

Urinary threshold considerations of dimethyl sulfoxide, dimethyl sulfone and hydrocortisone in the greyhound

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GENERAL INTRODUCTION

The human's efforts to improve the performance of athletes in sports through medication are as old as competitive sports themselves. The first report of "doping" in racing horses in England occurred at Worksop around 1666 (Tobin, 1981). "Doping", by its original definition in the race industry, is generally deemed to be the use of substances other than normal nutrients that could affect a racing animal's performance at the time of racing. With increasing knowledge of doping, the term "doping" is broadened now. The British authorities redrafted their definition of doping in the late 1970s. The new definition is described as "the detection in its tissues, body fluids or excreta of any quantity of any substance which is either a prohibited substance or a substance the origin of which cannot be traced to normal and ordinary feeding and which by its nature could affect the performance of the racing animal." By this definition a prohibited drug is a substance originating externally, whether or not it is endogenous to the animal, which cannot be traced to normal and ordinary feeding and which can affect the performance of an animal (Tobin, 1981).

Many different drugs are open to abuse by people and animals involved in sports now. Most of them are exogenous compounds, others are naturally occurring substances, so called endogenous materials. An endogenous material is an organic substance which is produced within a plant or an animal; an exogenous material is a substance which is not

synthesized in the body or can not obtained from ordinary food and which must therefore be obtained from an exogenous source.

From the seventeenth century until the late of the nineteenth century, because of lacking efficient drug testing assays, the practice of doping in the horse-racing industry spread like wildfire throughout the world. Many trainers gave a variety of drugs to their horses to make them win or lose unfairly over the horses who were not given drugs. Doping and its prevention have been the main stimulus for studies of drug testing and drug metabolism in the horse. The other two species in which doping occurs to a significant extent are man and the dog. In the United States, the first serious effort at pre-race testing took place in Maryland, where professor James Munch introduced a pre-race test in the 1930s (Tobin, 1981). Since then, testing of horse for drug presence by chemical analysis of bodily fluids has been developed and introduced in the racing industry.

Today, the analytical detection of prohibited drugs in racing animals has evolved to a high state of art with the development of more sophisticated instrumentation. There is a wide variety of drugs testing techniques that can be used to analyze and detect various drugs in biological fluids of racing animals. Collection of such fluids should not in general interfere with the animal, so that its racing performance might be affected. This limits the analyst to samples of urine, blood, saliva and sweat.

Urine is the most commonly collected biological fluid sample in racing chemistry. Chemical analysis of urine samples usually requires the following general steps: sample

preparation; screening of sample for the suspected drug; and final confirmation and quantitation.

A sample preparation procedure is usually to provide a relatively clean sample for analysis (thereby improving selectivity and precision) and to concentrate the sample (improving the sensitivity of analysis). Liquid-liquid extraction remains the most commonly used sample preparation technique in analytical chemistry. Besides liquid-liquid extraction, solid phase extraction and other sample preparation techniques are also available. The commonly utilized screening techniques in drug testing include: (1) direct methods, such as gas chromatography (GC), high performance liquid chromatography (HPLC), gas chromatography and mass spectrometry (GC/MS), high performance liquid chromatography and mass spectrometry (HPLC/MS); (2) indirect method, such as newly developed immunoassay techniques.

As previously mentioned, the principal use of drugs in race-animals is to make them win (or lose) unfairly. Many racing organizations are concerned with the abuse of drugs to influence the racing performance of animals, and this is reflected in the nature of the compounds studied. There is a big difference between exogenous drug testing and endogenous drug testing. For exogenous compounds since the results of drug tests are normally reported as either positive or negative, presence of the drug in body fluids, once confirmed, will provide strong evidence for doping. However, for endogenous drugs, it is very difficult to determine if a positive result is due to administration of the drug or natural levels of the drug in the animal.

In order to differentiate between normal endogenous levels and dosed exogenous levels of the drug in biological samples, including urine, it is necessary to investigate and determine a population "normal" value for an endogenous substance which could not be traced to ordinary feeding. A population normal level for an endogenous material is a threshold level which can be obtained by analyzing a great variety of biological samples at normal physiological state without exogenous contamination of the substance.

The primary purpose of this study was to investigate the threshold concentrations of two endogenous compounds, dimethyl sulfoxide (DMSO) and hydrocortisone, and their metabolites. The drug concentrations were determined by using the optimum analytical method of choice. The threshold values were evaluated to differentiate between normal concentrations and exogenous dosed concentrations of these two compounds in dog urine.

SECTION I. DIMETHYL SULFOXIDE

INTRODUCTION

Dimethyl sulfoxide (DMSO) and its metabolite dimethyl sulfone (DMSO₂) are both naturally occurring compounds with potent anti-inflammatory and analgesic action in man and animal. In the racing community, however, DMSO/DMSO₂ might be used exogenously as stimulants to the central nervous system of racing animals (Tobin, 1982).

In order to prevent the possible misuse of DMSO and DMSO₂, the chemist must differentiate between naturally occurring levels of DMSO and DMSO₂ and levels which arise due to exogenous administration of the compounds. Because of this, very sensitive and selective analytical methods are necessary to determine the naturally occurring level of these two endogenous compounds in the urine of greyhounds.

By understanding the natural levels, the analyst can begin to explore analytical thresholds which can be used to scientifically distinguish between endogenous and exogenous levels of these two compounds in the racing animal. Few studies of threshold determination involving endogenous drugs of possible abuse have been reported. Law et al. (1991) investigated the threshold concentrations of DMSO and DMSO₂ that occur normally in horse urine and plasma. They concluded that DMSO occurs normally in horse urine and plasma and arises mainly (if not entirely) from the diet, particularly lucerne hay. The proposed urinary DMSO threshold was 15 ug/ml, the proposed plasma DMSO threshold was 1.0 ug/ml. They indicated that DMSO₂ was also a normal constituent of equine urine and plasma.

The purpose of this study was to determine the threshold concentrations of DMSO and DMSO₂ and differentiate between normal concentrations and exogenous dosed concentrations in dog urine.

The object of the first part of this work was to determine method characteristics such as analyte recovery, detection ranges, specificity, reliability, precision and accuracy for the analysis of DMSO and DMSO₂ in biological samples. The second objective of this work was the determination of endogenous values of DMSO and its metabolite DMSO₂ in greyhound urine using the optimum method of choice.

LITERATURE REVIEW

General Characteristic and Pharmaceutical Effects

General characteristic

Dimethyl sulfoxide (DMSO) is a naturally-occurring organic compound within the food chain. All plant materials of marine origin contain trace quantities of DMSO (Herschler *et al.*, 1982). At present, there are only limited reports of DMSO in nature. Traces of DMSO, or its metabolites, have been found in spearmint oil, nonfat milk, water, and in a variety of fruits, vegetables, grains and beverages (Pearson *et al.*, 1981). It also has been suggested that DMSO and its metabolites are normal constituents of urine and plasma of man, equine, bovine, canine, and other animals (Hucker *et al.*, 1967; Gerhards and Gibian, 1967; Tiews *et al.*, 1975; Law *et al.*, 1991). DMSO₂, also called methyl sulfonyl methane (MSM) or dimethyl sulfone, a stable metabolite of DMSO, may be necessary at low levels in mammals to maintain normal health, structure and function (Jacob, 1982). The responses of DMSO and DMSO₂ level to equine diet were reported by Law *et al.*, 1990. The results revealed that a high-lucerne-hay diet led to a significant increase in urinary DMSO, showing that the substance was mainly of dietary origin, rather than endogenous. DMSO₂ also increased appreciably on feeding lucerne hay, much more so than DMSO. The increase was too large to be due to the original DMSO₂ alone and most of it must have arisen metabolically.

Chemically, DMSO is a dipolar, aprotic, hygroscopic solvent. The dipolar,

nucleophilic character of the molecule is due to the available free electron pairs at the S and O terminals. In most cases aprotic solvents like DMSO in various liquid media act neither as an acid nor a base. Pure DMSO is nearly odorless and has a slightly bitter taste. It is a clear, colorless to yellow liquid that freezes at 18.5 °C, and boils at 189 °C. Its molecular weight is 78.13. DMSO₂'s molecular weight is 94.33. Boiling point is 238 °C (Ogden, 1967; Rammler and Zaffaroni, 1967; Kharasch and Thyagarajan., 1975; Brayton, 1986).

DMSO was first prepared by the oxidation of dimethyl sulfide by Alexander Saytzeff in 1867. Commercially DMSO was produced by the Stepan chemical company in the 1950's (David, 1972). DMSO can act as both an oxidizing and a reducing agent. When acting as an oxidant, DMSO is reduced to dimethyl sulfide (DMS), and when acting as a reducing agent, DMSO is oxidized to dimethyl sulfone (DMSO₂). At present, in the United States, DMSO is commercially produced from lignin, a byproduct of the paper-making industry.

Pharmaceutical effects

In the early 1960s, the scientific community began to show serious interest in the medical potential of DMSO. In the early 1970s, DMSO was approved by the FDA for veterinary use in musculoskeletal injuries in dogs and horses. In 1978, the FDA authorized the use of 50% DMSO for the treatment of interstitial cystitis, a rare bladder disease, in humans (Brayton, 1986). Experimental studies in animals have shown that

dimethyl sulfoxide can produce a variety of biological effects. Properties that are considered to be particularly important to its pharmacological effects including:

(1) Penetration In man and lower animal, DMSO readily penetrates most tissue membranes and enhances penetration of other substances across biologic membranes. In man, radiolabeled DMSO can be detected in the blood within five minutes of cutaneous application, Within twenty minutes, DMSO can be found in all organs of the body. After one hour, radioactivity can be detected in bones and teeth (Kolb *et al.*, 1967).

DMSO most effectively penetrates skin in concentrations of 80-100%. Its penetrating ability is believed to be due to its exchange and interchange for water in biological membranes, causing a reversible configurational change of the skin protein, and accounting for the rapid passage of DMSO through the skin (Jacob *et al.*, 1971).

DMSO also penetrates mucous membranes (blood-brain barriers, cell and organelle membranes (Kocsis *et al.*, 1968). DMSO can serve as a vehicle for many substances and facilitate their penetration across membranes, a property which may be used to the benefit of or abused to the detriment of the recipient. For instance, in racing animals, DMSO can be used to facilitate percutaneous transport of therapeutic or prohibited drugs into the body. DMSO carries substances rapidly and deeply into the horny layer of the skin (Sulzberger *et al.*, 1967). DMSO has been shown to be an effective diuretic due to an increase in the urinary excretion of sodium and potassium (Koller, 1976; Blythe *et al.*, 1986b).

(2) Anti-inflammation DMSO apparently has significant anti-inflammatory

properties. The mechanisms involved here are complex. Some researchers indicated that the anti-inflammatory effect of DMSO may be due to its ability to scavenge inflammation-triggering free radicals and its ability to inhibit the neutrophil bactericidal activity (Jacob and Herschler, 1986).

In racing animals, the most popular uses of DMSO are for treatment of arthritic, tissue injuries, musculoskeletal injuries, acute trauma, and many of the common injuries (Richardson., 1973; Averkin *et al.*, 1975; Gorog and Kovacs, 1975; Koller, 1976; Wong and Reinertson, 1984).

(3) Analgesic action One of the most prominent pharmacologic actions of DMSO is the reduction of pain. Numerous clinical studies have been reported about pain relief action of DMSO. DMSO was reported to produce analgesia both locally and systemically, suggesting that DMSO either has been carried by the circulation to the affected area to act locally, or has depressed the central nervous system pain centers--or both (Haigler and Spring, 1981).

DMSO reversibly decreases the conduction velocity of nerves. One study, carried out by Sams (1967), showed that the conduction velocity of the isolated frog's sciatic nerve was decreased 40% by immersion for several hours in 6% DMSO. This effect was totally reversed by washing the nerve in a buffer for one hour. DMSO injected subcutaneously (10% concentration) into cats produced a total loss of the central pain response. Two milliliters (50% DMSO) injected into the cerebrospinal fluid led to total anaesthesia of the animal for 30 minutes without apparent adverse reactions (Jacob and

Herschler, 1986).

Analgesia by DMSO may be more comparable to narcotic analgesia than to local anesthesia by procaine derivative drugs. The "hot plate" and "tail flick" tests are standard tests of narcotic analgesia in rats. After studying the effect of DMSO in these tests, Haigler and Spring (1981) reported DMSO produces an analgesia effect comparable in magnitude to morphine, but it is longer lasting and is not reversible by administration of naloxone. They concluded that the mechanism of analgesic action of DMSO is apparently different from that of morphine because naloxone, a specific narcotic antagonist, does not block the analgesic effect of DMSO. This indicates that opiate receptors are not involved in DMSO-induced analgesia. One mechanism suggested is a chemical block of small nerve fibers (the C fibers on nonmyelinated nerve fibers). The block is reversible; the effect usually continues for four to six hours. Shealy studied peripheral small fiber after-discharge in the cat. Concentrations of 5-10% DMSO eliminated the activity of C fibers within one minute; activity of the fibers returned after the DMSO was washed away (Shealy, 1966; Jacob, 1982).

(4) Other central nervous system effects Tranquilizing or sedating effects have been noticed after topical, oral, or parenteral administration of DMSO in several species, including man. Braude and Monroe (1967) gave intraperitoneal injections of 5 g/kg of DMSO to mice, and found a decrease in the spontaneous motor activity and an increase in the hexobarbital sleeping time. Other authors had similar discovery (Kocsis *et al.*, 1968; Kocsis *et al.*, 1975). Intravenous DMSO reduced the dose of chloralose and

methohexital required to maintain anaesthesia in dogs (Peterson and Robertson, 1967).

(5) Respiratory effect The primary respiratory effect reported is increased rate and/or depth when high doses of DMSO (5000 or 10000 mg/kg in 25% solution) are administered to lightly anesthetized dogs (Peterson and Robertson, 1967). Rowed and De La Torre (1973) also indicated that DMSO may act as a respiratory stimulant. Marin (1975) reported that DMSO therapy on chronic respiratory insufficiencies appears to be very effective. Klein *et al.* (1983) demonstrated some improvement in adult respiratory distress syndrome after DMSO administration. One possible conclusion is that DMSO may act as a respiratory stimulant. But pure DMSO (200 mg/kg) has been reported to cause respiratory depression and apnea (DiStefano and Klahn, 1965).

(6) Toxicity of DMSO DMSO's systemic toxicity is considered to be low. In dog, the single dose intravenous LD-50 was reported as 2.5 g/kg body weight (Rubin, 1983). Dermal and oral LD-50 for dog were greater than 11 g/kg and 10 g/kg respectively (Smith *et al.*, 1967). The therapeutic intravenous dose is approximately 1.0 g/kg in a 10-45% solution, administered slowly to humans (Waller *et al.*, 1983), dogs (De La Torre *et al.*, 1975; Rucker *et al.*, 1983) and horses (Mayhew and Mackay, 1982).

Metabolism and Elimination

The major pathways of DMSO metabolism in man and animal are illustrated in Figure 1 (Rammler and Zaffaroni, 1967). Both DMSO₂ and dimethyl sulfide (DMS) have

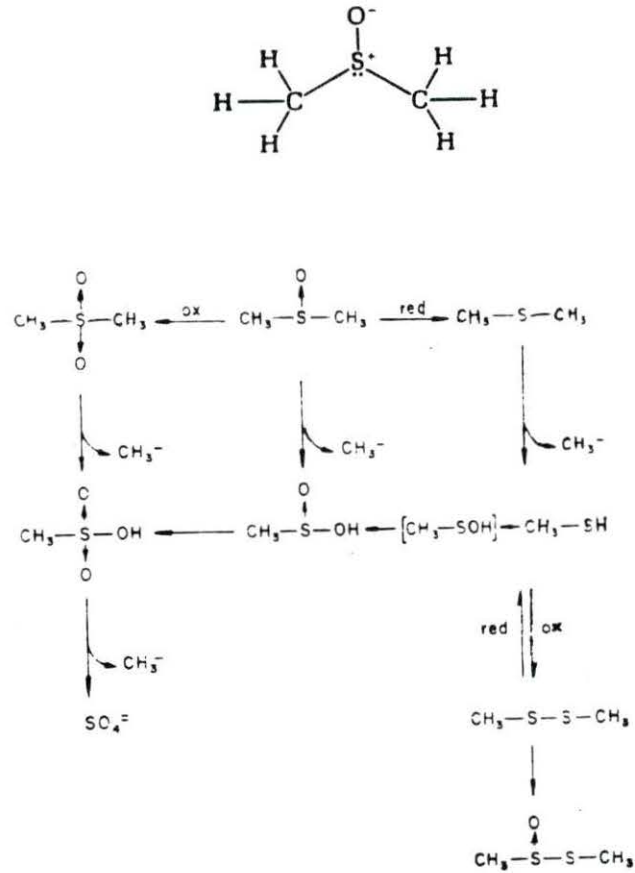


Figure 1. The major path ways of DMSO metabolism (Rammler and Zaffaroni, 1967)

been identified as metabolites of DMSO in man and animal (Gerhards and Gibian, 1967). Metabolism and urinary excretion of the parent drug are the major methods of elimination.

It was suggested that DMS, which unlike DMSO_2 , can be reconverted to DMSO (Williams *et al.*, 1966). Jacob *et al.* (1971) demonstrated that DMSO and DMSO_2 were present in all examined tissues of rats 2 hours after administration of $\text{DMSO-}^{35}\text{S}$ including both hard (i.e. lens and bone) and soft tissues, Hard tissues, i.e., lens and bone, accumulated approximately one-sixth the radioactivity that most of the soft tissues accumulated in the same period of time.

Unchanged DMSO is most prevalent in tissues, blood, feces and urine, with DMSO_2 also present, but in much smaller percentage. It has been estimated that dimethyl sulfide (DMS) accounts for less than 5% of the administered dose in most species reported. Approximately 3-6% of the original dose of DMSO is reduced to DMS and exhaled. The distribution phase of DMSO is very rapid. Regardless of the route of administration of DMSO, a remarkable amount of radioactive DMSO is found in the plasma after only 30 minutes. Cutaneous application of DMSO results in lower plasma levels than when DMSO is administered intravenously. Maximal blood concentrations of DMSO applied cutaneously are reached in two hours. An oral dose of 1 g/kg results in peak plasma level in 4-6 hours and is detectable for 400 hours. An intravenous administration at the same dosage achieves a higher plasma level and is more rapidly distributed to all tissues (Yellowlees *et al.*, 1980). One and one-half to two hours after

cutaneous administration of DMSO, the highest concentration is found in the kidneys, indicating that the primary route of excretion of DMSO is through the urine (Kolb *et al.*, 1967; Wong and Reinertson, 1984).

In beagles, elimination of DMSO after single and repeated cutaneous, oral and intravenous administration was studied. Results revealed that elimination depends on the way of application. After intravenous injection, one third of the injected dose was eliminated via the kidneys, indicating that the kidney is one of the organ of excretion. The elimination half time was about 36 hours whether by intravenous or oral administration. If DMSO was applied cutaneously, only 12 to 25% was eliminated in the dog urine during the first twenty four hours, and 37-48% during a seven-day test period. Only a small fraction (0.5-2%) of an original dose of DMSO was excreted in the feces, regardless of the manner of application (Kolb *et al.*, 1967).

The biological half life of DMSO after it is topically administered was reported to be greater than 10 hours in rats or dogs and 11 to 14 hours in people (Hucker *et al.*, 1967; Kolb *et al.*, 1967). The elimination half time in rats was 6 hours by intravenous injection and 8 hours by cutaneous application, while in humans it was about four days (Kolb *et al.*, 1967). In horses, the biological half life of DMSO was determined to be 9-10 hours by IV administration while urinary excretion of the parent drug accounted for approximately 26% of the dose over the first 12 hours (Blythe *et al.*, 1986a).

In all species, DMSO appeared in the urine earlier than DMSO₂. Hucker *et al.* (1967) suggested that this might simply indicate differences in renal clearance or that

DMSO₂ was bound to tissues and slowly liberated. Another study also showed that DMSO was eliminated more rapidly than DMSO₂ in dogs. A dermal-applied, single dose required two days to clear DMSO and eight days for DMSO₂. Multiple doses required 3 days to clear DMSO, 8-11 days to clear DMSO₂ (Hyde *et al.*, 1989). Law *et al.* (1991) reported that DMSO could be detected for 19 to 22 hours in horse urine after topical administration.

From these facts, it can be summarized that DMSO and its metabolites are endogenous constituents of urine and plasma of man and animal. Its major pharmacological action is anti-inflammatory and analgesia. When administered to dogs, DMSO produces a variety of effects including anti-inflammation, analgesia, increased respiratory rates and depth, and stimulation of the central nervous system. The primary route of elimination of DMSO is through the urine. The major metabolite of DMSO is DMSO₂, which also has been proposed as an analgesic, anti-inflammatory compound. As analgesic, anti-inflammatory agents, these two compounds are of concern in pari-mutuel activities as possible performance-altering drugs. In order to prevent the possible misuse of DMSO and DMSO₂, all racing animals should be required by racing commission rules to have their bodily fluids analyzed for abnormal concentrations of DMSO and DMSO₂ obtained from exogenous sources and not traceable to normal and ordinary feeding.

Analytical Consideration

Numerous research papers have been published about the detection of DMSO and its metabolite DMSO₂ in biological samples using a variety of methods. The specific methods chosen by a laboratory will depend on a number of factors, including cost, workload, turnaround time, sensitivity required, and reliability. In nearly all applications of urine drug detection, a confirmation analysis is essential for all specimens screened positive. A different type of analytical methodology should be used for the confirmation analysis.

Analytical methods used in most laboratories for the detection of drugs in body fluids can be classified into two main categories--immunoassay and chromatography--generally used respectively for screening and confirmation (Hawks and Chiang, 1986). Examples of these two types of methodologies are presented, including sections describing how they are used for screening and confirmation purposes.

Sample preparation

This step usually is to extract the DMSO, and its metabolites from the urine sample through either liquid-liquid extraction; solid-phase extraction, or any other means of separation from the biological matrix. This procedure is necessary to provide a relatively clean sample for analysis, thereby improving selectivity and precision and to concentrate the analyte, improving the sensitivity of analysis.

Liquid-liquid extraction remains a commonly used sample preparation technique in analytical chemistry. Drugs are extracted from biological samples by partitioning between two immiscible solvents. Since DMSO is an aprotic and a high polarity compound, which makes the extraction of DMSO from aqueous media more difficult, an inorganic salt (sodium chloride or potassium pyrophosphate) must be added to the sample first. This is called the salting-out technique (References from Racing chemistry laboratory, veterinary diagnostic laboratory, ISU., Law *et al.*, 1990). Salting out can be used generally in solvent extraction. There are various reasons why salting out may be successful: (1) the high background electrolyte concentration alters the activities of the extracted species in a manner favoring extraction; (2) the salting out agent is solvated by water molecules. Because of its high concentration, a significant proportion of "free" water molecules are removed and the effective concentration of the extractable species in the aqueous phase is increased. When an organic extraction solvent, such as dichloromethane or ethyl acetate is added and completely dispersed, the vast majority of the compound is extracted into the organic layer. Repeating the process of extraction three times, with a fresh volume of the organic solvent each time, ensures the maximum recovery of the compound from the urine. This is known as a exhaustive salt out extraction. The combined extract is concentrated to a small volume under nitrogen at room temperature, and the concentrated extract can then be utilized for further screening procedure.

In the experiment of present studies, the polar organic extract should not be evaporated to dryness at temperatures higher than room temperature because both DMSO

and DMSO_2 are volatile compounds and may vaporize at higher temperature. The disadvantages of exhaustive salt out extraction are the time required for multi-step extractions and the variable recovery of the analyte.

Optional sample preparation method for DMSO is protein precipitation by perchloric acid or methanol following by neutralization techniques (Garretson and Aitchison, 1982) or protein precipitation by acetone followed by separation on a porapak Q column (Mehta and Peaker, 1986). These techniques are reported to give clear solution results, and consequently there is less protein built-up in the injection port of gas chromatography.

Thin layer chromatography

Thin layer chromatography (TLC) has been regarded traditionally as a simple, rapid, and inexpensive screening method for the initial separation, tentative identification, and visual semiquantitation of a wide variety of substances in biological sample. When compared with instrumental techniques such as gas chromatography and high performance liquid chromatography, TLC usually has not been considered to be as sensitive or quantitative.

TLC is a mode of chromatography in which the sample is applied as a small spot to the origin of an adsorbent (stationary phase), such as silica gel, cellulose supported on a glass plate. A solvent mobile phase moves through the stationary phase by capillary action, allowing the substances to separate. The separated substances can then be

identified by sprayed with chemical reagents for characteristic color reaction. Drugs visualized in this way are identified on the basis of (a) reference values (ratio between the distance the mobile phase moves up the plate and the distance the compound moves from the point of application), (b) metabolic patterns (parent drug and characteristic metabolite), and (c) functional group analysis (chemical characteristics as defined by the color reaction with the spray reagent). The R_f value will vary according to the varying of the mobile phase strength and stationary phase.

Advantages of using TLC include: (1) low cost of equipment, (2) rapid analysis, and (3) ability to detect more than one drug or metabolite per analysis. Relatively small amounts of drugs can be detected, usually as low as of 0.5-1.0 micrograms per milliliter. Some of the disadvantages of TLC are that it provides only fair specificity and sensitivity. It does require practice to recognize patterns of drugs and their metabolites by the visualized colored spots (Hawks and Chiang, 1986).

Gas liquid chromatography

Gas liquid chromatography (GLC) is based upon the partition of the analytes between the stationary phase (solid support coated with a thin layer of a liquid phase) and the inert mobile carrier gas phase. Analytes must be thermally volatile and stable. For low volatility compounds, derivatization may be needed to render the analytes more volatile. Packed columns and capillary columns are commonly used in GC.

The detectors of GC generally are of three basic types: (1) Flame ionization

detector; (2) Electron capture detector; (3) Simplified mass spectrometric detectors such as the mass selective detector and ion trap detector. Since the flame detector and mass spectrometric detector are commonly used in our study, their principles will be discussed in detail later.

The basic working principle of GC is based upon the injected sample volatilizing to a gas on the column resulting in the various fractions of the gaseous sample moving through the column at different rates due to physical interaction with the stationary column phase resulting in the analyte appearing at different times (RT value) to the detector. The length of time for each maximum of the peaks to appear on the strip chart recorder, from the injection time, is the retention time (RT). RT is characteristic of the substance of interest and the liquid phase at a given temperature with proper flow and temperature control. The degree to which a GC system can reproduce R_t must be characterized. The retention time, therefore, qualitates the substance in the sample and the peak area quantitates the amount of each of the fractions present. The minimum detectable quantity is that amount which gives a detector response equal to twice or three times the noise level (McNair and Bonelli, 1969).

A number of methods have been proposed for quantitating DMSO, using gas chromatography for the final stage of isolation and quantitation with variations in sample preparation, columns, and detectors. Various chromatographic parameters, column, and obtaining various results for the analysis of DMSO and DMSO_2 are listed in Table 1.

In the literature, the commonly used columns for DMSO and DMSO_2 analysis are:

Table 1. Operating conditions and detection limits of dimethyl sulfoxide and dimethyl sulfone concentrations measured by gas liquid chromatography using flame ionization detection.

ARTICLE	COLUMN SPECIFICATIONS	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT	DETECTOR TYPE
Harold <i>et al.</i> , 1966	4 ft.x.25 inch I.D.	220 °C	Helium	22 ng/ml	FID
Andreae, 1980a	6 m x 4.8 mm I.D.	not given	Helium	0.02 ng/ml	FID/FPD
Garretson and Aitchison, 1982	64 cm x .2 cm I.D.	190 °C	Nitrogen	10 ng/ml	FID
Mehta and Peaker, 1986	2 m x 3.0 mm I.D.	240 °C	Nitrogen	100 ug/ml	FID

(1) packed glass columns (Mehta and Peaker, 1986; Garretson and Aitchison, 1982), (2) capillary column (Law *et al.*, 1990; Law *et al.*, 1991), (3) packed stainless column (Andreae, 1980a).

The commonly used detectors are: (1) flame ionization detector (FID) or flame photometric detector (FPD), (2) mass spectrometry with selective ion monitoring (MS-SIM). The FID is an ionization detector that exhibits a nearly universal response to all organic compounds. The basic principle of operation is based upon the introduction of an organic compound into a hydrogen-rich or hydrogen-air flame jet resulting in the compound's ionization and an increase in the detector current above the background level between the flame jet and a collector electrode above the flame. In the literature, the reported detection limit of DMSO concentration employing a flame ionization detector ranges from 0.02 ng/ml S (DMSO) (Andreae, 1980a) to 100 ng/ml (DMSO) (Garretson and Aitchison, 1986).

In terms of DMSO detection, it is possible to use a FPD (sulfur-phosphorus flame photometric detector), because DMSO contains sulfur atoms (Patterson *et al.*, 1978). This detector operates on the principle that S_2 molecules from sulfur containing compounds, and HPO molecules from phosphorus compounds, emit characteristic light at specific wavelengths (blue and green) in hydrogen-air flame. A filter is used to screen out all other wavelengths so that only the proper wavelength reaches the photomultiplier tube (PMT). A current is generated by the PMT which corresponds to analyte peak in the chromatogram.

Adequate reproducibility (precision) of the method was indicative of a coefficient of variation (C.V.) of 5%-10%. A linear regression analysis was performed for X as the peak area ratio and Y as the known concentration. A correlation coefficient of 0.99 or greater is indicative of excellent linearity.

GLC is a sensitive technique, and small amounts of drugs can easily be detected and identified by determining their respective retention times as compared with known drug standards under optimum instrument conditions. Some of the limiting factors are (1) the slowness of analysis, (2) the expertise required in conducting the tests, and (3) the sample preparation time. Additionally, GLC suffers from the deficiency that the retention time, which provides only a single parameter, cannot be used as an unequivocal identification in many cases (Hawks and Chiang, 1986).

The analytical technique of gas chromatography mass spectrometry (GC/MS) combines the efficient separating power on gas chromatography with the high sensitivity and specificity of mass spectrometric detection. GC/MS is generally considered to be the most conclusive method of confirming the presence of a drug in urine (Hawks and Chiang, 1986). Within most forensic racing laboratories, drug confirmation by GC/MS is performed using quadrupole mass spectrometry with positive ion collection and electron impact (EI) ionization techniques. The application of a mass spectrometer as a universal, yet extremely selective and sensitive detector in gas chromatography has revolutionized the identification and measurement of organic compounds.

The principle of operation of the mass selective detector is based on the fact that

when the sample enters a high vacuum ion source via the capillary column where the molecules can freely move in the evacuated space, they are bombarded into their constituent fragment ions by electrons emanating from the filament. The positively charged ions produced are propelled out of the source and focused into the quadrupole mass filter. When specific voltages are applied to the quadrupole rods, only ions of a specific mass-to-charge ratio pass from the ion source through the quadrupole to the detector without annihilation. After the ions are separated on the basis of mass-to-charge ratio, they are then counted as they enter the detector. The detected ions can be plotted graphically with the mass on the x axis and the counted ions (abundance) on the y axis to give a mass spectrum. Also, the abundance of all the ions can be plotted as a function of time to give a total ion chromatogram or, alternatively, selected ions can be monitored and plotted as a function of time (selected ion chromatogram).

There are several different modes of operating a GC/MS. It can be operated in the "full scan" mode which provides a complete mass spectrum for each component of the urine extract that passes through the gas chromatography. Since a complete mass spectrum represents a "fingerprint" pattern that is unique for each drug, this mode of operation will give the most conclusive identification if there is a sufficiently high concentration of the drug to provide a good quality mass spectrum. In selected-ion-monitoring mode (SIM), ions specific for the compound of interest are monitored rather than the entire AMU range. This mode of operation affords far higher sensitivity, but provides a less specific pattern for identification.

Table 2. Operating conditions and detection limits of dimethyl sulfoxide and dimethyl sulfone concentrations measured by gas liquid chromatography using mass spectrometric detection.

ARTICLE	COLUMN	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT
Garretson and Aitchison, 1982	107 cm x .2 cm I.D.	170 °C	Helium	10 ng/ml
Law <i>et al.</i> , 1990	10 m x .2 mm I.D.	180 °C	Helium	3-5 ng/ml
Law <i>et al.</i> , 1991	10 m x .53 mm I.D.	150 °C	Helium	3-5 ng/ml

The choice of which modes of operation to be used depends on what drugs are to be detected, the minimum concentration of the drug that constitutes a positive identification, and whether or not the concentration of the drugs is to be quantitatively determined. The reliability of a GC/MS assay is also dependent on the skill and experience of the operator, as well as on the method used for extraction of the drug(s) from the urine and for preparation of the extract for injection into the GC/MS.

Several papers in the literature described detection of DMSO and DMSO₂ using GC/MS techniques (Garretson and Aitchison, 1982; Law *et al.*, 1990; Law *et al.*, 1991). Quantitative analyses were performed by selected ion monitoring (m/e 63 and m/e 66 for DMSO; m/e 79 and m/e 82 for DMSO₂) (Law *et al.*, 1990; Law *et al.*, 1991). Their studies have concluded that the proposed urinary DMSO threshold in horses was 15 ppm. Various other important chromatographic parameters and the detection limits for the analysis of DMSO and DMSO₂ are listed in Table 2.

High performance liquid chromatography

High performance liquid chromatography (HPLC) has been used as a drug screening method for some time. It is a highly versatile technique that provides very good resolution and, unlike GC, is not adversely affected by the analyte's polarity or lack of volatility.

HPLC can be easily interfaced with visible, ultraviolet (UV) and fluorescence detectors. Other detectors of limited use are electrochemical and mass spectrometric

detectors. The combination of HPLC with UV detection offers a good balance between efficiency and specificity. Every compound exhibits a characteristic ultraviolet or visible spectra which can be used to qualitatively identify it, and each compound will have a characteristic HPLC RT value associated with it. Being able to obtain both retention and spectral information about a compound within the same analytical process is an efficient way to gain information about a compound's identity. HPLC has the advantage that polar drugs requiring derivatization on GLC systems can be assayed directly on HPLC. Its disadvantages are similar to those of GLC.

