tube to reconstitute the sample so a representative aliquot of the sample can be analyzed.

Solid phase extraction involves adding the sample to a column which contains beads coated with an organic compound. The sample is pulled by vacuum though the column with the organic components of the sample adhering to the column. This column may be rinsed with an inorganic solvent to eliminate inorganic compounds which may have been retained in the column. An organic solvent is added to the column to rinse the organic components from the sample into a test tube. This test tube is inserted into the evaporator and reconstituted in an organic solvent. This sample is ready for analysis.

Gas Chromatography: Gas chromatography involves the separation and detection of analytes in the gaseous state. A sample aliquot is injected into a heated port on the gas chromatograph called an injector. The injector quickly volatilizes the liquid sample into a gas. This gaseous sample is carried out of the injection port and through the gas chromatography column by a carrier gas. The carrier gas is called the mobile phase and is usually helium or nitrogen.

The column has a thin organic interior coating. The coating is denoted as the stationary phase. The column is contained in an oven that increases the temperature of the column during the separation or run. This temperature increase is termed the temperature gradient. As the gaseous sample passes though the column, it is absorbed into and out of the stationary phase thousands of times prior to reaching the detector. This action lengthens the amount of time the sample requires to travel from the injection port to the detector. This length of time is called the retention time (RT).

The length of time for each adsorption and desorbtion cycle varies depending upon the chemical composition of the analyte. Therefore, different analytes will have different RTs. This time is characteristic of that analyte for that particular column coating, length and diameter of column, thickness of column coating, type of column coating and temperature gradient.

The analytes after extraction/concentration process are derivatized in order to be heat stable and detectable in this method. The derivitization process adds a compound to the molecule to improve its stability at elevated temperatures. This changes the mass to charge ratio (m/z) of the analyte. The derivatization process can lead to a variety of end products.

In an experiment to determine cortisol concentrations using methoxyamine-trimethylsilyl (MO-triTMS) derivatives, the deuterated compounds yielded two different compounds and were encapsulated in two different chromatographic peaks (Ralston, 1990). For an ideal derivitization, this process should yield one compound and therefore one chromatographic peak. The splitting of the peaks reduces the peak area making quantification more difficult. Obtaining two different compounds makes analyte identification more difficult. The undeuterated cortisol had two chromatographic peaks also, due to formation of both anti and syn isomers (Ralston, 1990).

Another experiment demonstrated varying amount of derivatized fractions depending upon temperature used in the derivatization process (Rodchenkov, 1988). For betamethasone, a derivitisation temperature of 80 ^{0}C for 3 hours yielded a mono-

methoxyamine (MO) derivative. Derivitization overnight at 57 ^{0}C yielded a bi-methoxyamine derivative. This experiment also demonstrated that betamethasone required a more rigorous derivatization procedure than dexamethasone required. (Rodchenkov, 1988). Schnazner (1994) stated that steric hindrance created by the 9-fluoro group retards derivitization of the 11- beta hydroxyl group.

Different derivatization agents provide different results for various glucocorticoids. Schanzer *et al* (1994) reported on this phenomenon using N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) to derivatize dexamethasone and betamethasone which produced better results than MO-tri TMS. However, MO-triTMS worked better for prednisolone and prednisone than MSTFA (Schanzer, 1994).

Mass spectrometry detection: Mass spectrometry can be divided into two different ionization categories, hard and soft. Hard ionization involves adding a great deal of energy to a molecule, which leads to fragmentation and possible rearrangement of the molecule. Hard ionization processes leave little of the original molecule. **E**lectron **I**mpact (EI) is an example of a hard ionization technique. Figure 4 illustrates this ionization technique.

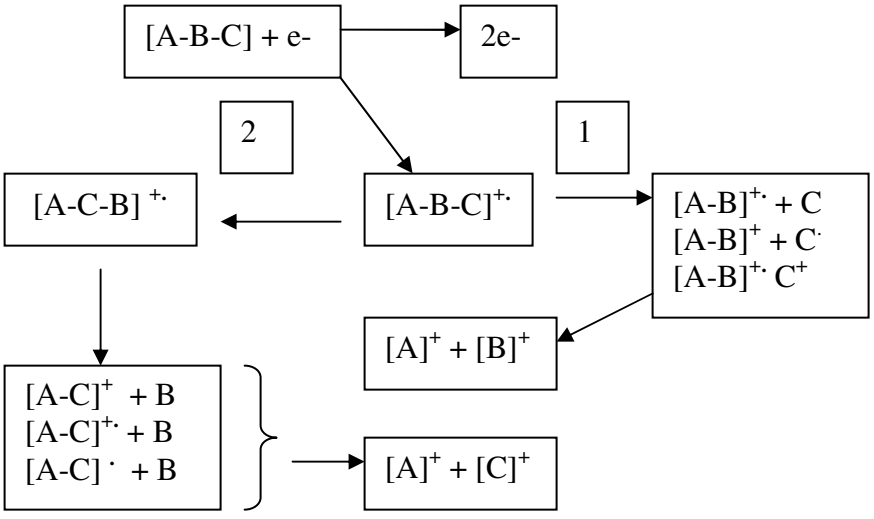

Figure 4. Illustration of electron impact for molecule ABC (Crews, 1998)

Figure 4 illustrates molecule ABC being ionized by one electron that also displaces an electron from the molecule. This molecule either fragments such as in path 1 or rearranges and then fragments as in path 2.

Chemical **I**onization (CI) is an example of a soft ionization technique. This technique ionizes a reagent gas such as methane or ammonia and this gas reacts with the analyte to create an ion of the analyte and heat. This process is outlined in figure 5.

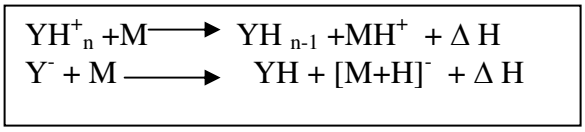

Figure 5. Diagram of chemical ionization reactions (Crews, 1998)

In figure 5, the reagent gas is denoted as Y and the analyte as M. Soft ionization techniques also produce molecular fragments that aid in identifying compounds of identical molecular weights since different compounds will exhibit different fragmentation patterns.

The use of CI leads to more certain identification spectra than EI using the M+1 weight (Signh *et al,* 1989). Singh *et al* analyzed 17 different steroid compounds, including metabolites, using CI and EI with GC/MS. Using EI, the base peak of 13 of these compounds was m/z 73. This fragment is the trimethylsilyl from the derivitization agent N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). A base peak is the largest m/z peak in the mass spectra. There were five base peaks for the 17 compounds (Signh *et al,* 1989).

The CI had 12 different base peaks for the 17 compounds, which allows for easier identification than the 5 base peaks in EI. Two testosterone metabolites were not detected with CI. The reason these peaks were not detected was not stated. There also were two pairs of isomers in the group. The EI yielded peak ratio intensities that could be used for identification between alpha and beta-cortol and alpha-cortolone and beta- cortolone. The CI peak ratio intensities for the cortolone isomers could be used for identification. The peak intensity ratios for the cortol isomers were identical for CI (Signh *et al,* 1989).

Thin layer chromatography (TLC): Thin layer chromatography is a useful technique in a qualitative sense, but in a quantitative use, it is subjective. This technique involves placing an aliquot of extracted sample near the bottom edge of a coated plate. This plate is placed nearly vertical in a volume of eluting solvent. The eluting solvent is absorbed up the plate moving the analyte up the plate as well. The plate is removed from the eluting solvent before the eluting solvent reaches the top of the plate. The position of the eluting solvent is marked.

The plate is dried and sprayed with various dyes. The analyte and dye combination will produce different colors depending upon the analyte. The color intensity of the dye analyte combination correlates to analyte concentration. The ratio of distance the analyte traveled divide by the distance the eluting solvent traveled is characteristic of the analyte. This ratio is defined as the retardation factor, R_f (Skoog, 1985).

This technique qualifies compounds by the distance the compound travels up a plate carried by the eluting solvent and by the color produced when sprayed with different dyes. The subjectivity in this method is due to the experimenters quantifying the color intensity, which corresponds to analyte concentration (Fritz and Shenk, 1987).

The analyte dye combination may absorb ultraviolet (UV) light which produces dark spots on the plate when viewed under UV light. This phenomenon defined as quenching is characteristic of some compounds. The analyte dye combination may also fluoresce, which is characteristic of other compounds.

Gallicano *et al* (1985) performed an experiment using thin layer chromatography as a qualitative tool for separating methylprednisolone and its metabolites. The solvent system used was 9:1(v/v) chloroform/methanol with 0.25 mm precoated silica gel 60 F-254 plates. The compounds were located by the observance of fluorescence or quenching of UV 254 light. After spraying the plate with phosphotungstic acid (PTA) spray reagent (15%w/v in ethanol), some compounds yielded a yellow color. Spraying the plate with tetrazolium blue (TZB) (0.07%w/v in 1:2v/v of ethanol/ 10% NaOH) turns some compounds purple. For example, methylprednisolone in this experiment reacted with both

dyes. Methyprednisone only reacted with TZB. The TZB reacts with the alpha – ketol group, however, a reduced c-20 keto group prevents color formation. The R_f values (methyprednisolone $R_f = 0.37$, methyprednisone $R_f = 0.54$) along with the spray results allowed compound identification. These plates were not used for quantification (Gallicano *et al,* 1985).

Enzyme-Linked ImmunoSorbent Assay (ELISA): ELISA is an assay that uses antibodies and enzymes to create a complex that affects color concentration in proportion to the concentration of the analyte (Lewis, 1998). ELISA is a technique that has low detection limits for many drugs. For example, d, l- methadone can be detected at 0.05 ng per ml of urine or 1pg /well. This technique has a range up to 125 pg/well with a 1:100,000-antiserum titre.

One of the problems with ELISA screening, however, is false suspicious (cross reactivity) results. In this instance, if d, l-methadone has a nominal value of 100%, the cross reactivity was 42% for l-methadone, 84% for d-methadone and 450% for alpha, beta-d, methadol –hemisuccinate. The cross reactivity for other corticoids such as codeine and morphine was < 0.01% (Schultz *et al,* 1998). A current manufacturer of dexamethasone ELISA kits lists cross-reactivities for prednisolone = 42.6%, isoflupredone = 27.8 % and triacinolone =10% (Bio-X Diagnostics, 2005).

Dexamethasone had a detection range in an ELISA experiment from 0.01 ng/ml to 50 ug/ml. The cross reactivity with flumethasone however was 100 % (Zhu *et al,* 1992). Anti-sera from treated rabbits were used to develop this test. Three mares that were injected with 5 mg of dexamethasone had their urine, serum and plasma analyzed for this study. Dexamethasone was detectable for 1 week in the urine.

Another drawback to ELISA testing is that some drugs may not be detected if the correct test battery is not selected. This makes prescreening of samples prior to ELISA useful, but does require extra time and resources.

Liquid Chromatography using Mass Spectrometry detection (LC/MS): Liquid chromatography involves separating liquid analytes with a liquid chromatography column.

The liquid sample is injected into liquid stream denoted as the mobile phase. The mobile phase and sample are pumped into a liquid chromatography column. This column is packed with particles that are coated with an organic substrate. The coating on these particles is the stationary phase. Similar to gas chromatography, the analyte is absorbed and desorbed from the stationary phase as it passes through the column. This process lengthens the time for the analyte to transverse the column, leading to a retention time for that particular analyte.

Liquid chromatography's principle advantage over gas chromatography is that samples do not need to be derivatized. This prevents formation of various derivatized compounds from one analyte, which allows for less ambiguity in the analysis.

LC/MS in the past had been criticized as not being sensitive enough to detect very low levels of corticosteroids. However, improvements in LC/MS and the use of tandem MS has greatly improved these detection limits. Mass spectrometry to the nth power (MSn) allows for more specificity. Parent ions fragment in unique patterns in the mass spectrometer. The fragment ions (daughters) of the parent ion lead to a more certain identity when dealing with larger numbers of analytes.

Detection limits have improved greatly in recent years. In 1994, Samuels *et al* detected 10 ng/ml of hydrocortisone in horse urine. A later study detected hydrocortisone and cortisone at 2 ng/ml using Liquid Chromatography with tandem Mass Spectrometry detection (LC/MS/MS) (Taylor *et al,* 2002). One of the main problems of LC/MS is isolating individual peaks from each other when many different substances are in a given sample.

Atmospheric Pressure Chemical Ionization (APCI) is an ionization technique that is less dependent on buffers and buffer strength than other ionization techniques. APCI and **E**lectro**S**pray **I**onization (ESI) are classified as soft ionization techniques. Molecular weight data can be obtained using soft techniques such as APCI and ESI. APCI is better than ESI with non-polar compounds such as steroids. Electrospray Ionization (ESI) is

better for more polar compounds (Thermo Electron Corp, 2003). The mechanism of the APCI technique is diagrammed below.

- $N_2 + e^- \longrightarrow N_2^{+\cdot} + 2e^-$
- $N_2^{\cdot} + H_2O \longrightarrow N_2^{+} + H_2O^{+}.$
- $H_2O^{+}. + H_2O \longrightarrow H_3O^{+} + HO.$
- $H_3O^{+} + M \longrightarrow (M+H)^{+} + H_2O$ (Thermo Electron Corp, 2003)

Summary of literature review: Glucocorticoids in the body are produced in the adrenal gland to maintain homeostasis. Glucocorticoids introduced into a subject from an outside source can cause health problems such as adrenal gland atrophy and decreased disease resistance for the subject as well as depress cortisol levels in the individual. These compounds can also have therapeutic effects for the animal such as reduced inflammation and pain. Glucocorticoid compounds can affect the athletic performance of animals, which could cause individuals to dose animals with these compounds to profit in pari-mutuel races.

Various techniques have been used throughout the years to identify and quantify glucocorticoids. Many of these techniques are being replaced by less subjective although more expensive diagnostic procedures. These newer techniques have lower detection limits and are a more legally defensible procedure than the procedures the industry has relied upon in the past.

Introduction to research project: This research project developed an analytical procedure for qualification and quantification of glucocorticoids in different matrices in equine plasma and urine as well as canine urine. The liquid\ liquid extraction is a variation of an extraction done by Luo *et al* (2005). Experimentation with this extraction involved pH adjustment and sample to solvent ratio changes. The analytical technique utilizes LC/MSn with an APCI probe. Definitions are provided for standard calibration curve and internal standard calibration.

Drug elimination studies were performed to test the method. The elimination studies provide data on the concentration of analytes in the urine and the speed the compounds are eliminated from the body. Elimination studies using dexamethasone were performed using canine and equine subjects. A fludrocortisone elimination study was performed utilizing a canine subject.

CHAPTER 3. METHODS AND MATERIALS

The following reagents and equipment were utilized to conduct this research.

Reagents:

MTBE: Fisher HPLC Grade

Methanol: Fisher HPLC Grade

Acetonitrile: Fisher HPLC Grade

Formic acid: Fisher 88%, certified ACS grade

Type 1 water: MilliQ Water System (Millipore) 18 megaohm reagent water.

Ammonium Formate: Fisher Lot # 046776

Hydrochloric acid: Fisher certified ACS plus

Ammonium hydroxide: Fisher certified ACS

6-alpha methylprednisololone: Sigma M0639 Lot # 107F-800

16-Beta methylprednisone: Steraloids P05800-000Batch # 6591

20-dihydro-6-alpha-methyprednisolone: synthesized from Sigma M-o639 Lot# 107F-0800

Amcinonide: SigmaA2428 Lot 079F0936

Beclomethasone: Sigma T-1643 Lot #24F-5950

Betamethasone: Sigma Lot# 126F0384

Clobetasone butyrate: Sigma C5548 Lot # 080H0759

Corticosterone: Sigma C2505 Lot# 016K1300

Cortisone: Sigma C2755 Lot #063K3517

Deoxycorticosterone: Steraloids Q3460-000 Batch # L1804

Desoxymethasone: Sigma D6038 Lot#025K1047

Dexamethasone: Sigma D9184 Lot# 036K1031

Dichlorasone acetate: Sigma D-6163 Lot # 100H0445

Fluadrenolide: Sigma F1642 Lot# 079F0708

Flucinonide: Sigma R 201596 Lot# C26H32F207

Fludrocortisone: Steraloids Q1280-000 Batch # B0255

Flumethasone: Sigma F-9507 Lot # 116F-0481

Fluocinolone acetonide: Sigma F8880 Lot# 043K1167

Fluorometholone: Sigma Lot# 109F0549

Hydrocortisone: Sigma H-4001 Lot #38F-0863

Medrysone: Sigma M0388 Lot# 058F02711

Paramethasone acetate: USPC Lot # F-1

Prednisolone: Sigma P-6004 Lot # 68F0511

Prednisone: Sigma P-6254 Lot # 117F-0426

Triamicinolone: Sigma T-1518 Lot# 24F-5955

Triamicinolone acetamide: Sigma T-1643 Lot # 24F-5950

Extraction equipment:

pH meter: Omega pH meter model # pHb-115

Rotorap: Bellco cell production roller apparatus. Range 0- 4 rpm. This device continuously mixes racks of test tubes by inverting the racks on a set of rollers.