To detection of DMSO in environmental water samples, Ivey and Haddad used ion exclusive chromatography with a polymeric cation-exchange column and a UV detector (Ivey and Haddad, 1987). The detection limit was reported to be 10 ppb with a 200 μ l injection volume. DMSO has E_{\max} of 195 nm with strong absorbance of UV light (Ivey and Haddad, 1987). No publication was found in the literature about analysis of DMSO in biological samples using HPLC technique.

Comparative studies

Many studies have been done using different methods to detect DMSO and its metabolites on an individual basis (Paulin et al., 1966; Jursik, 1967; Andreae, 1980a; Mehta and Peaker, 1986; Ivey and Haddad, 1987; Hyde et al., 1990; Law et al., 1990). In addition, one publication has reported the results of GC vs. isotope dilution-mass spectrometry comparative study (Garretson and Aitchison, 1982). The author indicated

that there was no difference in the ability to ascertain DMSO presence in biological samples between the two assay methods.

EXPERIMENTAL

Materials and Instrumentation

Instrumentations of GC and GC/MS

Perkin Elmer model 3920B GC instrument equipped with a sulfur-phosphorus flame photometric detector (FPD) was utilized in obtaining GC/FPD results. Samples were separated using a DB-WAX capillary column (15 m X 0.53 mm fused silica with 1 μm film thickness, J&W Scientific, Rancho Cordova, CA).

A Hewlett Packard model 5890 GC instrument connected to a Hewlett Packard 5988A quadrupole mass spectrometer was used in quantitative analysis of drugs. A HP data station was equipped with the GC/MS for mass spectral collection (electron ionization and positive ion collection). All GC/MS analyses were performed using a DB-WAX capillary column, 15 m x 0.25 mm fused silica with 0.3 μm phase loading.

Solvents, chemicals and reagents

Dimethyl sulfoxide utilized for dosing and for preparation of stock standard and spike was obtained from Fisher Scientific, Inc. (Fair Lawn, NJ). Dimethyl sulfone used for stock standard and spike preparations were obtained from Sigma Chemical Company, Inc. (St. Louis, MO).

Hexamethyldisilazine (HMDS) and pyridine for silanization of glassware were acquired from Pierce Chemical Company (Rockford, IL) and Aldrich Chemical

Company, Inc. (Milwaukee, WI) respectively.

All organic solvents used in this research were Fisher HPLC grade or equivalent and chemicals/reagents used were reagent grade or better. Most chemicals and reagents were purchased from Fisher Scientific (Fair Lawn, NJ) including: sodium chloride, dichloromethane, potassium permanganate, acetonitrile and methanol. Potassium pyrophosphate was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI)

All gases (nitrogen, air, helium, hydrogen, and high purity helium) used in the GC and GC/MS were obtained from Air Products, Des Moines, IA.

Other supplies

Graduated wide mouth specimen containers for initial urine collection and final storage were purchased from Cole-Parmer Instrument Company, Inc. (Chicago, IL). Screw top test tubes (16 x 125 mm) with PTFE lined caps used in the liquid-liquid extraction steps were purchased from Fisher Scientific. Thin layer chromatography separations were performed on Kiesel silica gel 60 plates (normal phase) with F₂₅₄ fluorescence indicator purchased from EM Science, Gibbstown, NJ.

Pipetmen precision microliter pipettes, utilized in all procedures for liquid dispensation were purchased from Rainin Instrument Company, Inc. (Woburn, MA).

Drug Administration and Sample Collection

Canine dosing experiments

Nineteen retired racing greyhounds, were used for this study. The animals ranged from 2-5 years in age and weighed from 15-28 kg. These animals received daily walking exercise. The dogs were held in a isolation without drug treatment for two weeks prior to any study to assume they had no drug bodyburden. They were held in caged mini-runs with water provide. A high protein, low fat, dry dog chow (Science diet) was fed once daily. Urine was obtained by a cup-on-a-stick volunteer collection at approximated 10:00 a.m daily. The samples were capped, labeled, and stored at -10 °C.

Dimethyl sulfoxide administration studies

Two retired racing greyhounds, # 8180 and # 10325, weighing approximately 22 kg were chosen for a study of 1 ml/ per animal dermal exposure. Dogs # 8180 and # 9577 were chosen for a study of 200 ul/ per animal dermal exposure. The DMSO liquid was applied directly to the test subject's skin (along the dorsal hair line). Due to the volatility of the DMSO, some of the drug evaporated into the surrounding air. No attempt was made to eliminate inhalation of this volatile fraction as a source of exposure in the test dogs. Urine was collected using the same procedure previously mentioned. Following DMSO administration, urine was collected at the times post administration indicated in Table 3. Prior to use, all glassware was rinsed thoroughly with acetone to remove any

Table 3. Collection times of urine following 1 ml/per dog and 200 ul/per dog DMSO administration from dogs # 8180, # 10325 and # 9577, respectively

Time Post Dose	Urine (DMSO 1 ml)		Urine (DMSO 200 ul)	
	#8180	#10325	#8180	#9577
0 hour	xx	xx	xx	xx
2 hour	xx	xx	xx	xx
4 hour	xx		xx	xx
6 hour	xx		xx	xx
8 hour	xx		xx	xx
24 hour	xx	xx	xx	xx
48 hour	xx		xx	
72 hour			xx	xx
96 hour	xx		xx	xx
120 hour	xx		xx	xx
144 hour	xx		xx	

xx indicates urine sample was collected at the time

interfering chemicals. To prevent drug adsorption to glass, all micro vials utilized in GC and GC/MS analyses were initially silanized by heating at 60 °C with a 5% HMDS in pyridine solution for one hour. The vials were then rinsed with acetone and used in the analyses.

Analytical Methods

Liquid-liquid extraction (DMSO administrated and blank urine)

In order to extract DMSO and its metabolites from urine (both dosed and blank) by classical liquid-liquid extraction, an exhaustive salt-out extraction procedure was employed. When extracting dosed and blank urine samples for analysis via TLC and GC/MS, two ml of urine at each collection time for both dogs were used.

After thawing in warm water, 2 ml of urine were dispensed into each of labeled screw top test tubes. One gram of sodium chloride was added to each tube and vortexed for 5 seconds. The samples were extracted three times with 4 ml of methylene chloride (DCM). Extracts was combined and the DCM layer was concentrated in pre-graduated nipple vials to 200 μ l under nitrogen, then capped and vortexed for TLC and GC/MS analysis. For GC/MS analysis, 1 μ l of the final extract was injected into the GC/MS. For TLC analysis, 5 μ l or 10 μ l of the extract were plated on a TLC plate.

Liquid-liquid extraction (DMSO spiked water and urine)

After thawing in warm water, 2 ml of blank urine or water was spiked at the following concentrations of DMSO/DMSO₂: 0.5 ppm, 1.5 ppm, 2.5 ppm, 5 ppm, 10 ppm, 15 ppm, and 20 ppm. Extraction of the DMSO/DMSO₂ spikes was performed using the same extraction procedure previously defined.

Thin-layer chromatographic techniques

All TLC analysis was performed on Kiesel silica gel 60 TLC plates. For DMSO/DMSO₂ analysis, the extracts were developed on the plate for a distance of 5 centimeters from the origin with Davidow development system, (ethyl acetate:methanol:ammonia 8.5 : 1.0 : 0.5), made fresh daily.

Following development, plates were visualized under short and long wave ultraviolet light for quenching and fluorescence respectively, Then, the plates were sprayed with potassium permanganate (1% in H₂O). DMSO was oxidized by potassium permanganate to give a yellow color spot, DMSO₂ is not oxidized by potassium permanganate and is not observed by TLC.

Gas chromatographic techniques and conditions

All GC-FPD analyses of DMSO/DMSO₂ were done using a J&W DB-WAX fused silica column (15 m x 0.53 mm with a 1 micron loading phase), with a Perkin-Elmer 3920 B gas chromatograph equipped with a sulfur-phosphorus FPD detector.

Conditions for GC/FPD were:

Detector temp.	200 °C
Oven temp.	90 °C
Carrier gas flow(nitrogen)	30 ml/min.
Hydrogen	50 psi
Air	50 psi.
Injection mode	on column

All GC/MS analyses were done using a J&W DB-WAX fused silica columns (15 m x .25 mm with a 0.3 micron loading film thickness). All extracts were injected without derivatization or thin-layer clean up. The analyses were performed with a Hewlett Packard 5890A gas chromatograph connected to a Hewlett Packard 5988 quadrupole MS.

Conditions for GC/MS were:

Injector temp:	200 °C
Oven temp:	70 °C hold for 1 min, programmed at 20 °C/min to 150 °C
Carrier gas (He):	1 ml/min flow rate
Injection mode:	Splitless
Electron energy:	70 eV
Source temp:	265 °C
Ionizer vacuum:	$< 10^{-6}$ torr

Blank and dosed urine were analyzed by GC/MS in full scan (40-100 AMU) mode with EIC (extract ion chromatogram) or TIC (total ion chromatogram) data interpretation techniques and SIM mode with SIC (selected ion chromatogram) data interpretation technique. The ions monitored were m/z 63 and 78 for DMSO and m/z 79 and 94 for DMSO_2 .

The GC/MS instrument was operated in the EI mode, the quadrupole mass axis was calibrated using the ions m/e 69, 219 and 502 from the perfluorotributylamine (PFTBA) with an ion source of 200 °C and an electron energy of 70 eV.

RESULTS AND DISCUSSION

Analytical Methodology

The choice of a valid analytical procedure is an obvious and important factor in achieving results of high quality. The specificity, sensitivity, and reproducibility of the method must be known in order to achieve the quality goals selected previously.

Limit of detection

The ability of any assay to detect low levels of drugs has an inherent limit. The concentration of drug in the urine sample below which the assay can no longer be considered reliable is the "detection limit". By comparing the detection limits of the various methods, some knowledge can be obtained regarding the useability of the respective methodology. In this work, it is especially important to obtain the accurate information about the detection limits of the various methods.

TLC For all thin-layer work, the TLC plates were developed for a distance of five centimeters with a solvent consisting of ethyl acetate:methanol:ammonia (8:5:1). Following plating and development of DMSO standards on TLC plates, neither quenching or fluorescing were observed under SWUV and LWUV visualization. Lastly, the plates were sprayed with potassium permanganate, and the spots of interest were then visible on the plate. DMSO₂ was not observed by TLC. The R_f value for DMSO is 0.22.

Concentrations of 4, 3, 2, 1, and 0.5 μg DMSO standard present on the TLC

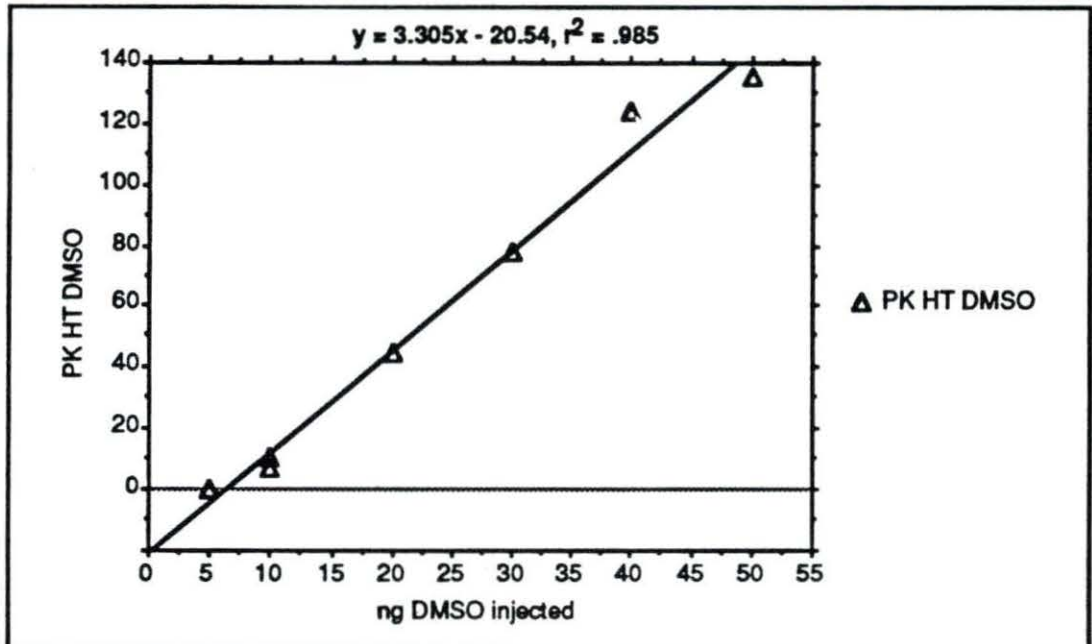


Figure 2. DMSO standard curve by GC using flame photometric detector, n=1

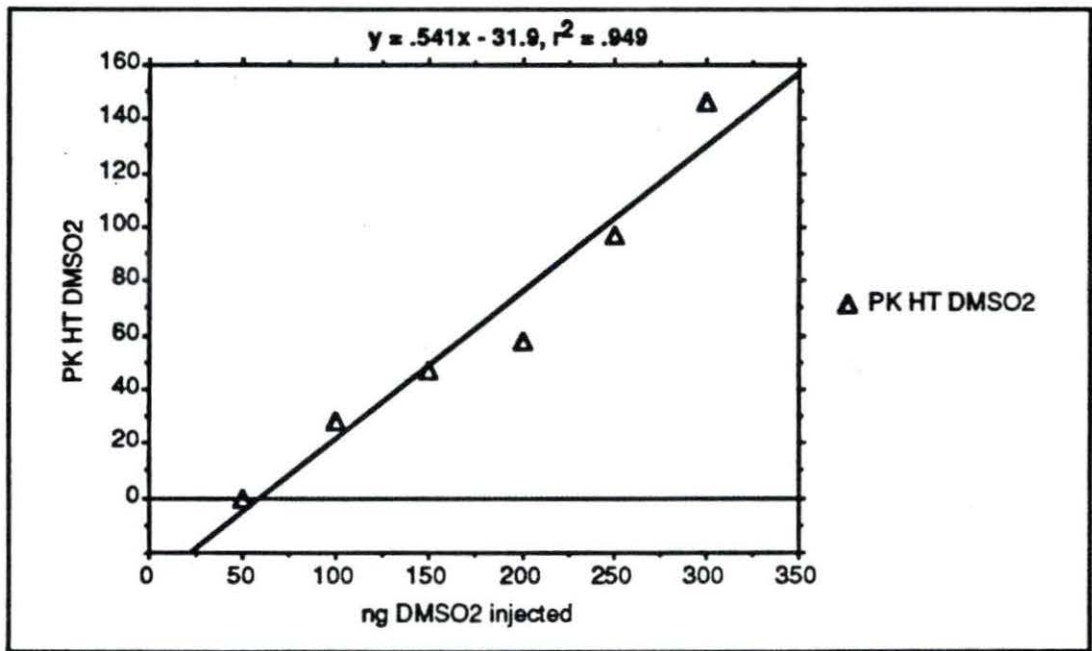


Figure 3. DMSO₂ standard curve by GC using flame photometric detector, n=1

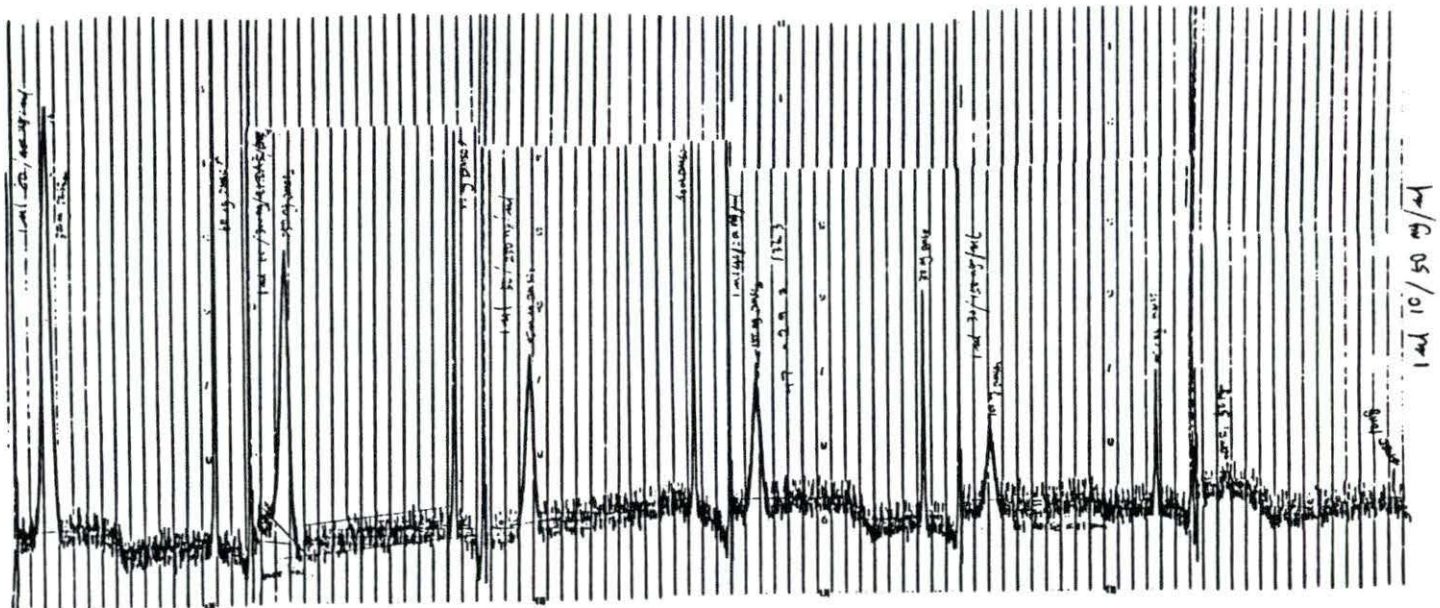


Figure 4. GC/FPD typical output of DMSO/DMSO₂ standard curves

plate were developed, visualized under UV light, and then sprayed with the spray reagent. All concentrations higher than, and including 1 μg , were visible to the naked eye at the Rf value of interest. Below 1 μg DMSO the ability to visualize the spot of interest with the naked eye was very difficult. When taken into consideration that an actual TLC plate would contain a large number of samples, each with endogenous constituents becoming visible following spraying, the lowest detectable level of DMSO would have to be classified as approximately 1 μg on the TLC plate.

GC/FPD Gas chromatography with sulfur-phosphorus detection had a detection limit for DMSO and DMSO₂ equal to approximately 20 ng and 100 ng on column with a signal/noise (peak height) ratio equal to or larger than three, respectively. Below 20 ng DMSO and 100 ng DMSO₂ the signal/noise was smaller than two. Figure 2 and 3 illustrate a DMSO standard curve and a DMSO₂ standard curve by GC/FPD. Figure 4 shows a typical GC/FPD output.

GC/MS Analysis of DMSO and DMSO₂ were done using the total abundance of ions m/e 63, m/e 78, m/e 79, m/e 94 and TIC (total ion chromatogram) in full scan and SIM mode. Both compounds were detected by the presence of major two ions. For DMSO, the base ion is m/e 63, and the molecular ion m/e 78 is present at 50% of the base ion. For DMSO₂, the base ion is m/e 79, and the molecular ion m/e 94 is present at 50% of the base ion. Gas chromatography/mass spectrometry detection in full SCAN and SIM mode had detection limits for DMSO equal to approximately 3.0 ng and 0.6 ng with a signal/noise (peak area) ratio equal to or larger than three, respectively. Detection

limits in full scan and SIM mode for DMSO₂ were found to be 3.0 ng and 0.6 ng with a signal/noise (peak area) ratio equal to or larger than three, respectively. Below 3.0 ng and 0.6 ng the signal/noise ratio was smaller than two. Figure 5a, 5b, 6a, 6b, 7a, 7b, 8a, and 8b illustrates a DMSO standard curve and a DMSO₂ standard curve under both full scan mode and SIM mode. Curve b is the enlarged lower range of curve a.

Reproducibility

Precision studies have to be performed in the analytical measurements for quality assurance. The reproducibility of the each method was studied by calculating the standard deviation (SD) and coefficient of variation (CV) at each standard level.

GC/FPD The method precision was obtained by quantitatively analyzing two DMSO/DMSO₂ standards: 20/100 and 30/150 ng. Table 4 represents the statistical analysis, mean, SD and CV of the within day precision at each of the two concentrations tested. Results seem to indicate that the analysis method has a poor reproducibility. Within-day coefficients of variation (C.V.) ranged from 7.61 to 41.13%. No day-to-day precision analysis was performed in this study. Figure 9 shows a typical GC/FPD output.

GC/MS The GC/MS precision was obtained by quantitatively analyzing five DMSO/DMSO₂ standards: 30/30, 50/50, 60/60, 70/70, and 80/80 ng in full scan mode. The within-day precision was obtained by injecting each standard five times within 24 hour period. Table 5 represents the statistical analysis, mean, SD, CV of the within-day precision of the five standards tested. DMSO peaks were integrated using the

Table 4. Statistical analysis of within-day precision study by GC/FPD.

Number of Analyses	Level of Standards	Mean of Peak height	SD of Peak height	CV, %
6	20 ng DMSO	4.55	± 0.67	14.7
6	100 ng DMSO ₂	2.68	± 0.20	7.61
8	30 ng DMSO	7.36	± 3.01	41.13
8	150 ng DMSO ₂	3.95	± 1.21	30.74

Table 5. Statistical analysis of within-day precision study by GC/MS in full scan mode (DMSO and DMSO₂ peaks were integrated using total ion chromatogram).

Number of Analyses	Level of Standards	Mean of Peak area	SD of Peak area	CV, %
5	30 ng DMSO	4545.2	± 266.9	5.9
5	50 ng DMSO	7438.2	± 143.3	1.9
5	60 ng DMSO	10487.8	± 111.0	1.1
5	70 ng DMSO	11571.4	± 441.0	3.8
5	80 ng DMSO	13436.8	± 536.5	4.0
5	30 ng DMSO ₂	2853.6	± 122.9	4.3
5	50 ng DMSO ₂	4969.8	± 166.7	3.4
5	60 ng DMSO ₂	6995.6	± 151.6	2.2
5	70 ng DMSO ₂	8517.4	± 133.2	1.6
5	80 ng DMSO ₂	8651.2	± 348.3	4.0

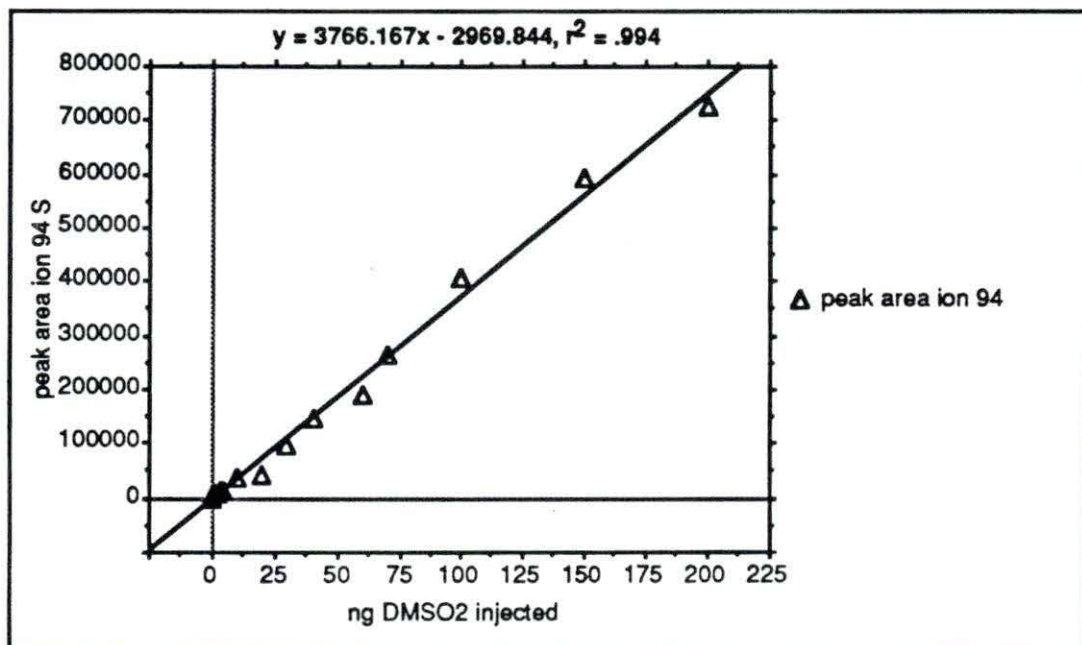


Figure 5 (a). DMSO₂ standard curve by GC/MS in SIM mode, n=1

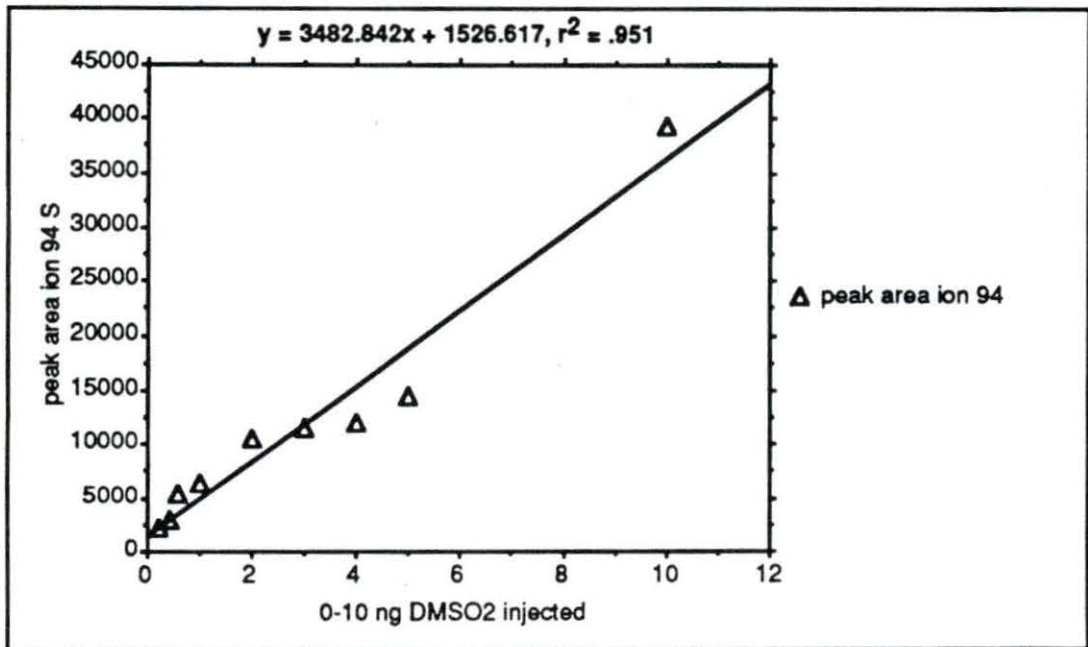


Figure 5 (b). DMSO₂ standard curve by GC/MS in SIM mode, n=1

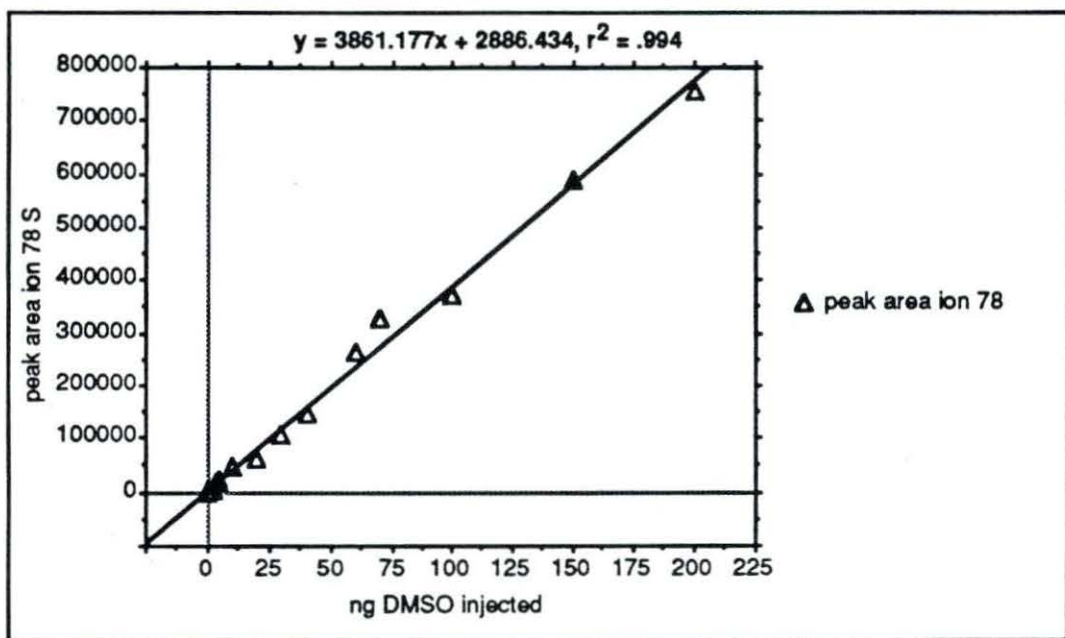


Figure 6 (a). DMSO standard curve by GC/MS in SIM mode, n=1

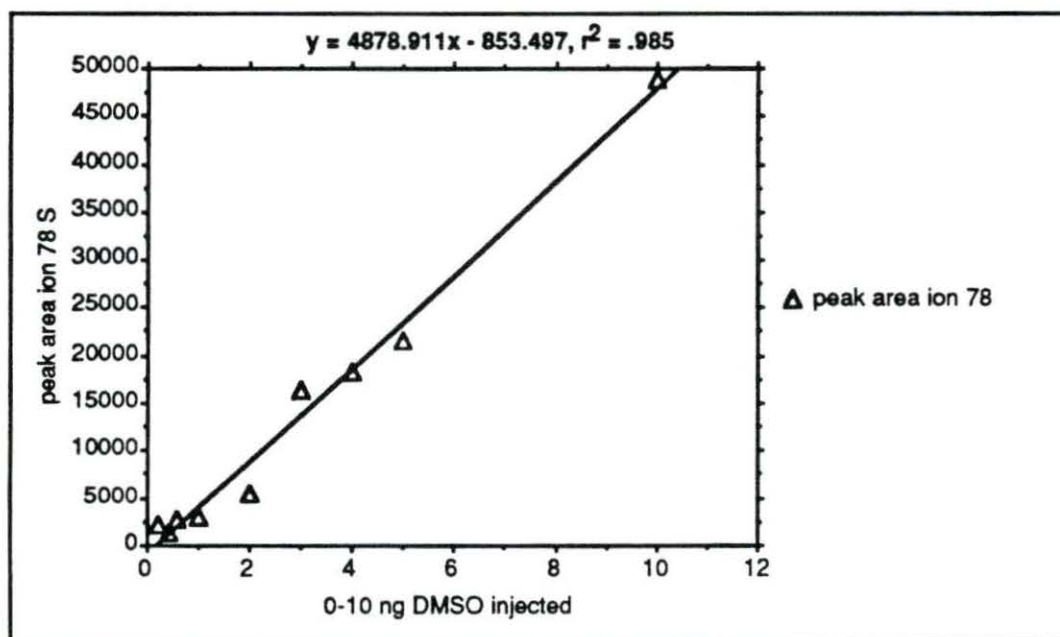


Figure 6 (b). DMSO standard curve by GC/MS in SIM mode, n=1

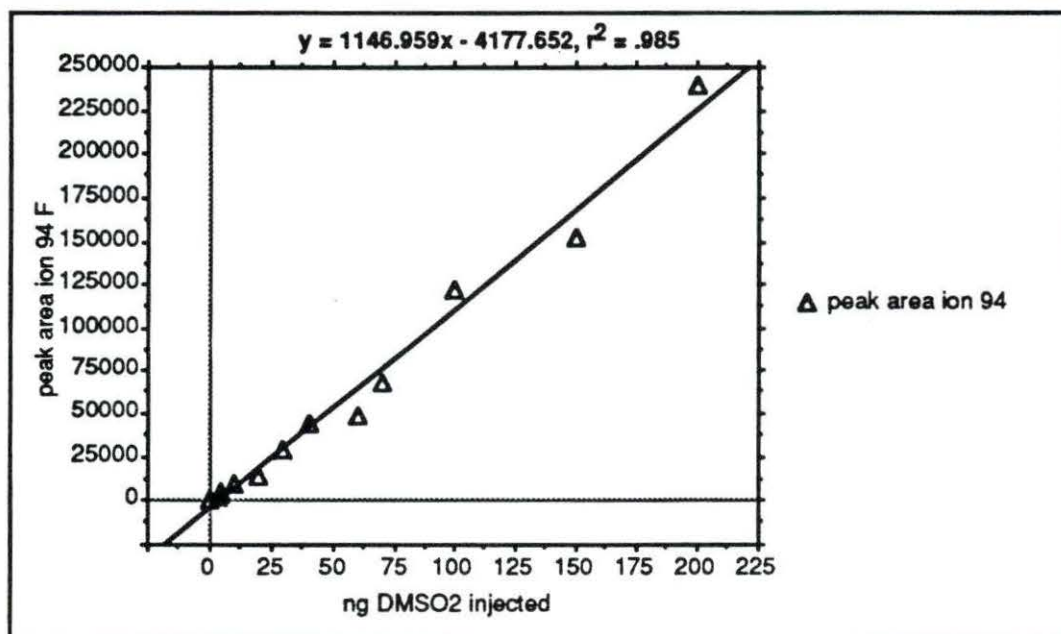


Figure 7 (a). DMSO₂ standard curve by GC/MS in full scan, n=1

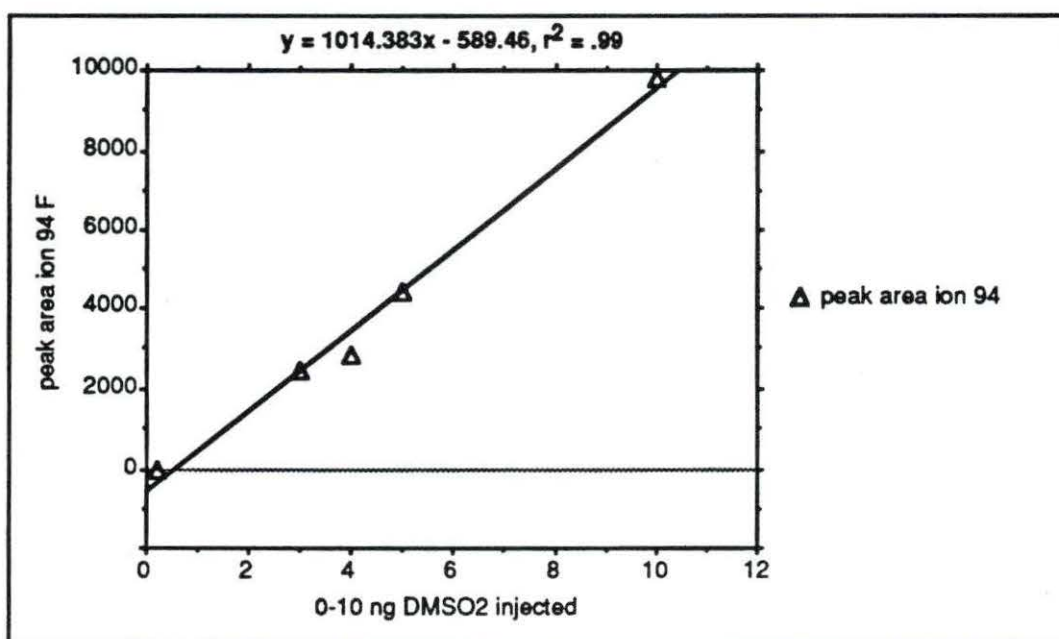


Figure 7 (b). DMSO₂ standard curve by GC/MS in full scan, n=1

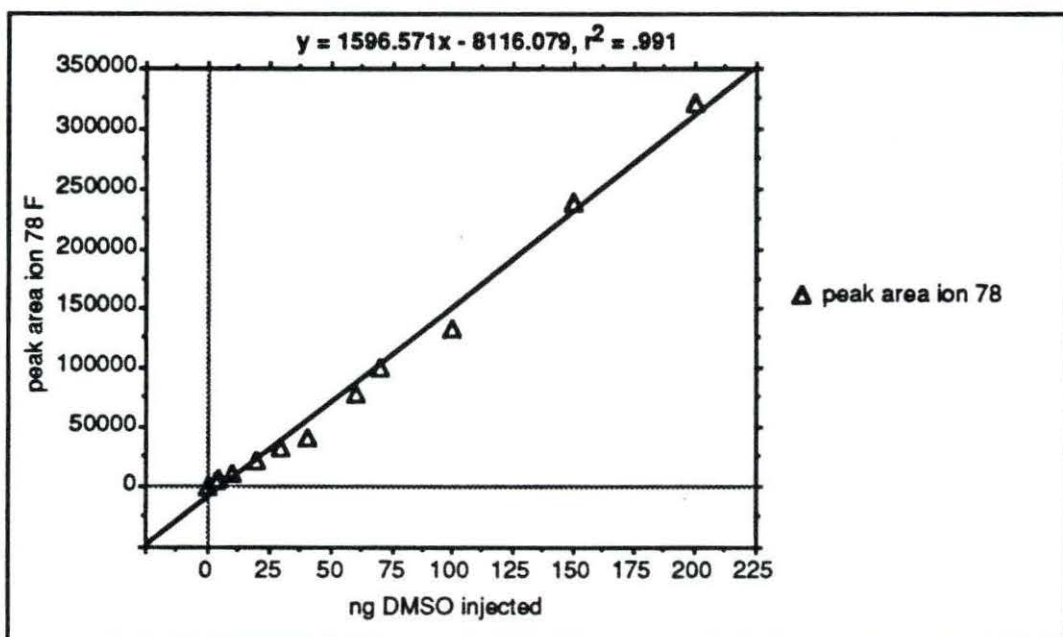


Figure 8 (a). DMSO standard curve by GC/MS in full scan, n=1

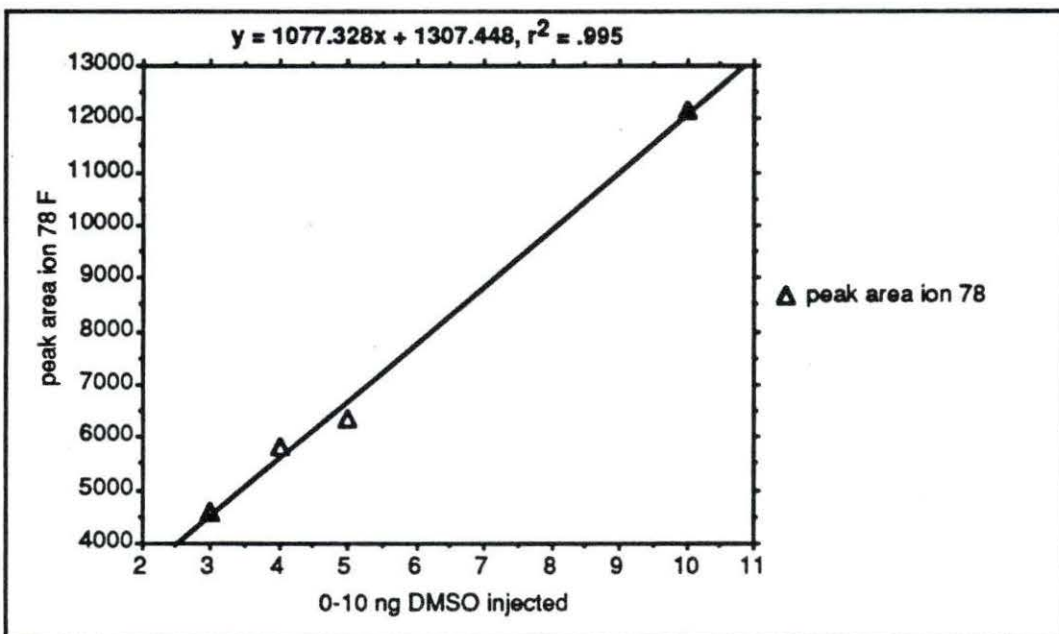


Figure 8 (b). DMSO standard curve by GC/MS in full scan, n=1

molecular ion m/e 78, DMSO_2 peaks were integrated using the molecular ion m/e 94.

Reproducibility of the method was verified by five standards with within-day coefficients of variation (C.V.) of 1.1 to 5.9% for DMSO and C.V. of 1.6 to 4.3% for DMSO_2 . A four-point calibration curve was prepared for every batch of samples. A urine blank and two urine samples spiked with a known concentrations of DMSO and DMSO_2 (10/10, 15/15 ppm) were included in every batch of samples as control samples.

The day-to-day precision was verified by injecting five standards within five days, respectively. Table 6 gives the statistical analysis, mean, SD, CV of the day to day precision of the five standards tested (50/50, 100/100, 150/150, and 200/200 ng). Both DMSO and DMSO_2 peaks were integrated using total ion chromatogram (TIC). Day-to-day coefficients of variation (C.V.) ranged from 3.3 to 9.2% for DMSO, and 3.5 to 7.3% for DMSO_2 . A four-point calibration curve was constructed every day. A blank urine sample and two urine spikes (10/10, 15/15 ppm) were also included in every day analysis. Two stock DMSO and DMSO_2 standards ($1 \mu\text{g}/\mu\text{l}$) were used for all standard curves preparation and spiking.

Average spike recoveries and day-to-day coefficients of variation were determined with these urine spikes. Results are given in Table 7. Figure 10 and Figure 11 represent the typical results of within-day precision study by showing a DMSO and a DMSO_2 standard curve with error bars indicating 2 SD, $n=5$.

Sample spikes

Extraction of DMSO, DMSO₂ aqueous spikes and urine spikes was performed to determine the extraction efficiency and reproducibility of the method for the analyte of interest in biological and aqueous matrices. Spike recoveries and within day coefficients of variation were determined using these spikes.

TLC Two ml of blank dog urine were spiked with DMSO and then extracted by the sodium chloride exhaustive salt-out procedure. Urine was spiked at the following concentrations: 5, 10, 25, 50, 75, and 100 ppm. After extraction and concentration, 5 μ l of the final 200 μ l extract were spotted on the TLC plate. For reference, DMSO standard was spotted on each side of the TLC plate. The TLC plate was then treated with the approximately solvents and sprays indicated in the Materials and Methods. Typical pictures of DMSO water and urine spikes on a TLC plate are represented in figure 12 and 13. All concentrations greater than or equal to 50 ppm were visible to the naked eye at the R_f value of interest. Below 25 ppm DMSO the ability to observe the color spot of interest with the naked eye was greatly impaired. The lowest detectable level of DMSO urine spikes on TLC plate was estimated at approximately 50 ppm. For aqueous spikes, using the same procedure, the detection range of DMSO water spikes was extended to approximately 25 ppm.

For both aqueous spikes and urine spikes visible on TLC plates, after spraying, the color reaction can be seen immediately. However, it is obvious that the detection range of the aqueous spike is lower than that of the urine spike. It is assumed that the

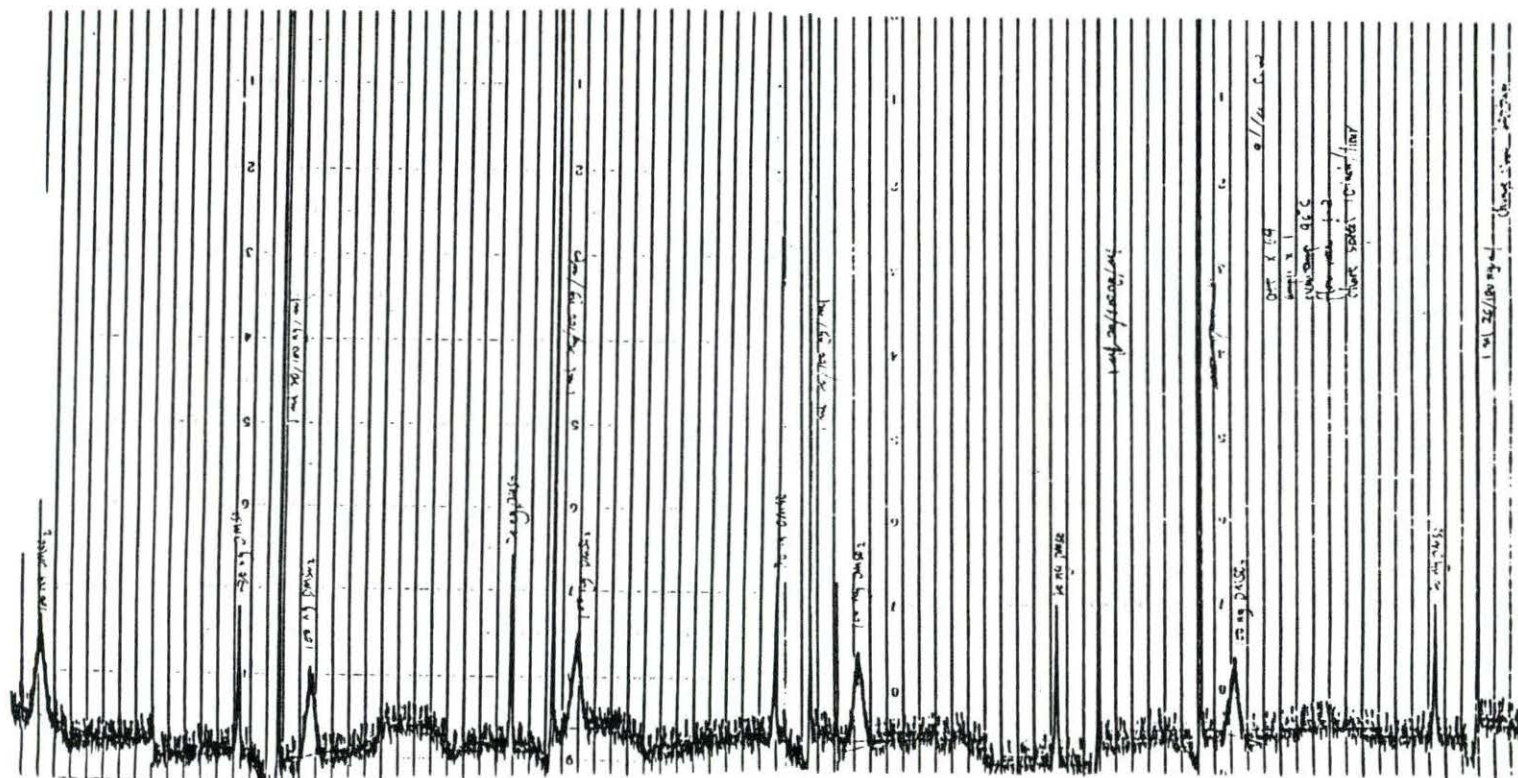


Figure 9. GC/FPD output of reproducibility

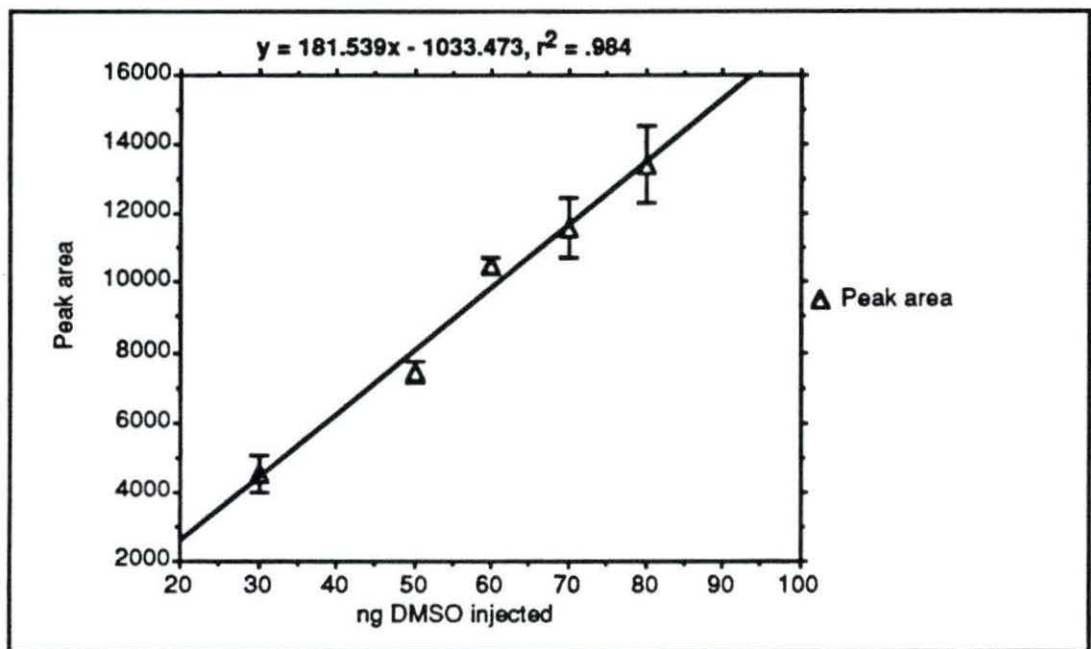


Figure 10. DMSO standard curve by GC/MS in full scan mode, error bars indicating 2 SD, n=5

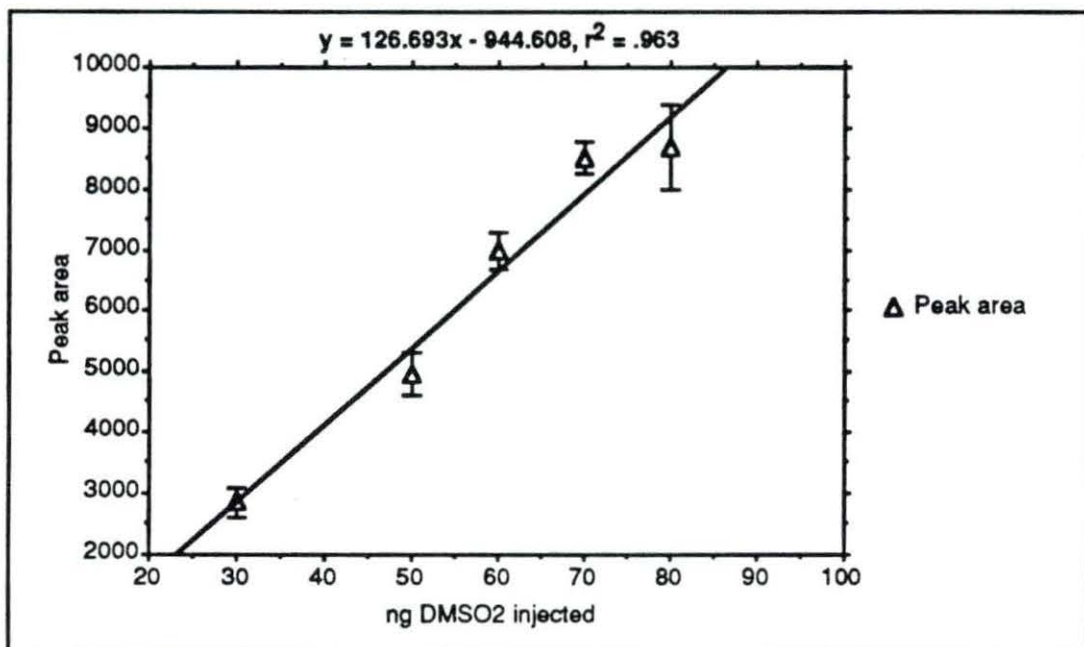


Figure 11. DMSO2 standard curve by GC/MS in full scan mode, error bars indicating 2 SD, n=5

Table 6. Statistical analysis of day-to-day precision study by GC/MS in full scan mode (DMSO peaks were integrated using ion m/e 78, DMSO₂ peaks were integrated using ion m/e 94).

Number of Analyses	Level of standards	Mean of peak area	SD of Peak area	CV %
6	60 ng DMSO	53352.2	± 2734.4	5.1
6	100 ng DMSO	115514.2	± 3823.8	3.3
6	150 ng DMSO	151976.5	±10314.5	6.8
6	200 ng DMSO	218499.0	±20285.8	9.2
6	60 ng DMSO ₂	50869.0	± 1997.2	3.9
6	100 ng DMSO ₂	93125.3	± 3246.8	3.5
6	150 ng DMSO ₂	144906.7	± 8079.9	5.6
6	200 ng DMSO ₂	219550.5	±16154.5	7.3

Table 7. Percent recovery and day-to-day coefficients of variation of spiked urine samples by GC/MS in full scan mode.

Number of Analyses	concentration (ug/ml)	Mean of recovery %	SD of recovery	CV, %
DMSO				
8	10	47.9	±6.5	18.3
8	15	48.9	±5.2	15.1
DMSO ₂				
8	10	107.5	±13.4	19.9
8	15	109.5	±14.1	21.5

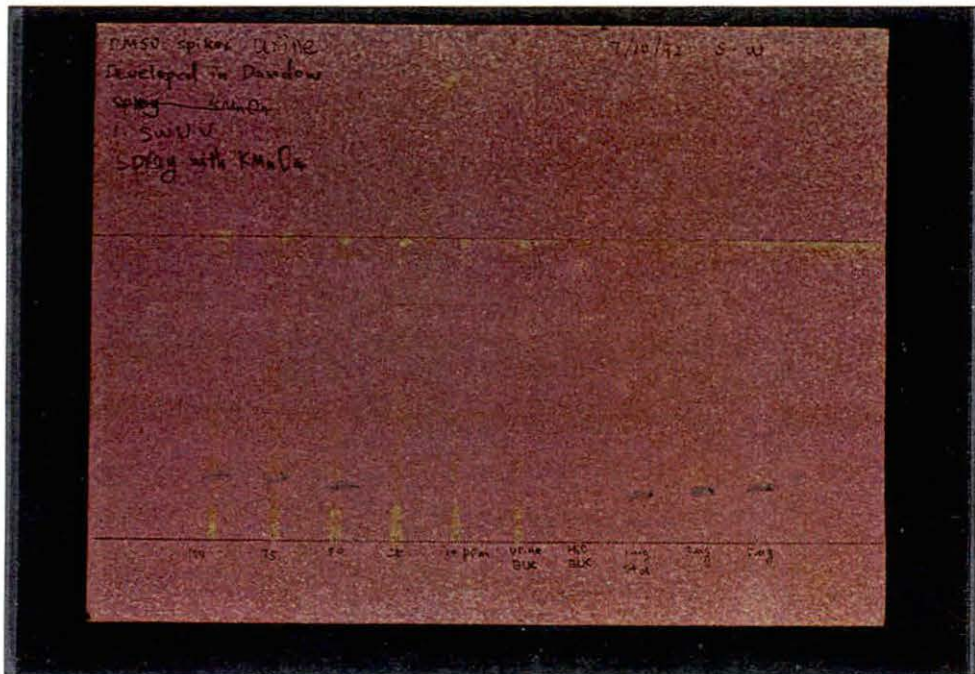


Figure 12. Thin-layer chromatographic results of DMSO spikes following salt out extraction of 2 ml urine.
 Spray sequence = 1 % potassium permanganate.

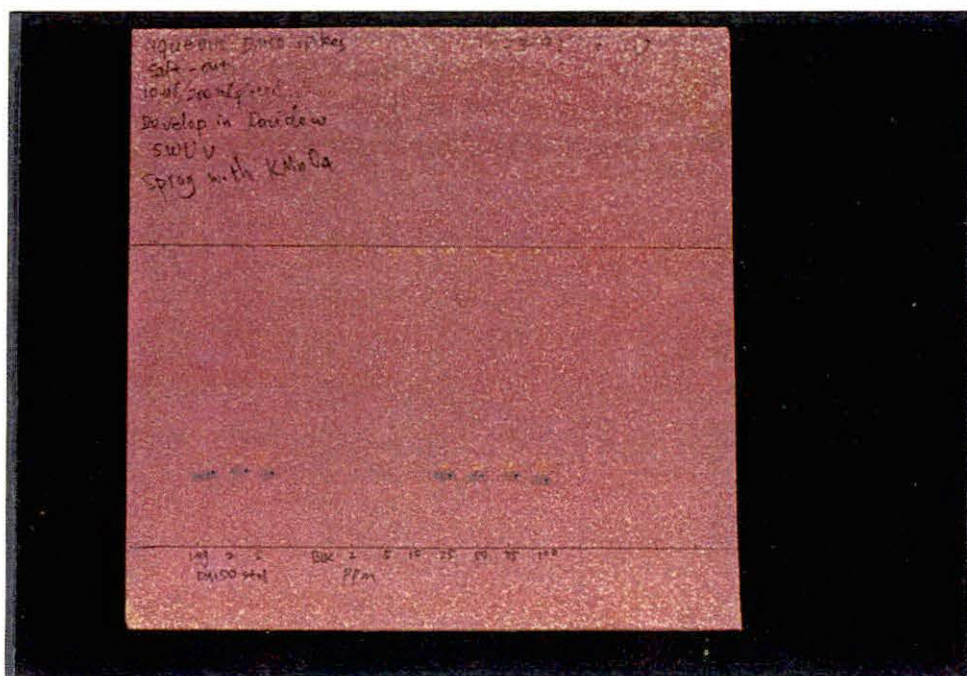


Figure 13. Thin-layer chromatographic results of DMSO spikes following salt out extraction of 2 ml water.
Spray sequence = 1 % potassium permanganate

result is reasonable because the endogenous background in urine spike may interfere with the extraction. In order to increase spike recovery of DMSO, these same spikes were also extracted by potassium pyrophosphate exhaustive salt-out extraction procedure according to the extraction procedure of the Law *et al.* (1990). They believed that better spike recovery might be obtained using potassium pyrophosphate instead of using sodium chloride, because the latter is more soluble in DMSO. However, in our experiment, the detection ranges of two methods were the same for both DMSO urine and aqueous spikes on TLC plates. In comparison, sodium chloride salt-out procedure was easy to perform. So, this method was chosen for all future DMSO/DMSO₂ extraction.

GC/FPD No gas chromatography with sulfur-phosphorus detector analysis of urine spikes was performed in this study due to the lack of acceptable analytical reproducibility.

GC/MS GC/MS was chosen for the DMSO/DMSO₂ spike recovery analysis because of the high degree of specificity, accuracy, and precision within the system. Two ml of the blank dog urine was spiked with DMSO/DMSO₂ at the following concentrations: 0.0, 0.5/0.5, 1.5/1.5, 2.5/2.5, 5/5, 10/10, 15/15, and 20/20 ppm. Spikes were then extracted by the salt-out exhaustive extraction procedure, and 1 μ l of the final extract was injected into the GC/MS. For DMSO urine spikes, quantitative analysis was done by SIM mode with peak area of the molecular ion m/e 78 used to determine percent recovery. For DMSO₂ urine spikes, peak area of the molecular ion m/e 94 was used to determine percent recovery. Aqueous spikes were extracted and treated using the same

procedure and data was integrated in the same way. In urine, the spikes were detectable down to 0.5 ppm for DMSO and 1.5 ppm for DMSO₂. In water, the spikes were detectable down to 0.5 ppm.

Table 8 and Table 9 represent the percent recovery and within day coefficients of variation of the DMSO and DMSO₂ in urine and aqueous matrix by GC/MS in the SIM mode. The results indicate that at all concentrations listed, recovery of DMSO₂ urine spikes varied from 104.5 % at 1.5 ppm to 156.8 % at 20.0 ppm, recovery of DMSO urine spikes varied from 20.4 % at 0.5 ppm to 74.8 % at 20.0 ppm. The possible causes of spike recovery variability are: (1) multistage exhaustive extraction method might cause variable recovery of the analyte; (2) loss of analyte during the concentration process might cause a lower recovery at lower spike level.

Since both DMSO and DMSO₂ are possible endogenous compounds in dog urine, A blank urine sample (0.0 ppm) was also analyzed for the presence of both DMSO and DMSO₂, the background values of DMSO and DMSO₂ in blank urine were subtracted from final spike recovery calculation. DMSO spike recoveries were obviously lower than that of DMSO₂ at each spike level. The possible explanations for this phenomenon are (1) salting out does not improve extraction because many inorganic salts highly soluble in dimethyl sulfoxide, (2) the loss of DMSO during the concentration process because DMSO is more volatile than DMSO₂. However, within the forensic racing laboratories, this extraction method is sensitive enough for DMSO/DMSO₂ analysis of dog urine because it is relevant to levels found in doped dogs.

Table 8. Percent recovery of DMSO and DMSO₂ urine spikes by GC/MS in SIM mode (n=1).

Spike (ppm)	0.0	0.5	1.5	2.5	5.0	10	15	20
DMSO ^a	4.4	8.9	27.8	47.9	99.9	227.6	377.6	584.2
DMSO ^c STD	0.0	22.1	61.4	99.4	196.1	389.1	582.0	775.0
% Recovery	--	20.4	38.4	43.7	48.7	57.4	64.1	74.8
DMSO ₂ ^b	287.4	209.1	343.2	428.2	641.0	794.9	1086.8	1463.7
DMSO ₂ ^d STD	0.0	16.9	53.4	91.2	185.3	373.6	561.9	750.3
% Recovery	--	--	104.5	154.6	190.8	135.8	142.3	156.8

Within day spike recovery coefficient variation (C.V. % is 19.2 for DMSO₂, 21.7 for DMSO)

^aDMSO sample spike (area X 1000).

^bDMSO₂ sample spike (area X 1000).

^cDMSO standard (area X 1000).

^dDMSO₂ standard (area X 1000).

Table 9. Percent recovery of DMSO and DMSO₂ water spikes by GC/MS in SIM mode (n=1).

Spike (ppm)	0.5	1.5	2.5	5.0	10	15	20
DMSO ^a	5.8	29.3	46.7	78.0	217.6	271.4	397.6
DMSO ^c STD	22.2	61.0	99.6	196.1	389.0	582.0	775.1
% Recovery	26.2	48.1	46.9	39.8	55.9	46.6	51.3
DMSO ₂ ^b	12.3	64.8	88.1	160.4	382.2	542.7	670.3
DMSO ₂ ^d STD	15.6	53.5	91.1	185.3	373.6	561.9	750.3
% Recovery	78.8	120.9	96.6	86.6	102.3	96.6	89.0

Within day spike recovery coefficient variation (C.V. % is 14.1 for DMSO₂, 21.3 for DMSO)

^apeak area (m/e 78) X 1000 of sample spike

^bpeak area (m/e 94) X 1000 of sample spike

^cpeak area (m/e 78) X 1000 of DMSO standard

^dpeak area (m/e 94) X 1000 of DMSO₂ standard

Endogenous and Dosed Drug Concentrations

Administration urine

TLC Two ml of urine sample from dog # 8180 given DMSO at two dose levels (1 ml and 200 μ l pure DMSO) were extracted with the salt-out exhaustive extraction procedure. These two dosages were chosen because it is important for us to know at which dosage DMSO levels can be distinguished from endogenous levels at certain postdose times in dog urine.

Typical pictures of TLC results are represented in figure 14 and 15. The results show that at 1 ml/per dog dose level, presence of the DMSO can be detected according to the currently accepted thin-layer parameters up to approximately 24 hours post dose when 10 μ l of the extract are plated on TLC plate. After 24 hours post dose, the presence of DMSO on the plate was not visible to the naked eye. At 200 μ l/per dog dose level, visualization to the naked eye of original DMSO administration was not possible.

GC/MS Gas chromatography mass spectrum analysis of the administration urine from dog # 8180 and # 9577 at 200 μ l dose level and from dog #8180 at 1 ml dose level was performed in full scan and SIM mode. A four-point standard curve and two control samples (spikes) were included in every batch of samples. The total peak area of ions were monitored for quantitation at m/e 63, m/e 78 for DMSO and m/e 79, m/e 94 for DMSO₂. When analyzing the total abundance of ions m/e 63, m/e 78, m/e 79, and m/e 94 in full scan mode, dog # 8180 reached maximum excretion of parent drug (DMSO) in

the urine at 6 hours post administration at both dose levels (1 ml and 200 μ l). It reaches maximum excretion of DMSO₂ in the urine at 8 hours post administration at both dose levels. Dog # 9577 reached maximum excretion of DMSO at 6 hours, and it attained maximum excretion of DMSO₂ at 8 hours post administration at 200 μ l dose level. When analyzing the total abundance of ions m/e 63, m/e 78, m/e 79, and m/e 94 in SIM mode, at 1 ml/per dog dose level, dog # 8180 reached maximum excretion of DMSO in the urine at 6 hours post dose. It reached maximum excretion of DMSO₂ at 8 hours post dose. At 200 μ l dosage, dog # 8180 reached maximum excretion of DMSO in the urine at 8 hours post administration. It reached maximum excretion of DMSO₂ at 8 hours post dose. Results from pre-administration urine indicate both DMSO and DMSO₂ are endogenous constituents of greyhound's urine. From these results, it is obvious that in most cases the peak excretion of DMSO in dogs occurred at 6 hour post dose, the peak excretion of DMSO₂ happened at 8 hours post dose.

Reproducibility and stability of the GC/MS method in full SCAN mode for administered sample analysis were verified by injecting two administration samples three times within a 24 hour period (24 hours and 96 hours at 1 ml/per dog dose level).

Table 10 represents the statistical analysis results. When the variable analyte recovery factor is considered, this statistical results indicate that the method employed has a reasonable reproducibility and short term stability for administered sample analysis. The urinary excretion profiles for DMSO and DMSO₂ in two greyhounds after topical administration of DMSO (1 ml, 200 μ l per dog dose level) in full SCAN mode and SIM

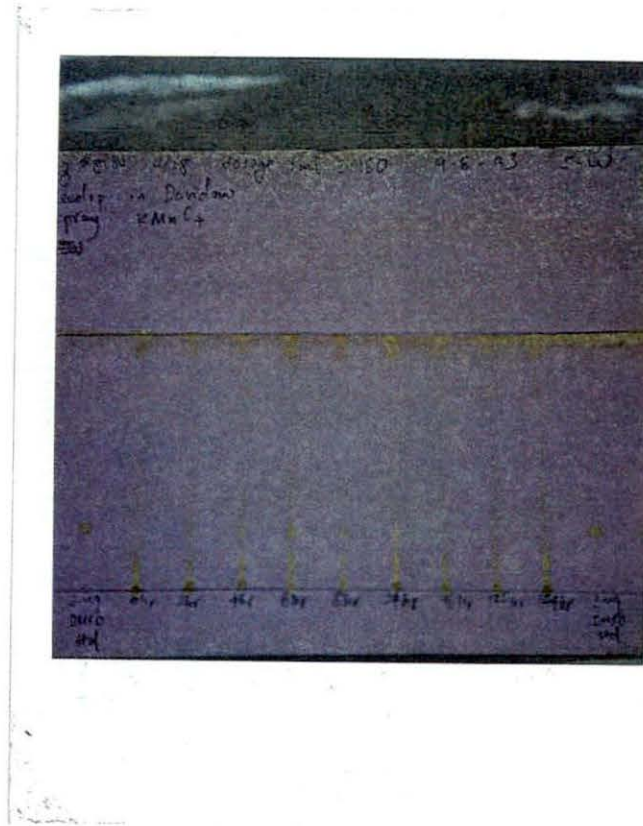


Figure 14. Thin-layer chromatographic results from dog #8180 (1 ml per dog) following salt out extraction of 2 ml urine
 Spray sequence = 1 % potassium permanganate
 Arrows indicate the distance DMSO standard moves from the point of application

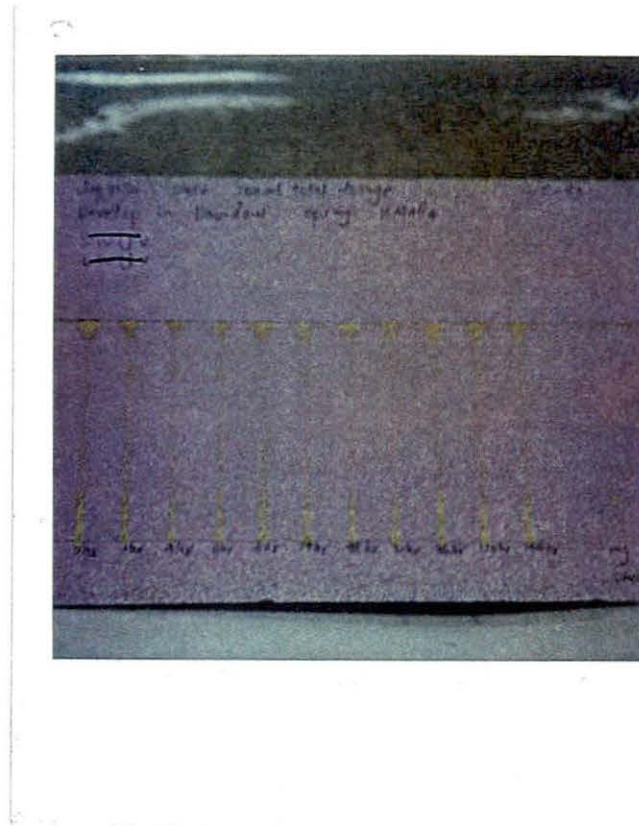


Figure 15. Thin-layer chromatographic results from dog #8180 (200 ul per dog) following salt out extraction of 2 ml urine
Spray sequence = 1 % potassium permanganate
Arrows indicate the distance DMSO standard moves from the point of application

Table 10. Statistical analysis of reproducibility and short term stability study by GC/MS in full scan for administered samples.