Centrifuge: IEC Centra-8. Separates liquids by density though centrifugal force.

Evaporator: Zymark turbovap LV evaporator.

Pipettes: Fisher glass Pastuer pipettes 5¾ in.

Pipette: Gilson 1 ml pipetman

Pipette: Eppendorf 10-100 ul reference pipette

Vortex apparatus: Fisher Vortex Genie 2, cat # 12-812

Test tubes for evaporation: Fisher 16mm X 100 mm

Screw top tubes: 16mm x 125mm. 10 ml or larger capacity.

Analytical instrumentation:

Column: Zorbax SB C8 3.5u 3.0 mm X 15 cm P/N 863954.306

HPLC: Agilient 1100 with degasser and autosampler. The degasser eliminates dissolved gases making analysis more reproducible and more sensitive.

Column heater: Phenomenex HPLC column heater Thermasphere TS 130

Mass Spec: Finnagan LTQ

HPLC mobile phase and diluent

HPLC mobile Phase A: 0.1 % Formic acid in H_2O

B: 0.1 % Formic acid in Acetonitrile

Diluent: Combine 40 ml of 2 mM of ammonium formate in type 1 water with 60 ml of methanol.

Standard calibration curve: A standard calibration curve is composed of aliquots of a blank matrix that contain differing amounts of the desired analyte in increasing concentration. These calibration samples are extracted and analyzed using the same procedures used for the actual samples. The analyte areas are compared to the known concentrations using linear regression in the case of a linear calibration curve. This linear regression will produce a formula for the line computed for the standard concentration and corresponding areas. The analyte areas for each sample are incorporated into the equation that calculates the concentration of the analyte in the unknown sample.

Internal standard calibration: The internal standard should be chemically similar to the analytes so the extraction process will produce similar recoveries between the internal standard and the analytes. This similarity will also yield retention times that will be within the run time of the analysis. The internal standard cannot be a substance that would be contained in the sample.

Desoximethasone is the internal standard used for these studies. The internal standard is added to the standards and samples at the beginning of the extraction process. The aliquots of internal standard which are added to the standards and samples are the same concentration and therefore the same volume is added to each sample and standard.

The peak areas of the internal standard should be similar after extraction in the standards and samples. The internal standard areas in the calibration curve are averaged to produce a benchmark area that is compared to the internal standard peak areas in the samples. The internal standard area average of the calibration curve and the internal standard area of the sample are converted to a ratio (calibration standard internal standard area /sample internal standard average area). This ratio is multiplied by the concentration

of the desired analyte calculated using the standard curve for that analyte. The internal standard calibration corrects inconsistencies in several areas.

The first area of the analytical procedure that the internal standard calibration can correct is in the extraction process. If the extraction process yielded lower or higher recoveries for all of the analytes including the internal standard, all of the samples would be corrected for that percent difference. Such recovery problems can be caused by contamination, poor solvent quality or incorrect pipette settings when the sample is reconstituted after the turbovap evaporation step.

The second area that internal calibration can correct is errors in the analytical run. Problems such as incorrect sample volume uptake and changes in instrument temperatures or pressures or ionization parameters can be corrected or at the very least detected, so the problem can be corrected. Since the analyst would be aware of the area counts of the internal standard and the retention time of the internal standard, the erroneous instrument parameters can be corrected after the first sample of the run if the parameters are suspect. This knowledge also helps detect eluent flow rate changes from previous runs due to different tubing or column configuration.

Extraction procedure benefits: The extraction procedure eliminates the matrix of the sample and concentrates the analytes in the sample. This improves detection by eliminating compounds that create high background noise. High background noise could make detection of the desired analyte more difficult or impossible. Extraction also improves detection by increasing the concentration of the desired analytes, increasing the analyte areas. This improves the signal to noise ratio for that peak which improves quantification and identification.

The extraction procedure reduces the number of times the instrument needs to be cleaned by eliminating much of the matrix which can contaminate interior components. This lack of matrix leads to more consistent instrument performance. Analyzing extracted samples, as opposed to non-extracted samples, increases instrument component lifetime since these components need cleaning less frequently.

The pH of the sample matrix needs to be optimized for urine and plasma samples to optimize recovery of the analytes. This adjustment makes the analytes less water soluble and easier to extract. The pH of the samples was adjusted using 50% ammonium hydroxide or 3N HCl. Four analytes were selected that were representative of the group of corticosteroids to be analyzed. These analytes, prednisone, betamethasone, dexamethasone and dichlorisone acetate span the range of retention times and molecular weights in the run.

Once the pH was determined, variations of the extract were performed. Type 1 water was added to an aliquot of sample to improve the extraction recovery by lowering the salt concentration in the inorganic phase. This involved adding one milliliter of type 1 reagent water to the one milliliter of sample prior to extraction but after pH adjustment. Another variation involved increasing the amount of Methyl-tert Butyl Ether (MTBE) from 5 ml to 7 ml with the water added to use with 1 ml of sample.

Internal standard preparation: A 1 ug/ul stock standard of desoximethasone was made using powdered desoximethasone and reagent grade methanol. This standard was diluted to 10 ng/ul for use as the internal standard.

Stock and working standard preparation: Stock standards of 24 corticosteroids were made from solid compounds in 1 ug/ul concentrations diluting with methanol or acetonitrile. These standards were diluted in methanol to make the working standards. The working standard concentrations used are 0.01, 0.02, 0.05, 0.1, 0.5, 1.0, 10.0, and 20 ng/ul.

Calibration standard preparation: Drug-free animal urine was collected and pooled to be processed into stripped urine. Stripped urine is urine which was taken through the extraction process three times to eliminate any possible drugs in the urine from endogenous or exonogeous sources. Ten microliters of a working standard are added to one milliliter of stripped urine or plasma to make the calibration standards that have concentrations of 0.1, 0.2, 0.5, 1.0, 5.0, 10, 100 and 200 ng/ml. After this point, standards and samples are treated identically though the extraction process. Ten microliters of

internal standard were added to the samples and standards before extraction. The standards were extracted with the samples to assure uniformity in reagents and mixing times.

Extraction procedure: **Caution** MTBE is an extremely flammable liquid and vapor. Vapor may cause a flash fire. MTBE can cause central nervous system depression and can cause kidney and liver damage and tumors. Consult MSDS before using. Use in fume hood with gloves and safety goggles (Fisher, 2007).

1. Adjust urine pH to 9.0 +/- 0.1 units. (Adjust plasma pH to 8.0 +/- 0.1 units.)
2. Pipette 1 ml of urine or plasma into a screw top tube.
3. Add ten microliters of 10 ng/ul internal standard solution.
4. Mix on rotorap for 10 min at 2 rpm.
5. Add 1 ml Type 1 H_2O to tube. Vortex 10 sec.
6. Add 5 ml of Methyl-tert Butyl Ether (MTBE).
7. Rotorap for 10 min. at 2 rpm.
8. Centrifuge at 3000 rpm for 10 min.
9. Extract top MTBE layer using a Pasteur pipette. Dispense in tube (16mm x100mm).
10. Evaporate MTBE in turbovap at 46 C for 14 min.
11. Reconstitute in 100 ul of diluent. Sample is ready for analysis.

Instrumentation parameters: For working standards, 2 ul aliquots were injected. For calibration standards and samples, 20 ul were injected which corrects for dilution since 1 ml of sample was concentrated to 0.1 ml. The HPLC gradient ramp is described in table 2.

Table 2. HPLC gradient with mobile phase composition gradient

		Composition of mobile phase gradient ramp	
Time (min)	Flow rate (ml/min)	%A	%B
0	0.6	70	30
4	0.6	70	30
12	0.6	50	50
16	0.6	5	95
20	0.6	5	95
21	0.6	70	30
25	0.6	70	30

Mass Spectrometer parameters

MS run time: 20 minutes

Other parameters are in table 3 on page 28.

Dexamethasone elimination study #1

Most dexamethasone treatment therapies call for 0.2 to 2 mg/kg as the initial dose and additional doses of lower amounts over the next few days of treatment. An eight-year-old male greyhound weighing 36 kg was orally dosed once with 0.1 mg/kg dexamethasone (Sigma D9184-100mg Lot # 036k1031). Voluntary urine elimination samples were collected pre-dosing and at 2, 4, 8, 24, 36, 48, 60, 72, 84 and 96 hours after dosing.

Food and water were available *ad libitium* to the greyhound. The test animal was housed in a separate kennel to prevent carry over to other animals. Kennels are cleaned daily.

Dexamethasone elimination study # 2:

Dexamethasone elimination study in equine urine administration sample #63. An administration sample is a sample that was collected from an animal which had been administered a known amount of a drug. In the equine study, 20 mg of dexamethasone was dosed orally in two horses weighing 596 kg and 561 kg, ages unknown. Pre-dose urine was collected by catheter. Post dose urine was collected by catheter at 2, 4, 8, 24, 48 hours. The drug administered was dexamethasone (Sigma Reference grade: D-1756, Lot #87F-0740).

Fludrocortisone elimination study

An eight-year-old male greyhound weighing 35 kg was orally dosed once with five 0.1 mg fludrocortisone tablets (Barr Laboratories Lot # 6080391 exp. 8-31-08). Voluntary urine elimination samples were collected pre-dosing and at 2, 4, 8, 24, 48, 72, and 96 hours after dosing. Care conditions for the animal were identical to the canine dexamethasone study.

CHAPTER 4. RESULTS

The HPLC gradient in table 2 on page 24 was used to determine retentions times for the compounds in table 3. This gradient was optimized to prevent coeluting peaks as much as possible. Diluted glucocorticoid standards were injected into the mass spectrometer to optimize ionization parameters and analyte ions. The analyte ions are presented in table 3 and the ionization parameters are presented in the table 4 on page 28. Structures for these compounds are in the appendix starting on page 56.

Table 3. Ions and retention times (RT) using LC/MSn

Compound	Mw	Ionization and ions	RT #	RT(min)
triamacinolone	394.4	pos ms2 359, ions 359, 341, 339,321	1	3.04
fludocortisone metabolite	384	pos ms2 384 ions 249,267,309	2	3.71
20 dihydro 6 alpha methylprednisolone	376	neg ms3 421, 375 ions 357, 345, 327	3	4.65
hydrocortisone	362.5	pos ms2 363 ions (345, 327, 309, 267)	4	4.98
prednisolone	360.4	pos ms2 361.3 ions 265.3,279.3,289,307,325.2,343.1,362	5	5.16
fludrocortisone	380.5	pos 2 381 ions (295,313,333,349,361,381)	6	5.29
prednisone	358.4	pos ms2 359 ions (213.2,313.3,323.1,341)	7	5.39
cortisone	360.4	pos ms2 361.3 , ions(163,307,325,341,343)	8	5.56
6 alpha methylprednisolone	374.5	neg ms3 419, 343 ions 327, 309, 294	9	7.38
betamethasone	392.5	neg ms3 437, 361 ions 345, 327,310,325, 307, 292	10	7.82
flumethasone	410.5	neg ms 2 379 ions 290,305.1,325.1,328,343.1,363.1	11	7.88
dexamethasone	392.5	neg ms3 437, ms 361 ions 345, 325, 307,292	12	8.21
16 b methyprednisolone	372.5	neg ms2 341 ions 282.3,299.3,323.2,341.2	13	8.44
corticosterone	346.5	pos ms2 347.5 ions 269,293,311,329,347	14	9.44
beclomethasone	408.9	pos ms3 409, 391 ions 237,279,319,337.2,355.2,373.3	15	9.53
flurandrenolide	436.5	pos ms2 436.0 ions 303,315,331,359,361,377,417	16	10.04
fluocinolone (acetonide)	452.5	pos ms2 453 ions 337.1,341,413.2,433.1	17	10.44
triamacinolone acetamide	434	pos ms 2 436 ions 321.2,339.2,357.1,393.3,397.2,415.2	18	10.72
fluorometholone	376.5	pos ms2 377.5 ions 279.2,321,339,357,377	19	10.72
desoximethasone	376	pos ms2 377.5 ions 279.3,303.3,321.3,339,357	20	11.31
paramethasone acetate	418.5	pos 2435 ions 291.2,319.2,337.3,379.1,397.2,417.1	21	12.73

Table 3 (continued)				
Compound	**Mw**	**Ionization and ions**	**RT #**	**RT(min)**
deoxycorticosterone (21)	330.5	pos ms2 331 ions 331,313,295, 277, 267	22	13.93
medrysone	344.5	pos ms3 345,327 ions 309,291, 269, 225	23	14.95
flucinonide	494.5	pos ms2 495 ions 219.3,319.2,337.2,417.1,455,475	24	15.17
dichlorisone acetate	455.4	pos ms2 456 ions 253.1,319.2,337.2,397.2	25	15.28
amcinonide	502.6	pos ms2 503 ions 321.1,339,399.1,483.1	26	16.50
clobetasone butyrate	479	pos ms2 480 ions 389.2, 371,343, 317, 279	27	17.26

Tune parameters

Tune parameters are instrument parameters which when properly applied give optimal results for an analyte. One analyte may be used to create a tune file for other compounds. Tune parameters for triamicinolone were based on triamicinolone tune file. For the other positive ion scans, the cortisone tune file was used. For the negative ions, the two methyprednisolone tune files were used. Parameters common to all five tune files are listed below.

Vaporizer temp: 430^{o}C

Injection control parameters AGC settings: Full MS target 30000, SIM target 10000.0, MSn Target10000.0, Zoom Target 3000.0

Other Tune parameters are in table 4 on page 28.

Scan segments: A scan segment is a user modifiable part of the analytical program that is set for a particular retention time range. In this retention time range, ions are selected for compounds that elute in the chosen time range. Since there are relatively few ions chosen per scan segment, the detector scans the selected ions for a longer time period than would be possible in full scan mode. This increased analysis time creates larger peak areas which makes peak identification and quantification more exact.

The detector also spends practically no time on ions that are not listed in the scan segment. This eliminates much of the noise that can mask analyte peaks for compounds at low concentration levels. The details of the scan segments are listed on page 28.

Table 4. Mass spectrometer tune parameters

parameter\tune file	Triamcinolone	MpredAPCI061306	Cortisone APCI
APCI source			
Sheath gas Flow rate(arb)	50.00	55.00	45
Aux Gas Flow rate (arb)	5.00	5.00	5
Sweep Gas flow Rate (arb)	0.00	0.00	0
Discharge Current (uA)	5.00	5.00	5.00
Capillary Temp (^{o}C)	275.00	275.00	275.00
Capillary Voltage (V)	7.00	-8.00	25.00
Tube Lens (V)	50.00	-65.00	80.00
Ion Optics			
Multipole 00 offset(V)	-4.25	3.50	-4.25
Intermultipole Lens 0 Volt.(V)	-4.00	4.00	-4.00
Multipole 0 offset(V)	-5.00	5.00	-5.25
Intermultipole Lens 1 Volt.(V)	-15.00	12.00	-11.00
Gate LensVoltage (V)	-64.00	68.00	-38.00
Multipole 1 offset(V)	-11.50	14.00	-11.00
Multipole RF Amplitude (Vp-p)	400.00	400.00	400.00
Front Lens(V)	-6.00	5.25	-6.25

parameter\tune file	MpredAPCIFloAdj	cortisoneAPCILOW
APCI source		
Sheath gas Flow rate(arb)	50.00	45.00
Aux Gas Flow rate (arb)	5.00	5.00
Sweep Gas flow Rate (arb)	0.00	0.00
Discharge Current (uA)	5.00	5.00
Capillary Temp (^{o}C)	275.00	275.00
Capillary Voltage (V)	-41.00	25.00
Tube Lens (V)	-65.00	80
Ion Optics		
Multipole 00 offset(V)	4.00	-4.25
Intermultipole Lens 0 Volt.(V)	4.00	-4.00
Multipole 0 offset(V)	5.00	-5.25
Intermultipole Lens 1 Volt.(V)	8.00	-11.00
Gate LensVoltage (V)	46.00	0.00
Multipole 1 offset(V)	9.50	-11.00
Multipole RF Amplitude (Vp-p)	400.00	400.00
Front Lens(V)	5.00	-6.25

Scan segment details are listed below.