Number of Analyses	Post dose Hours	Analytes	Mean ^a of peak area	SD of peak area	CV %
3	24	DMSO ₂	2273.4	± 58.5	2.6
3	96	DMSO ₂	951.1	± 140.3	14.8
3	96	DMSO	71.9	± 7.5	10.4

^apeak area X 1000

mode are showed in Figures 16, 17, 18, 19, 20, 21, 22, 23, 24, and 25.

Endogenous levels of DMSO and DMSO₂ in the greyhound urine

Nineteen greyhounds ranged from 2-5 year in age were chosen for this study. Urine samples were drawn from each dog at 10 a.m every day. The research dogs were not exposed to any drugs during the urine collection period. Samples were stored at -10 °C until analyzed. After thawing in warm water, 2 ml of urine sample from each dog were then extracted by the salt-out exhaustive extraction procedure. Total extract was concentrated down to approximately 200 μ l and transferred to a pre-graduated autosampling vial, 1 μ l injected into the GC/MS by autosampler. Samples were analyzed in full scan and SIM mode for dimethyl sulfoxide and dimethyl sulfone.

A four-point calibration curve was constructed for every day analysis. The correlation coefficient was always better than 0.99. A urine blank and two urine spikes were also included in every batch of samples as control samples.

Usually, the initial mass spectral analysis of suspicious samples is routinely performed in full scan mode. The scan run allows for all ions within the sample to be detected, and then when a suspicious drug is thought to be present SIM mode will be performed by selectively looking at specific ions within the chromatogram. When analyzing the total abundance of ions m/e 63, m/e 78, m/e 79, m/e 94 in full SCAN mode, DMSO₂ can be detected by the presence of both major ions (m/e 79, m/e 94) with

the molecular ion m/e 94 present at 50% of the base ion m/e 79 in all of the blank samples, which make us believe that DMSO_2 is a normal constituent in dog urine. DMSO can also be detected by the presence of both major ions (m/e 78, m/e 63) with the molecular ion m/e 78 present at 50% of the base ion m/e 63 in all of the blank samples, which indicates that DMSO is also an endogenous constituent in dog urine. In SIM mode, both DMSO and DMSO_2 were detected by the presence of two major ions (m/e 78, m/e 63 for DMSO, m/e 79, m/e 94 for DMSO_2) in all blank dog urine samples. However, DMSO_2 concentrations were at much higher level compared to that of DMSO. It is believed that the increase on DMSO_2 level is too large to be due to the original DMSO_2 alone and most of it must have arisen metabolically.

Quantitative analysis of DMSO and DMSO_2 was done in SIM mode by integrating peak area of the molecular ion m/e 78 for DMSO, the molecular ion m/e 94 for DMSO_2 .

Without applying the spike recovery factors, the absolute endogenous DMSO concentrations from 19 research dogs were presented in Table 11 with an average of 552.5 ppb (SD=346.5). Two DMSO absolute concentrations (51694.8 and 2237.0 ppb) which do not fall into the 3 SD range were omitted. From urine spike recovery Table 8, it is obvious that spike recoveries of DMSO in urine were concentration dependent and varied predictably from 20.4 % at 0.5 ppm concentration to 74.8 % at 20.0 ppm concentration. After corrected by the spike recovery factors, endogenous DMSO concentrations from 19 research dogs covered a range from 1258.8 to 3827.8 ppb (Table 11). The average endogenous concentration of DMSO is 2383.4 ppb

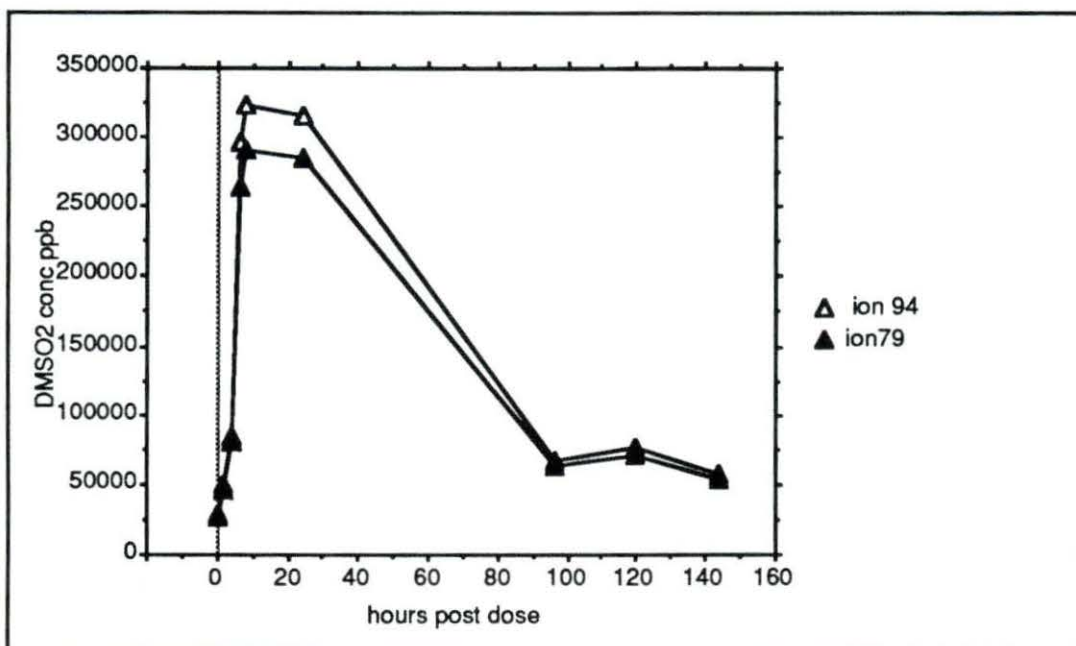


Figure 16. DMSO₂ urinary extraction profile of dog #8180 following administration of 1 ml DMSO by GC/MS in full scan, n=1

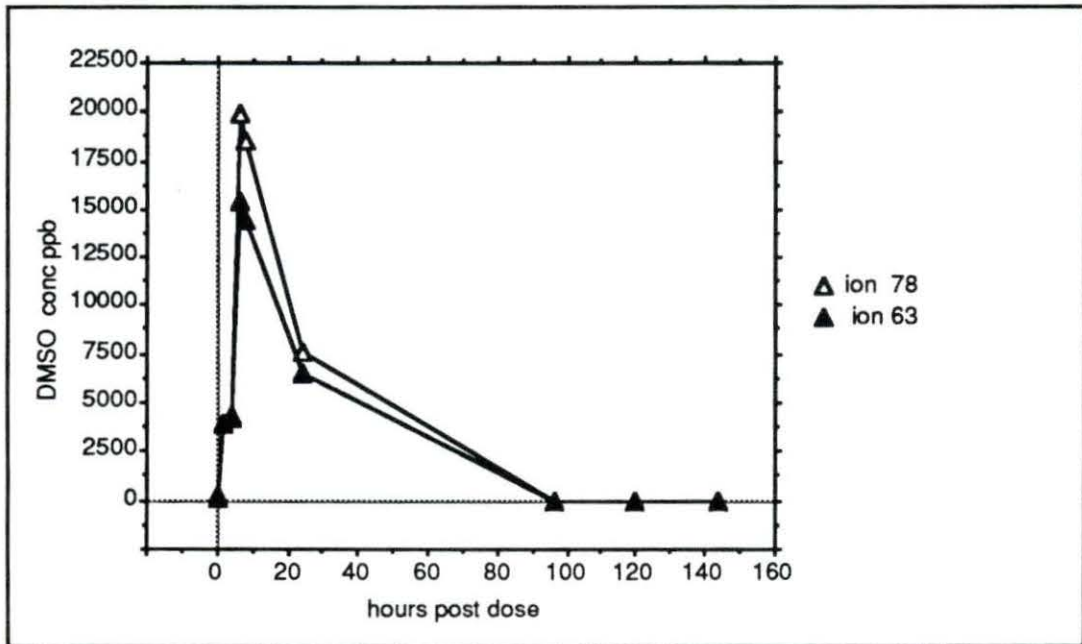


Figure 17. DMSO urinary extraction profile of dog #8180 following administration of 1 ml DMSO by GC/MS in full scan, n=1

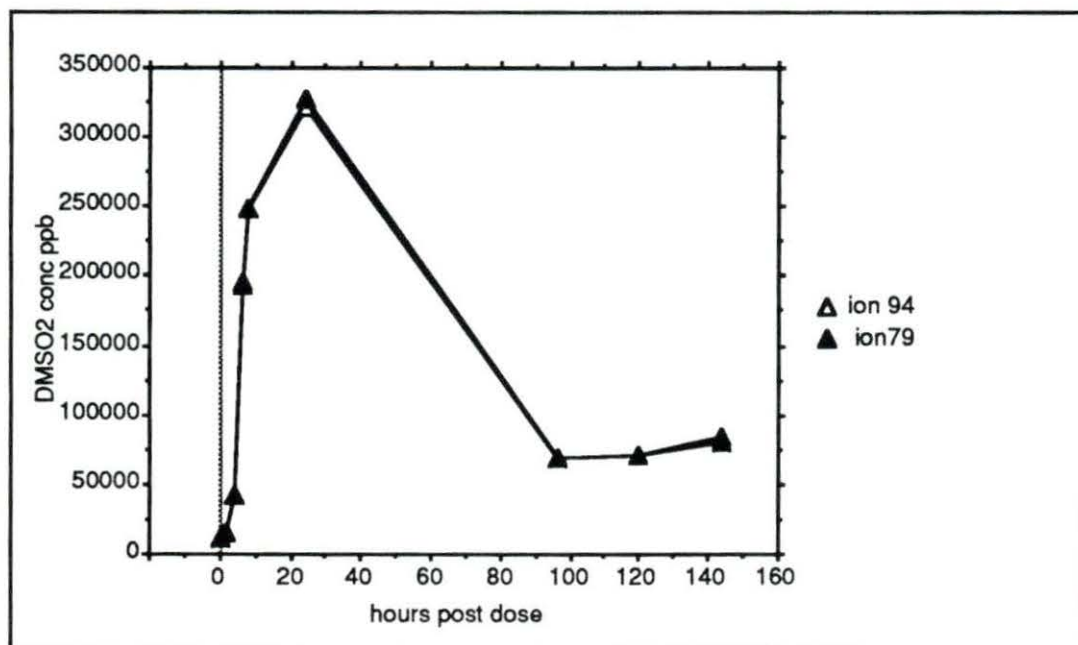


Figure 18. DMSO₂ urinary extraction profile of dog #8180 following administration of 1 ml DMSO by GC/MS in SIM mode, n=1

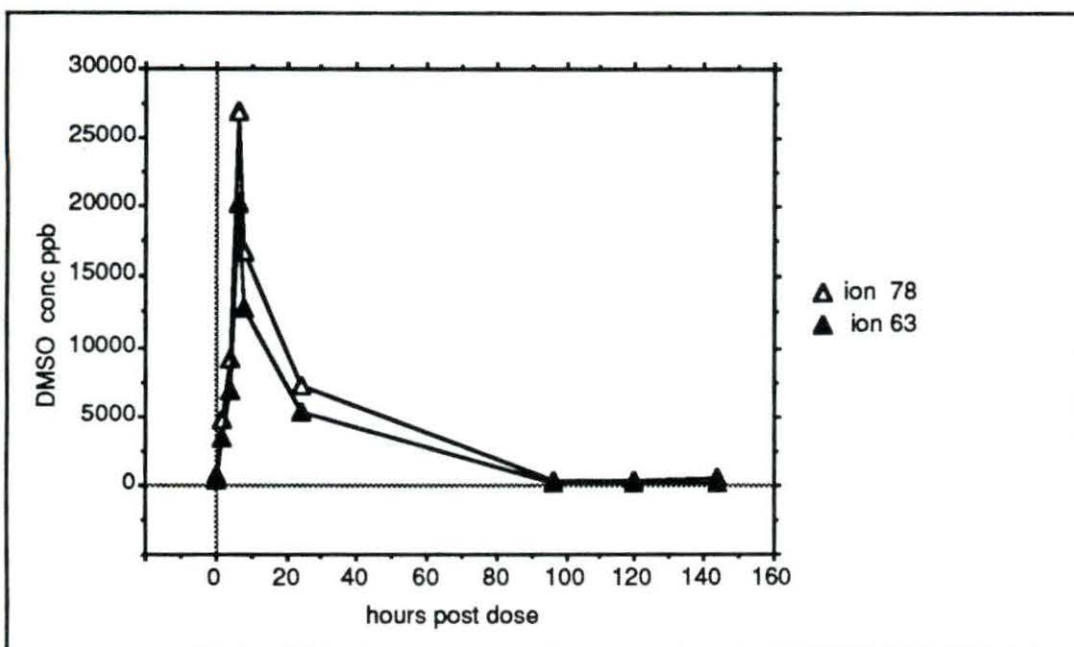


Figure 19. DMSO urinary extraction profile of dog #8180 following administration of 1 ml DMSO by GC/MS in SIM mode, n=1

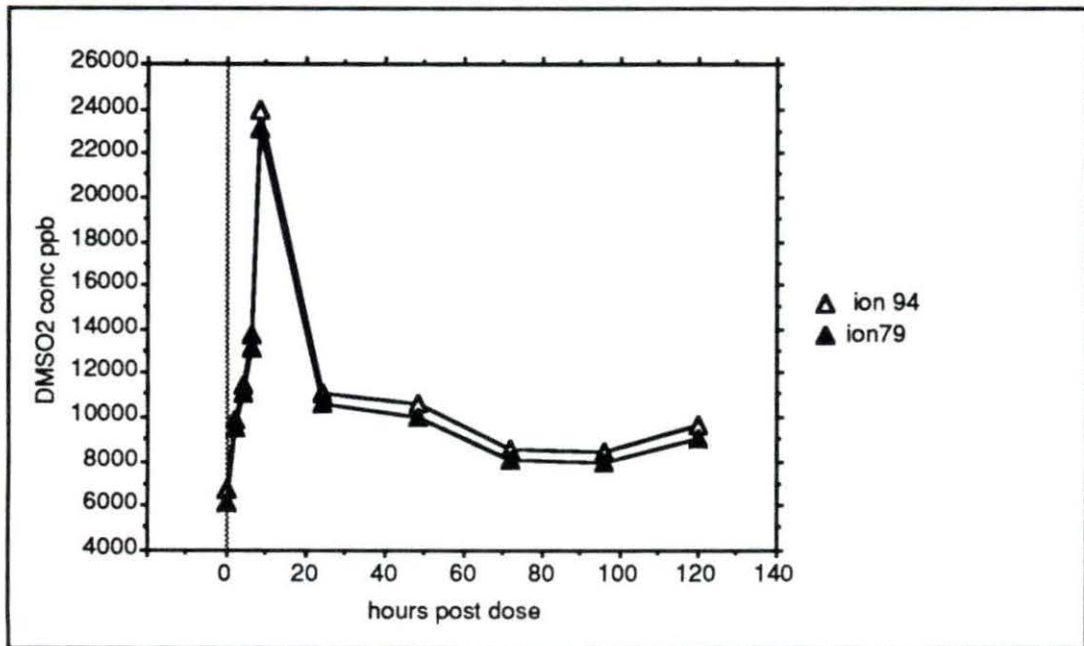


Figure 20. DMSO₂ urinary extraction profile of dog #8180 following administration of 200 ul DMSO by GC/MS in full scan mode, n=1

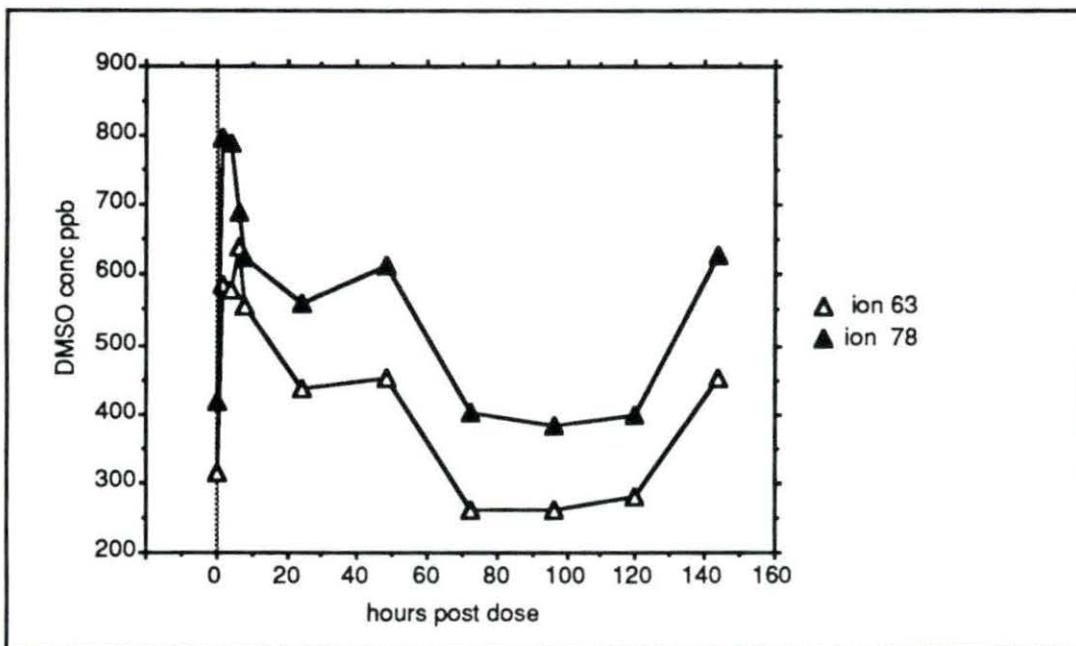


Figure 21. DMSO urinary extraction profile of dog #8180 following administration of 200 ul DMSO by GC/MS in full scan mode, n=1

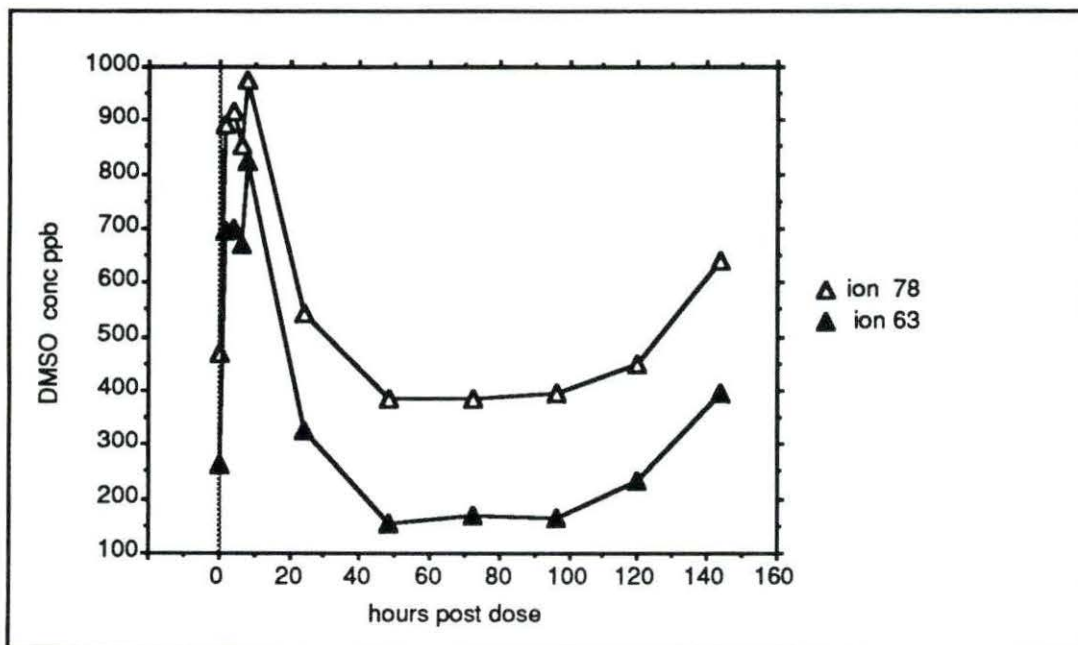


Figure 22. DMSO urinary extraction profile of dog #8180 following administration of 200 ul DMSO by GC/MS in SIM mode, n=1

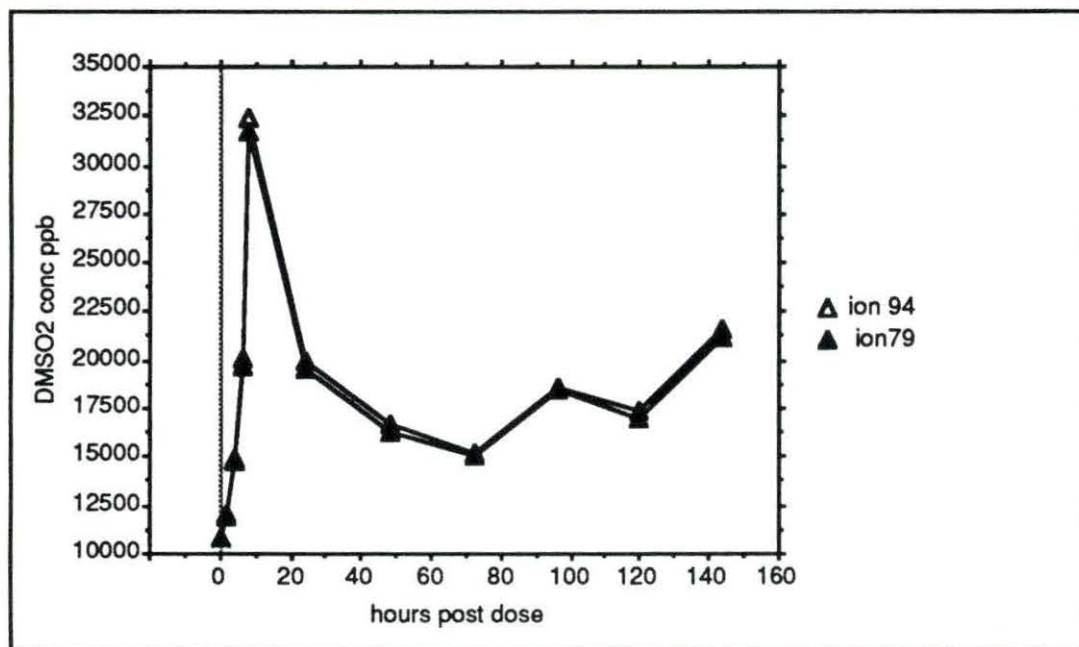


Figure 23. DMSO₂ urinary extraction profile of dog #8180 following administration of 200 µl DMSO by GC/MS in SIM mode, n=1

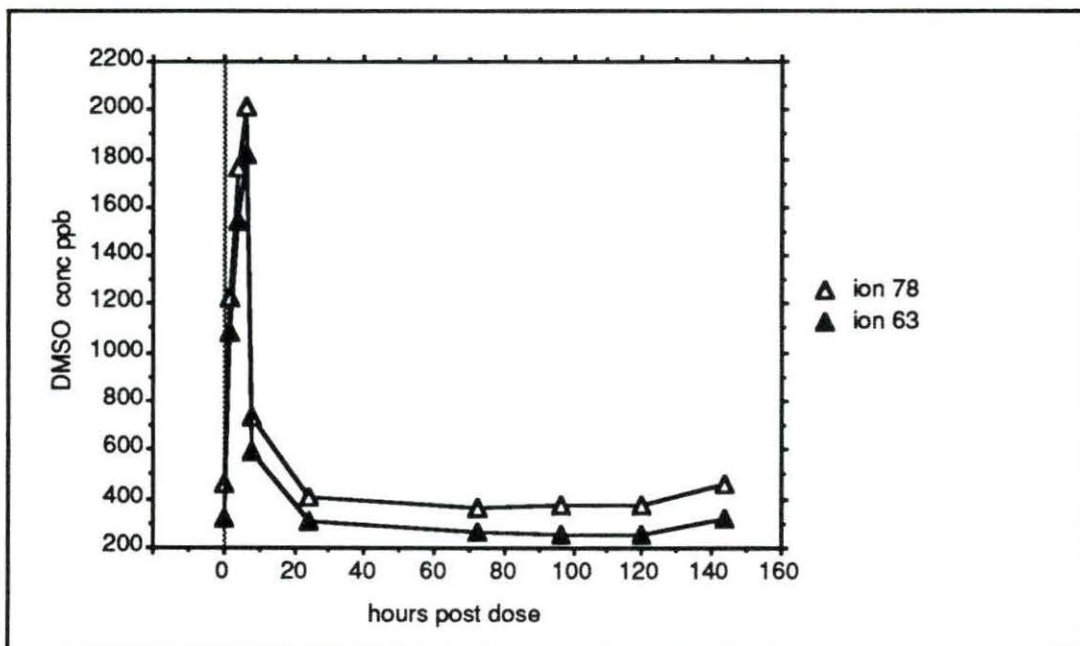


Figure 24. DMSO urinary extraction profile of dog #9577 following administration of 200 ul DMSO by GC/MS in full scan mode, n=1

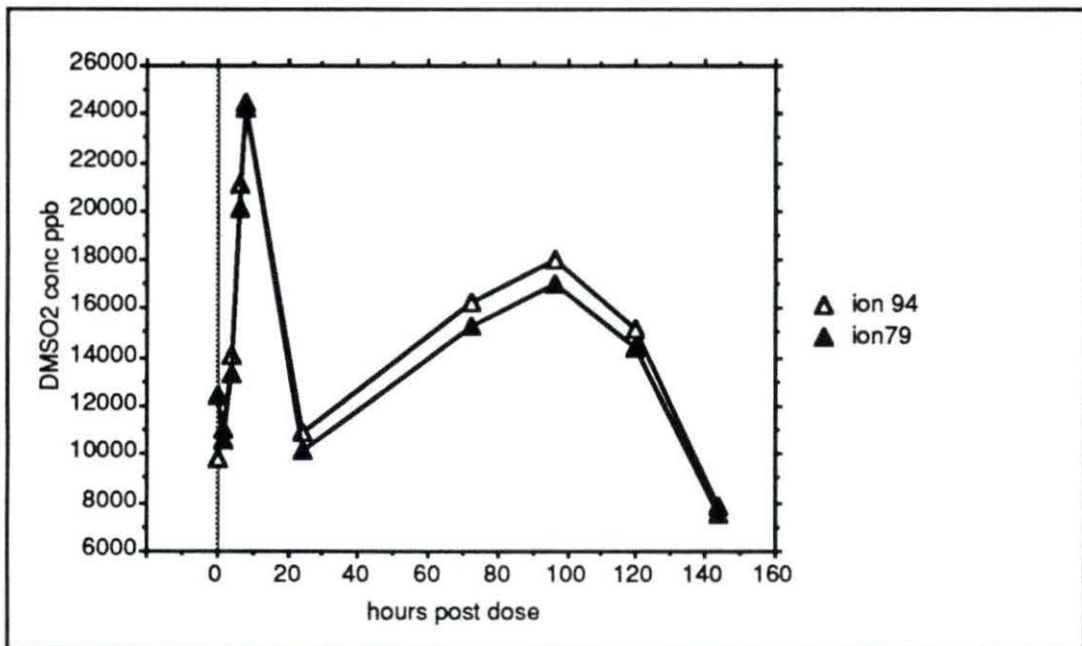


Figure 25. DMSO₂ urinary extraction profile of dog #9577 following administration of 200 ul DMSO by GC/MS in full scan mode, n=1

(SD=798.2). Endogenous concentration range of DMSO₂ in nineteen dogs varied from 2.2 ppm to 164.8 ppm. From urine spike recovery Table 8, it can be seen that the percent recovery of DMSO₂ urine spike was above 100% at each spike level. One DMSO₂ concentration (309093.4 ppb) which falls to outside of the 3 SD range was omitted. The average endogenous concentration of DMSO₂ is 70.4 ppm (SD=50.4). The reasonable explanation of the variability are (1) variable detector, (2) variable analyte recovery.

Figure 26 shows the comparative study between normal DMSO levels of 19 research dogs and dosed DMSO levels for 144 hours post dose period of dog #8180 and #9577 at two subtherapeutic use levels (DMSO 1 ml and 200 ul/dog). From the figure, it can be seen clearly that at 200 ul dosage all dosed DMSO levels of dog #8180 for 144 hours post dose period are within the 2 SD range of normal DMSO level, However, 4 and 6 hours post dosed levels of dog #9577 are not within the 2 SD range of normal levels. At this point, it is hard to draw a conclusion regarding the differentiation between the dosed DMSO levels and normal DMSO levels at 200 ul subtherapeutic dosage. Further statistical analysis and more administration study are necessary for reaching the final conclusion. At 1 ml dose level, the farthest post dose time point at which DMSO level does not fall into the two standard deviation range of normal DMSO levels is 24 hours, which indicates that normal DMSO levels can be easily distinguished from dosed DMSO levels for 24 hours post dose even at subtherapeutic use level 1 ml per dog.

Figure 27 represents the similar comparative study between normal DMSO₂ levels

Table 11. Endogenous urinary concentrations of DMSO in 17 research dogs

Absolute concentrations ppb	Corrected concentrations ppb
186.3	1258.8
1068.5	3561.7
903.9	3263.2
501.5	2458.3
873.7	3223.9
226.5	1461.3
766.0	3039.7
439.0	2286.5
1416.3	3827.8
276.6	1686.6
195.0	1308.7
403.0	2155.1
247.1	1563.9
595.8	2708.2
344.6	1957.9
433.2	2256.3
514.6	2498.1

of 19 research dogs and dosed DMSO₂ levels for 144 hours post dose period of dog #8180 and dog #9577 at two subtherapeutic use levels (DMSO 1 ml and 200 ul/dog). From the figure, it is obvious that all dosed DMSO₂ levels of two dogs for 144 hours post dose at 200 ul dosage are within the 2 standard deviation range of normal DMSO₂ levels. The results mean that dosed DMSO₂ levels in dog #8180 and #9577 at 200 ul subtherapeutic dosage can not be distinguished from normal DMSO₂ levels within the 2 SD range. At 1 ml dosage, the farthest post dose time point at which DMSO₂ urine level does not fall into the 2 SD range of normal DMSO₂ levels is 24 hours, which also indicates that normal DMSO₂ levels can be easily distinguished from dosed DMSO₂ levels for 24 hours post dose at subtherapeutic use level 1 ml per dog. These results provide very useful information for this work regarding urinary clearance kinetic and therapeutic use levels of DMSO and DMSO₂ in dogs at rest state.

The results also help us to identify the important post dose time point regarding the differentiation between normal DMSO/DMSO₂ urinary levels and dosed DMSO/DMSO₂ urinary levels. However, for accurate determination of this crucial time point, more statistical analysis and administration study of DMSO and DMSO₂ in dogs will be needed in future work.

Practicability

The procedure and techniques utilized in this work could be utilized in a routine drug testing program with these conditions. Chromatographic screening and confirmation

often necessitates extensive extraction and cleanup procedures prior to injection into the instrument, greatly increasing the time required for the analysis. The analysis of a large number of samples usually requires several days to finish just the extraction process and need more time to do the final confirmation and quantitation so that the results can be finalized. If when automatic instruments are available, the time can be greatly reduced.

The chromatographic instrumentation such as GC/FPD and GC/MS require some degree of training in order to fully operate the instrument to its capacity and solve the problems encountered.

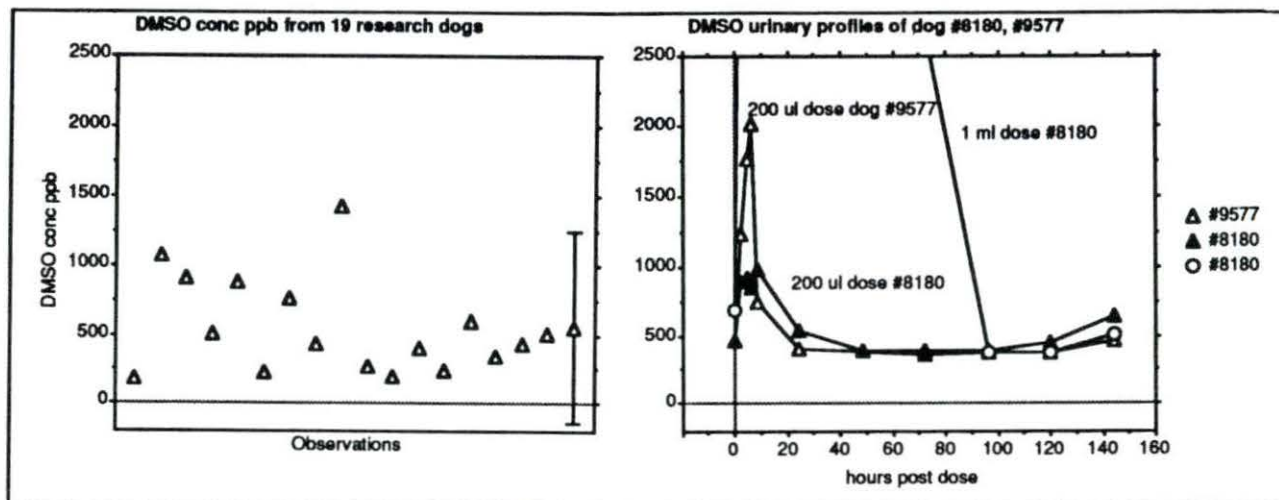


Figure 26. Comparative study between normal DMSO urine levels and dosed DMSO urine levels

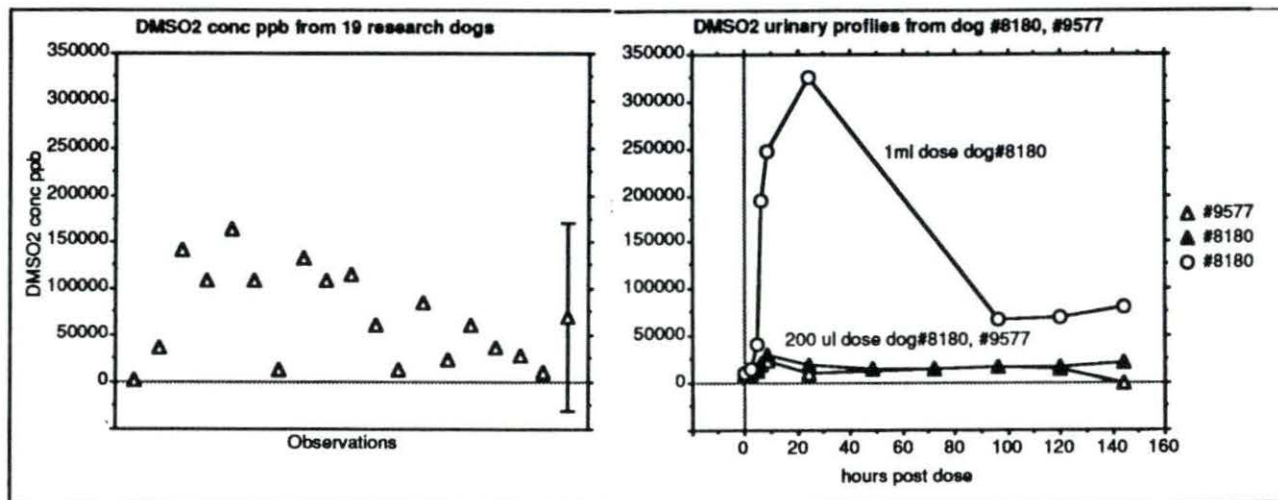


Figure 27. Comparative study between normal DMSO₂ urine levels and dosed DMSO₂ urine level

SUMMARY

This study investigated the characteristics of analytical methods such as analyte recovery, detection ranges, specificity, reliability and precision, and determined endogenous levels of DMSO and DMSO₂ in a selected sampling of greyhound urine using the optimal method of choice.

Gas chromatography with sulfur-phosphorus flame photometric detection method had poor sensitivity and reproducibility with standards, which make GC/FPD an undesirable method for detection of DMSO and DMSO₂. Thin layer chromatography was also indicated as a poor detection method in terms of sensitivity with standard and spikes. Gas chromatography/mass spectrometry showed much better reproducibility than the GC/FPD analytical technique. GC/MS in full scan or SIM results in acceptable curves with useful detection limits and specificity. The lowest detection limit of 0.6 ng for both DMSO and DMSO₂, was accomplished by GC/MS in SIM mode detection.

GC/MS method was also indicated as being superior in terms of analytical specificity, sensitivity and reproducibility with pure standards and aqueous/urine-based spikes with DMSO and DMSO₂. With spikes, the detection limit can be extended as low as 0.5 ppm in urine-based spikes for DMSO; 1.5 ppm for DMSO₂, and 0.5 ppm in aqueous-based spikes for both DMSO and DMSO₂.

Among the classical analytical methods investigated, exhaustive salt out liquid liquid extraction assay was relevant to spiked and dosed urine samples.

A spike recovery study was done in SIM mode by analyzing both aqueous and urine spikes. Spike recoveries were concentration dependent and varied predictably from 26.2% at 0.5 ppm to 51.3% at 20.0 ppm for aqueous DMSO spikes, 78.8% at 0.5 ppm to 89.0% at 20.0 ppm for aqueous DMSO₂ spikes; For urine DMSO spikes, spike recoveries varied predictably from 20.4% at 0.5 ppm to 74.8% at 20.0 ppm. For urine DMSO₂ spikes, spike recoveries were above 100% at every spike levels.

Administration study of DMSO and DMSO₂ in dogs was done in both full scan and SIM mode. Two subtherapeutic dosages (1 ml and 200 ul/per dog) of DMSO liquid were topically administrated on the skin of two dogs of each group. In most cases, the peak excretion of parent compound DMSO occurred at 6 hours post dose, and the peak excretion of metabolite DMSO₂ happened at 8 hours post dose.

Endogenous levels of DMSO in 17 research dogs averaged at 2383.4 ppb with a SD of 798.2 after corrected by spike recovery factors. Without correction of spike recovery factors, endogenous concentrations of DMSO averaged at 552.5 ppb with a SD of 346.5. Endogenous concentrations of DMSO₂ in 18 dogs averaged at 70.4 ppm with a SD of 50.4.

In terms of distinguishing between normal DMSO/DMSO₂ urine levels and dosed DMSO/DMSO₂ urine levels, GC/MS techniques can detect presence of both DMSO and DMSO₂ in all of the blank urine samples and dosed urine samples analyzed. At 200 ul per dog dose level, dosed DMSO₂ levels of two dogs can not be easily distinguished from normal DMSO₂ levels; dosed DMSO levels of dog #8180 were also within the 2 SD

range of normal DMSO levels. However, at same dosage, not all of dosed DMSO levels of dog #9577 were within the 2 SD range of endogenous DMSO levels. At 1 ml per dog dose level, normal DMSO levels were easily distinguished from dosed DMSO levels for 24 hours post dose time; normal DMSO₂ levels also can be distinguished from dosed DMSO₂ levels for 24 hours post dose.

SECTION II. HYDROCORTISONE

INTRODUCTION

Hydrocortisone is an endogenous steroid compound occurring in man and animal. As well as having potent anti-inflammatory effects, cortisol also has potent effects on protein and carbohydrate metabolism. In man and animal, cortisol is often used clinically as an anti-inflammatory agent. In the racing community, exogenous administration of cortisol and its synthetic analogues can be used to affect the performance of the racing animals. Because of this, very sensitive and selective methods are necessary to establish endogenous cortisol levels in biological samples.

Among today's variety of analytical technology, GC/MS is usually regarded as one of the best confirmation methods for drug detection in racing chemistry. Newly developed immunoassay techniques also offer advantages of ease of performance, and rapidity, sensitivity and specificity.

The objective of this study was to investigate urinary threshold levels of hydrocortisone in the dog. The work consisted of several stages. The first part of this research work was to determine the characteristics of different analytical methods such as detection ranges, specificity, reliability, precision and accuracy. The second part of this work was to focus on the determination of urinary endogenous values of cortisol in the greyhound using the optimum method of choice.

LITERATURE REVIEW

General Characteristics and Pharmaceutical Effects

Hydrocortisone ($11\beta,17\alpha,21$ -trihydroxy-4-pregnene-3,20-dione, cortisol or compound F) is the primary glucocorticoid secreted by the canine adrenal cortex to limit the body's response to stress and regulate protein and carbohydrate metabolism. It is synthesized originally from cholesterol by a series of reactions. Its molecular weight is 362.47. UV max is 242 nm (Baxter and Rousseau, 1979).

Cortisol and its synthetic analogous--prednisolone, methylprednisolone dexamethasone, betamethasone, triamcinolone, and paramethasone are known to exhibit many important physiological and biochemical effects. In humans, cortisol has been used clinically since the early 1950's as an anti-inflammatory agent. In dogs, the corticosteroids are used for a variety of conditions. Examples of these are, arthritis, dermatitis, eczema, and ocular inflammations (Egan *et al.*, 1977).

The pharmacological use of cortisol in man and animal has been summarized as follows:

- (1) anti-inflammatory uses
- (2) uses in skin diseases
- (3) uses in infectious conditions
- (4) uses in arthritics
- (5) uses in shock

(6) uses in immunology.

The precise mechanism of the anti-inflammatory effects of the corticosteroids is not known, but certain characteristics of this action can be enumerated. It produces its anti-inflammatory effect by inhibiting vasodilation, by decreasing capillary permeability, by modifying the activity of the white cells, and by affecting the connective tissues (Melby, 1977). As well as having a potent anti-inflammatory effect, the cortisol also has equally potent effects on protein and carbohydrate metabolism. These two effects appear equally important and inseparable.

Most mammalian species are reported to secrete predominantly cortisol. Many investigations of adrenocortical secretion in the dog have shown that the main steroid produced is cortisol, followed by corticosterone (Adlin and Channick, 1966). One paper showed that in the post-race urine samples of 110 horses, the free cortisol measured by enzyme-linked-immunoassay showed a mean concentration of 950 nmol/L and covered a wide range from 64-3281 nmol/L. The possible causes of the variability were summarized as: (1) breed; (2) variability in the adrenal response to excitement and exercise; (3) the effects of vasopressin (antidiuretic hormone) which showed a marked, but variable increase during a simulated race day (Irvine *et al.*, 1988). Studies also indicated that the large variation in the quantities of corticosteroids secreted by different dogs under uniform experimental conditions are possible (Hechter *et al.*, 1955). Several studies have been done to determine the serum hydrocortisone values in normal dogs by different immunoassay techniques (Richkind and Edqvist, 1973; Becker *et al.*, 1976;

Chen *et al.*, 1978; Peterson *et al.*, 1986).

Cortisol secretion varies with time of day in many species. Diurnal variation in urine or plasma levels in different species have been reported by several researchers. In human, plasma cortisol is highest in early morning and lowest near midnight. In equine and canine, cortisol plasma levels were high in the morning and low in the evening (Harwood and Mason, 1956; Zolovick *et al.*, 1966; Hoffsis *et al.*, 1970a; Larsson *et al.*, 1979). In nocturnal animals such as the rat, plasma cortisol concentration secretion may depend on the sleep-wake or light-dark transition of the subject (Bush, 1953). In Beagles and Greyhounds, one paper indicated that there was no diurnal variation of peripheral plasma levels of corticosteroids (Richkind and Edqvist, 1973).

As previously mentioned, cortisol and its synthetic analogues have been used both locally and systemically as anti-inflammatory agents and antiarthritic drugs since the 1950's. When administered to racing animals systemically, however, the corticosteroids have a number of pharmacological effects that might be considered useful in improving the performance of a racing animal. These agents may improve a racing animal's performance through reduction of inflammation, relief of stress, an increase in the availability of glucose, and possibly because of the euphoria which is often induced. The agents stimulate the utilization of fat and amino acids and thereby increase blood glucose levels, which glucose is then available as extra energy for the animal (Tobin, 1981).

The glucocorticoids also stimulate the appetite and produce an elevation of "mood". These actions make cortisol useful in racing animals that are under severe stress

and are probably have led to the use of this drug in illegal medicine (Tobin, 1981). One study reported the influence of synthetic corticosteroids on racing performance by concluding that the synthetic corticosteroids have the potential to change the racing performance of horses. Race horses medicated inconsistently with synthetic corticosteroids, from one start to the next, tend to have an inconsistent racing form in over half of the cases (Watrin, 1988).

Metabolism and Elimination

In plasma, usually around 95% of the cortisol present in the circulation is bound to plasma protein. Most of this binding (77%) is to a specific corticosteroid-binding globulin (CBG or transcortin) and a lesser amount (15%) is to albumin, with about 8% in an unbound form. This circulating steroid is largely inactivated in the liver upon conjugation with glucuronic acid and sulfuric acid (Tietz, 1970).

The liver is the important site of catabolism of steroids, and the enzymes concerned are located in the sub-cellular fractions. Five major routes exist for cortisol metabolism: (1) reduction of the 4-5 double bond, hydroxylation of the 3-keto group; (2) hydroxylation at the C₂; (3) conversion of the 11-hydroxyl to a 11-keto group; (4) reduction of carbonyl group at C₂₀ to two isometric alcohols; (5) hydroxylation at C₆ (Baxter and Rousseau, 1979).

The major pathways of cortisol metabolism in man are illustrated in Figure 1.

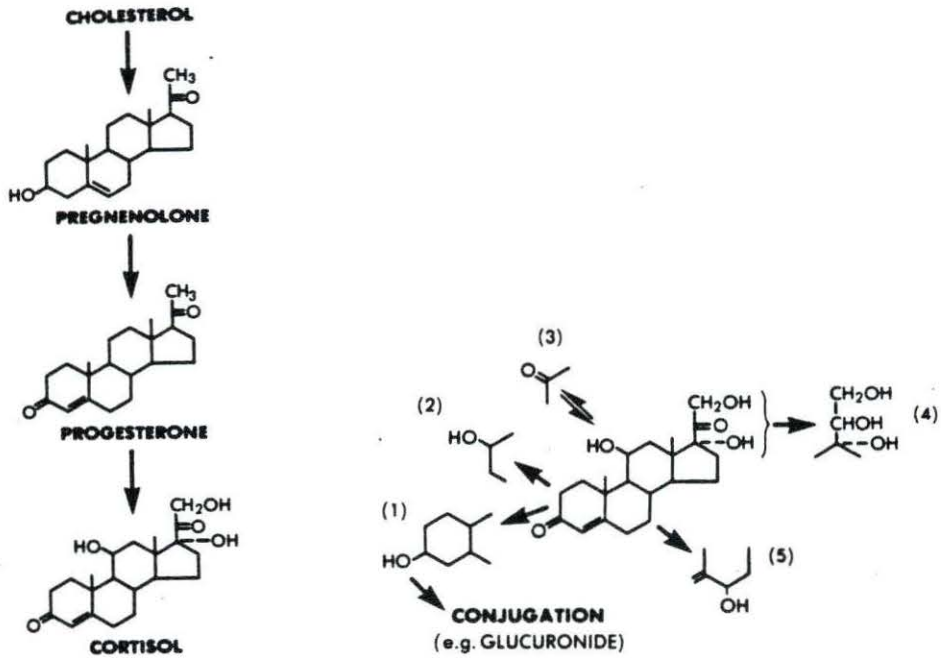


Figure 1. The major path ways of cortisol metabolism in man (Baxter and Rousseau, 1979)

Extra-hepatic catabolism of glucocorticosteroids has been reported to occur in the lung (Sowell *et al.*, 1971), skin (Hsia and Hao, 1966), eyes, pituitary (Brown *et al.*, 1957) and kidney (Ganis *et al.*, 1971).

Excretion is the end compartment of a process that starts with biosynthesis or administration and continues through absorption, distribution and metabolism in the various body tissues. The different end products of steroid catabolism are usually made more water-soluble by conjugation with polar acidic substances before they are eliminated from the organism. A small proportion (1%) of circulating blood cortisol is excreted in the urine in the unconjugated or free state. The excretion of free cortisol in the urine is remarkably limited by plasma protein binding which lessens cortisol filtration at the glomerulus, and also by the tubular reabsorption of filtered cortisol (Beisel *et al.* 1964; Scurry and Shear, 1969), and this fraction increases or decreases in accordance with adrenal output.

Cortisol is rather rapidly removed from the circulation, having a plasma half-life of approximately 60 minutes in humans. Endogenous steroids and their synthetic analogues are excreted, almost completely, in both urine and feces mainly as the conjugates and in small amounts as free steroids. Steroids metabolites excreted in the feces can arise from the unabsorbed drug after oral administration or from their biliary secretion. After biliary secretion into the intestine, steroids can be reabsorbed and pass into systemic circulation (Hyde and Williams, 1957).

The route of excretion of glucocorticosteroids varies with the nature of the steroids

and the species. In humans, the major proportion of the metabolites of endogenous cortisol are excreted in urine as conjugates of glucuronic acid and a minor proportion as sulphoconjugates (Kornel and Saito, 1975). The latter are mainly eliminated in the feces (Hellstrom *et al.*, 1969). The majority of the urinary metabolites (50-55%) were conjugated with glucuronic acid, about 13-15% appeared as unconjugated materials and less than 10% was found in the sulphate fraction. In dogs, after administration of ^{14}C -corticosterone, 55% of the metabolites are excreted in urine, 32% in feces. The cortisol analogues have longer plasma half lives (Baxter and Rousseau, 1979).

In animals, some experiments have been done to demonstrate the response of the cortisol secretion to age, sex, pregnancy, diet, and exercise. In equine, one study indicated that there were no significant differences in the mean plasma cortisol level according to age, sex, and pregnancy (Hoffsis *et al.*, 1970a). Ralston *et al.* (1988) studied the concentrations of cortisol in blood and urine samples taken from horses after racing. They concluded that the sex of the horse had no significant effect on the plasma cortisol levels. Urine volume and pH had no significant influence on the urinary cortisol concentration, however 9.5% of the urinary cortisol variation could be explained due to the influence of plasma cortisol concentration.

Another study has demonstrated responses of blood cortisol concentration to common equine diets. In equine, blood cortisol concentration showed no meal-related responses to any of the equine diets within its expected circadian rhythm (Stull and Rodiek, 1988). In canine, studies showed that there were no marked differences due to

age, sex, body weight, or breed in the blood hydrocortisone values by radioimmunoassay (Murphy, 1967; Siegel, 1968; Campbell and Watts, 1973; Chen *et al.*, 1978).

Cortisol levels can increase in normal animals due to stress, or be very low in normal resting animals (Siegel, 1968; Campbell and Watts, 1973; Becker *et al.*, 1976; Ling *et al.*, 1979; Feldman, 1985). Aging effects on canine adrenocortical response to exogenous adrenocorticotrophic hormone (ACTH) were also investigated by fluorometric measurement of plasma corticosteroids. Statistical analyses failed to reveal significant sex or age differences between samples (Breznock and McQueen, 1970). In man and animals, exercise seems to increase the rate of uptake of cortisol. When the work load exceeds a critical level, stimulation of the adrenal cortex results in a massive secretion of cortisol which is sufficient to raise the plasma level (Few, 1974; Foss *et al.*, 1971).

Plasma cortisol concentrations in dogs were significantly depressed given either dose of dexamethasone or dexamethasone sodium phosphate by posttreatment hour two and concentrations remained suppressed for at least 16 hours (Kemppainen *et al.*, 1989).

When ACTH (adrenocorticotrophic hormone) was administered, a significant increase of the plasma cortisol level was observed 60 to 90 minutes after the injections (Richkind and Edqvist, 1973). The administration of ACTH caused relatively uniform increased excretion of urinary cortisol in normal dogs of different ages, breeds, and body weights were also reported (Siegel, 1968; Rijnberk *et al.*, 1968). In horses, dexamethasone has a half life of 53 minutes whereas prednisolone has a half life of 99.5 minutes (Toutain *et al.*, 1984).

In dogs, the mean exogenous cortisol half life was evaluated to be 52 ± 1.2 minutes (Eik-Nes *et al.*, 1953). It was reported that the half life of exogenous cortisol in a given dog was not very consistent from one day to another. Errors in timing of sample collections, or in the method itself, might account for the disparity (Kuipers *et al.*, 1958).

In rats, distribution patterns of injected cortisol have been reported. After 5 minutes almost 50% of the administered Cortisol-4-C₁₄ was found in the skeletal muscle. The total plasma contained only about 7% of the injected dose. It was rapidly released through the liver into excretory pathways so that 60 minutes after injection only 2% remained in the muscular tissue whereas 87% of the material injected was found in the gastrointestinal tract. The urine contained only 11% (Firschein *et al.*, 1957).

In horses, the biological half life of cortisol was estimated to be 2.1 ± 0.6 hours. The disappearance of cortisol was found to be biphasic, composed of redistribution and elimination phases. A redistribution phase occurred between 0 and 30 minutes. This phase represented the rapid loss of free and bound cortisol from the blood, followed by cortisol coming into the blood from tissue reservoirs between 0.5 and 2 hours. The second phase (elimination) occurred between 2 and 12 hours. It was a progressive linear decrease to nondetectable or near nondetectable values (Slone *et al.*; 1983).

Because of potential effect of corticosteroids on racing animals, many racing jurisdictions in the United States have rules prohibiting the exogenous use of cortisol and other synthetic corticosteroids. Since hydrocortisone is produced naturally by the body, it is difficult to determine whether additional amounts have been given to the animal

athletes, especially since the normal amounts of cortisol in urine have not been determined.

Analytical Consideration

Many research papers have been published about the detection of cortisol and its metabolites in biological samples using a variety of methods, including thin layer chromatography (TLC), gas chromatography with mass spectrometry detection (GC/MS), high performance liquid chromatography (HPLC), and immunoassay techniques.

Sample preparation

For analysis of steroids in biological sample, the first step of the analytical procedure usually should be hydrolysis, either acid or enzyme hydrolysis, a method which split (hydrolysis) such ether and ester linkages between glucuronic or sulfuric acid and cortisol, enabling the analysis of both free and conjugated cortisol. However, in this study, only free state of urinary cortisol level will be analyzed. So, the hydrolysis procedure will be omitted.

The second step is extraction of steroids. In this step, the selection of the organic solvent is based on the polarity of the steroid hormones in question. Cortisol and its metabolites are steroids with a common 4-ring system and with three or more oxygens. They are quite polar, and extract best with solvents such as chloroform, dichloromethane,

or ethyl acetate. To improve recovery of compounds, the extraction process is repeated three times. The combined extract is blown down to dryness under nitrogen and redissolved for further analysis (Tietz, 1970).

Solid phase extraction of cortisol has also been reported, involving a partitioning of compounds between a stationary phase and a mobile phase. The extracted compound has a stronger affinity for the solid phase than the sample matrix, yet can be easily removed from the stationary phase with a small volume of solvent. For hydrocortisone, several solid phase extraction methods have been reported (Shackleton and Whitney, 1980; Schoneshofer *et al.*, 1980; Diamandis and D'costa, 1988; Park *et al.*, 1990). Of these, the sep-pak C₁₈ cartridges are most widely used for rapid sample preparation prior to quantitation. This technique is convenient, faster, and results in better recovery of analytes. But the following disadvantages still exist in this and all other solid-phase techniques: (1) complex elution sequences are not practicable, (2) the relatively expensive cartridges are generally used once, (3) each new cartridge has to be activated prior to extraction (Dhar and Schoneshofer, 1987).

Thin layer chromatography

Thin layer chromatography has been regarded traditionally as a screening method. Screening methods are fairly rapid, less sensitive, and contain a lower degree of accuracy. In terms of cortisol analysis, TLC can be used as an acceptable screening method for initial separation, tentative identification, and visual semiquantitation. In the

literature, the reported detection limit of cortisol, employing silica gel G TLC was 1.2 μg with carbazole-sulfuric acid spray reagent (Ghosh and Thakur, 1982).

Gas liquid chromatography

In most forensic laboratories, gas liquid chromatography usually be used for drug confirmation by utilizing various detection methods, such as FID, ECD, or mass spectrometric detection. Of the three, GC/MS remains one of the most commonly used confirmation method.

The integration of gas chromatography with mass spectrometry also made possible the analysis of steroids in the low nanogram range and resulted in the most powerful technique available for structural elucidation of steroids in biological samples.

There were four reports in the literature about the detection of hydrocortisone by gas chromatography mass spectrometry using glass capillary columns (Singh, 1989; Ralston *et al.*, 1990; Ulick *et al.*, 1991). One report cited use of a packed column (Lantto, 1982).

However, analyses of corticosteroids by GC/MS methods, while highly sensitive and specific, require derivatization prior to injection because the compounds are thermally instable and their volatility is low for direct GC and GC/MS analysis. Some chemical derivation methods are usually needed to convert the cortisol to thermally stable compound. O-methyloxime-trimethylsilyl ether derivatives of cortisol and its metabolites have been used prepared before GC injection (Lantto, 1982; Singh, 1989; Ralston *et al.*,

1990; Ulick *et al.*, 1991). In general, the ions present at m/e 73 and m/e 75 in the mass spectra of most steroids TMS derivatives are formed by fragmentation of the trimethylsilyl groups and carry a high ion current relative to the other ions in the spectrum yet yield virtually no structural information. Various other important chromatographic parameters for cortisol are listed in Table 1.

Compared to other chemistry methods, however, gas chromatography cortisol assays always involve lengthy sample preparation/extraction/derivatization procedures. Moreover, elevated-temperature separation may decompose some drug molecules. In addition, the successive derivatization procedures can form multiple products due to incomplete reactions which in turn produce mass spectra with low abundance molecular ions and fragmentation patterns that are dominated by the type of derivative rather than the drug's structure (Houghton *et al.*, 1981).

High performance liquid chromatography

HPLC with UV detector procedure was reported for detection of synthetic and natural corticosteroids in biological fluids. Because there is no possibility of thermal decomposition, HPLC offers the advantage that the corticosteroids can be analyzed without derivation. HPLC interfaced to a mass spectrometer offers the further advantage of a highly specific detection of steroids in biological extracts. All corticosteroids show the E_{max} of 246 nm based on the Δ^4 -3-keto group (Park *et al.*, 1990). Other LC

Table 1. Operating conditions and detection limits of hydrocortisone concentrations measured by gas liquid chromatography using mass spectrometric detection.

ARTICLE	COLUMN	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT
Lantto, 1982	1.5 m x 2 mm I.D.	250 °C	Helium	NA
Singh, 1989	not given	280 °C	Helium	NA
Ulick <i>et al.</i> , 1991	30 m x .25 mm I.D.	280 °C	Helium	NA
Ralston <i>et al.</i> , 1990	30 m x .25 mm I.D.	310 °C	Helium	NA

NA mean not given

detection procedures have also been reported for steroid analysis. Included are (1) by using derivatization reaction with fluorescent compounds (Seki and Yamaguchi, 1983; Nozaki *et al.*, 1991; Haegele and Wade, 1991), (2) by using high performance liquid affinity chromatography (Nilsson, 1983), (3) by using LC/MS techniques which offers the further advantage of highly specific detection of these steroids in biological sample (Skrabalak *et al.*, 1984; Park *et al.*, 1990).

There are two possible liquid chromatography methods for the separation of cortisol in biological samples: normal phase HPLC (Cavina *et al.*, 1973; Sayegh and Vestergaard, 1978; Rose *et al.*, 1979; Schoneshofer *et al.*, 1980b; Nakamura and Yakata, 1985; Haegele and Wade, 1991), and reversed phase HPLC (Okumura, 1981; Lantto, 1982; Seki and Yamaguchi, 1983; Skrabalak *et al.*, 1984; Ralston *et al.*, 1988; Diamandis and D'costa, 1988; Nozaki *et al.*, 1991; Park *et al.*, 1990; Shalaby and Shahjahan, 1991; Wade and Haegele, 1991).

In reverse phase chromatography, a non-polar stationary phase, such as silica gel, chemically bonded with a C₁₈ chain or other organic functional groups, and a polar mobile phase, such as acetonitrile or methanol/water, are used. The organic analytes interact with the stationary alkyl chains. In normal phase chromatography, a polar stationary phase, such as of silica gel, and a non-polar mobile phase, such as methanol or acetonitrile, are employed. Separation is due to the interaction between the "active" silanol groups and the analytes. Normal phase is very useful in resolving structurally similar molecules. Various other chromatographic parameters and the detection limits for

the analysis of cortisol are listed in Table 2.

High performance liquid chromatography can detect cortisol levels in the low nanogram range (Schoneshofer *et al.*, 1980; Nakamura and Yakata, 1985; Rose and Jusko, 1979; Park *et al.*, 1990; Park *et al.*, 1990; Wade and Haegele., 1991). The major disadvantages indicated by some authors were that HPLC method is time consuming, and it requires large sample volumes.

Fluorometric analysis

Free cortisol can be measured by fluorometric techniques in the presence of sulfuric acid (Breznock and McQueen, 1970; Ratliff and Hall, 1973). Fluorometric methods also exhibit some drawbacks. Since cortisol is a small molecule, it is structurally similar to many steroids found in biological fluids, and it is present in low concentration (ppb) in body fluids, so fluorometric techniques often overestimate the true amount of cortisol present. These methods also have been reported to show poor reproducibility (Frankel *et al.*, 1966).

Immunoassay techniques

All the immunoassay are based upon the selective reactivity of an antigen to an antibody which has been raised to that antigen. All formats utilize a detectable tag such as an enzyme, a fluorophore or radioisotope which is attached to either the antigen supplied with the test or attached to antibody provided with the test. All immunoassay measure the

Table 2. Operating conditions and detection limits of hydrocortisone concentrations measured by high performance liquid chromatography.

ARTICLE	COLUMN	MOBILE PHASE	DETECTOR TYPE	DETECTION LIMIT
Lantto, 1982	not given Nucleosil-NO ₂ , Supelco	DCM/ethanol (97:3)	UV 254 nm	not given
Park <i>et al.</i> , 1990	100 x 4.6 mm I.D. Hypersil-ODC	H ₂ O/acetonitrile	Diode array UV 246 nm	10 ng/ml
Schoneshofer <i>et al.</i> , 1980	250 x 3 mm I.D. Diol-sil, Merck	n-hexane/i-propanol (75:25)	UV 254 nm	4.9 pg
Ralston <i>et al.</i> , 1988	25 x .46 mm I.D. C18	H ₂ O/acetonitrile (40:60)	UV 245 nm	not given
Wade and Haegele, 1991	250 x 2 mm I.D. Keystone-C8	Acetonitrile/methanol /water (10:40:50)	UV 240 nm	0.5 ng /ml
Rose and William, 1979	250 x 4.6 mm I.D. Zorbax-sil, DuPont	methanol/methylene chloride (3:97)	UV 254 nm	15 ng

Table 2. (continued)

ARTICLE	COLUMN	MOBILE PHASE	DETECTOR TYPE	DETECTION LIMIT
Cavina <i>et al.</i> , 1973	300 x 2 mm I.D. Silicic-acid, Bio-Rad	methanol/chloroform	UV 240 nm	not given
Diamandis and D'Costa, 1988	300 x 3.9 mm I.D. Bondapak-C18, Waters	tetrahydrofuran/methanol /water	UV 254 nm	not given
Nilsson, 1983	250 x 5 mm I.D. Lichrosorb RP-18	methanol/water (60:40)	UV 254 nm	not given
Nakamura and Yakata, 1985	250 x 4.6 mm I.D. Zorbax-CN, DuPont	methanol/water (40:60)	UV 242 nm	40 ng
Shalaby and Shahjahan, 1991	150 x 4.6 mm I.D. Ultrasphere-ODS	acetonitrile/phosphate buffer (6:4)	UV 250 nm	not given

concentration of tag present after natural sample antigen has an opportunity to react with kit antigen and/or antibody. The amount of tag detected at the end of a test is correlated to natural antigen present initially in the sample. The immunoassay techniques are usually sensitive, simple, rapid, and inexpensive methods for chemical analysis including corticosteroids. Several formats of immunoassay are employed in drug detection, such as radioimmunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA), enzyme-linked immunosorbent assay (ELISA), etc.

In RIA, a "specific" antibody to a drug molecule is used to bind both a sample of radiolabeled drug and whatever unlabeled drug may be added with the unknown plasma or urine sample. The basic principle of RIA is outlined as follows:



where

L^* = the radiolabeled ligand or bound tracer,

L = the unlabeled ligand, or the drug molecules,

Ab = the antibody.

In the presence of limited binding sites of the antibody, L^* and L undergo competitive binding. After reaching equilibrium at the end of the incubation period, L and L^* will form Lab and L^*Ab , the "antibody-bound" fraction, while the remaining L and L^* will remain as the "free" fraction. The bound and free fraction are separated and the radioactivity of the bound portion is measured. If a significant amount of nonradioactive drug is added to the system, enough drug is displaced for the reduction of

radioactivity in the system to be measurable. The quantity of drug present in the original sample will be inversely related to the amount of radioactivity measured. The radioactive tracer used to compete with the unlabeled drug can be either ^{125}I , ^3H , or ^{14}C , ^{75}Se , etc.

In the literature, there were numerous reports about cortisol analysis using RIA technique (Hasler *et al.*, 1976; Chen *et al.*, 1978; Johnston and Mather, 1978; Schoneshofer *et al.*, 1980b; Lantto, 1982; Peterson *et al.*, 1986; Ralston *et al.*, 1988). The reported lowest detection limit for cortisol was 10 pg (Chen *et al.*, 1978). Method selectivity or degree of cross reactivity varied according to the antibody which was being used.

Other immunoassay techniques are also available for the analysis of cortisol. Included are: Enzyme-immunoassay analysis (EIA). Its principle is similar to RIA's except that the steroids (the antigens) compete with an enzyme labeled analog for the antibodies against the antigen being analyzed. There were several schemes of the enzyme immunoassay for the analysis of cortisol reported (Pearson *et al.*, 1981; Markina *et al.*, 1989; Karaseva *et al.*, 1990), each of which has its advantages and drawbacks. Peroxidase, β -galactosidase, or dehydrogenase are most often used as the marker enzymes of cortisol.

A competitive protein-binding (radiotransinassay RTA) method was also being used for cortisol measurement (Hoffsis *et al.*, 1970a; Fiorelli *et al.*, 1972; Peterson *et al.*, 1986; Pearson *et al.*, 1981). Cortisol competes with a radioactive labeled tracer for

binding sites on indigenous plasma proteins or "transins" (e.g., transcortin, globulin). The bound portion remains in the aqueous phase while the displaced tracer passes into the scintillator phase, where its radioactivity is counted. The lowest detection limit was reported as 100 pg (Pearson *et al.*, 1981).

A fluorescence polarization immunoassay (FPIA) was employed in the present studies. If a small fluorescent antigen with rapid movement becomes bound to an antibody, rotational relaxation time will be increased as a result of formation of the antigen-antibody complex with slow movement. Thus the polarization is increased. When an unlabeled antigen is added to this system, it competes with fluorescent antigen for binding to the antibody and the amount of free fluorescent antigen with rapid movement is increased. Thus the rotational relaxation time is decreased and the polarization decreased. If the amount of antibody is constant, the degree of polarization will be inversely correlated with the amount of added unlabeled antigen. In other words, the added unlabeled antigen can be determined from the degree of decreased polarization (Ngo, 1988). However, these methods all have the disadvantages of the synthesis of either radiolabeled or enzyme labeled cortisol tracers, cross reactivity, as well as the possibility of radioactive contamination.

Enzyme-linked immunosorbent assay (ELISA) is a newer immunoassay technique available for cortisol screening test (Lewis and Elder, 1985; Irvine *et al.*, 1988; Ford *et al.*, 1990). When cortisol (when present in the sample) binds to a cortisol antibody in a plate, which is washed after formation of the antibody-cortisol complex. Antibody

specific for a different site on the cortisol is then added, and is again washed. This second antibody is attached to an enzyme such as alkaline phosphatase or peroxidase. This enzyme can rapidly convert an added colorless substrate into a colored product which can be detected by a colorimetric procedure. Substrate conversion is monitored by absorbance change at a UV wavelength appropriate for different substrates. The amount of second antibody bound to the plate is proportional to the quantity of cortisol in the sample. This method is superior to RIA in terms of expense, time, simplicity, and instrumentation. It does not require handling of radioactive material.