Segment 1: Duration = 4 min, number of scan events = 1,

tune method= triamcinaolone.

Scan event details 1 ITMS + c norm = (359.0) ->0 (95.0-500.0)

MS/MS: CE 20 .0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 2: Duration = 1 min, number of scan events = 1,

tune method=MpredAPCIFloAdj.

Scan event details 1 ITMS - c norm = (421.0) -> (375.0) ->0 (100.0-500.0)

MS2: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

MS3: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 3: Duration = 1.2 min, number of scan events = 4,

tune method= cortisoneAPCI.

Scan event details 1: ITMS + c norm = (381.0) ->o (100.0-500.0)

MS/MS: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 2: ITMS + c norm = (361.3) ->o (95.0-500.0)

MS/MS: CE 30 .0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 3: ITMS + c norm = (3590) ->o (100.0-500.0)

MS/MS: CE 30 .0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 4: ITMS + c norm = (363.5) ->o (100.0-500.0)

MS/MS: CE 30 .0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 4: Duration = 2.8 min, number of scan events = 4,

tune method= MpredAPCI061306

Scan event details 1: ITMS - c norm = (437.0) -> (361) ->o (95.0-600)

MS2: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

MS3: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 2: ITMS - c norm = (419.0) -> (343.0) ->o (90.0-500.0)

MS2: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

MS3: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 3: ITMS - c norm = (379.0) ->o (100.0-600.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 4: ITMS - c norm = (341) ->o (100.0-600.0)

MS/MS: CE 30.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 5: Duration = 1.0 min, number of scan events = 2,

tune method= cortisoneAPCI

Scan event details 1: ITMS + c norm = (347.5) ->o (95.0-500.0)

MS/MS: CE 25 .0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 2: ITMS + c norm = (409.0) -> (391) ->o (105.0-500.0)

MS2: CE 30.0%, Q=0.25, time=30.0 Iso W= 5.0

MS3: CE 30.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 6: Duration = 2.0 min, number of scan events = 3,

tune method= cortisoneAPCI

Scan event details 1: ITMS + c norm = (436.0) ->o (120.0-500.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 4.0

Scan event details 2: ITMS + c norm = (409.0) -> (391) ->o (105.0-500.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 3: ITMS + c norm = (453.5) ->o (120.0-550.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 7: Duration = 1.75 min, number of scan events = 1,

tune method= cortisoneAPCI

Scan event details 1: ITMS + c norm = (435.0) ->o (115.0-500.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 8: Duration = 1.00 min, number of scan events = 1,

tune method= cortisoneAPCI

Scan event details 1: ITMS + c norm = (331.0) ->o (90.0-500.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 9: Duration = 1.25 min, number of scan events = 3,

tune method= cortisoneAPCI

Scan event details 1: ITMS + c norm = (345.0) -> (327.0) ->o (90.0-500.0)

MS2: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

MS3: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 2: ITMS + c norm = (495.0) ->o (135.0-500.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Scan event details 3: ITMS + c norm = (456.0) ->o (125.0-550.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 10: Duration = 1.00 min, number of scan events = 1,

tune method= cortisoneAPCI.

Scan event details 1: ITMS + c norm = (503.0) ->o (135-600.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 11: Duration = 2.50 min, number of scan events = 1,

tune method= cortisoneAPCI.

Scan event details 1: ITMS + c norm = (480.0) ->o (130-550.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Segment 12: Duration = 0.50 min, number of scan events = 1,

tune method= cortisoneAPCILOW.

Scan event details 1: ITMS + c norm = (480.0) ->o (130-550.0)

MS/MS: CE 25.0%, Q=0.25, time=30.0 Iso W= 2.0

Using the LC gradient in table 2 and ions listed in table 3, the previously listed tune parameters and scan segments, the chromatograph in figure 6 on page 32 is obtained for a canine urine standard spiked at 100 ng/ml for the compounds listed in table 3 on page 26.

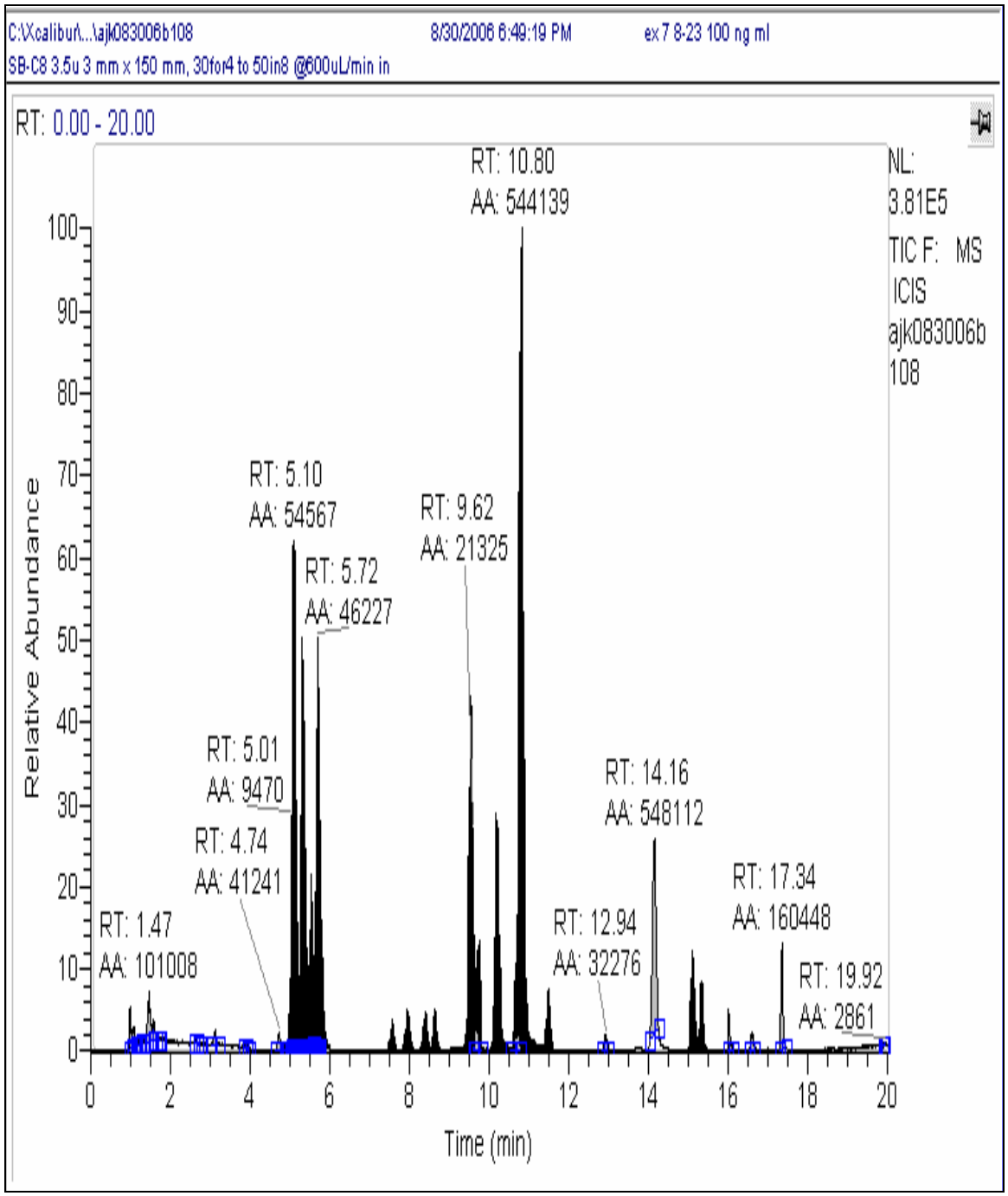

Figure 6. Total Ion chromatograph of 100 ng/ml standard in canine urine.

Using the ion selection filters for cortisone and hydrocortisone, the chromatogram presents a clearer picture of these two compounds in figure 7 on page 33.

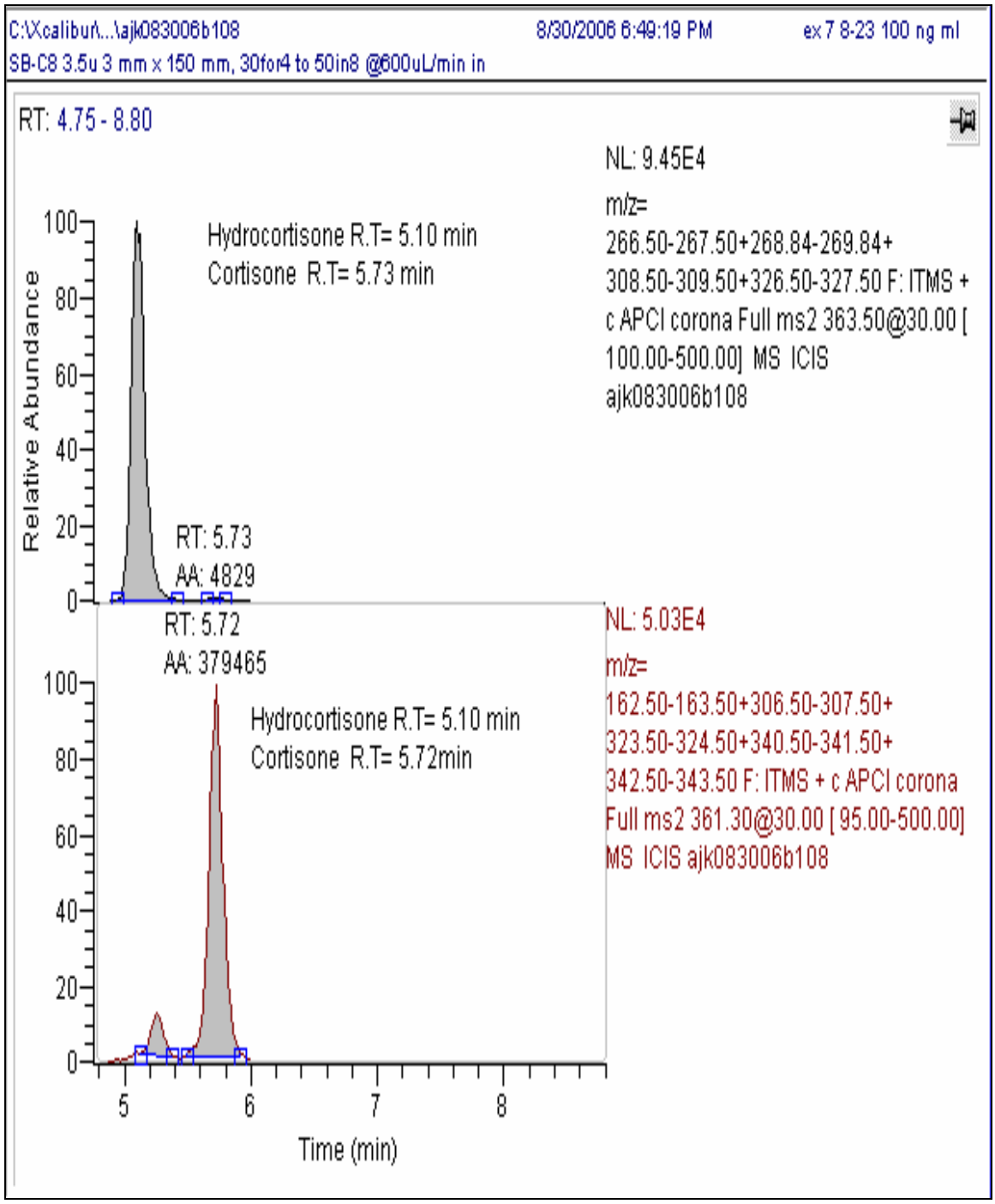

Figure 7. Ion chromatograph using filters for cortisone and hydrocortisone.

The mass spectra are presented with the ion chromatographs of cortisone and hydrocortisone in figure 8 on page 34.

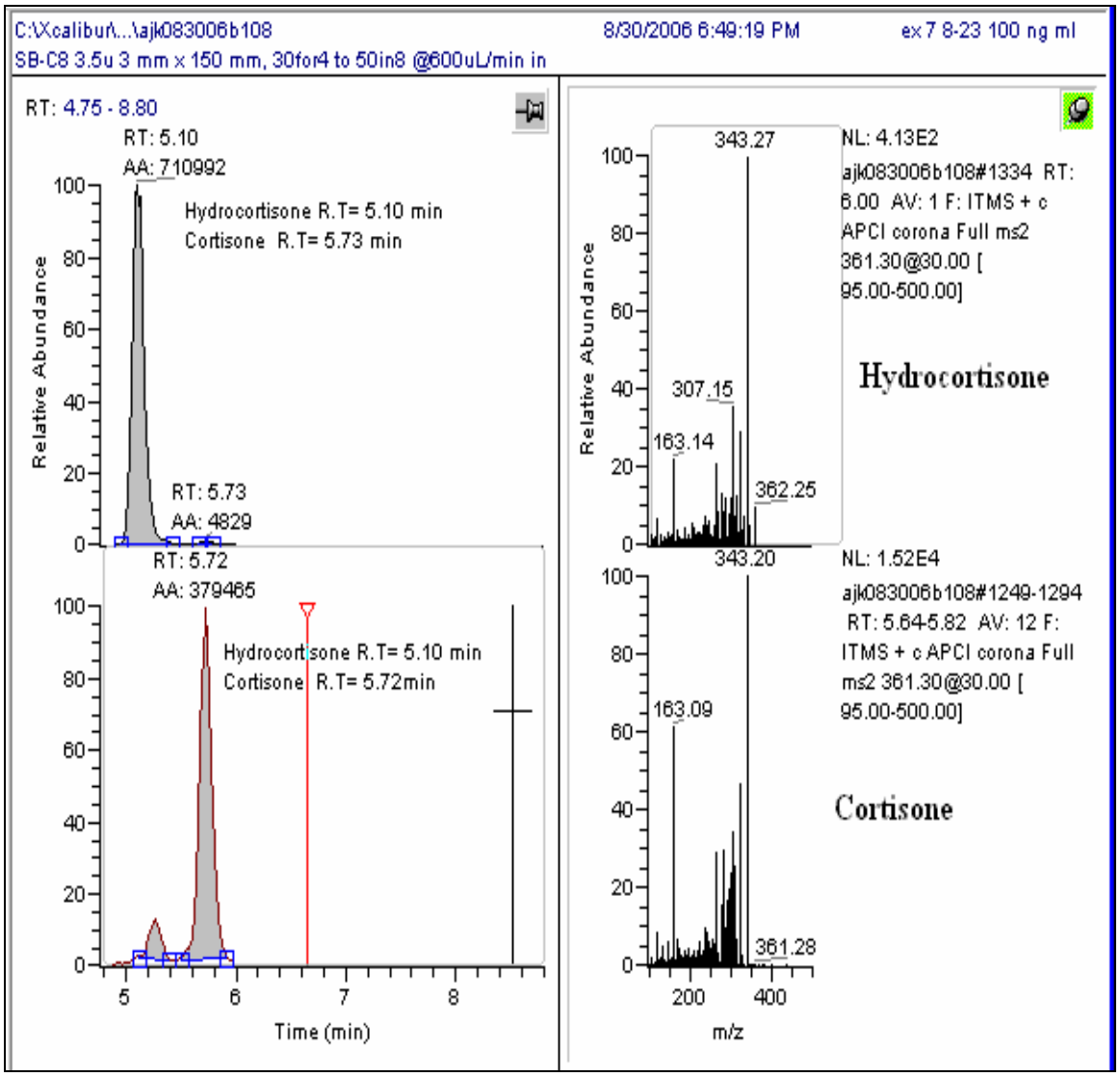

Figure 8. Ion chromatograph and mass spectra of cortisone and hydrocortisone

Figure 9 on page 35 contains the chromatograph and mass spectra of dexamethasone and betamethasone. These chemicals have the same molecular formula and are structurally identical except for a methyl group on c-16 that has an alpha orientation in dexamethasone and a beta orientation in betamethasone as shown in figure 3 page 6.

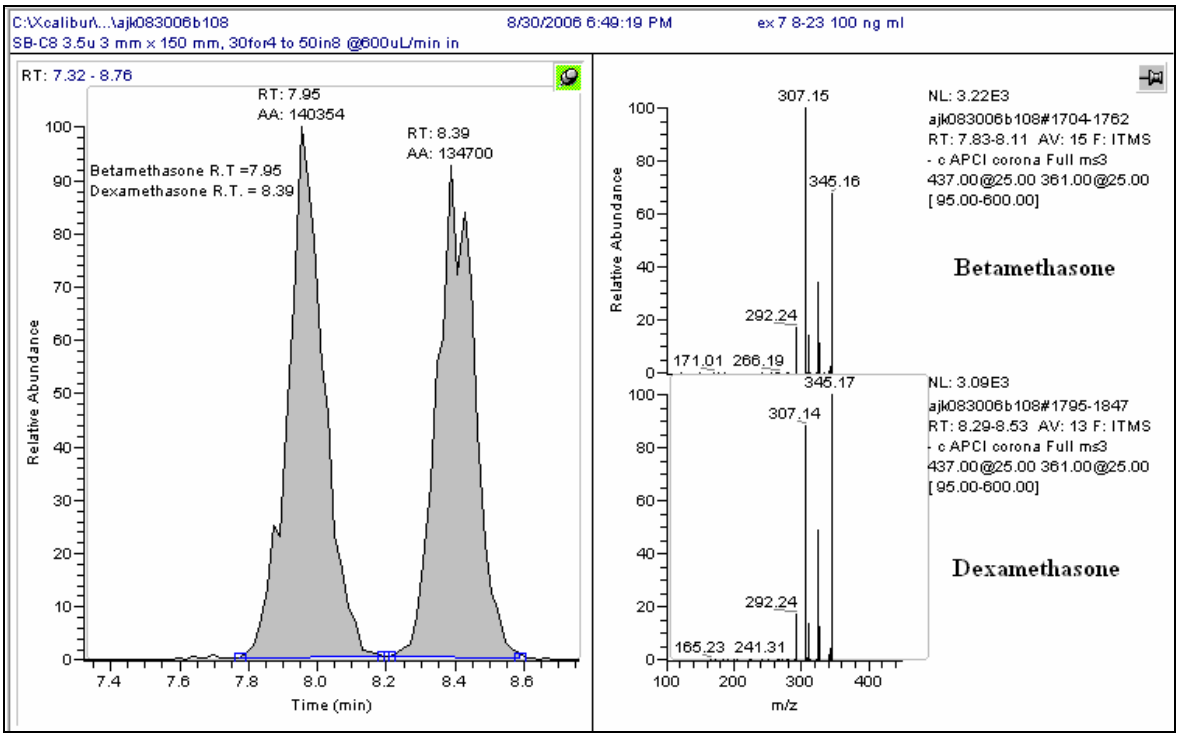

Figure 9. Chromatograph and mass spectra of dexamethasone and betamethasone.

The recoveries of dexamethasone, betamethasone, prednisolone, and dichlorisone acetate for pH range from 5.0 to 10.0 in canine urine are shown in Table 5. The control sample was the canine urine sample without pH adjustment.

Table 5. Extraction recoveries of select analytes in canine urine at various pH levels.

Sample Name	% recovery				
Matrix	**A**	**B**	**C**	**D**	**E**
ph 5	3	2	50	4	18
control ph 6.2	4	3	41	7	16
ph 6.5	3	6	52	10	21
ph 7.0	9	8	62	16	26
ph 7.5	15	15	54	30	28
ph 8.0	18	31	55	49	35
ph 8.25	10	19	69	29	33
ph 8.50	6	10	54	16	23
ph 8.75	8	27	50	35	28
Legend: A=Prednisone, B=Dexamethasone and Betamethasone, C= Dichlorisone acetate					
D= summation of recoveries for the dexamethasone betamethasone coelution and prednisolone					
E= Average recoveries for the dexamethasone betamethasone coelution and prednisolone					

Table 5. (continued)					
Sample Name	**% recovery**				
Matrix	**A**	**B**	**C**	**D**	**E**
ph 8.75	8	27	50	35	28
ph 9.0	17	36	57	53	37
ph 9.25	7	30	56	37	31
ph 9.50	0	26	34	26	20
ph 9.75	3	14	46	17	21
ph 10.0	8	20	42	28	23
ph 9 w h20	13	39	65	53	39
pH 9 w h20 and 7 ml. MTBE	11	31	64	42	35
ph 9.0 duplicate	9	32	62	41	34
9ml with 1 ml naso4 extracted 1 ml urine	8	36	67	44	37

Table 6 summarizes the average analyte recoveries and linear correlation coefficient (r^2) of the standards used in the standard curve for canine urine. The r^2 value range is 0-1 with a value of exactly 1 indicating an ideal linear relationship between the parameters in the calculation. The calibration curves in this study are calculated using a linear equation. The origin is ignored as opposed to being included in the calculation or using a forced zero equation. The calibration curves are not weighted so that each calibration point has the same amount of influence on the calibration curve calculation as any other point. The analyses summarized in this table were performed after the mass spectrometer had been optimized for each analyte. The analyses in table 5 were performed prior to mass spectrometer optimization.

Table 6. Average recoveries* and r^2 of standards 0.5- 200 ng/ml in stripped canine urine

	20-dihydro-6-alpha_		
triamicinalone	**methyprednisolone**	**hydrocortisone**	**fludrocortisone**
$R^2 = 0.9994$	$R^2 = 0.9975$	$R^2 = 1.0000$	$R^2 = 1.0000$
% recovery = 41.	% recovery= 57.	% recovery = 78.	% recovery = 66.
prednisone	**cortisone**	**prednisolone**	**6_alpha methyl prednisolone**
$R^2 = 0.9974$	$R^2 = 0.9998$	$R^2 = 0.9997$	$R^2 = 0.9984$
% recovery = 72.	% recovery = 62.	% recovery = 65.	% recovery = 73.

Table 6 continued.			
betamethasone	**flumethasone**	**dexamethasone**	**16 beta methylprednisolone**
$R^2 = 0.9922$	$R^2 = 0.9991$	$R^2 = 0.9994$	$R^2 = 0.9998$
% recovery= 70.	% recovery = 67.	% recovery = 88.	% recovery = 75.
corticosterone	**beclomethasone**	**flurandrenolide**	**fluocinilone acetamide**
$R^2 = 0.9999$	$R^2 = 1.0000$	$R^2 = 1.0000$	$R^2 = 0.9996$
% recovery= 82.	% recovery = 79.	% recovery = 75.	% recovery = 95.
triamicinolone_			
acetamide	**fluorometholone**	**paramethasone_ acetate**	**deoxycorticosterone**
$R^2 = 1.0000$	$R^2 = 0.9998$	$R^2 = 1.0000$	$R^2 = 0.9999$
% recovery= 94.	% recovery = 89.	% recovery = 105.	% recovery =100.
medrysone	**flucinonide**	**amcinonide**	**clobetasone_butyrate**
$R^2 = 0.9999$	$R^2 = 0.9999$	$R^2 = 0.9965$	$R^2 = 0.9974$
%recovery =104.	% recovery = 98.	% recovery =103.	% recovery= 112.
* Recoveries are +/- 5%.			

Equine plasma was also optimized for pH. In this later experiment, all 24 analytes were used to obtain the optimal pH using a standard to simulate 100 ng/ml of each analyte. The results are included in table 7.

Table 7. Extraction recoveries of select analytes in stripped equine plasma at varying pH levels at 100 ng/ml.

pH	triamicinalone	20-dihydro-6-alpha_methyprednisolone	hydrocortisone	prednisolone
7.5	151	71	15	30
8.0	130	83	91	81
8.5	108	67	99	85
9.0	138	69	95	85
9.5	90	78	89	79
pH	fludrocortisone	prednisone	cortisone	6_alpha_methylprednisolone
7.5	84	53	69	83
8.0	81	79	89	88
8.5	90	38	91	83
9.0	84	81	79	92
9.5	81	40	67	83

Table 7 (continued)

pH	betamethasone	flumethasone	dexamethasone	16beta-methylprednisolone
7.5	103	100	85	83
8.0	88	105	85	85
8.5	100	106	82	80
9.0	109	99	90	85
9.5	102	98	88	85
pH	corticosterone	beclomethasone	flurandrenolide	fluocinilone_acetamide
7.5	82	97	90	107
8.0	85	89	96	104
8.5	88	98	90	97
9.0	89	87	98	109
9.5	85	77	102	111
pH	fluorometholone	triamicinolone_acetamide	paramethasone_acetate	deoxycorticosterone
7.5	103	99	92	115
8.0	102	102	99	122
8.5	95	95	99	103
9.0	106	102	100	121
9.5	110	105	90	100
pH	medrysone	flucinonide	dichlorisone_acetate	amcinonide
7.5	136	124	100	68
8.0	128	107	119	123
8.5	106	97	103	123
9.0	124	110	93	141
9.5	101	88	106	85
pH	clobetasone_butyrate			
7.5	71			
8.0	144			
8.5	124			
9.0	138			
9.5	87			

Figures 10-12 on pages 39-40 show the recoveries of the glucocorticoids studied in this method. All of the percent recoveries represented in the figures were from samples spiked with 100 ng/ml of the analytes. These samples were extracted and analyzed in the same run. The compounds are separated into three graphs to make the data more legible. The first set of analytes is the set of analytes with the shortest retention times. The second set of compounds contains the next set of compounds based on retention time. The third set contains the compounds with the longest retention times.

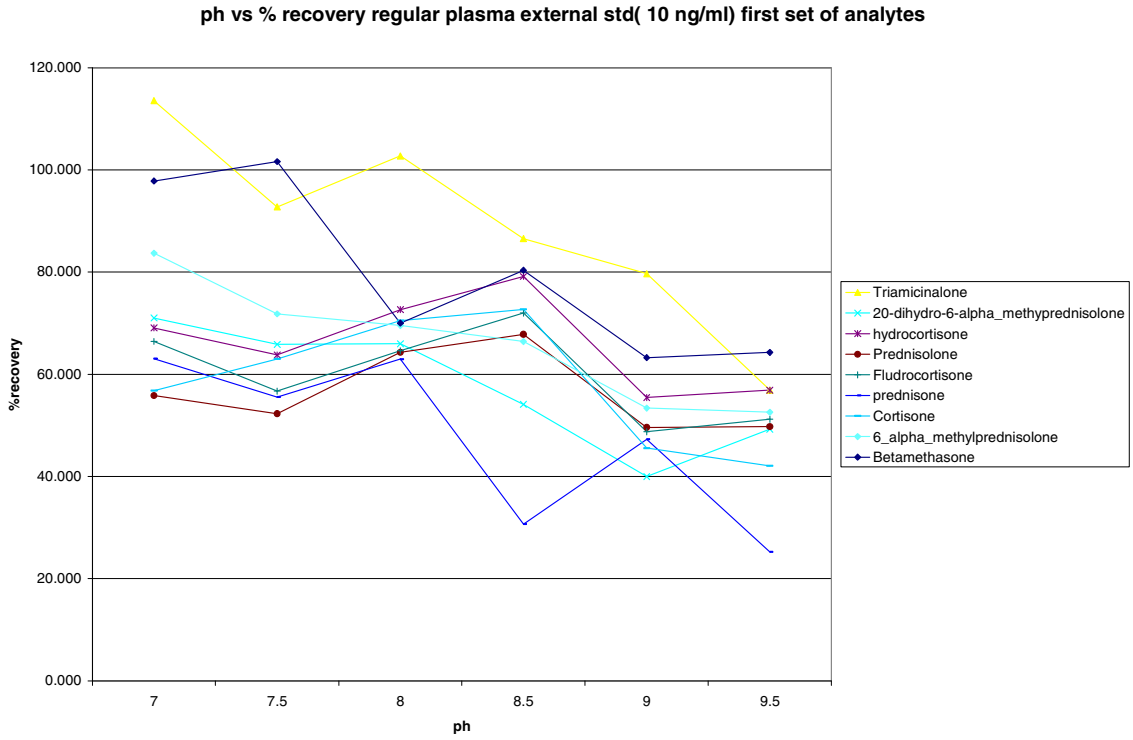

Figure 10. pH vs. drug recovery for first set of analytes (100 ng/ml) in equine plasma.

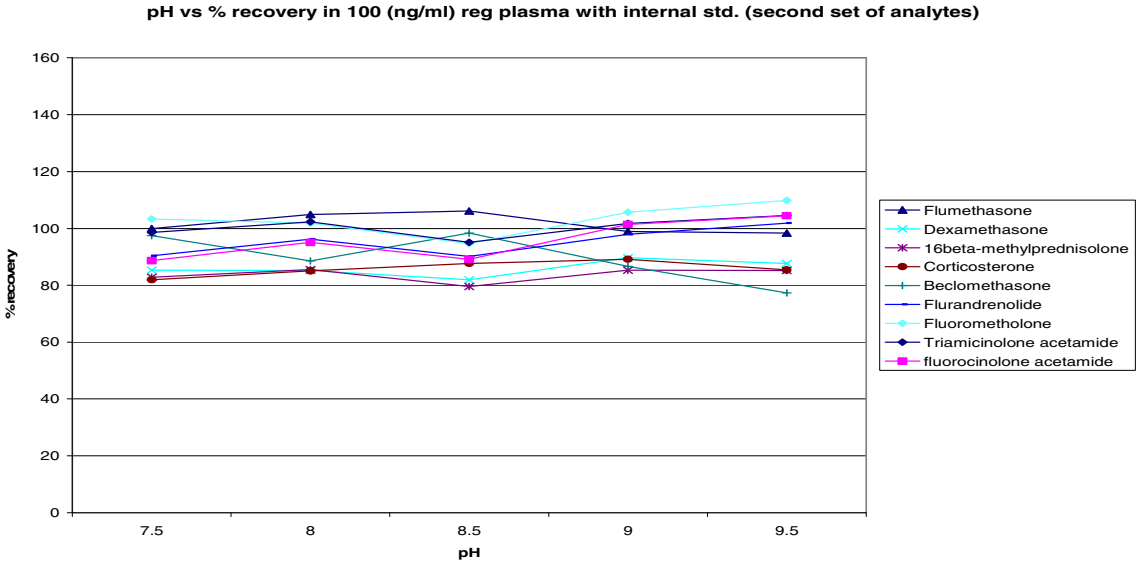

Figure 11. pH vs. drug recovery for second set of analytes (100 ng/ml) in equine plasma.