The major drawback of various immunoassay techniques is that antibodies partially cross-react with endogenous cortisol as well as synthetic steroids. These techniques cannot unequivocally distinguish similar synthetic or endogenous corticosteroids and false determinations are frequently encountered. One report by Pearson and his colleagues (1981) showed that some of the immunoassay methods (RIA; RTA with four different antibodies and three transins) used for cortisol detection without chromatography grossly overestimate the amount of cortisol in urine. Schoneshofer *et al.* (1980a,b) made a similar discovery. Specific estimation urinary cortisol by radioimmunoassay requires a preceding chromatographic technique of high efficiency, such as HPLC or GC/MS.

Comparative studies

In the literature, various investigators have analyzed the capability of numerous procedures to detect cortisol presence on an individual basis. In addition, there has been a comparison study between the competitive protein-binding assay vs. radioimmunoassay for plasma cortisol detection (Peterson *et al.*, 1986). The author indicates that there was excellent correlation between the cortisol values obtained by the two assay methods. The detection limit of both the competitive protein-binding assay and the RIA was 1.0 ng/ml. Lantto (1982) has reported the compared results by three different routine methods for analysis of free urinary cortisol: (1) isotope dilution-mass spectrometry (ID-MS); (2) HPLC; (3) two radioimmunoassay methods, one involving direct assay and one involving extraction.

The results indicated that the HPLC method turned out to be the most nearly accurate of the different routine methods tested (regression coefficient 0.86); ID-MS gave less accurate results. Further purification did not change the results obtained. Two RIA methods gave considerably higher values than those achieved with the comparison method. Extracting the samples before analysis did not improve the accuracy.

A comparison study of HPLC with a diode array detector vs. thermospray LC/MS techniques for analysis of corticosteroids in urine was also reported by Park *et al.* (1990). Their results indicated that HPLC allows the simultaneous determination of at least ten corticosteroids with a simple, reproducible, and reliable analytical procedure. Detection limit by diode array detector (DAD) was 10 ng/ml in 5 ml of urine. It seems to indicate

that the thermospray LC/MS technique produced useful information concerning the molecule weight of corticosteroids without the need for derivatization and could give a lower detection limit 10 to 50 ng in the scan mode and 1 to 5 ng in SIM mode. Their results also indicated that the extraction recovery of each corticosteroid from the spiked urine by liquid-liquid extraction at pH 9 using diethyl ether was greater (85%) than the result obtained by solid-phase extraction with a sep-pak C₁₈ cartridge (79%).

Singh (1989) reported comparison results of electron impact vs. chemical ionization gas chromatography-mass spectrometry for screening of steroids in horse urine. His results revealed that chemical ionization mass spectrometry was more sensitive than the electron impact ionization mass spectrometry for cortisol analysis. The extraction efficiency by liquid-liquid used in this study without TLC clean-up was approximately 60-70% from urine and reduced to 45-55% with TLC clean-up.

In addition, a comparison of the quantitation of urinary free cortisol from post race thoroughbred standardbred horses has been made using radioimmunoassay (RIA) and gas chromatography mass spectrometry (GC/MS) (Ralston *et al.*, 1990). Their results showed that there was a good correlation between the absolute urinary cortisol values obtained by GC/MS and the values obtained by RIA. As such, the cortisol RIA method did appear to be an acceptable quantitative screening method for cortisol in urine.

EXPERIMENTAL

Materials and Instrumentation

Gas chromatography and mass spectrometer

Hewlett Packard model 5890 gas chromatography connected to Hewlett Packard 5988A quadrupole mass spectrometer-data station equipped for electron ionization with positive ion collection was used for mass spectral collection. All GC/MS analysis were performed using a DB-5 capillary column, 15 m x 0.25 mm fused with 0.25 μm film thickness, purchased from J&W Scientific, Rancho Cordova, CA.

TDx FLx (FPIA) immunoassay

The fluorimeter used for quantitating fluorescence polarization immunoassay is commercialized by Abbott laboratories (North Chicago, IL). It can measure automatically polarization components and compute a polarization value after correcting for background and optical bias. The TDx FLx cortisol kits were also purchased from Abbott Laboratories. Only the reagents supplied in the respective kit were used in the analyses. Systems cuvettes (No.9518-06), tri-well sample cartridges (No.9560-05), carousels (No.9518-11) and TDx FLx analyzer (No.9520-XX) were provided by Abbott Laboratories, (North Chicago, IL).

Materials and supplies

All organic solvents used in this study were Fisher HPLC grade or equivalent and chemicals/reagents used were reagent grade or better. Most chemicals and reagents were purchased from Fisher Scientific (Fair Lawn, NJ) including: ethyl acetate, methanol, acetone, acetonitrile, sodium borate, chloroform, potassium permanganate, ethanol, and concentrated sulfuric acid.

Hexamethyldisilazine (HMDS) and pyridine for silanization for glassware were acquired from Pierce Chemicals Company (Rockford, IL) and Aldrich Chemical Company, Inc. (Milwaukee, WI) respectively.

Pipemen precision microliter pipettes, utilized in all procedures for liquid dispensation were purchased from Rainin Instrument Company, Inc. (Woburn, MA).

Hydrocortisone standard used for standard and spike preparations was obtained from Sigma Chemical Company, Inc. (St. Louis, MO). Screw top test tubes (16 x 125mm) with PTFE lined caps used in the liquid-liquid extraction steps were purchased from Fisher Scientific, (Fair Lawn, NJ). Thin layer chromatography separations were performed on Kiesel silica gel 60 plates with F₂₅₄ fluorescence indicator purchased from EM Science, (Gibbstown, NJ).

Hydrocortisone sodium succinate was obtained from the Upjohn Company (Kalamazoo, MI.) Graduated wide mouth specimen containers for initial urine collection and final storage were purchased from Cole-Parmer Instrument Company, Inc. (Chicago, IL).

Drug Administration and Sample Collection

Blank urine collection for hydrocortisone threshold analysis

a. Five dogs, # 8445, # 9494, # 9485, # 9487, and # 9491, weighed from 20-25 kg. Urine samples were collected from each dog at 10:00 a.m. every morning consistently for five days.

b. Three dogs, # 8445, # 9494, and # 9485, weighed approximately from 20-25kg. Urine samples were obtained by cup-on-a-stick volunteer collection at 0 , 2, 4, 6, 8, 12, 18, and 24 hour respectively. The samples were capped, labeled, stored at -10 °C until analyzed.

Canine administration and sample collection

Two retired racing greyhounds, # 9485 and # 9491, weighed approximately 24 kg were administrated intravenously via the left front leg, 50 mg hydrocortisone sodium succinate. Urine was obtained by cup-on-a-stick volunteer collection before cortisol exposure and at regular intervals for several days thereafter. The samples were capped, labeled, and stored at -10 °C until analyzed.

Analytical Methods

Liquid-liquid extraction (Cortisol administrated and blank urine)

A steroid urine (SU) extraction procedure was used in this study to extract hydrocortisone out of dog urine (both dosed and blank). 9 ml urine at each collection time for both dogs was used when extracting dosed and blank urine for TLC and GC/MS analysis.

After thawing in warm water, 9 ml of urine were added to each of labeled screw cap test tubes. One ml of saturated sodium borate solution was added to each tube for hydrolysis of urine. Urine were extracted three times with 3.0 ml of ethyl acetate. Extracts were combined in a clean tube, 1.0 ml of 15% sodium sulfate/1.0 N NaOH was added to the extracts, vortexed well, and the organic phase was transferred and evaporated to dryness under nitrogen. Residue was dissolved in 50 μ l of ethyl acetate, capped and vortexed for TLC and GC/MS analysis. For TLC analysis, 5 μ l of the final extracts were plated on TLC plate. For GC/MS analysis, 1 μ l of the final extracts was injected into the GC/MS system.

Liquid-liquid extraction (cortisol spiked urine and water)

After thawing in warm water, 9 ml of urine or water was spiked at following concentrations of hydrocortisone: 10 ppb, 25 ppb, 50 ppb, and 100 ppb. Extraction of cortisol spikes was performed using the same extraction procedure previous mentioned.

Thin-layer chromatography techniques

For hydrocortisone analysis, the extracts were developed on the plate for a distance of 5 centimeters from the origin with chloroform:ethyl acetate:methanol (50:45:5). After drying, the plates were developed again with Davidow for a distance of 7 centimeters from the origin. Following development, plates were visualized under short and long wave ultraviolet light for quenching and fluorescence respectively. Lastly, the plates were sprayed with 50% sulfuric acid : ethanol, heated gently on hot plate until standard turns grey. Hydrocortisone reacted to give a yellow color spot on plate under LWUV light.

Gas chromatographic techniques and conditions

For GC/MS analyses, DB-5 fused silica columns, 15m x 0.25mm with 0.25 μ m micron loading film thickness were used. Prior MO-TMS derivatization of extract was necessary before injection to the GC/MS system. Procedures for MO-TMS derivatization were:

- (1) Put 10 ug hydrocortisone into a 2 dram vial. Blew dry under nitrogen.
- (2) Added 50 ul methoxamine in pyridine. Capped tightly. Heat at 80 °C for 30 minutes.
- (3) Evaporated with nitrogen plus heat. (4) Added 50 ul N-methyl-N-TMS-trifluoroacetamine (MSTFA), Capped and foiled tightly. Derivatized for 2 hours at 80 °C.
- (5) Added 0.5 ml water and 2 ml ether. Vortexed well. (6) dry ether over pipet with sodium sulfate into 1 dram vial. (7) Evaporated under nitrogen and heat. (8) Transferred to nipple vial with 50 ul acetonitrile.

All GC/MS analyses were performed with a Hewlett Packard 5890 A gas chromatography connected to a Hewlett Packard 5988 quadrupole MS in electron impact ionization, with positive ion collection at 70 eV.

Conditions for GC/MS were:

Injector temp.	265 °C
Oven temp.	Start at 70 °C Hold for 1 minute, then programmed at 20 °C per minute to 300 °C.
Carrier gas (Helium).	1 ml/min flow rate
Injection mode	Splitless
Electron energy	70 eV
Source temp.	250 °C

TDx FLx fluorescence polarization immunoassay method

No pretreatment was necessary for urine sample of TDx FLx cortisol assay. Urine to be analyzed was added directly to sample cartridge. The cortisol calibrators accompanying the kit were used for instrumental calibration and standard preparation. Three cortisol control samples were also supplied with the kit. The exact procedure used for the kit is listed in the appendix. All reagents utilized in the TDx FLx method were supplied by Abbott Laboratories. All working standards were prepared in blank dog urine by dilution of hydrocortisone stock solution (1 $\mu\text{g}/\mu\text{l}$ hydrocortisone in methanol). The excitation and emission wave lengths were 485 nm and 525 to 550 nm, respectively.

RESULTS AND DISCUSSION

Classical Techniques

Limit of detection

TLC For cortisol screening on TLC plate, the plate were developed for a distance of five centimeters from the origin with chloroform:ethyl acetate:methanol (50:45:5). After drying, the plate was developed again with Davidow for a distance of seven centimeters from the origin. Following plating and development of hydrocortisone standards on the TLC plate, quenching was observed under SWUV. Lastly, the plate was sprayed with 50% sulfuric acid:ethanol, heated gently on hot plate until standard turns grey, and the spots of interest were then visible on the plate. Rf value of hydrocortisone is 0.77.

Concentrations of 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, 0.05, and 0.025 μg of hydrocortisone standard present on the TLC plate were developed, visualized under UV light, and sprayed with the spray reagent. All concentrations higher than, and including 0.25 μg , were visible to the naked eye at the Rf value of interest. Below 0.1 μg the ability to visualize the spot of interest with the naked eye was very difficult. When taken into context that an actual TLC plate would contain a large number of samples, each with endogenous consistent becoming visible following spraying, the detection limit of hydrocortisone would have to be classified as approximately 0.25 μg on the TLC plate. Figure 2 shows the typical results of cortisol standards on TLC plate.

GC/MS For hydrocortisone analysis by GC/MS, some sample preparation and derivatization procedures have been studied. No result is available.

HPLC For hydrocortisone analysis by HPLC. little work has been done. No result is available at this point.

Sample spikes

Extraction of hydrocortisone aqueous and urine spikes was performed to determine if the extraction procedure used were efficient and reproducible. Also, some knowledge about matrix effects would be obtained.

TLC Nine ml of blank dog urine was spiked with hydrocortisone and then extracted following the steroid urine extraction procedure. Urine were spiked at following concentrations: 0.1 ppm, 0.25 ppm, 0.5 ppm, 1 ppm, 2 ppm, 5 ppm, and 10 ppm. After extraction and evaporation, 5 μ l out of 50 μ l extract were plated on the TLC plate, hydrocortisone standard was also spotted on each side of the plate. The plate was then developed, visualized and sprayed with proper solvents and reagents. For aqueous spikes, all concentrations higher than, including 0.25 ppm were visible to the naked eye. Below 0.1 ppm the ability to visualize the spot of interest with the naked eye was very difficult. For urine spikes, all concentrations higher than, including 0.25 ppm were visible to the naked eye. The lowest detectable concentration of hydrocortisone in urine matrix on TLC plate would have to be determined as 0.25 ppm. In water matrix, the lowest detectable concentration of hydrocortisone on TLC plate would be determined as 0.25 ppm also.

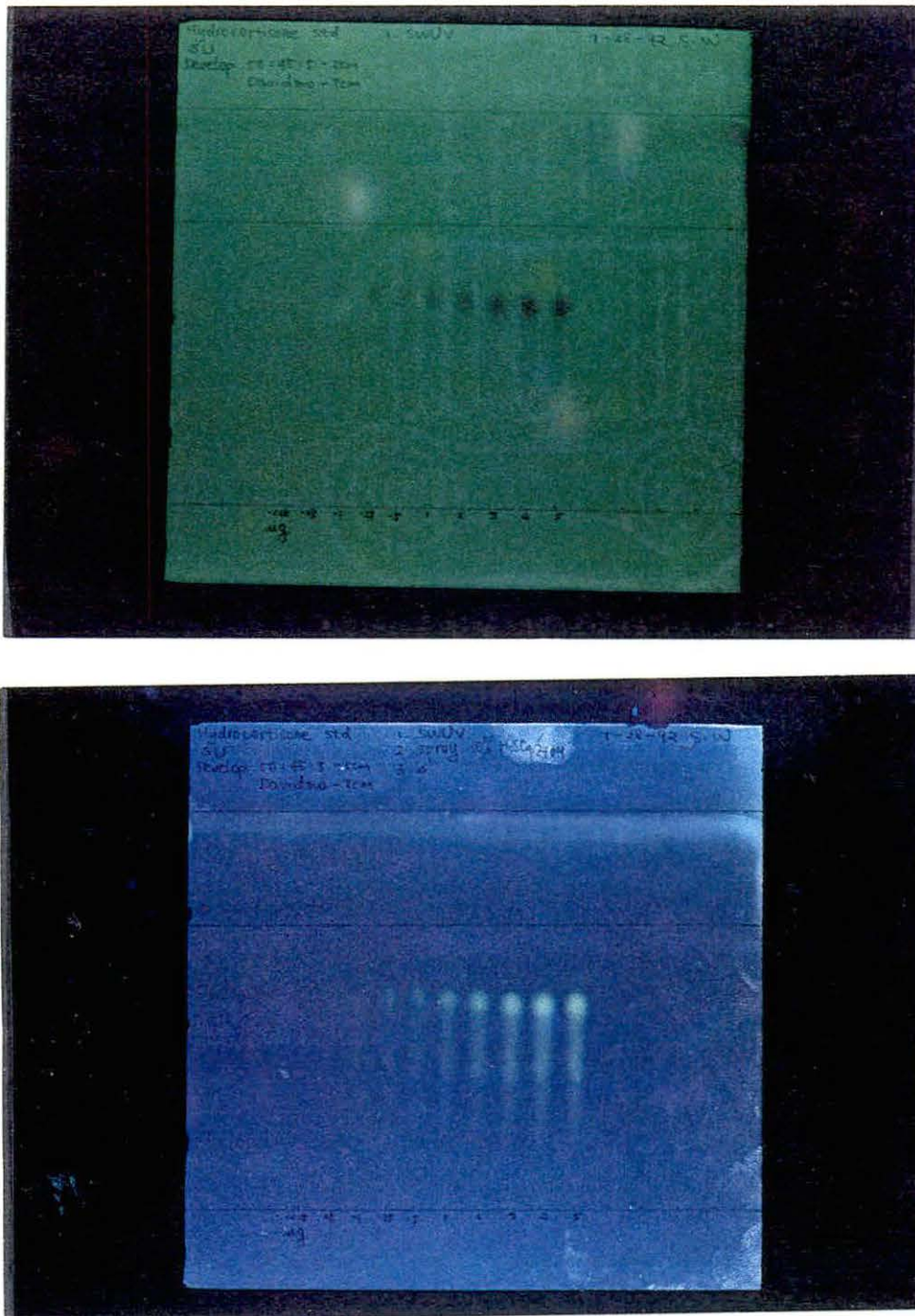


Figure 2. Thin-layer chromatographic results of cortisol standard

Spray sequence = 50% sulfuric acid:ethanol

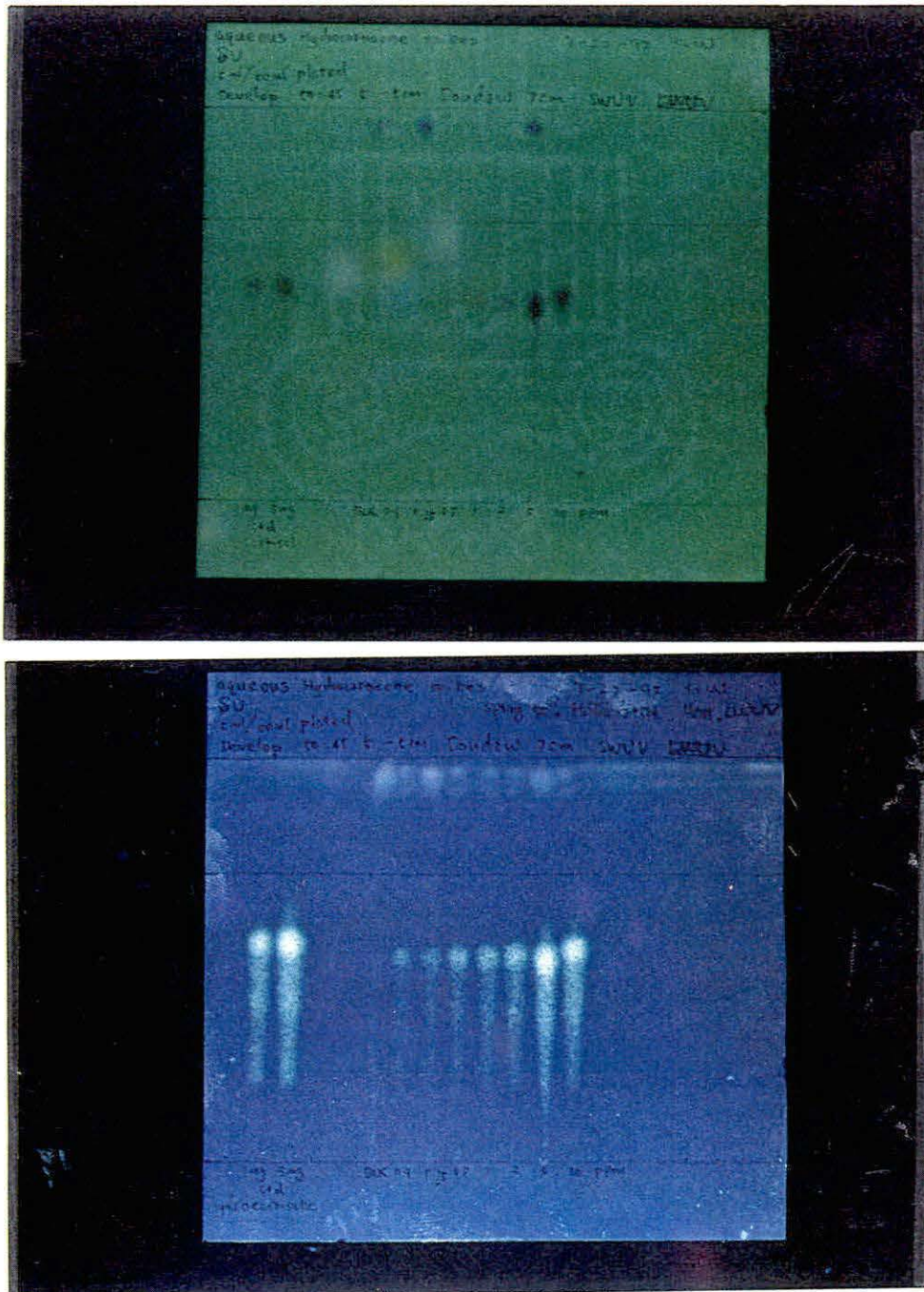


Figure 3. Thin-layer chromatographic results of cortisol spikes following SU extraction of 9 ml water
 Spray sequence = 50% sulfuric acid:ethanol

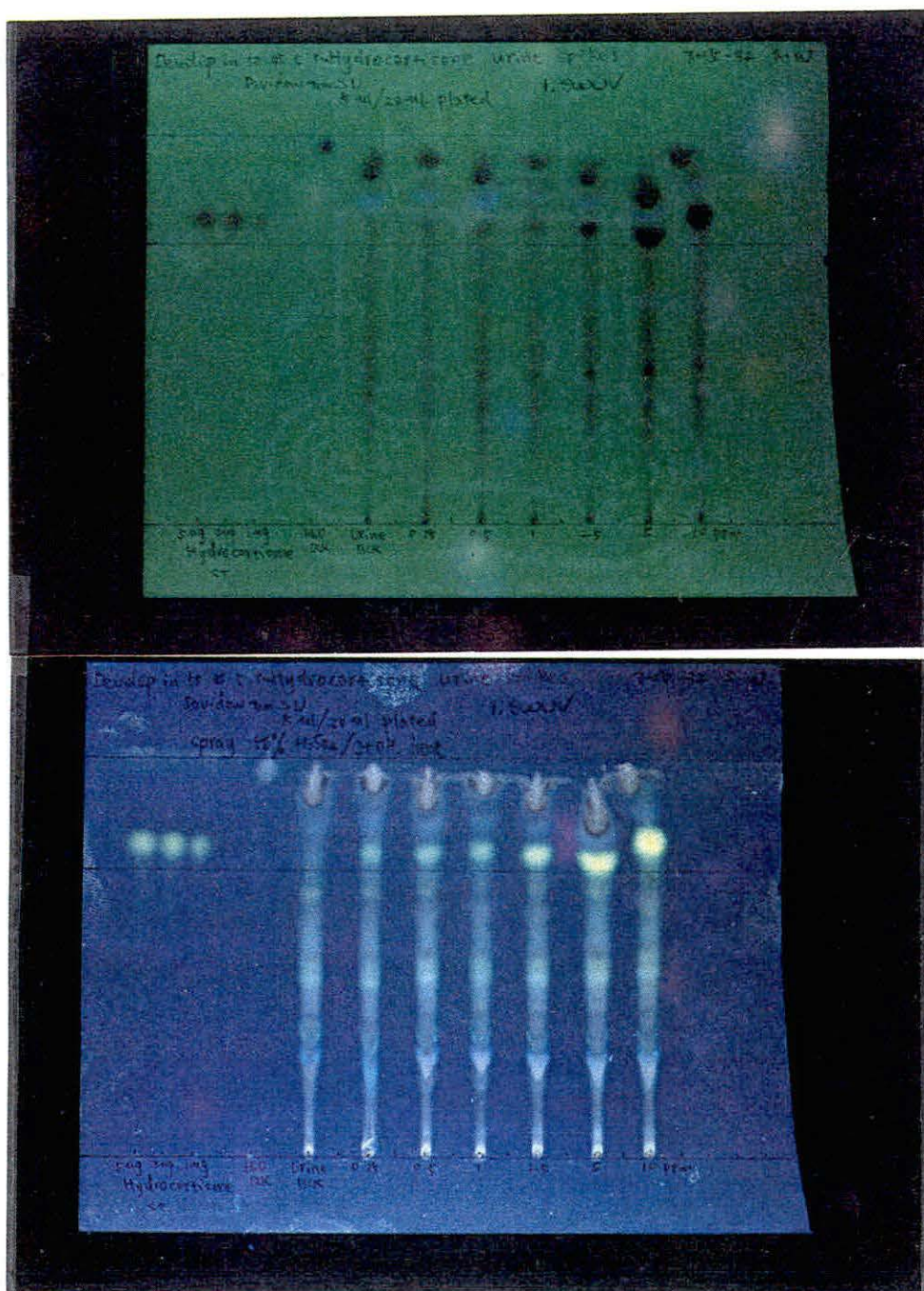


Figure 4. Thin layer chromatographic results of cortisol spikes following extraction of 9 ml urine

Spray sequence = 50% sulfuric acid:ethanol

The results indicated that biological matrix has no obvious impact on spike recovery. Figure 3, 4 shows the typical results of cortisol water and urine spikes on TLC plates.

Immunoassay Techniques

The TDx FLx fluorescence polarization immunoassay (FPIA) is a simple, and relatively sensitive method for the quantitative analysis of urinary free hydrocortisone with or without organic solvent extraction and chromatographic purification. The assay also can be used to detect conjugated hydrocortisone in urine. The majority of the hydrocortisone and its metabolites are excreted in the urine as the water-soluble conjugates of glucuronic acid and sulfuric acid. In order to detect conjugated cortisol levels in urine by FPIA method,, the splitting (hydrolysis) of such ether and ester linkages is an obligatory first step. Following hydrolysis, the free cortisol become sparingly soluble in aqueous solution, and then the cortisol assay can be utilized to determine cortisol levels. However, like most immunoassay methods, the major drawback of FPIA technique is that antibody partially cross-react with endogenous cortisol as well as synthetic steroids. So, determination of cross-reactivity of the assay is also an important part of this work.

Standard curves

(1) FPIA standard curve in serum was constructed by using the calibrators accompanying with the kit as standards, diluted with 0.0 calibrator. The concentrations of cortisol calibrators were: 1, 5, 10, 25, 50, 100, 250, and 600 ppb. All the standards were analyzed in triplicate. Figure 5 shows a typical standard curve of cortisol in serum.

(2) FPIA Standard curve in urine was constructed by using stock cortisol standard ($1 \mu\text{g}/\mu\text{l}$), diluted it with blank dog urine as following concentration: 0, 5, 10, 25, 50, 100, 250, and 500 ppb. All the standards were analyzed in triplicate. Figure 6 shows the standard curve of cortisol in urine.

Sensitivity

The lowest measurable level is defined as that concentration which can be distinguished from zero at the probability < 0.05 significance level. It was determined by one group T test to be 5 ppb ($P = 0.045$), in serum, and 10 ppb ($p = 0.009$) in urine. This is consistent with the results of the manufacture who reported a detection limit of 4.5 ppb in serum.

Precision

Table 3 shows the results of assay reproducibility study by the kit manufacturers. In our studies, for each batch of samples, two or three of control samples accompanying with the kit were included. A four-point standard curve was also included in every day

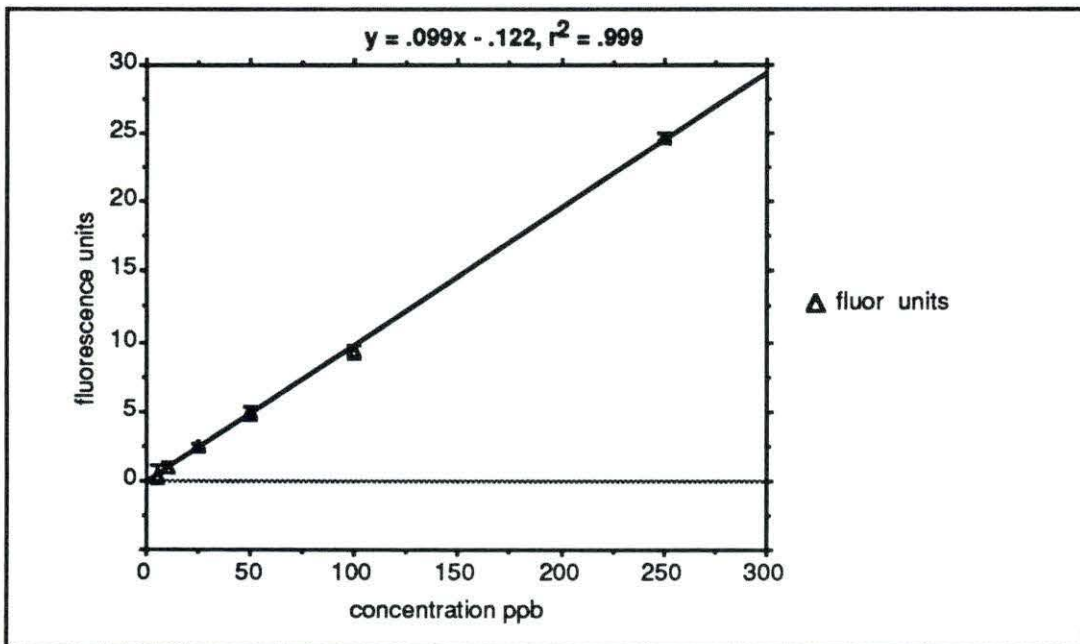


Figure 5. Hydrocortisone standard curve in serum by TDx FLx with error bars indicating 2 SD

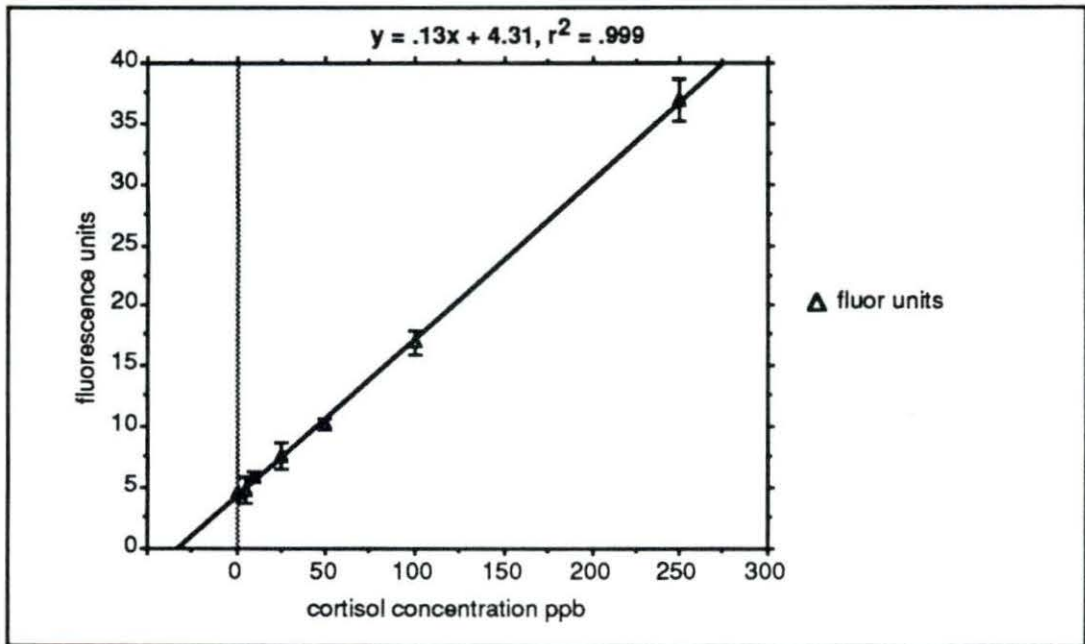


Figure 6. Hydrocortisone standard curve in urine by TDx FLx with error bars indicating 2 SD, n=3

Table 3. Statistical analysis of within-run and between-run precision study by TDx kit manufacturers.

Target value	Concentration ($\mu\text{g}/\text{dl}$)		
	4.0	15.0	40.0
mean	4.20	14.60	40.44
SD within run	0.43	0.65	1.16
CV within run %	10.73	4.35	2.90
SD between run	0.39	0.75	1.94
CV between run %	9.73	4.97	4.86

analysis. The correlation coefficients were always better than 0.99. All the control samples and standards were analyzed in triplicate. CV and SD of daily and day to day variation of standards and control samples in our studies are given in Table 4.

Table 4. Statistical analysis of within-day and day to day precision study by TDx (ours).

Number of Analyses	Mean of Fluor units	Conc of Standard	SD of Fluor units	CV %
Within-day precision study (standards)				
3	7.72	25 ppb	.58	7.48
3	10.25	50 ppb	.25	2.39
3	16.97	100 ppb	.56	3.27
3	36.92	250 ppb	.85	2.29
Day to day precision study (standards)				
43	9.04	25 ppb	1.41	15.67
43	11.75	50 ppb	1.16	9.90
43	17.64	100 ppb	1.34	7.64
43	36.45	250 ppb	2.51	6.88
Day to day precision study (control samples)				
48	4.52	4 $\mu\text{g}/\text{dl}$	0.71	15.56
51	15.29	15 $\mu\text{g}/\text{dl}$	1.29	8.43
27	41.15	40 $\mu\text{g}/\text{dl}$	2.78	6.75

Results from day to day precision studies typically yield CV's of less than 16% for the low control (4 $\mu\text{g}/\text{dl}$) and the low standards (25 ppb) and less than 10 % for the medium and high controls and the high standards (above 25 ppb), which are consistent with manufacturer's results which yielded CV's of less than 15% for the low control and less than 9% for both the medium and high controls.

Determinations of cross-reactivity with various compounds

Cross-reactivity was tested with endogenous or synthetic steroids whose chemical structure could cause interference with the Cortisol assay. Cross-reactivity of a compound was determined by assaying it in blank urine at the following concentrations: 10, 25, 50, 100, 250, and 500 ppb. All the standards were prepared from 1 $\mu\text{g}/\mu\text{l}$ stock standards and analyzed in triplicate. Percent cross-reactivity = $100 \times$ (measured concentration of cortisol divided by the concentration of the test compound, determined at 500 ppb).

Table 5 shows the results of cross-reactivity studies in this study and the kit manufactures. The two sets of results are consistent. The results show that the cross-reactivity of the cortisol antibodies with triamcinolone is quite high. Figure 7 represents the cortisol cross-reactivity curves by FPIA.