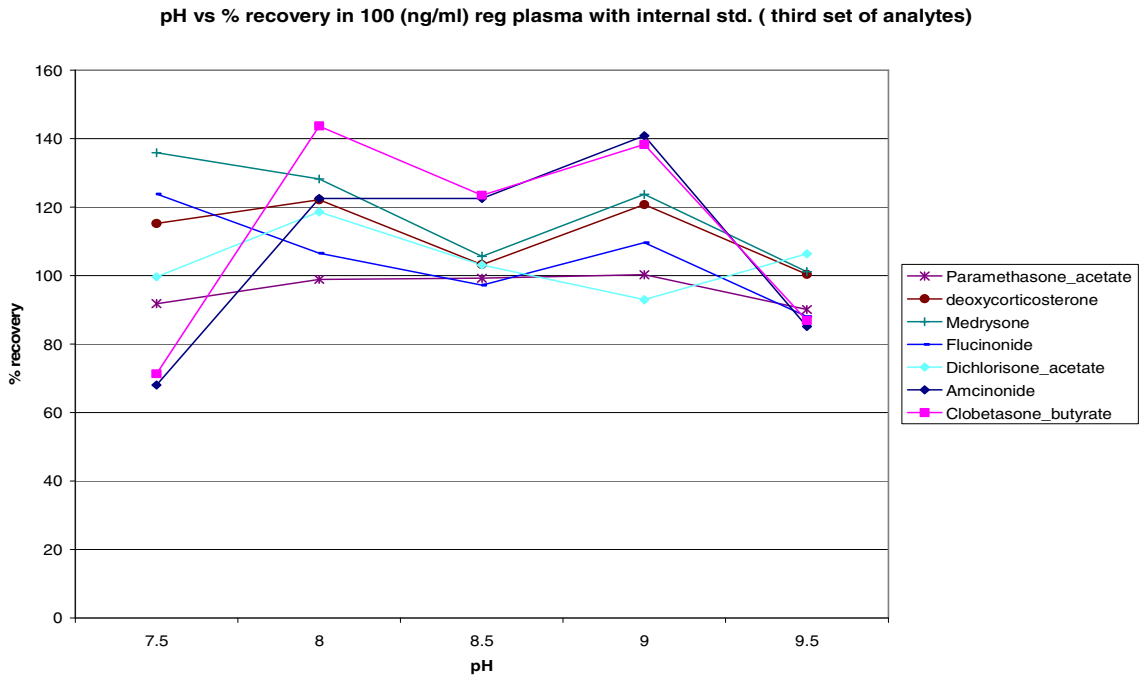

Figure 12. pH vs. drug recovery for third set of analytes (100 ng/ml) in equine plasma.

An example of a calibration curve is presented in figure 13. This particular calibration curve was produced using dexamethasone standards made from stripped canine urine.

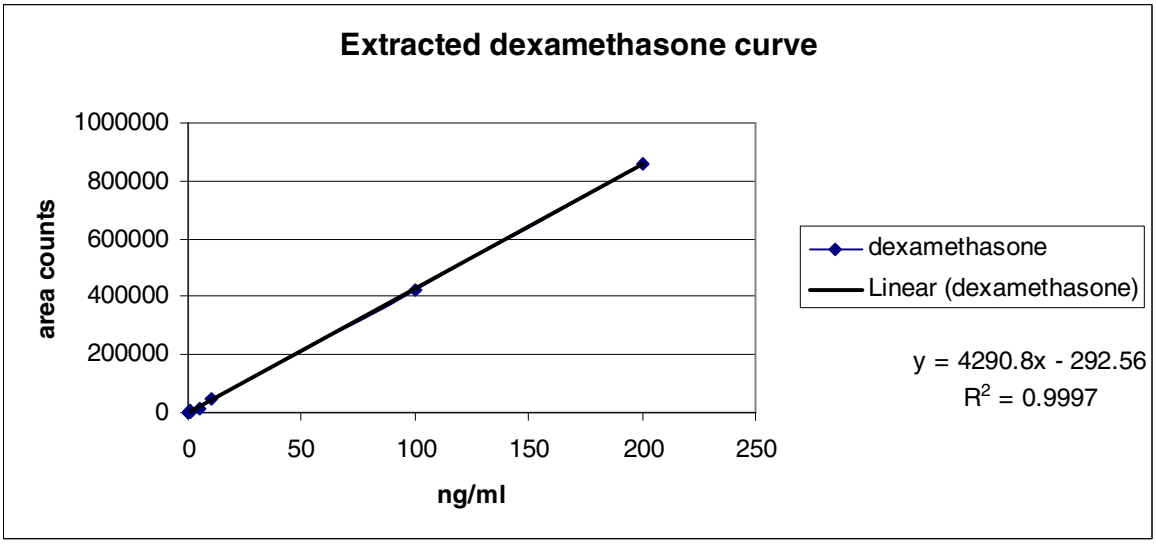

Figure 13. Extracted dexamethasone calibration curve in canine urine.

Each analyte in a study should produce a calibration curve similar to figure 13. Table 8 contains the volume and pH values of canine urine in the dexamethasone elimination study.

Table 8. Canine urine volume and pH value in dexamethasone study.

Hours Post Dose	Volume of Urine collected (ml)	initial pH	Final adjusted pH
0	20	5.83	9.05
2	54	6.14	9.03
4	17	8.47	9.04
8	128	7.9	9.08
24	104	6.53	9.07
36	30	6.06	9.02
48	78	5.74	9.06
60	36	6.39	9.08
72	66	6.8	9.06
84	77	7.31	9.05
96	18	5.95	9.05

The results in table 9 on page 42 are from three sets of extracted canine urine samples from the dexamethasone elimination study. The first two data sets were re-extracted from urines that had the sample pH adjusted to 9.0 +/- 0.1 six weeks prior to the extraction. These samples were originally extracted shortly after the pH adjustment but due to instrument malfunction on a few runs, the volume of several extracted samples was depleted. The third set of samples were stored at the original pH, extracted after pH adjustment, and analyzed after the instrument had been repaired. There is no result for T=0 for the third set as the original urine sample had been depleted.

The first two sets were extracted in the same run as the third set. It is speculated that sample degradation occurred due to storage at pH 9. Degradation studies were not performed on the samples. Extracts (ex) 32 and 33 were extracted using the current in house method using 9 ml of sample and extracted with ethyl acetate. The concentration was corrected to compare to using 1 ml of sample.

Table 9. Dexamethasone in canine urine results

Dexamethasone	Equation Y = 0.00440369+0.0100207*X R^2 = 0.9996			
Sample Name	Calc Amount	Dexamethasone Area	internal standard Area	RT(min.)
ex 1 blank 11-30 stripped urine	N/F		641384.	
ex 2 0.1ng/ml mix 24 11-30 stripped urine	< 1	538.	636221.	8.42
ex 3 0.2 ng/ml mix 24 11-30 stripped urine	< 1	1195.	606411.	8.44
ex 4 .5 ng/ml mix 24 11-30 stripped urine	< 1	3272.	563529.	8.54
ex 5 1.0 ng/ml mix 24 11-30 stripped urine	< 1	7404.	603559.	8.58
ex 6 5.0 ng/ml mix 24 11-30 stripped urine	5.	22767.	413061.	8.42
ex 7 10 ng/ml mix 24 11-30 stripped urine	10.	35660.	352018.	8.46
ex 8 100 ng/ml mix 24 11-30 stripped urine	103.	422065.	405430.	8.45
ex 9 200 ng/ml mix 24 11-30 stripped urine	198.	861041.	432372.	8.46
ex 10 t=0 urine first set	N/F		422180.	
ex 11 t=2 hr	101.	155045.	152227.	8.44
ex 12 t=4hr	46.	180638.	387321.	8.46
ex 13 t=8hr	40.	146295.	360749.	8.46
ex 14 t=24	15.	71385.	471358.	8.48
ex 15 t=36	4.	20248.	411233.	8.46
ex 16 t=48	< 1	3936.	376144.	8.46
ex 17 t=60	< 1	1407.	365458.	8.44
ex 18 t=72	< 1	415.	403574.	8.50
ex 19 t = 84	< 1	535.	408240.	8.46
ex 20 t =96	< 1	196.	384888.	8.46
ex 21 t=0 second set	N/F		466435.	
ex 22 t=2	97.	251434.	256773.	8.44
ex 23 t=4	50.	200074.	396759.	8.44
ex 24 t=8	51	122942.	240484.	8.44
ex 25 t=24	16.	70758.	443015.	8.46
ex 26 t=36	5.	20003.	381294.	8.48
ex 27 t=48	< 1	4251.	403897.	8.42
ex 28 t=60	< 1	615.	320896.	8.48
ex 29 t=72	< 1	1038.	434722.	8.38
ex 30 t=84	< 1	553.	456711.	8.44
ex 31 t=96	< 1	318.	504732.	8.46
ex 32 steroid ex t=8	43.	1034232.	264576.	8.44
ex 33 steroid ex t=48	< 1	13590.	132916.	8.43
ex 34 ph t=2 third set	129.	275360.	212018.	8.46
ex 35 ph t=4	55.	247675.	442203.	8.46
ex 36 ph t=8	58.	238456.	410197.	8.44
ex 37 ph t=24	23.	89049.	384433.	8.46
ex 38 ph t=36	6.	19938.	305767.	8.46
ex 39 ph t=48	< 1	5874.	411052.	8.48
ex 40 ph t=60	< 1	1280.	305325.	8.48
ex 41 ph t=72	< 1	1109.	369995.	8.48
ex 42 ph t=84	< 1	443.	405181.	8.46
ex 43 ph t=96	< 1	309.	536835.	8.54
ex 44 blank	N/F		490099.	

Average internal standard area= 406577.

Note RT = retention time for the analyte, not the internal standard.

Average dexamethasone retention time (min.) = 8.46. Standard deviation= 0.034

NF= Peak Not Found

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The area counts for dexamethasone in canine urine are plotted against the hours post dose in figure 14.

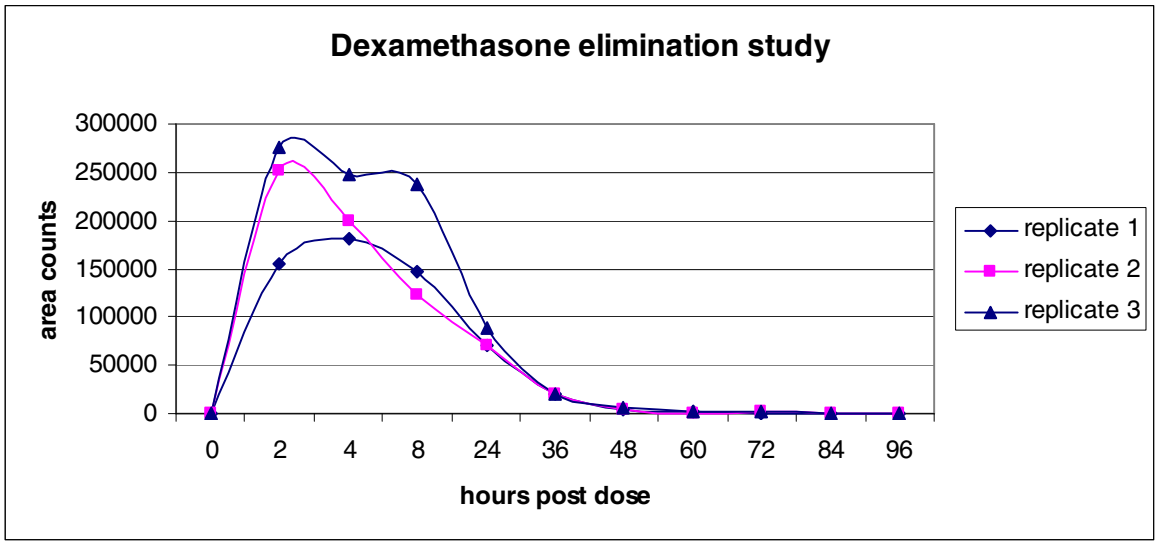

Figure 14. Dexamethasone elimination study area counts vs. hours post dose

Figure 15 illustrates the area counts of the latter part of the elimination study on a smaller scale.

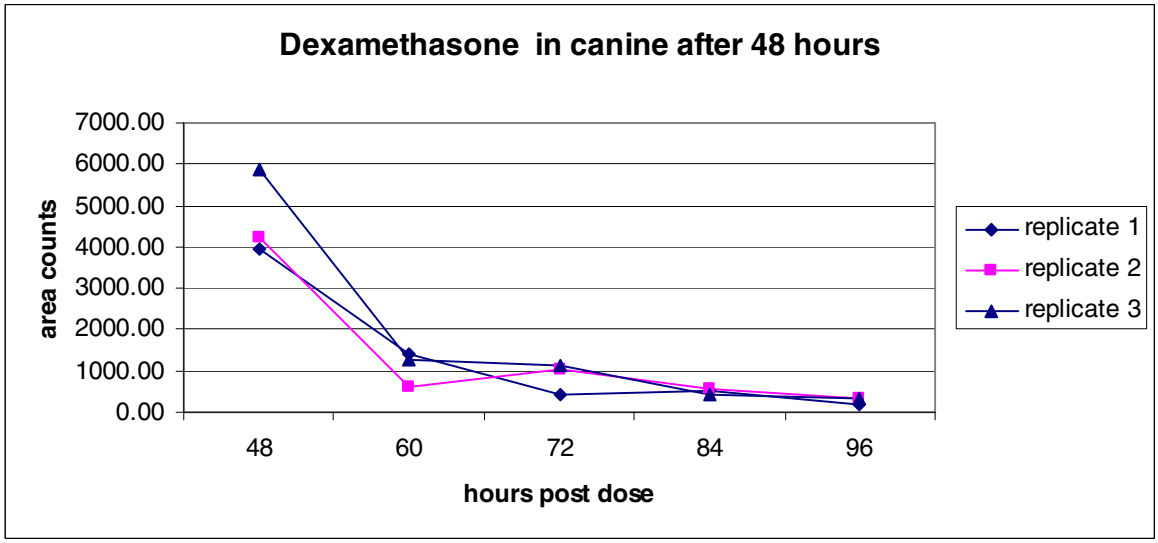

Figure 15. Dexamethasone elimination study results last 48 hours on smaller scale

Figure 16 plots dexamethasone concentration against hours post dose.

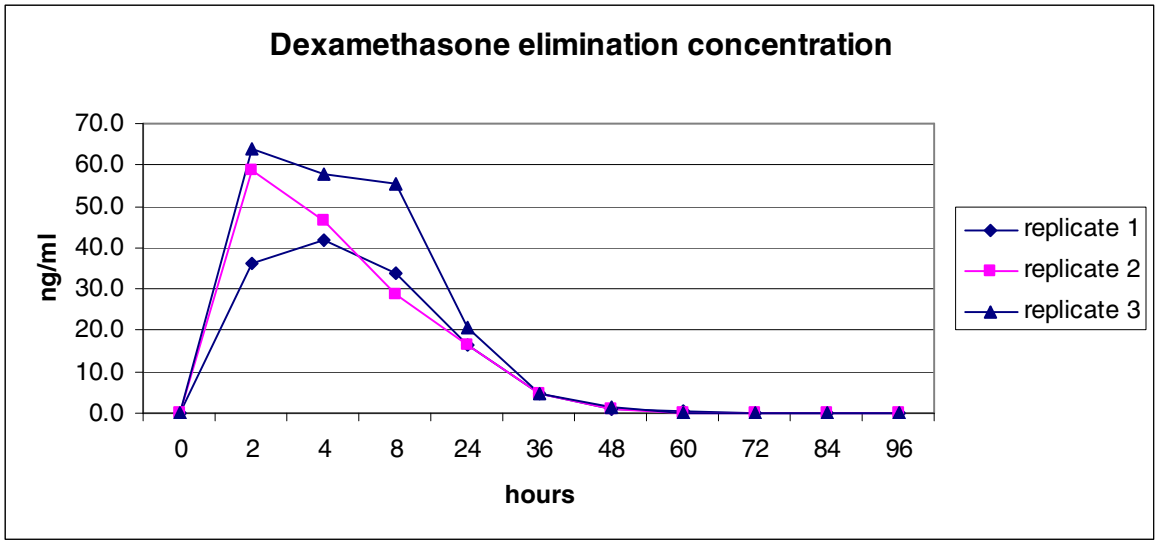

Figure 16. Dexamethasone elimination study concentrations vs. hours post dose

Tables 10-12 summarize the hydrocortisone, cortisone and dexamethasone concentrations in the dexamethasone equine urine elimination study.

Table 10. Hydrocortisone in equine urine dexamethasone study

Hydrocortisone				
Y = 0.00898139+0.0256703*X	R^2= 1.000			
Sample Name	Calculated concentration(ng/ml)	Analyte Area	Internal standard Area	RT(min.)
solvent		N/F		??
blank stripped urine 1-2-07	< 1	3104.	503282.	5.11
0.1 ng/ml mix 24 1-2 stripped urine	< 1	4464.	543407.	5.16
0.2 ng/ml mix 24 1-2 stripped urine	< 1	4413.	440830.	5.22
0.5 ng/ml mix 24 1-2 stripped urine	< 1	9059.	488035.	5.13
1.0 ng/ml mix 24 1-2 stripped urine	1.	13639.	382978.	5.16
5.0 ng/ml mix 24 1-2 stripped urine	5.	65724.	441449.	5.16
10.0 ng/ml mix 24 1-2 stripped urine	10.	114183.	432052.	5.15
100 ng/ml mix 24 1-2 stripped urine	100.	1162568.	451378.	5.17
0 hr a63 1-2	15.	185016.	462209.	5.16
2hr a63 1-2	17.	196463.	433068.	5.16
4hr a63 1-2	8.	94020.	466255.	5.14
8 hr a63 1-2	3.	37645.	474876.	5.13
24 hr a63 1-2	< 1	11144.	466664.	5.16
48 hr a63 1-2	21.	258915.	481614.	5.20
0 hr a63 1-2 dup	17.	187814.	417141.	5.22
2hr a63 1-2 dup	14.	173039.	449517.	5.27

Table 10 (continued)				
Sample Name	Calculated concentration(ng/ml)	Analyte Area	Internal standard Area	RT(min.)
4hr a63 1-2 dup	11.	116098.	411359.	5.23
8 hr a63 1-2 dup	3.	36649.	428728.	5.20
24 hr a63 1-2 dup	< 1	10805.	410182.	5.15
48 hr a63 1-2 dup	25.	277768.	427757.	5.15

Table 11. Cortisone in equine urine dexamethasone study

Cortisone				
Y = 0.00721512+0.0090433*X	R^2= 0.9998			
Sample Name	Calculated concentration(ng/ml)	Analyte Area	Internal standard Area	RT(min.)
solvent	N/F		??	
blank stripped urine 1-2-07	N/F		503282.	
0.1 ng/ml mix 24 1-2 stripped urine	N/F		543407.	
0.2 ng/ml mix 24 1-2 stripped urine	N/F		440830.	
0.5 ng/ml mix 24 1-2 stripped urine	< 1	3238.	488035.	5.73
1.0 ng/ml mix 24 1-2 stripped urine	< 1	4862.	382978.	5.73
5.0 ng/ml mix 24 1-2 stripped urine	5.	24366.	441449.	5.72
10.0 ng/ml mix 24 1-2 stripped urine	11.	45058.	432052.	5.72
100 ng/ml mix 24 1-2 stripped urine	100.	411117.	451378.	5.71
0 hr a63 1-2	11.	51214.	462209.	5.77
2hr a63 1-2	14	56510.	433068.	5.70
4hr a63 1-2	6.	29442.	466255.	5.72
8 hr a63 1-2	3.	14591.	474876.	5.72
24 hr a63 1-2	N/F	10631.	466664.	
48 hr a63 1-2	17	76543.	481614.	5.75
0 hr a63 1-2 dup	13.	52635.	417141.	5.79
2hr a63 1-2 dup	11.	46277.	449517.	5.84
4hr a63 1-2 dup	9.	38197.	411359.	5.79
8 hr a63 1-2 dup	2.	12556.	428728.	5.77
24 hr a63 1-2 dup	2.	9880.	410182.	5.72
48 hr a63 1-2 dup	22.	89330.	427757.	5.73

Table 12. Dexamethasone in equine urine study

Dexamethasone				
Y = 0.00453013+0.00854919*X	R^2 = 0.9994			
Sample Name	Calculated concentration(ng/ml)	Analyte Area	Internal standard Area	RT(min.)
solvent	N/A	18.	??	8.42
blank stripped urine 1-2-07	N/F		503282.	
0.1 ng/ml mix 24 1-2 stripped urine	< 1	256.	543407.	8.38
0.2 ng/ml mix 24 1-2 stripped urine	< 1	449.	440830.	8.38
0.5 ng/ml mix 24 1-2 stripped urine	< 1	1849.	488035.	8.42
1.0 ng/ml mix 24 1-2 stripped urine	< 1	3485.	382978.	8.38
5.0 ng/ml mix 24 1-2 stripped urine	6.	22845.	441449.	8.38
10.0 ng/ml mix 24 1-2 stripped urine	12.	45996.	432052.	8.40

Table 12 continued.				
100 ng/ml mix 24 1-2 stripped urine	100.	387130.	451378.	8.41
0 hr a63 1-2	N/F		462209.	
2hr a63 1-2	18.	66786.	433068.	8.40
4hr a63 1-2	30.	120077.	466255.	8.40
8 hr a63 1-2	13.	53058.	474876.	8.38
24 hr a63 1-2	1.	7385.	466664.	8.42
48 hr a63 1-2	< 1	146.	481614.	8.40
0 hr a63 1-2 dup	N/F		417141.	
2hr a63 1-2 dup	14.	57472.	449517.	8.52
4hr a63 1-2 dup	37.	132964.	411359.	8.50
8 hr a63 1-2 dup	16.	59182.	428728.	8.42
24 hr a63 1-2 dup	1.	6184.	410182.	8.42
48 hr a63 1-2 dup	<1	123.	427757.	8.44

Figures 17 -19 are graphical depictions of the data presented in tables 10-12.

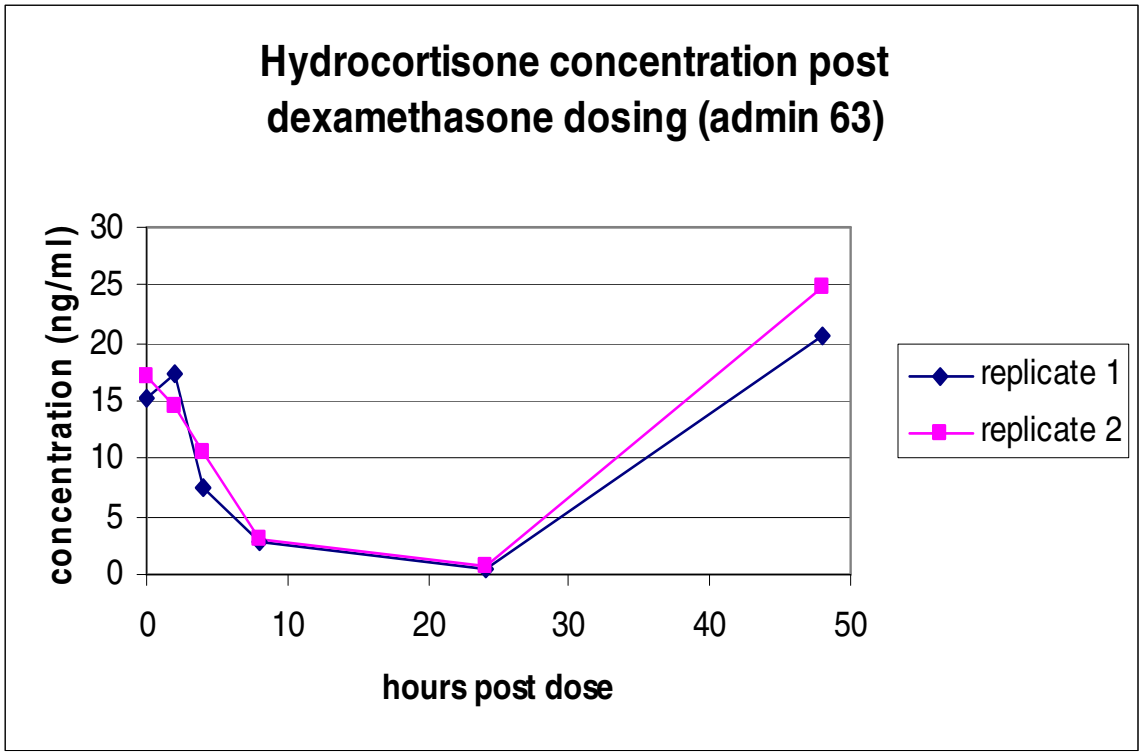

Figure 17. Hydrocortisone concentration in equine dexamethasone study.

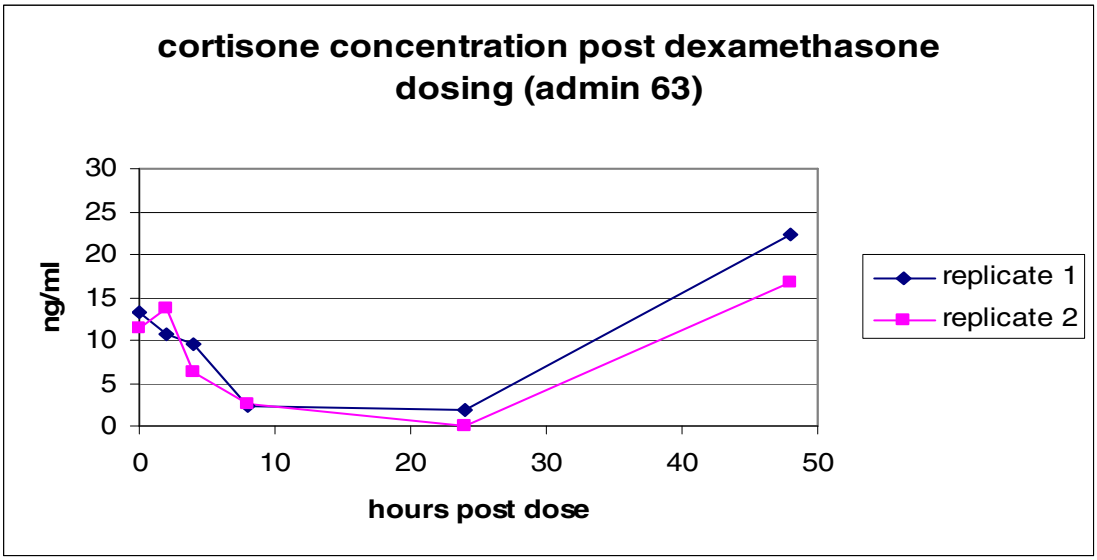

Figure 18. Cortisone concentration in dexamethasone equine urine study

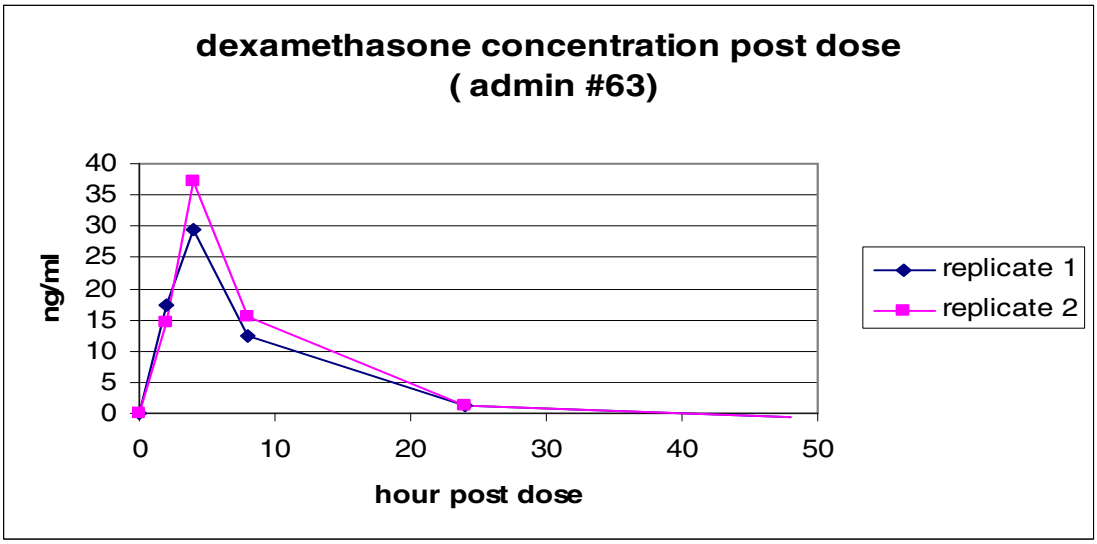

Figure 19. Dexamethasone concentration in dexamethasone equine urine study.

The increased dexamethasone concentration in figure 20 on page 48 corresponds to decreased hydrocortisone and cortisone concentrations. The concentrations of these

compounds return to a level higher than the original baseline values after the dexamethasone has been eliminated. This effect was published by Toutain in 1984.

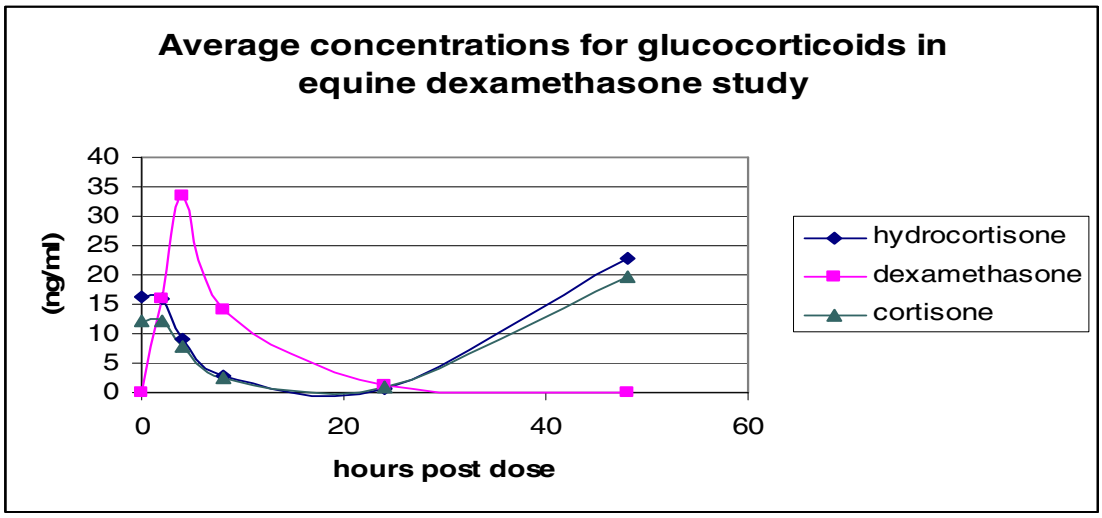

Figure 20. Comparison of dexamethasone concentration to cortisone and hydrocortisone

Table 13 contains the data used for the calibration curves for fludrocortisone, hydrocortisone and cortisone in the fludrocortisone elimination study in canine urine.

Table 13. Calibration curve in fludrocortisone canine urine study

Fludrocortisone elimination study (standards are in stripped urine matrix)						
fludrocortisone			**hydrocortisone**		**Cortisone**	
R^2 = 0.9979			R^2 = 0.9975		R^2 = 0.9941	
Sample Name	**Calc Conc.**	**Area**	**Calc Conc.**	**Area**	**Calc Conc.**	**Area**
blank	N/F		N/F		N/F	
0.1 ng/ml	1.	270.	N/F		N/F	
0.2 ng/ml	1.	412.	N/F		N/F	
0.5 ng/ml	1.	1973.	N/F		3.	2465.
1.0 ng/ml	3.	3856.	3.	7362.	4.	3115.
5.0 ng/ml	6.	27674.	7.	58964.	7.	21355.
10.0 ng/ml	9.	43537.	10.	91594.	9.	30202.
100 ng/ml	92.	554509.	93.	1140799.	88.	395540.
200 ng/ml	204.	1257801.	204.	2589343.	206.	959560.

Tables 14 -16 on page 49 contain data from the fludrocortisone elimination study in canine urine. The different run numbers are replicates of the same samples extracted and analyzed at the same time.

Table 14. Fludrocortisone for runs 1-3 in canine urine

Fludrocortisone (ng/ml)			
hours post dose	run 1	run 2	run 3
0	N/F	N/F	N/F
2	3.	3.	4.
4	7.	8.	8.
8	5.	6.	5.
24	N/F	1.	N/F
48	N/F	N/F	N/F
72	N/F	N/F	N/F
96	N/F	N/F	N/F

Table 15. Hydrocortisone in fludrocortisone canine urine study

Hydrocortisone (ng/ml)			
hours post dose	run 1	run 2	run 3
0	9.	9.	8.
2	6.	7.	12.
4	5.	6.	6.
8	3.	4.	3.
24	7.	7.	7.
48	9.	9.	11.
72	13.	12.	13.
96	7.	7.	8.