Diurnal variation

Cortisol concentrations in dog urine may be subject to diurnal variations (daily variations). Diurnal variations can lead to cortisol values in dog urine which would be

Table 5. FPIA cortisol specificity study (cross-activity)

<u>STEROID</u>	<u>Cross-reactivity % by this study</u>
Endogenous:	
testosterone	0.98
Synthetic:	
dexamethasone	3.39
betamethasone	2.64
prednisone	2.36
prednisolone	28.46
methyl prednisolone	13.02
Triamcinolone	43.81
<u>Endogenous:</u>	<u>Cross-reactivity % by kit manufacturers</u>
11-deoxycortisol	4.7
corticosterone	4.5
cortisone	1.1
tetrahydrocortisol	0.8
17-OH-progesterone	0.5
11-deoxycorticosterone	0.4
6- β -OH-cortisol	0.2
progesterone	0.1
11- β -OH-progesterone	0.1
β -cortol	0.1
β -cortolone	0.1
testosterone	0.1
cortisol-21-glucuronide	0.1
<u>Synthetic:</u>	
prednisolone	23.8
6-methyl prednisolone	1.1
dexamethasone	0.6
prednisone	0.4

abnormal and have doubtful significance in correlation with cortisol threshold determination. Diurnal variation of cortisol in dogs was determined with the urine samples from dog # 8445, # 9485, and # 9494. Urine samples were collected in wide mouth containers at eight intervals during a 24 hours military time cycle. (0.00, 02.00, 04.00, 06.00, 08.00, 12.00, 18.00, and 24.00). All the samples were analyzed in triplicate, standards and controls were analyzed in duplicate.

Figure 8 shows the diurnal variation curves of cortisol level of three dogs. In dog #9494 (female), cortisol concentration is greater than that at any other given time at 24.00 hours. At 24.00 hours, the urine cortisol level is 66% greater than at 06.00 hours. Dog #9485 (female), the highest point of cortisol level is at 02.00 hours. The cortisol level at 02.00 hours is 60% greater than at 06.00 hour. In dog #8445 (male), cortisol levels do not change significantly throughout 24 hours, there is no significant rise in cortisol levels during 24 hours. For all three dogs, the lowest urine cortisol level is at 8.00 hours. It can be concluded that in two female dogs, cortisol urinary levels are the highest in the early or late morning hours and lowest in the afternoon.

There was no publication in the literature about diurnal variation pattern in dog urine. The absence of diurnal variation of peripheral plasma levels of corticosteroids was previously reported by Breznock and McQueen (1970) and Richkind and Edqvist(1973). However, in the dog, one study showed that total glucocorticosteroid levels were highest in morning hours (09.00 hours) in plasma (Harwood and Mason, 1956). Several researchers indicated that cortisol levels can increase in normal animals due to a number

of different stimulus such as environmental changing, stress or due to a fact that dogs be frightened during the experiment (Siegel, 1968; Becker *et al.*, 1976; Feldman, 1985).

Endogenous and Dosed Drug Concentrations

The normal concentrations of cortisol in research dog urine

Initially the normal concentration of urinary cortisol and its day to day variation were determined in the five research dogs (# 8445, # 9494, # 9485, # 9487, and # 9491) during a four days period by urine sampling at 9.00 to 10.00 a.m every day. Day to day variation curves of cortisol levels of five dogs are shown in Figure 9.

For each batch of samples, two controls were included, the controls were placed in the first and last position in the carousel. A four-point standard curve was constructed every day. All the standards and controls were analyzed in duplicate, all the samples were analyzed in triplicate. No day to day variation pattern of cortisol level was obtained from this work. The normal hydrocortisone concentrations in five dogs varied from 46.32 to 179.39 ppb, with a mean concentration of 101.8 ppb (SD = 37.1). Cortisol concentrations in three female dogs varied from 78.34 to 179.39 ppb, with a mean value of 120.94 ppb (SD = 29.58). Cortisol concentrations in two male dogs varied from 46.32 to 109.96 ppb, a mean value is 65.52 ppb (SD = 20.2). The possible causes of variability are (1) breed; (2) variability in the adrenal response to excitement.

A T test was carried out to determine if the basic mean cortisol concentrations are

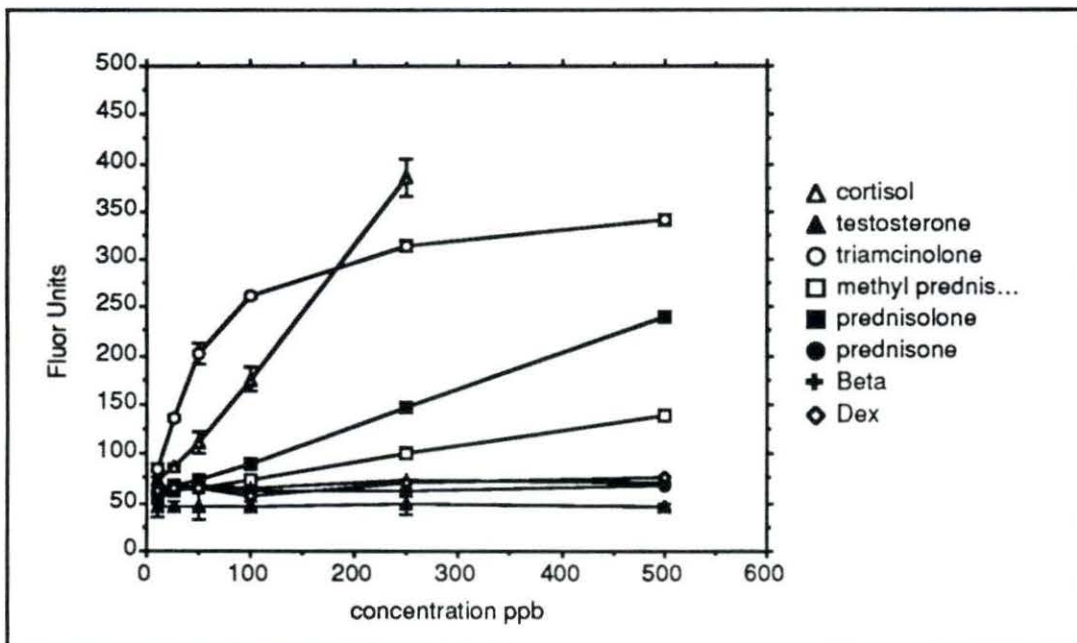


Figure 7. Hydrocortisone cross reactivity curves by TDx FLx with error bars indicating 2 SD, n=3

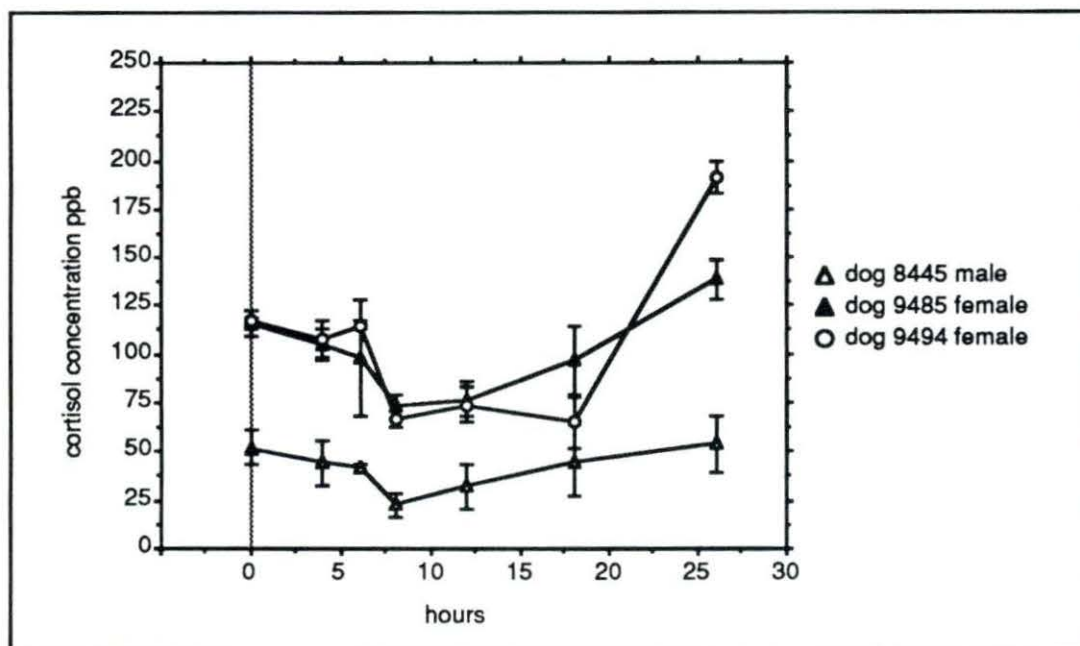


Figure 8. Diurnal variation curves of cortisol level of three dogs by TDx FLx, error bars indicating 2 SD, n=3

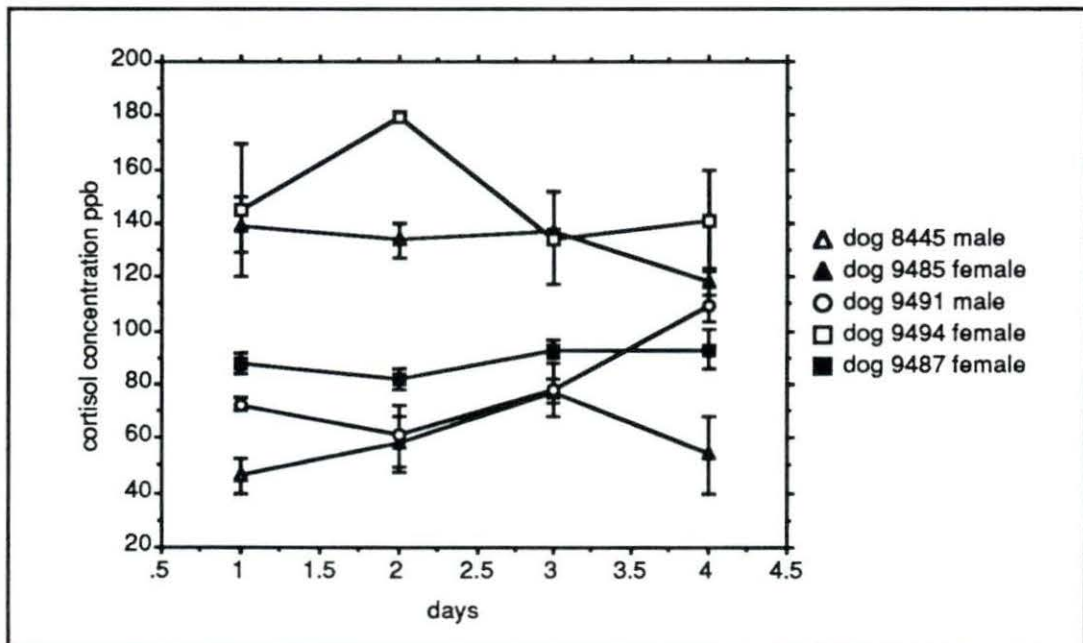


Figure 9. Day to day variation curves of cortisol level of five dogs by TDx FLx, error bars indicating 2 SD, n=3

influenced by gender. The results indicate that the average cortisol level of female dogs is significantly higher than that of the male dogs ($P = 0.002$). In literature, only one report indicated that basal female serum cortisol levels were significantly higher than levels in male normal dogs (Garnier *et al.*, 1990). Their explanation for this phenomenon was that males and females have different ability to become acclimatized to their housing condition. The acclimatization appeared to occur faster in males than in females.

Figure 10 represents the univariate scattergrams of cortisol levels of 20 blank urine samples from five research dogs, with error bar indicating 2 SD.

Administration urine

FPIA analysis of administration urine from two dogs # 9485 female and # 9491 male, each dog received an intravenous injection of 50 mg hydrocortisone sodium succinate. Urine samples were collected immediately before injection of cortisol and 2, 4, 6, 8, 12, 24, 26, 48, 72, 96, 120, 140, 164, and 196 hours after administration. Cortisol levels were determined in triplicate. Standards and controls were analyzed in duplicate.

Both dogs reached maximum excretion of cortisol in the urine at 2 hours post administration. In both dogs, the farthest time point post dose at which cortisol levels are not significantly different with that of at 0 hour pre dose, defined by T test, is 12 hours post dose ($P = 0.45$ dog #9485, $P = 0.07$ dog #9491). At 24 hours post dose, dosed cortisol levels of two dogs are also not significantly different with normal cortisol levels ($P = 0.35$ dog #9485, $P = 0.05$ dog #9491). Table 6a and 6b represents the T test data.

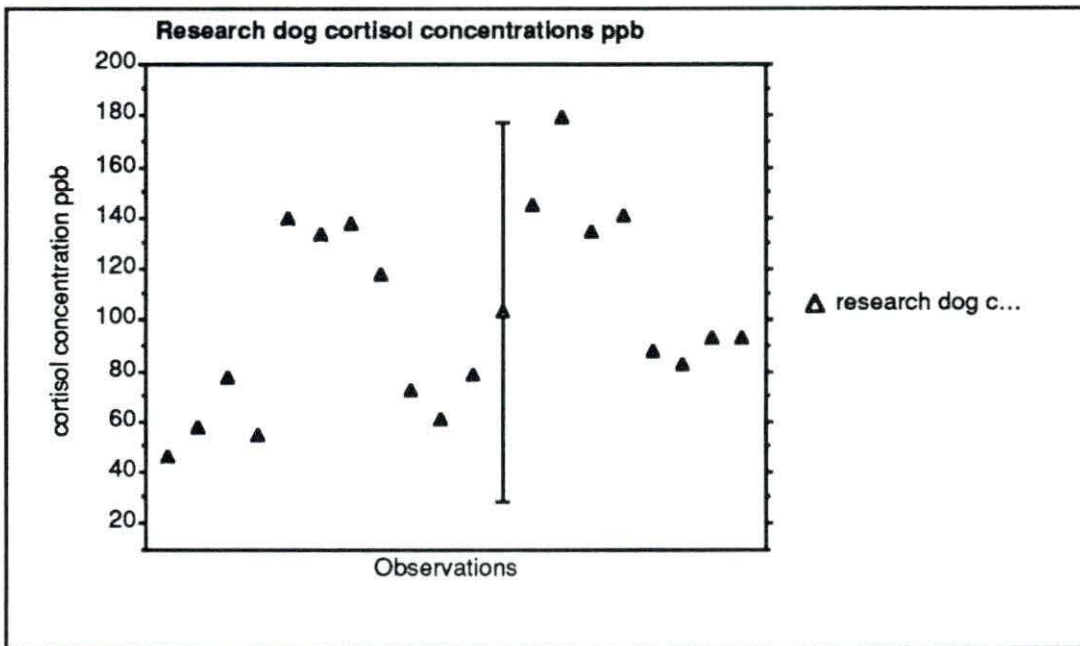


Figure 10. Univariate scattergrams of cortisol concentrations of 20 urine samples from five research dogs, error bar indicating 2 SD

Table 6a. Data from T test (12 hours post dose)

Unpaired t-Test X ₁ : Column 1 Y ₁ : Column 2				
DF:		Unpaired t Value:		Prob. (2-tail):
7		.797		.4514
Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Element 1	4	16.445	2.357	1.179
Element 2	5	14.252	5.024	2.247

Unpaired t-Test X ₁ : Column 1 Y ₁ : Column 2				
DF:		Unpaired t Value:		Prob. (2-tail):
9		-2.02		.0741
Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Element 1	4	8.342	3.155	1.577
Element 2	7	12.911	3.814	1.442

Table 6b. Data from T test (24 hours post dose)

Unpaired t-Test X₁ : compare groups Y₁ : cortisol intensity units

DF:	Unpaired t Value:	Prob. (2-tail):
7	.996	.3523

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
control	4	16.447	2.358	1.179
dosed	5	13.63	5.187	2.32

Unpaired t-Test X₁ : compared groups Y₁ : cortisol intensity units

DF:	Unpaired t Value:	Prob. (2-tail):
10	-2.272	.0464

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
control	4	8.342	3.155	1.577
dosed	8	12.268	2.665	.942

The results indicate that endogenous urinary cortisol levels can not be significantly distinguished from dosed urinary cortisol levels for 12 or 24 hours post dose. These statistical data provides very important information about cortisol urinary clearance kinetic in dogs, and it also helps us to identify the post dose time point regarding the differentiation between endogenous cortisol urinary levels and dosed urinary cortisol levels. Absence of these information can lead to cortisol values in dogs which would be incorrect or have doubtful significant in correlation with normal cortisol population determination and endogenous/exogenous cortisol levels differentiation.

The urinary excretion profiles of cortisol in two greyhounds after i.v administration are showed in Figure 11 and 12. Figure 13 shows portions of urinary excretion profiles (post dose 24 hours) from dog # 9485 and # 9491. The whole urinary excretion profiles of dog # 9485 before and after cortisol administration are showed in Figure 14. The mean concentration of cortisol of seven samples from dog # 9485 pre and post 24 hours administrated urine samples was 125.88 ppb (SD=27.52).

Figure 15 shows the comparative study between normal cortisol levels of research dogs and dosed cortisol levels for 196 hours post hours of dog #9485 and #9491 at therapeutic use level (50 mg hydrocortisone sodium succinate per dog i.v). From the figure, it can be clearly seen that at 12 hours or 24 hours post dose time, all dosed cortisol levels of both dogs are within the 2 SD range of normal cortisol levels. At 8 hours post dose, only dosed cortisol levels of dog #9485 are within the 2 SD range of normal levels, 8 hours post dosed level of dog #9491 is outside of the 2 SD range of

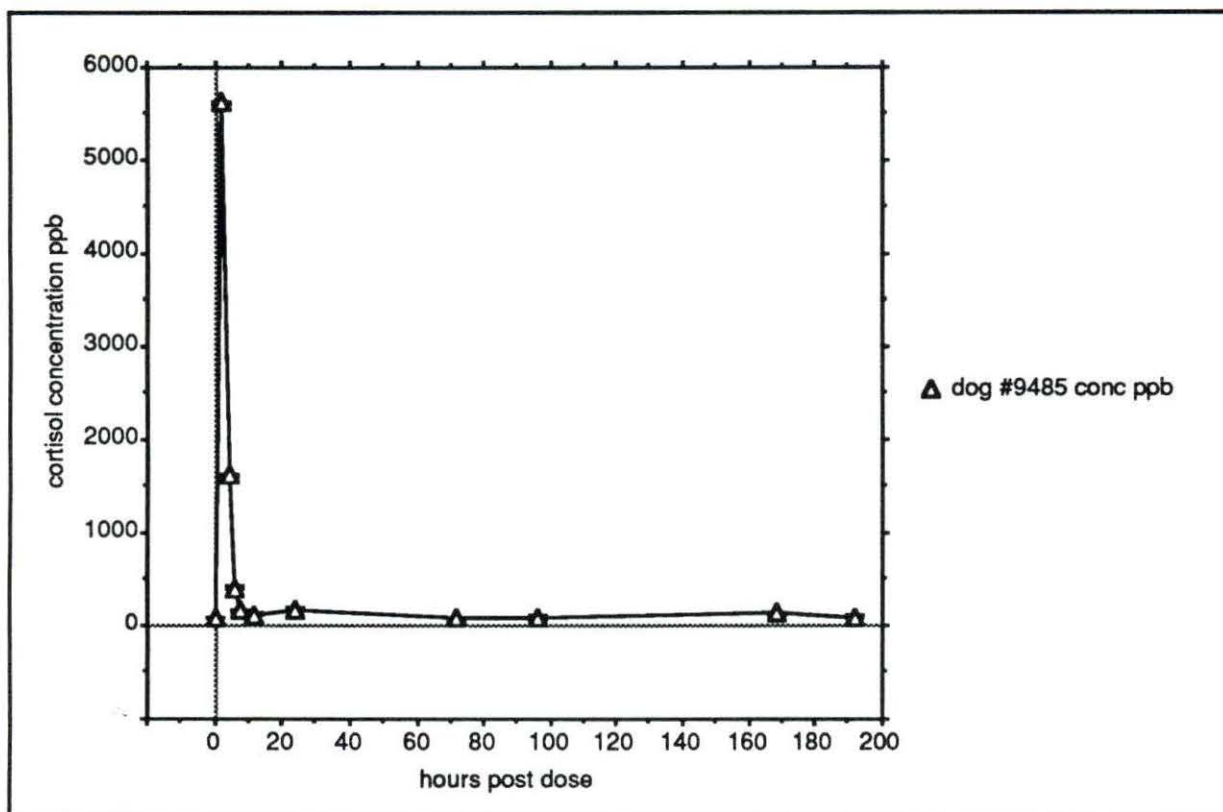


Figure 11. Urinary profile of dog #9485 following administration of 50 mg cortisol iv. by TDx FLx, error bars indicating 2 SD, n=3

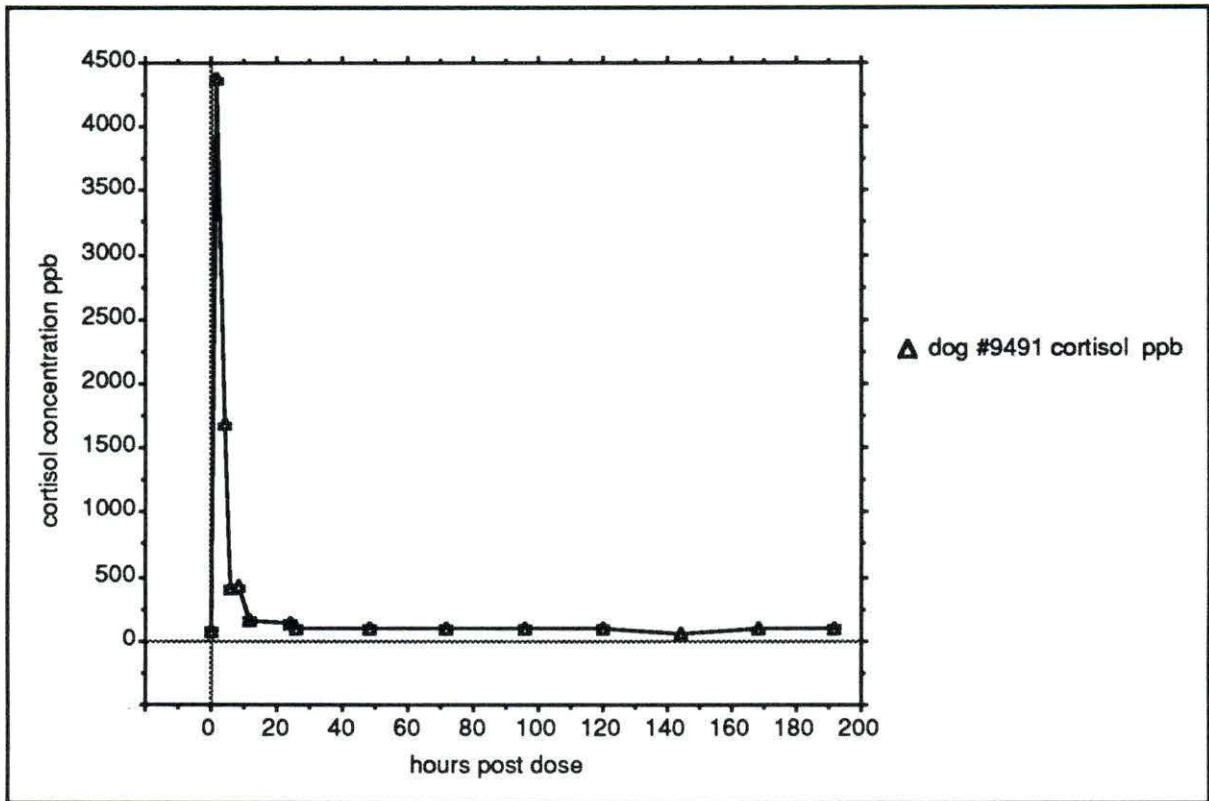


Figure 12. Urinary profile of dog #9491 following administration of 50 mg cortisol iv. by TDx FLx, error bars indicating 2 SD, n=3

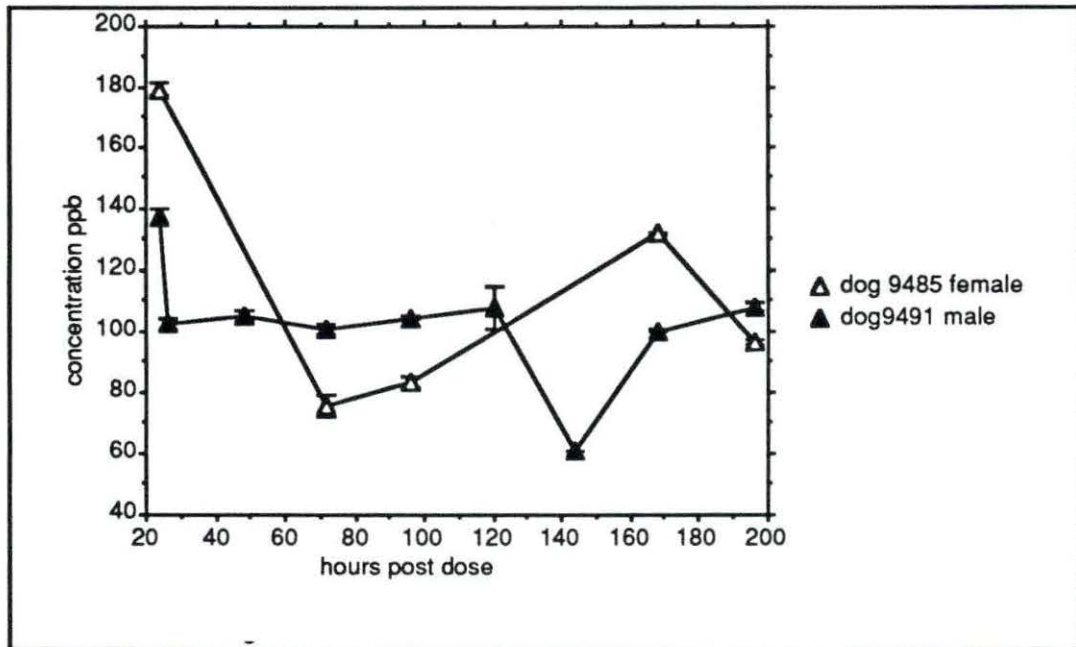


Figure 13. Urinary profiles (24 hr to 196 hrs) of dog #9485, #9491 following administration of 50 mg cortisol iv. by TDX FLx, error bars indicated 2 SD, n=3

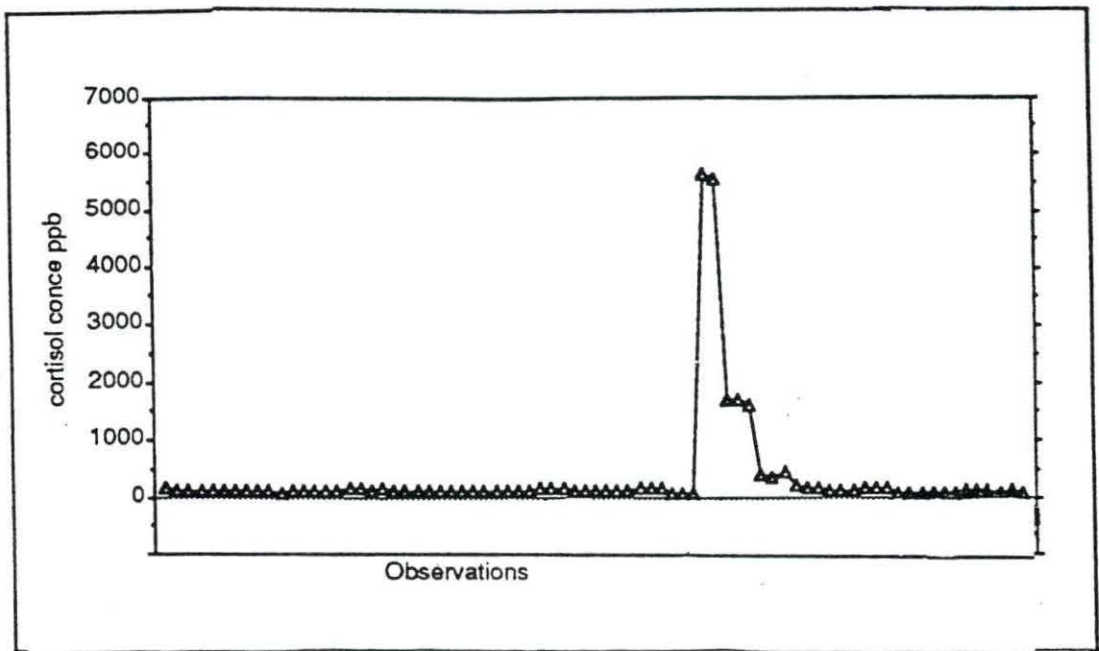


Figure 14. The whole urinary profile of dog #9485 following administration of 50 mg cortisol iv. by TDx FLx, n=3

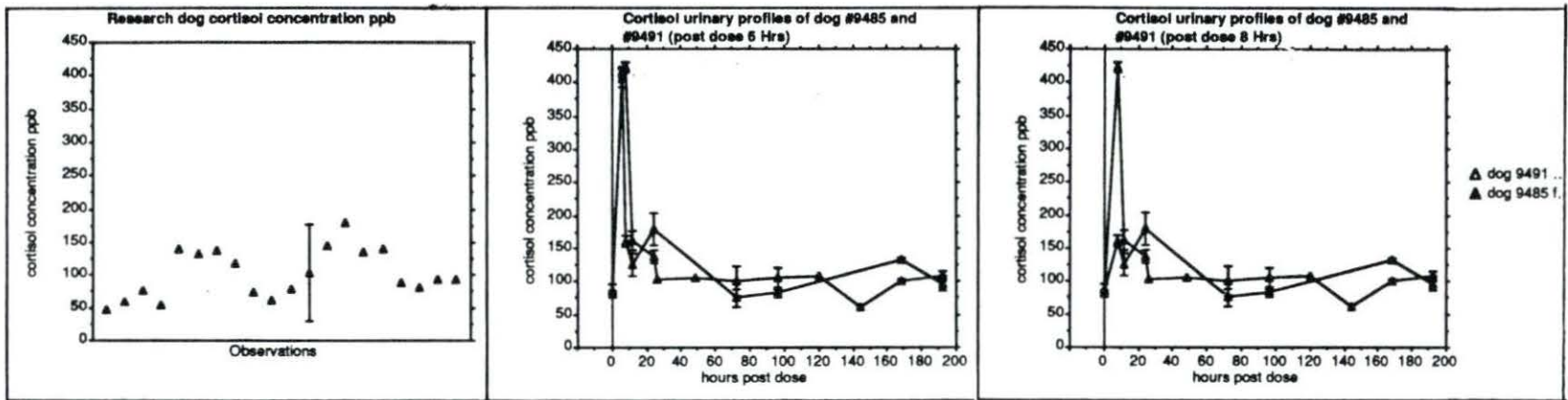


Figure 15. Comparative study between normal cortisol levels of research dogs and dosed cortisol levels of dog #9485 and #9491

normal cortisol levels. At 6 hours post dose, dosed cortisol levels of both dogs are outside of the 2 SD range of normal cortisol levels.

From the results presented above, we are able to conclude that normal cortisol levels can not be easily distinguished from dosed cortisol levels for 12 hours or 24 hours post dose, which consistent with the results of T test. The finding seems to indicate that 8 post dose hours is the time point at which normal cortisol levels can be statistically distinguished from dosed cortisol levels. For accurate determination of this crucial time point, more samples from administrated dogs will be needed.

The normal concentration of cortisol in track dog urine

102 track dog urine samples were chosen for this study. These samples have been monitored by TLC for cortisol presence prior to FPIA analysis to eliminate the influence of cortisol or corticosteroid treatment. Within TLC detection range, all of the samples turned out to be negative.

Calibration standards, consisting of cortisol (250, 100, 50, 25, and 10 ppb), were analyzed in duplicate with each batch of samples. Two or three control samples were included with each batch of samples. The mean concentration of cortisol of 96 track dog urine samples was 115.1 ppb (SD= 99.4) but covered a wide range from 10.1 to 343.0 ppb. Unreliable low response (below detection limit, not significantly different with 0 ppb) from five dogs were not included. Data from one sample (784.1 ppb) which is out of the 3 SD range was also not included.

In the literature, Irvine *et al.* (1988) measured the amounts of free cortisol in routine post-race urine samples from 110 horses and investigated causes of variability. They concluded that possible causes of the variability were: (1) Breeds; (2) Variability in the adrenal response to excitement and exercise; (3) The effects of vasopressin (antidiuretic hormone) which showed a marked but variable increase during a stimulated raceday. Other studies also indicated that the large variation in the quantities of corticosteroids secreted by different dogs under uniform experimental conditions are possible (Hechter *et al.*, 1955).

Figure 16 shows the univariate scattergrams of cortisol levels of 96 track dogs, with error bar indicating 2 SD. Figure 17 represents the comparative study between normal cortisol levels of track dogs and dosed cortisol levels for 196 hours post dose of dog #9485 and #9491. The results are consistent with the finding from comparative study between normal cortisol levels of research dogs and dosed cortisol levels of same two dogs. A T test has been done to determine if there is significant difference between normal cortisol levels of research dogs and normal cortisol levels of track dogs. The results indicate that statistically there is no difference between two groups ($P = 0.56$). Data from T test are given in table 7.