Table 16. Cortisone in fludrocortisone canine urine study

Cortisone (ng/ml)			
hours post dose	run 1	run 2	run 3
0	13.	14.	13.
2	9.	10.	16.
4	7.	8.	7.
8	5.	5.	5.
24	8.	8.	8.
48	10.	10.	12.
72	13.	12.	13.
96	8.	8.	9.

Fludrocortisone metabolite

After the initial LC/MSn run of the fludrocortisone samples, the samples were analyzed for metabolites. One metabolite was discovered at a retention time of 3.73 minutes and m/z 384. The base peak for this compound is m/z 267.25. Other ions used for qualification and quantification were m/z 249 and 309. The chromatograph and mass spectra of the fludrocortisone metabolite are in figure 21 on page 50.

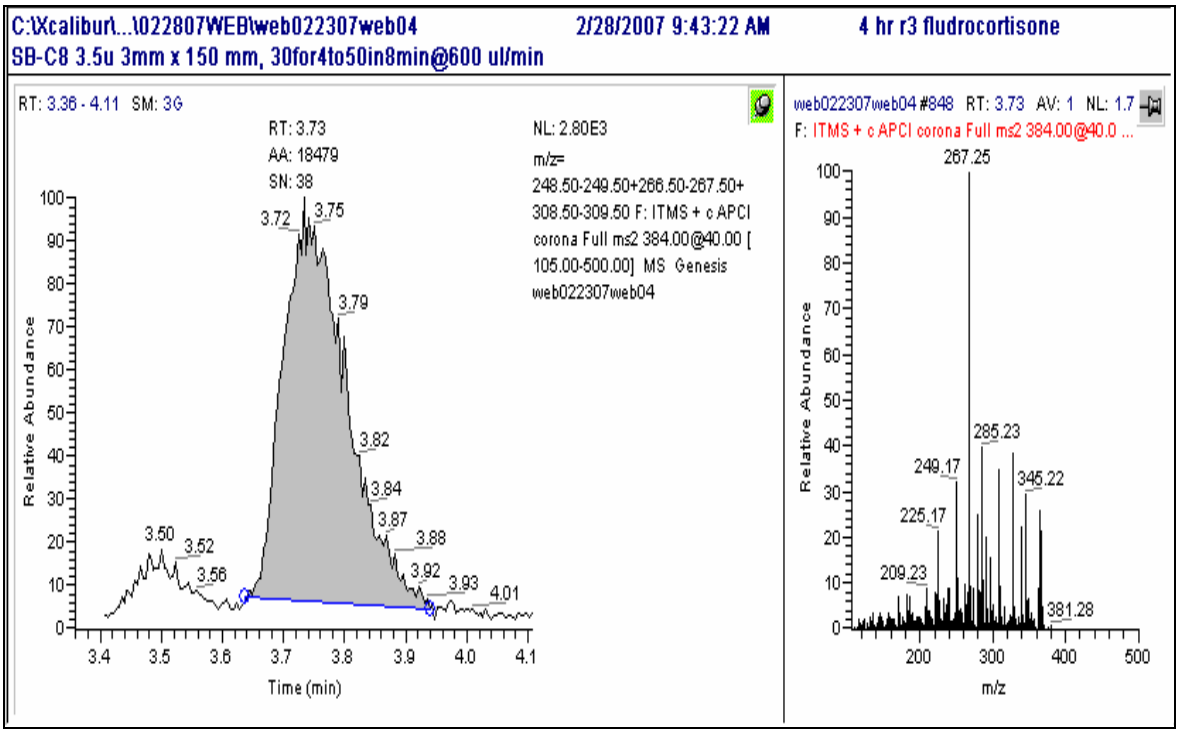

Figure 21. Chromatograph and mass spectra of fludrocortisone metabolite

Table 17 illustrates the fludrocortisone metabolite areas and signal to noise (S/N) ratio in the canine urine study

Table 17. Fludrocortisone metabolite areas in canine urine

Sample Name	Area	S/N
solvent	N/F	
0 hr	N/F	
2 hr	4323.	6.67
4 hr	18479.	37.94
8 hr	19858.	32.23
24 hr	N/F	

Figure 22 on page 51 is a side-by-side comparison of the chromatographs of fludrocortisone and its metabolite.

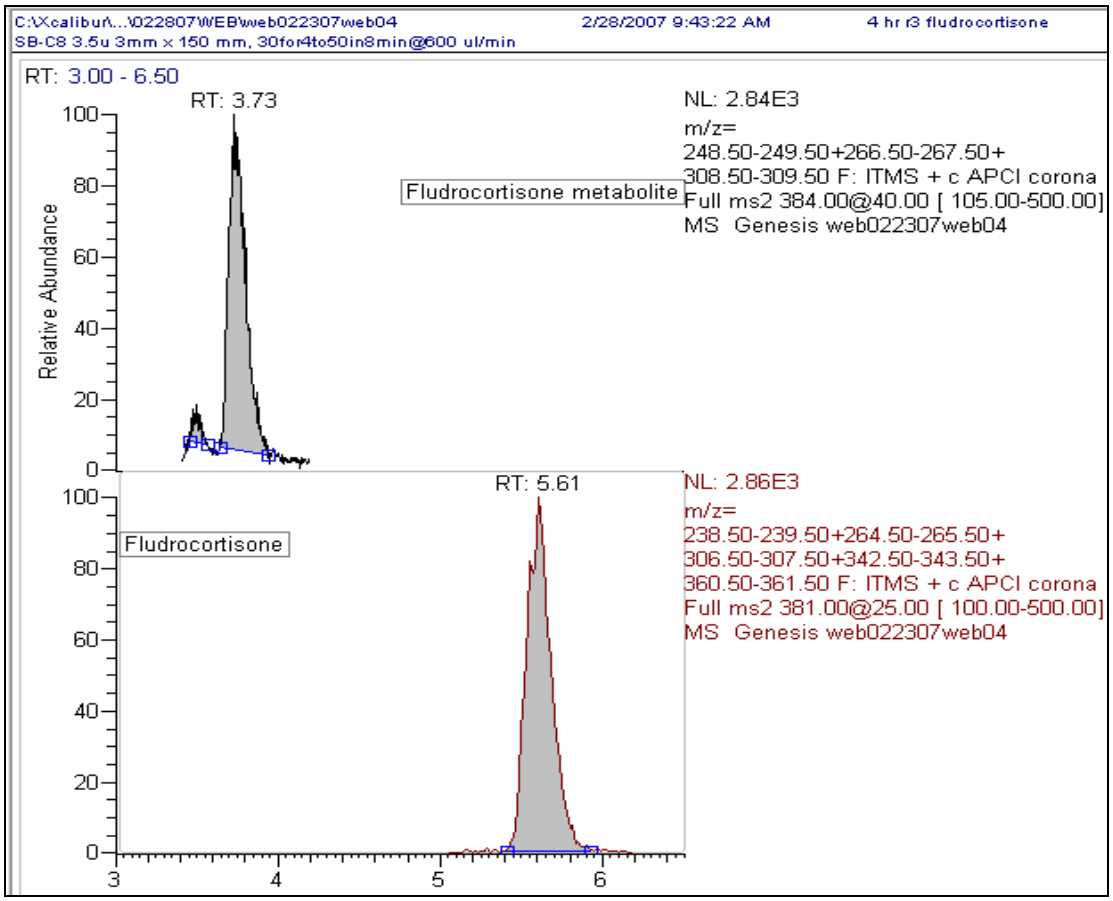

Figure 22. Chromatographs of fludrocortisone and metabolite

Figure 23 is the chemical structure of fludrocortisone.

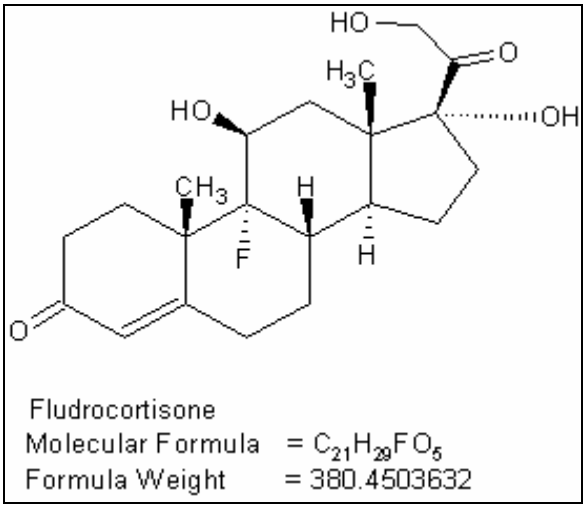

Figure 23. Chemical structure of fludrocortisone (Merck, 2001)

The structure in figure 24 was proposed after examination of mass spectra and possible metabolite routes described in Gower (1984). The name for the proposed fludrocortisone metabolite in figure 24 is 9-alpha -fluoro-3, 11-beta, 17-alpha, 20, 21 – pentahydroxy- 4- pregnene.

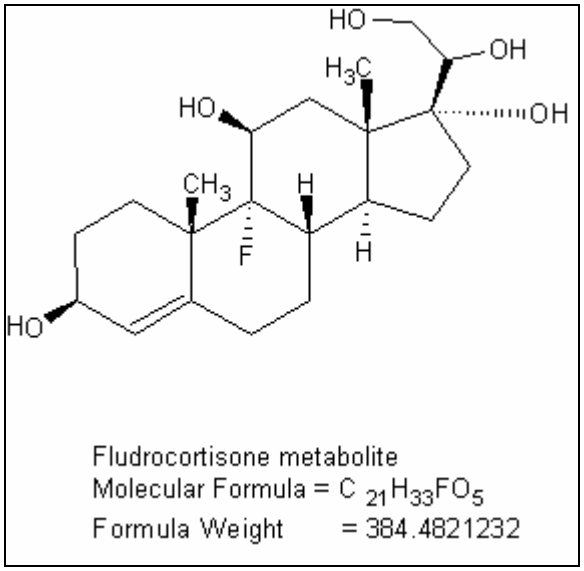

Figure 24. Proposed chemical structure of fludrocortisone metabolite

CHAPTER 5. SUMMARY AND INTERPRETATION

This research presents refined extraction and analytical techniques to identify and quantify 24 corticosteroids in canine urine and equine plasma and urine. The pure single compound analyte standards were injected into the mass spectrometer to optimize mass spectrometer operating parameters for the compounds. These parameters were used to determine the ions selected for identification and quantification.

The LC gradient was optimized to prevent co-elution of chromatographic peaks. Using this gradient, retention times for the analytes were determined. The combination of retention times and mass spectra ions enabled a LC/MSn analytical method to be created to determine the efficiency of the extraction process.

A liquid/liquid extraction process was used with 1 ml of sample and MTBE as the extractant. The initial pH study in canine urine provided evidence that the addition of 1 ml of type 1 water with a sample pH of 9 +/- 0.1 pH units produced the best average yield for all of the analytes compared to extractions using other pH values. Average recoveries for standards from 0.5 - 200 ng/ml ranged from 41% for triamicinolone to 112% for clobetasone butyrate. With the exception of triamacinolone and 20-dihydro-6-alpha methylprednisolone, recoveries were 60 % or higher. The lowest correlation coefficient for the 24 analyte standard curves was 0.9922.

The equine plasma pH study was performed using a pH range from 7.0 to 9.5. The results using a sample pH of 8.0 +/-0.1 pH unit yielded optimal glucocorticoid recoveries. Average recoveries for standards ranged from 63% for prednisone to 114% for clobetasone butyrate. The lowest r^2 in the plasma samples was 0.9709.

The dexamethasone elimination study in the canine demonstrated the practical use of this method. The calibration was performed using a stripped urine matrix spiked with the analytes. The study showed detection of dexamethasone to 48 hours post dose. This experiment also produced recoveries similar to the current in-house method. The method used in this research requires 1 ml of sample instead of 9 ml required for the in-house extraction procedure. This small amount of required sample allows testing of spikes and

duplicates using much less sample than required by other methods. The small sample size is also useful if the collected sample has low volume or if other analytical tests are required for the sample.

The study of dexamethasone in equine urine produced good recoveries for dexamethasone. The results of this study also demonstrated similar hydrocortisone suppression as had been cited from Toutain (1984). This study detected dexamethasone to 24 hours post dose. The hydrocortisone and cortisone concentrations remained depressed into the 24-48 hour time period. This study used a dexamethasone dose of 33 ug/kg. The Toutain study used 50 ug/kg, which explains the increased length of cortisol suppression in the Toutain study compared to this study.

The canine fludrocortisone study also produced hydrocortisone suppression. The hydrocortisone concentration in the urine returned to time = 0 concentrations after 24-48 hours. The cortisone concentration returned to time = 0 concentration between 48-72 hours.

All three extraction runs detected fludrocortisone for 2, 4 and 8 hours post dose. One extraction run detected fludrocortisone at 24 hours post dose. The fludrocortisone study also produced a metabolite, which was detected in the 2, 4 and 8-hour post dose samples. The metabolite area counts had approximately the same area counts as the peaks for the unmetabolized eliminated fludrocortisone. The retention time of the metabolite is 3.72 minutes compared to 5.61 minutes for fludrocortisone. This metabolite had m/z of 384. The molecular formula for the metabolite named 9-alpha -fluoro-3, 11-beta, 17-alpha, 20, 21 – pentahydroxy- 4- pregnene is $C_{21}H_{33}FO_5$.

CHAPTER 6. CONCLUSIONS

This report presents evidence of the effectiveness of this method for the analysis of glucocorticoids in different matrices. The majority of the work was on the analysis of glucocorticoids in canine urine due to subject availability and ease of sample collection. Recoveries of glucocorticoids using different extraction pHs were tested to obtain an optimal extraction pH of 9 for urine. Studies were also presented which varied the solvent to sample ratio from the final 5:1 ratio.

Studies also were performed on equine plasma samples. The optimal extraction pH was discovered using a pH range similar to that used for canine urine. The optimal pH for equine plasma for this technique is pH 8.

The glucocorticoid elimination studies in the canine and equine test subjects demonstrated a drug elimination pattern similar to what had been previously observed in the literature. These studies also produced cortisone and hydrocortisone suppression which had been demonstrated previously (Toutain, 1984). The fludrocortisone study produced an elimination pattern similar to other glucocorticoid studies and a metabolite, which had not been discussed in previous canine urine studies.

The extraction and analytical techniques presented in this research requires only a small amount of sample to be as effective as other methods requiring larger amounts of samples. The technique identifies and quantifies 24 glucocorticoids in a single analytical run in plasma and urine. This analysis should decrease cost of analysis and increase laboratory throughput due to time savings.

APPENDIX: COMPOUND STRUCTURES

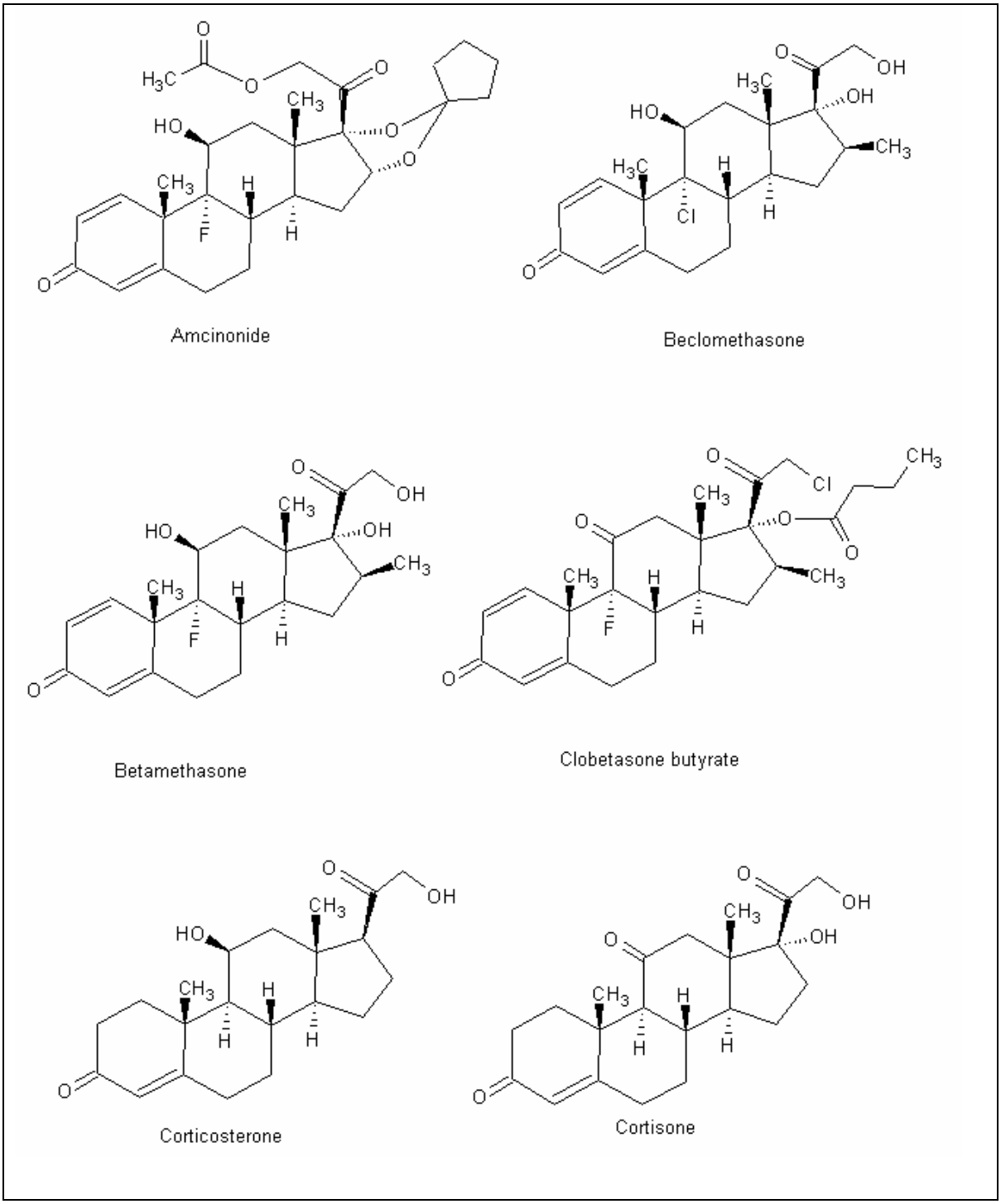

Figure 25. Structures of amcinonide through cortisone (Merck, 2001)

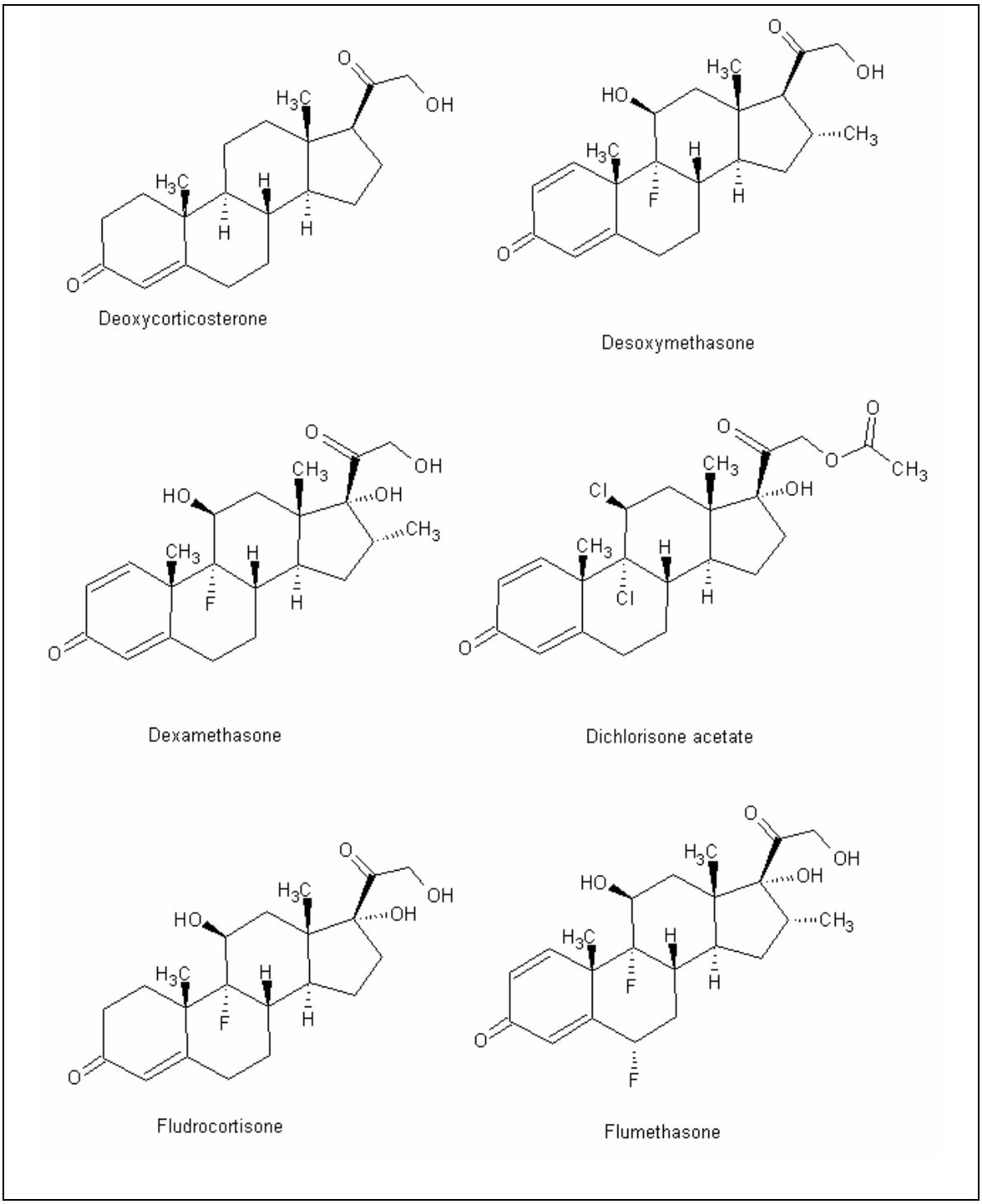

Figure 26. Structures of deoxycorticosterone to flumethasone (Merck, 2001)

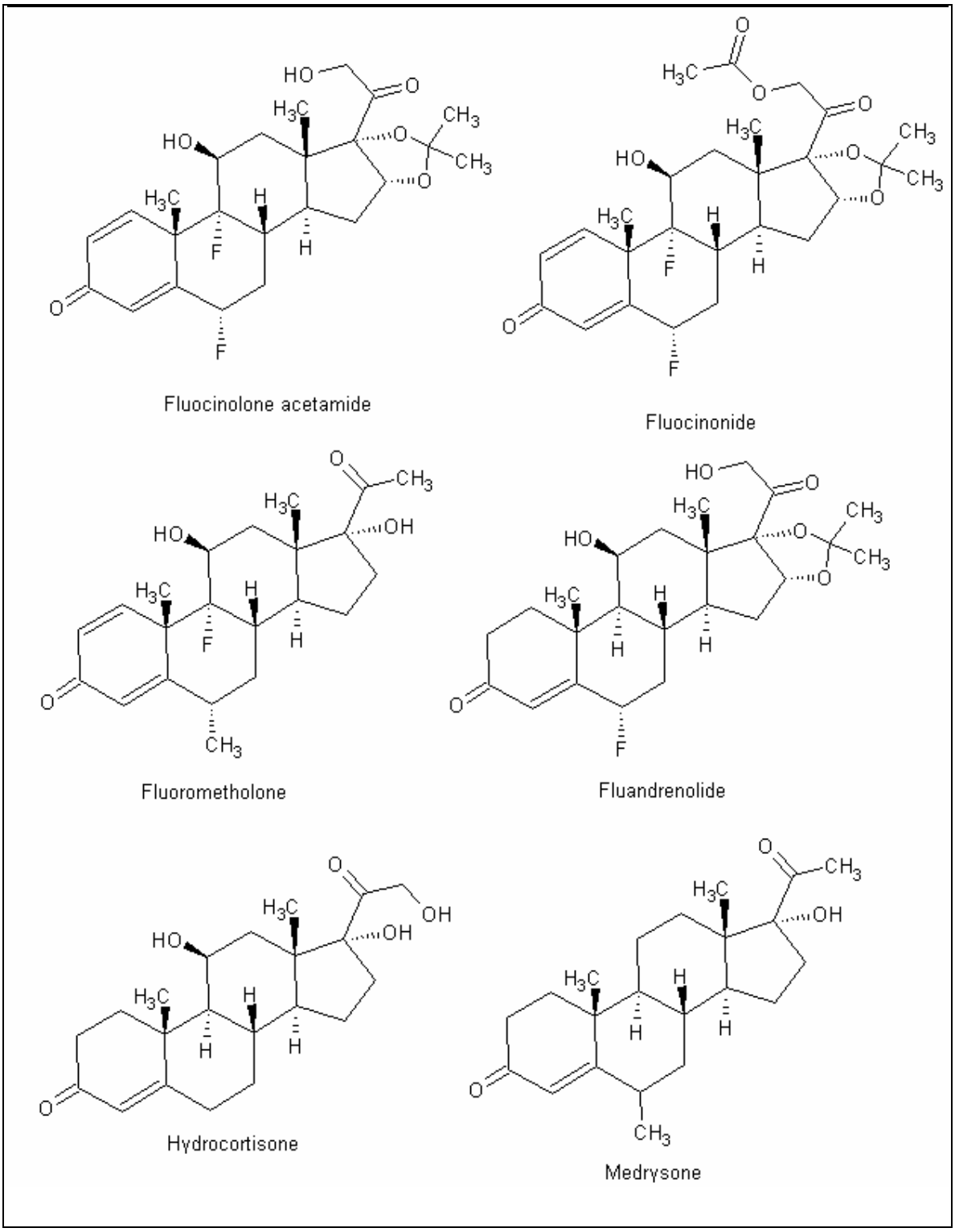

Figure 27. Structures of fluocinolone acetamide to medrysone (Merck, 2001)

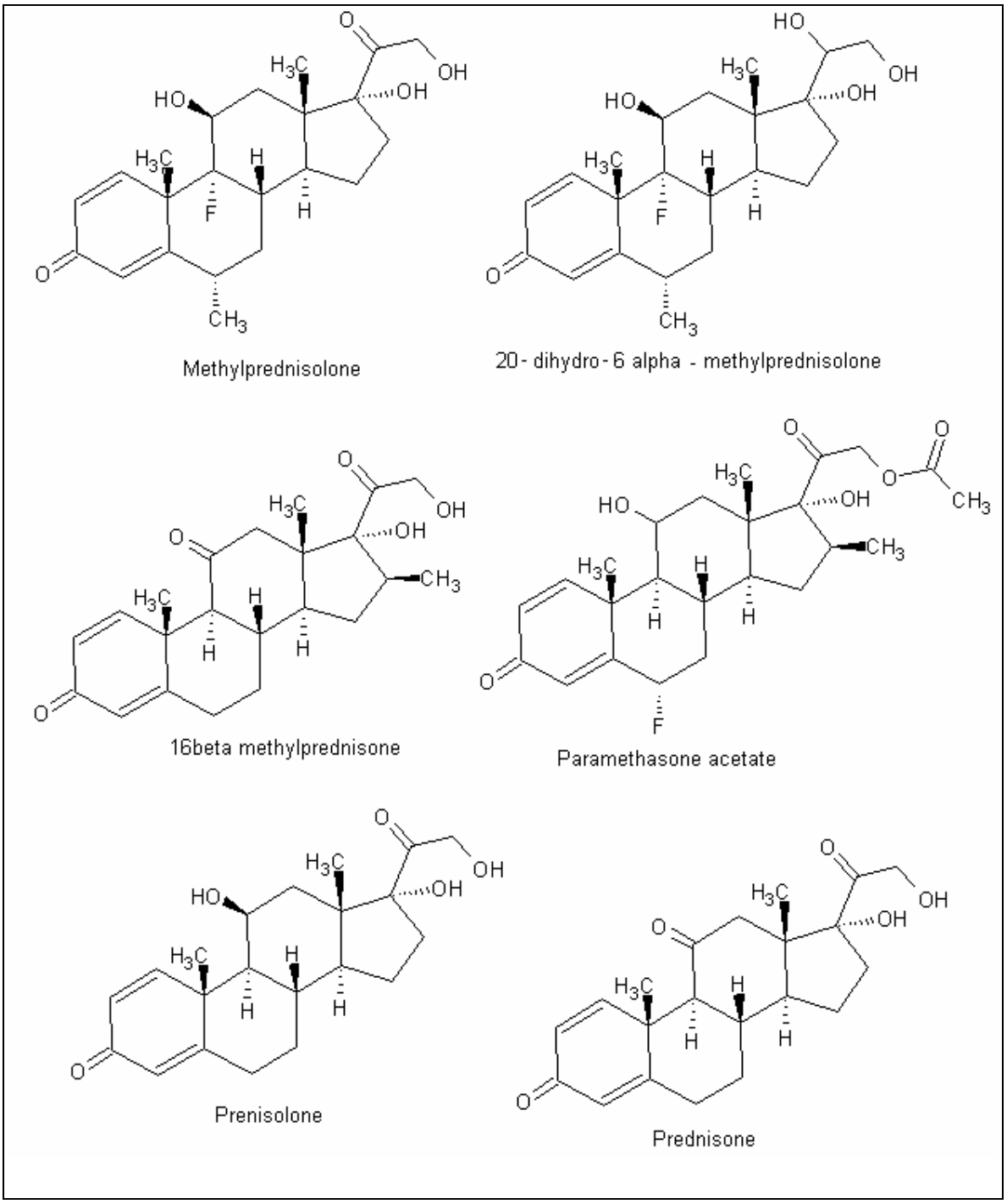

Figure 28. Structures of methyprednisolone to prednisone (Merck, 2001)

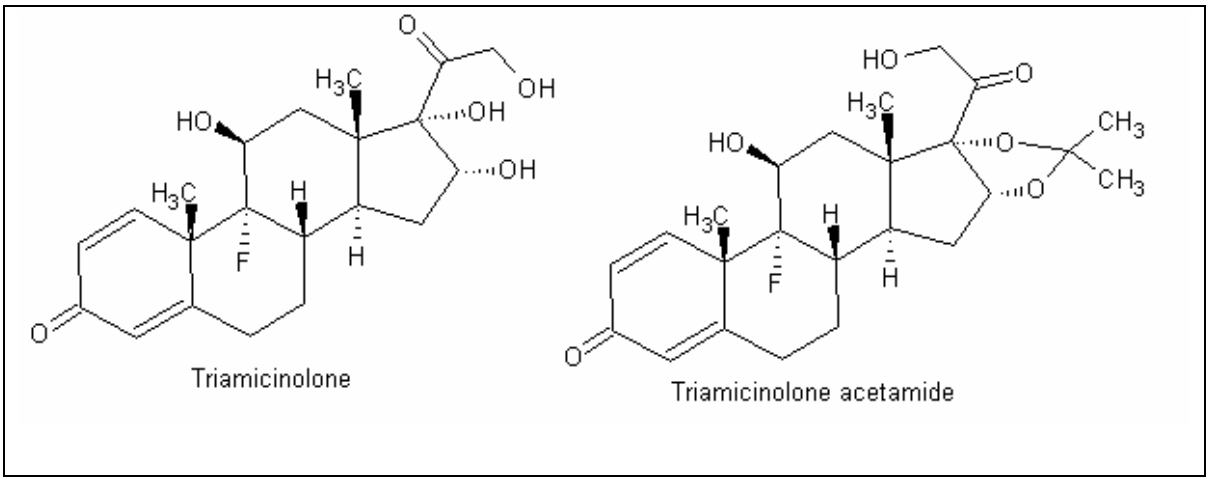

Figure 27. Structures of triamicinolone and triamicinolone acetamide

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