Practicability

FPIA assay is a very simple, fairly sensitive, reproducible and time saving method for cortisol analysis. Cross-reactivity studies also show it to be adequately

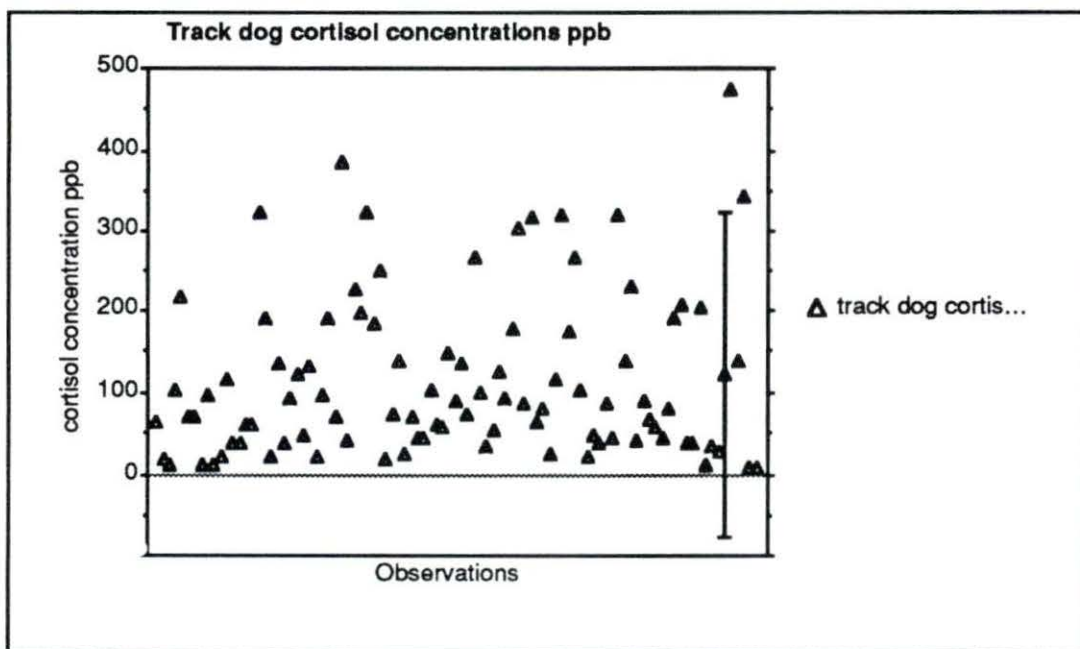


Figure 16. Univariate scattergrams of normal cortisol concentrations of 96 track dogs, error bar indicating 2 SD

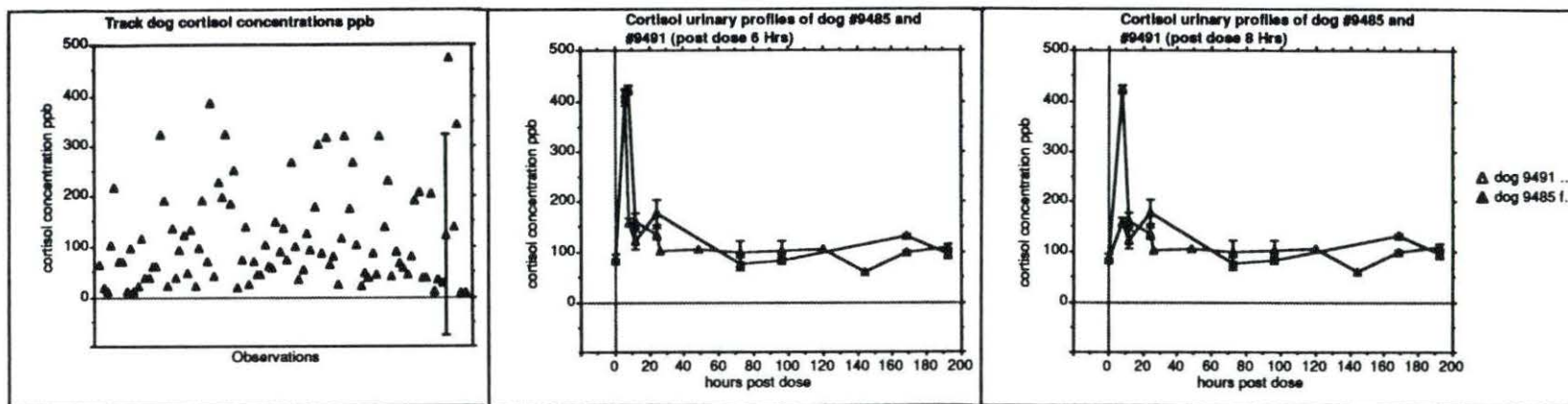


Figure 17. Comparative study between normal cortisol of track dogs and dosed cortisol levels of dog #9485 and #9491.

Table 7. Data from T test (Research samples and track samples)

Unpaired t-Test X ₁ : Column 8 Y ₁ : concentrations ppb				
DF:		Unpaired t Value:		Prob. (2-tail):
114		.591		.5556
Group:	Count:	Mean:	Std. Dev.:	Std. Error:
track dogs	96	115.118	99.401	10.145
rese dogs	20	101.753	37.051	8.285

specific for the quantitative measurement of cortisol. It does not need prior extraction and cleanup procedure. However, immunoassay in general are often considered a method with poor specificity because the antiserum used may cross react with other structurally similar compounds and as such absolute values for a single analyte cannot be assured.

In most racing chemistry laboratories, a reliable confirmation method such as GC/MS or HPLC are usually recommended to validate the use of FPIA and to assure the identity of analyte resulting in the endogenous concentrations observed. Regarding cortisol assays, the HPLC methods offer the advantage of a sufficient specificity. High resolution capillary GC/MS remains the method of choice for the final confirmation of cortisol in complex biological extracts with proper derivatization. Further comparisons between TDx assay and classical methods should be included in future study.

SUMMARY

This study investigated the characteristics of analytical method such as detection limit, specificity, reliability, precision and accuracy, and determined endogenous population level of cortisol in the greyhound urine using the optimal method of choice.

Gas chromatography/mass spectrometry is regarded as one of the confirmation method for cortisol analysis, but prior derivatization of cortisol is usually necessary before injection to GC/MS system because cortisol is not a thermal stable compound, which makes the analysis procedure more complex and difficult.

Through literature review, it is noticeable that high performance liquid chromatography is commonly used for cortisol analysis. It is reported that HPLC can detect cortisol levels in the low nanogram range (Schoneshofer *et al.*, 1980; Park *et al.*, 1990; Wade and Haegele., 1991). However, prior sample preparation and extraction procedures are usually time consuming, and the method requires large sample volumes.

Among the classical analytical methods investigated, Thin layer chromatography usually be used as initial screening method but not suitable for confirmation and quantitation.

Newly developed immunochemical assay FPIA offers the advantages that it is relatively easy to perform, can be performed rapidly, without the need for sample preparation, and has a fair degree of sensitivity, precision and specificity for overall confirmation of cortisol in biological samples.

The results generated by the FPIA assay for the screening and confirmation of cortisol in urine indicated that the assay had good intra and inter-assay reproducibility with a fair degree of sensitivity and specificity. The lowest detection limit was 10 ppb in urine. Diurnal variation of cortisol in dog was also included in this study, results showed that cortisol levels in dog urine might be subject to diurnal variations. In two female dogs, cortisol urinary levels were the highest in the morning hours and lowest in the afternoon. In one male dog, cortisol levels did not change significantly throughout 24 hours. A T test was carried out to determine if the cortisol levels were influenced by gender, the results indicated that the average cortisol level in female dogs was significantly higher than that of the male dogs. For accurate determination of diurnal variations and gender difference, more urine samples and statistical analysis are necessary. For threshold level determination, the FPIA assay detected presence of cortisol in all of the normal blank urine samples analyzed. The normal concentration of cortisol in research greyhound urine is 101.8 ppb (SD= 37.1). The normal concentration of cortisol in track greyhound urine is 115.1 ppb (SD= 99.4).

In terms of distinguishing between endogenous cortisol levels and exogenous cortisol levels, FPIA assay is able to detect the presence of cortisol in all of the blank urine samples and dosed urine samples analyzed. At 50 mg cortisol per dog dosage, endogenous urinary cortisol levels are not significantly different with exogenous urinary levels for 12 or 24 hours post dose.

SUGGESTIONS FOR FUTURE WORK

Future work could include further investigating and comparing extraction and sample preparation techniques to DMSO and its metabolites such as protein precipitation techniques. It would be of interest to know which of the various sample preparation techniques gives better spike recovery and clear solution results. More administration study of DMSO and DMSO₂ in dog and statistical analysis are also necessary regarding the differentiation between endogenous levels and dosed levels of these two drugs.

In terms of cortisol analysis, future work should include comparison study of the quantitation of urinary free cortisol using FPIA assay and gas chromatography /mass spectrometry with proper derivatization. It could then be determined if there is good correlation between the absolute values of free cortisol obtained by FPIA assay and the values obtained by GC/MS.

LITERATURE CITED

- Adair, H. S., D. O. Goble, S. Vanhooser, J. T. Blackford, and B. W. Rohrbach. 1991. Evaluation of use of dimethyl sulfoxide for intra-articular lavage in clinically normal horses. *AM. J. Vet. Res.* 52:333-336.
- Adlin, E. V., and Channick, B. J. 1966. Effects of prolonged inhibition of 11 β -hydroxylase in the dog. *Endocrinology.* 78:511-521
- Andreae, M. O. 1980a. Determination of trace quantities of dimethylsulfoxide in aqueous solutions. *Anal. Chem.* 52:150-153
- Andreae, M. O. 1980b. Dimethylsulfoxide in marine and freshwaters. *Limnol. Oceanogr.* 25:105
- Averkin, E., J. Kilian, T. O'Brien, and J. Sickles. 1975. Experimental design of clinical trials testing the efficacy of 90% DMSO* solution in injuries to the musculoskeletal system in the dog. *Vet. Med. Sm. An. Clin.* 2:177-179.
- Baucus, K. L., E. L. Squires, S. L. Ralston, A. O. McKinnon, and T. M. Nett. 1990. Effect of transportation on the estrous cycle and concentrations of hormones in mares. *J. Anim. Sci.* 68:419-426.
- Baxter, J. D., and G. G. Rousseau. 1979. Glucocorticoid hormone action. Springer-Verlag Berlin. Heidelberg. NY.
- Becker, M. J., D. Helland, and D. N. Becker. 1976. Serum cortisol (Hydrocortisone) values in normal dogs determined by radioimmunoassay. *Am. J. Vet. Res.* 37:1101-1102.
- Beisel, W. R., J. J. Cos, R. Horton, P. Y. Chao, and P. H. Forsham. 1964. Physiology of urinary cortisol excretion. *J. Clin. Endocr. Metab.* 24:887-893.
- Blythe, L. L., A. M. Craig, J. M. Christensen, L. H. Appell, and M. L. Slizeski. 1986a. Pharmacokinetics disposition of dimethyl sulfoxide administered intravenously to horses. *Am. J. Vet. Res.* 47:1739-1743.
- Blythe, L. L., A. M. Craig, L. H. Appell, and E. D. Lassen. 1986b. Intravenous use of dimethyl sulfoxide (DMSO) in horses: clinical and physiologic effects. Pages 441-

446 in F. J. Milne, eds., Proceedings of the thirty second annual convention of the American association of equine practitioners. College of Veterinary Medicine, Oregon State University, Corvallis, OR.

- Bossut, D. F. B., L.S. Leshin, M. W. Stromberg, and P. V. Malven. 1983. Plasma cortisol and beta-endorphin in horses subjected to electra-acupuncture for cutaneous analgesia. *Peptides*. 4:501-507.
- Braman, R. S., J. M. Ammons, and J. L. Bricker. 1978. Preconcentration and determination of hydrogen sulfide in air by flame photometric detection. *Anal. Chem.* 50(7):992-993.
- Braude, M. C., and R. R. Monroe. 1967. Effects of dimethyl sulfoxide and alphaglucochloralose on pentylenetetrazol convulsive thresholds in mice. *Ann. N. Y. Acad. Sci.* 141:248-253.
- Brayton, C. F. 1986. Dimethyl sulfoxide (DMSO): A Review. *Cornell Vet.* 76:61-90.
- Breznock, E. M., and R. D. McQueen. 1970. Adrenocortical function during aging in the dog. *Am. J. Vet. Res.* 31:1269-1273.
- Brotherton, H. O., and R. A. Yost. 1985. Rapid screening and confirmation for drugs and metabolites in racing animals by tandem mass spectrometry. *Am. J. Vet. Res.* 45(11):2436-2440.
- Brown, J. H. U., A. Anason, and J. Jacobs. 1957. In vitro metabolism of hydrocortisone in tissues of the adult rat. *Am. J. Physiol.* 190:259-264.
- Bush, I. E. 1953. Species differences in adrenocortical secretion. *J. Endocr.* 9:95-100.
- Cavina, G., G. Moretti, and A. Cantafora. 1973. Analysis of corticosteroids in mixtures by gradient elution liquid chromatography. *J. Chromatogr.* 80:89-100.
- Campbell, J. R., and G. Watts. 1973. Assessment of adrenal functions in dogs. *Br. Vet. J.* 129:134-143.
- Chapman, D. I., M. S. Moss, and J. Whiteside. 1977. The urinary excretion of synthetic corticosteroids by the horse. *Vet. Rec.* 100:447-450.

- Chen, C. L., M. S. A. Kumar, M. D. Williard, and T. F. Liao. 1978. Serum hydrocortisone (cortisol) values in normal and adrenopathic dogs as determined by radioimmunoassay. *Am. J. Vet. Res.* 39:179-180.
- Clifford, D. H., D. C. Lee, and M. O. Lee. 1983. Effects of dimethyl sulfoxide and acupuncture on the cardiovascular system of dogs. *Ann. N. Y. Acad. Sci.* 411:85-93.
- Colborn, D. R., D. L. Thompson, Jr., T. L. Roth, J. S. Capehart, and K. L. White. 1991. Responses of cortisol and prolactin to sexual excitement and stress in stallions and geldings. *J. Anim. Sci.* 69:2556-2562.
- Cottet-Emard, J. M., and L. Peyrin. 1977. An improved fluorimetric method for assay of dopa in urine and tissues and its use for determination of urinary dopa, at endogenous level, in different species. *J. Neural. Transmi.* 41:145-173.
- Craig, A. M., L. L. Blythe, L. H. Appell, and M. L. Slizeski. 1987. Evaluation of the potential for interference by dimethyl sulfoxide (DMSO) in drug detection in racing animals. *J. Vet. Pharmacol. Therap.* 10:298-304.
- David, N. A. 1972. The pharmacology of dimethyl sulfoxide. *Ann. Rev. Pharmacol.* 12:353-357.
- De La Torea, J. C., H. M. Kawanage, D. W. Rowed, C. M. Johnson, D. J. Goode, K. Kajihara, and S. Mullan. 1975. Dimethyl sulfoxide in central nervous system trauma. *Ann. N. Y. Acad. Sci.* 243:497-506.
- Devenport, L., A. Knehans, A. Sundstrom, and T. Thomas. 1989. Corticosterone's dual metabolic actions. *Life Sci.* 45:1389-1396.
- Dhar, T.K., and M. Schoneshofer. 1987. A fully automated method for sample clean-up prior to chromatographic and immunological analysis. *J. Clin. Chem. Clin. Biochem.* 25:241-244.
- Diamandis, E. P., and M. D'costa. 1988. selective determination of urinary free cortisol by liquid chromatography after solid-state extraction. *J. Chromatogr.* 426:25-32.
- Di Stefano, V., and J. J. Klahn. 1965. Observations on the pharmacology and hemolytic activity of dimethyl sulfoxide. *Toxicol. Appl. Pharmacol.* 7:660-666.
- Dobkin, A. B., and J. P. Su. 1966. Newer anesthetics and their uses. *Clin. Pharmacol.*

Ther. 7:648-682.

- Egan, P. S. J. A. 1977. Studies on the effect of short term corticosteroid therapy on serum electrolytes, S-GPT, and S-AP in the normal greyhound. *Ir. Vet. J.* 11:161-169.
- Eiler, H., D. Goble, J. Oliver. 1979. Adrenal gland function in the horse: effects of cosyntropin (synthetic) and corticotropin (nature) stimulation. *Am. J. Vet. Res.* 40(5):724-726.
- Eik-Nes, K., D. H. Nelson, and L. T. Samuels. 1953. determination of 17, 21-hydroxycorticosteroids in plasma. *J. Clin. Endocr. Metab.* 13:1280-1282.
- Farwell, O., and S. J. Gluck. 1979. Determination of sulfur-containing gases by a deactivated cryogenic enrichment and capillary gas chromatographic system. *Anal. Chem.* 51(6):609-615.
- Feldman, E. C., and J. B. Tyrrell. 1977. Adrenocorticotrophic effects of a synthetic polypeptide-alpha 1-24 corticotropin in normal dogs. *Am. Anim. Hosp. Assoc.* 13:494-499.
- Feldman, E. C., 1985. Evaluation of a combined dexamethasone suppression/ACTH stimulation test in dogs with hyper-adrenocorticism. *AM. J. Vet. Med. Assoc.* 182:506-510.
- Few, J. D. 1974. Effect of exercise on the secretion and metabolism of cortisol in man. *J. Endocrinology.* 62:341-353.
- Fiorelli, G., P. Piolanti, G. Forti, and M. Serio. 1972. Determination of plasma corticosteroids and urinary cortisol by a competitive protein-binding method using dextran-coated charcoal. *Clin. Chimi. ACTA.* 37:179-187.
- Firschein, H. E., F. Devenuto, W. M. Fitch, E. M. Pearce, and U. Westphal. 1957. Distribution of injected cortisol-4-C¹⁴ in normal and shocked rats. *Endocrinology.* 60: 347-358.
- Ford, E. J. H., I. P. Robinson, and J. Evans . 1990. An enzyme-linked immunoassay for the measurement of the concentration of cortisol in sheep plasma. *Res. in Vet. Sci.* 48:262-263.
- Foss, M. L., R. J. Barnard, and C. M. Tipton. 1971. Free 11-Hydroxycorticosteroid

- levels in working dogs as affected by exercise training. *Endocrinology*. 89:96-103.
- Frankel, I. A., B. Cook, and J. W. Graber. 1966. Determination of cortisterone in plasma by fluorometric techniques. *Endocrinology*. 80:181-194.
- Frantz, A. G., F. H. Katz, and J. W. Jailer. 1961. 6β -hydroxycortisol and other polar corticosteroids: measurement and significance in human urine. *J. Clin. Endocr.* 21:1290-1303.
- Fuller, B. J., A. L. Busza, and E. Proctor. 1989. Studies on cryoprotectant equilibration in the intact rat liver using nuclear magnetic resonance spectroscopy: A noninvasive method to assess distribution of dimethyl sulfoxide in tissues. *Cryobiology*. 26:112-118.
- Garnier, F., E. Benoit, M. Virat, R. Ochoa, and P. Delatour. 1990. Adrenal cortical response in clinically normal dogs before and after adaptation to a housing environment. *Lab. Anim.* 24:40-43.
- Ganis, F. M., L. R. Axelrod, and L. L. Miller. 1971. The metabolism of hydrocortisone by kidney tissue in vitro. *J. Biol. Chem.* 218:841-848.
- Garretson, S. E., and J. P. Aitchison. 1982. Determination of dimethyl sulfoxide in serum and other body fluids by gas chromatography. *J. Anal. Toxicol.* 6:76-81.
- Garretson, S. E. 1983. Comparison of dimethyl sulfoxide levels in whole blood and serum using an autosampler-equipped gas chromatograph. *Ann. N. Y. Acad. Sci.* 411:328-331.
- Gerhards, E., and H. Gibian. 1967. The metabolism of dimethyl sulfoxide and its metabolic effects in man and animals. *Ann. N. Y. Acad. Sci.* 141:65-76.
- Ghosh, P., and S. Thakur. 1982. Spray reagent for steroids and triterpenoids on thin-layer plates. *J. Chromatogr.* 240:515-517.
- Gorog, P., and I. B. Kovacs. 1975. Antiarthritic and antithrombotic effects of topically applied dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 243:91-97.
- Haegle, A. D., and S. E. Wade. 1991. Ultrasensitive differential measurement of cortisol and cortisone in biological samples using fluorescent ester derivatives in normal phase HPLC. *J. Liq. Chromatogr.* 14(6):1133-1148.

- Haigler, H. J., and D. D. Spring. 1981. DMSO (dimethyl sulfoxide), morphine and analgesia. *Life Sci.* 29:1545-1553.
- Haigler, H. J. 1983. Comparison of the analgesic effects of dimethyl sulfoxide and morphine. *Ann. N. Y. Acad. Sci.* 411:19-27.
- Harter, J. G. 1975. The status of dimethyl sulfoxide from the perspective of the food and drug administration. *Ann. N. Y. Acad. Sci.* 243:1-5.
- Harwood, T., and J. W. Mason. 1956. Effects of intravenous infusion of autonomic agents on peripheral blood 17-hydroxycorticosteroid levels in the dog. *Am. J. Physiol.* 196:445-452.
- Hasler, M. J., K. Painter, and G. D. Niswender. 1976. An ¹²⁵I-labeled cortisol radioimmunoassay in which serum binding proteins are enzymatically denatured. *Clin. Chem.* 22(11):1850-1854.
- Hawks, R. L., and C. N. Chiang. 1986. Urine testing for drugs of abuse. Department of health and human services. Rockville, ML.
- Hechter, O., I. A. Macchi, and H. Korman. 1955. Quantitative variations in the adrenocortical secretion of dogs. *Am. J. Physiol.* 182:29-34.
- Hellstrom, K., J. Sjovall, and R. Vihko. 1969. Plasma levels and excretion of solvolyzable steroids. *Acta. Endocr.* 60:501-511.
- Herschler, R. 1982. Proceeding of the symposium on dimethyl sulfoxide: chemistry and biological effects. *Vet. Med. Sm. An. Clin.* 3:367-369.
- Hill, D. W., T. R. Kelley, S. W. Matiuck, K. J. Langner, and D. E. Phillips. 1982. Single extraction for the recovery of basic, neutral and weakly acidic drugs from greyhound dog urine. *Anal. Lett.* 15(B2):193-204.
- Hillidge, C. J. 1985. The case for dimethyl sulfoxide (DMSO) in equine practice. *Equine. Vet. J.* 17:259-261.
- Hoesis, J. J., S. Harkaway, and R. Snyder. 1975. Biological effects of the metabolites of dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 243:105-109.
- Hoffsis, G. F., P. W. Murdick, V. L. Tharp, and K. Ault. 1970a. Plasma concentrations of cortisol and corticosterone in the normal horse. *Am. J. Vet. Res.* 31(8):1380-

1387.

- Hoffsis, G. F., and P. W. Murdick. 1970b. The plasma concentrations of corticosteroids in normal and diseased horses. *J. Am. Vet. Med. Assoc.* 157(11):1590-1594.
- Hohtari, H., K. Salminen-Lappalainen, and T. Laatikainen. 1991. Response of plasma endorphins, corticotropin, cortisol, and luteinizing hormone in the corticotropin-releasing hormone stimulation test in eumenorrheic and amenorrheic athletes. *Fertility and Sterility.* 55(2):276-280.
- Houghton, E., M. C. Dumasia, and J. K. Wellby. 1981. The use of combined high performance liquid chromatography negative ion chemical ionization mass spectrometry to confirm the administration of synthetic corticosteroids to horses. *Biomed. Mass Spec.* 8:558-64.
- Hsia, S. L., and Y. L. Hao. 1966. Metabolic transformations of cortisol-4-[¹⁴C] in human skin. *Biochemistry.* 5:1469-1474.
- Hucker, H. B., J. K. Miller, A. Hochberg, R. D. Brobyn, F. H. Riordan, and B. Calesnick. 1967. Studies on the absorption, excretion and metabolism of dimethylsulfoxide(DMSO) in man. *J. Pharmacol. Exp. Ther.* 155(2):309-317.
- Hyde, P. M., and R. H. Williams. 1957. Absorption and metabolism of hydrocortisone-4-C¹⁴. *J. Biol. Chem.* 227:1063-1081.
- Hyde, W., W. Duer, and D. Hill. 1990. DMSO and its metabolic methyl sulfonyl methane in the greyhound. Page 167-172 in J. P. Rodgers, and T. Toms, eds., *Proceeding of the 8th international racing greyhound symposium.* Harare, Zimbabwe.
- Irvine, C. H. G., R. J. Huun, and S. L. Alexander. 1988. The cortisol concentration in post-race urine of racehorses, and factors affecting it. Page 101-103 in T. Tobin, J. W. Blake, M. Potter, and T. Wood, eds., *Proceeding of the 7th International conference of Racing Analysis and Veterinarians.* Louisville, Kentucky.
- Irvine, C, H, G., S. L. Alexander, and R. A. Donald. 1989. effect of an osmotic stimulus on the secretion of arginine vasopressin and adrenocorticotropin in the horse. *Endocrinology.* 124:3102-3108.
- Ivey, J. P., and P. R. Haddad. 1987. Determination of dimethyl sulfoxide using ion-exclusion chromatography with ultraviolet absorption detection. *J. Chromatogr.*

3912:309-314.

Jacob, S. W., E. E. Rosenbaum, and D. C. Wood. 1971. Basic concepts of DMSO. Marcel Dekker, NY.

Jacob, S. Proceedings of the symposium on dimethyl sulfoxide. 1982. Vet. Med. Sm. An. Clin. 3:365-376. Jacob, S. W., and R. Herschler. 1986. Pharmacology of DMSO. Cryobiology. 23:14-27.

Jacob, S. W., and R. Herschler. 1986. Pharmacology of DMSO. Cryobiology. 23:14-27.

James, V. H. T., M. W. Horner, M. S. Moss, and A. E. Rippon. 1970. Adrenocortical function in the horse. Endocrinology. 48:319-335.

Johnston, S. D., and E. C. Mather. 1978. Canine plasma cortisol (hydrocortisone) measured by radioimmunoassay: clinical absence of diurnal variation and results of ACTH stimulation and dexamethasone suppression tests. Am. J. Vet. Res. 39(11):1766-1770.

Jones, C. A., K. R. Refsal, A. C. Lippert, R. F. Nachreiner, and M. M. Schwacha. 1990. Changes in adrenal cortisol secretion as reflected in the urinary cortisol/creatinine ratio in dogs. Domest. Anim. Endocr. 7(4):559-572.

Jursik, F. 1967. Ion-exchange separation and quantitative determination of dimethyl sulfoxide. J. Chromatogr. 26:336-338.

Karaseva, E. I., A. N. Eremin, and D. I. Metelitsa. 1990. Determination of cortisol by enzyme immunoassay using glucose-6-phosphate dehydrogenase and the antigen immobilized on nitrocellulose membranes. J. Anal. Chem. USSR. 45(4):536-542.

Karasek, F. W., and R. E. Clement. 1988. Basic gas chromatography-mass spectrometry principles and techniques. Elsevier. New York.

Kemppainen, R. J., J. L. Sartin, and M. E. Peterson. 1989. Effects of single intravenously administered doses of dexamethasone on response to the adrenocorticotrophic hormone stimulation test in dogs. Am. J. Vet. Res. 50(11):1914-1917.

Kharasch, N., and B. S. Thyagarajan. 1975. Structural basis for biological activities of dimethyl sulfoxide. Ann. N. Y. Acad. Sci. 243:391-401.

- Klein, H. A., S. Samant, B. L. Herz, and H. S. Pearlman. 1983. Dimethyl sulfoxide in adult respiratory distress syndrome: abstract of preliminary report. *Ann. N. Y. Acad. Sci.* 411:389-390.
- Knowles, R. P. 1967. Clinical experience with DMSO in small animal practice. *Ann. N. Y. Acad. Sci.* 141:479-483.
- Kocsis, J. J., S. Harkaway, and W. H. Vogel. 1968. Dimethyl sulfoxide: Breakdown of the blood-brain barrier. *Science.* 160:1427-1473.
- Kocsis, J. J., S. Harkaway, and R. Snyder. 1975. Biological effects of the metabolites of dimethylsulfoxide. *Ann. N. Y. Acad. Sci.* 243:104-109.
- Koller, L. D. 1976. Clinical application of DMSO by veterinarians in Oregon & Washington. *Vet. Med. Sm. An. Clin.* 5:591-597.
- Kolb, K. H., M. Kramer, and P. E. Schulze. 1967. Absorption, distribution and elimination of labeled dimethyl sulfoxide in man and animals. *Ann. N. Y. Acad. Sci.* 141:85-95.
- Kornel, L., and Z. Saito. 1975. Studies on steroid conjugates VIII: isolation and characterization of glucuronide-conjugated metabolites of cortisol in human. *J. Steroid Biochem.* 6:1267-1284.
- Kuipers, F., R. S. Ely, and V. C. Kelley. 1958. Metabolism of steroids: the removal of exogenous 17-hydroxycortisterone from the peripheral circulation in dogs. *Endocrinology.* 62:64-74.
- Lantto, O. 1982. Radioimmunoassay and liquid-chromatographic analysis for free cortisol in urine compared with isotope dilution-mass spectrometry. *Clin. Chem.* 28(5):1129-1132.
- Larsson, M., L. E. Edqvist, L. Ekman, and S. Persson. 1979. Plasma cortisol in the horse, diurnal rhythm and effects of exogenous ACTH. *Acta. Vet. Scand.* 20:16-24.
- Law, W. C., K. K. Leung, and D. L. Crone. 1990. Normally occurring dimethyl sulfoxide. page 1 to 18. personal communication.
- Law, W. C., K. K. Leung, and D. L. Crone. 1991. Urinary and plasma thresholds for dimethyl sulphoxide. Page 1-8 in the 44th meeting of the AORC. Cincinnati, OH.

- Lee, D. E., S. V. Lamb, and T. J. Reimers. 1991. Effects of hyperlipemia on radioimmunoassays for progesterone, testosterone, thyroxine, and cortisol in serum and plasma samples from dogs. *Am. J. Vet. Res.* 52(9):1489-1491.
- Lerner, H. J., and G. L. Beckloff. 1967. Effects of intravesical instillation of DMSO on the urinary bladder of the dog: preliminary report. *Ann. N. Y. Acad. Sci.* 173:254-260.
- Lewis, J. G., and P. A. Elder. 1985. An enzyme-linked immunosorbent assay (ELISA) for plasma cortisol. *J. Steroid Biochem.* 22(5):673-676.
- Ling, G. V., G. H. Stabenfeldt, K. M. Comer. 1979. Canine hyperadreno-corticism: pretreatment clinical and laboratory evaluation of 117 cases. *Am. J. Vet. Med. Assoc.* 174:1211-1215.
- Macharg, M. A., G. D. Bottoms, G. K. Carter, and M. A. Johnson. 1985. Effects of multiple intramuscular injections and doses of dexamethasone on plasma cortisol concentrations, and adrenal responses to ACTH in horses. *Am. J. Vet. Res.* 46(11):2285-2287.
- Markina, V. L., A. N. Eremin, and D. I. Metelitsa. 1989. Determination of microquantities of cortisol by enzyme-immunoassay using malate dehydrogenase and an antigen immobilized on nitrocellulose membranes. *J. Anal. Chem. USSR.* 44(12):1784-1790.
- Marin, R. E. 1975. Evaluation of dimethyl sulfoxide therapy in chronic respiratory insufficiency of bronchopulmonary origin. *Ann. N. Y. Acad. Sci.* 243:475-484.
- Mayhew, I. G., and R. J. Mackay. 1982. Neurologic disease in equine medicine and surgery. ed.3., Vol.2 Santa Barbara, Calif: American veterinary publications. p. 1201.
- McNair, H. M., E. J. Bonelli. 1969. Basic chromatography. Varian aerograph. Berkeley. CA.
- Mehta, A. C., and S. Peaker. 1986. Rapid gas chromatographic determination of dimethyl sulphoxide and its metabolite dimethyl sulfone in plasma and urine. *J. Chromatogr.* 383:400-404.
- Melby, J. C., 1977. Clinical pharmacology of systemic corticosteroids. *Annu. Rev.*

Pharmacol Toxicol. 17:511-527.

- Messerli, F. H., W. Nowaczynski, M. Honda, J. Genest, and O. Kuchel. 1976. Effects of ACTH on steroid metabolism. *J. Clin. Endocr. Metab.* 42(6):1074-1080.
- Metcalf, F. L. 1965. Laboratory and clinical evaluation of DMSO, Comments. *Modern Vet. Practice.* 8:36-37.
- Metcalf, J. 1983. MSM- a dietary derivative of DMSO. *J. Equine. Vet. Sci.* 3:148-175.
- Murphy, B. E. P., 1967. Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein-binding radioimmunoassay. *J. Clin. Endocr. Metab.* 27:973-990.
- Nakamura, J., and M. Yakata. 1985. Determination of urinary cortisol and 6 β -hydroxycortisol by high performance liquid chromatography. *Clinica. Chimica. Acta.* 149:215-224.
- Nilsson, B. 1983. Extraction and quantitation of cortisol by use of high-performance liquid affinity chromatography. *J. Chromatogr.* 276:413-417.
- Nozaki, O., T. Ohata, and Y. Ohba. 1991. Determination of serum cortisol by reversed-phase chromatography using precolumn sulfuric acid-ethanol fluorescence derivatization and column switching. *J. Chromatogr.* 570:1-11.
- Ogden, H. D. 1967. Experiences with DMSO in treatment of headache. *Ann. N. Y. Acad.Sci.* 141:647-648.
- Okumura, T. 1981. Application of thin-layer chromatography to high-performance liquid chromatographic separation of steroidal hormones and cephalosporin antibiotics. *J. liq. Chromatogr.* 4(6):1035-1064.
- Panaretto, B. A., J. Y. F. Paterson, and F. Hills. 1973. The relationship of the splanchnic, hepatic and renal clearance rates to the metabolic clearance rate of cortisol in conscious sheep. *J. Endocr.* 56:285-294.
- Park, S. J., Y. J. Kim, H. S. Pyo, and J. Park. 1990. Analysis of corticosteroids in urine by HPLC and thermospray LC/MS. *J. Anal. Toxicol.* 14:102-104.
- Parker, A. J., and C. W. Smith. 1979. lack of functional recovery from spinal cord

trauma following dimethyl sulfoxide and epsilon amino caproic acid therapy in dogs. *Res in Vet Sci.* 27:253-255.

- Paulin, H. S., J. B. Murphy, and R. E. Larson. 1966. Determination of dimethyl sulfoxide in plasma and cerebrospinal fluid by gas-liquid chromatography. *Anal. Chem.* 38(4):651-652.
- Patterson, P. L., R. L. Howe, and A. Abu-Shumays. 1978. Dual-flame photometric detector for sulfur and phosphorus compounds in gas chromatograph effluents. *Anal. Chem.* 50(2):339-344.
- Pearson, T. W., H. J. Dawson, and H. B. Lackey. 1981. Natural occurring levels of dimethyl sulfoxide in selected fruits, vegetables, grains, and beverages. *J. Agric. Food. Chem.* 29(5):1089-1091.
- Pearson Murphy, B. E., L. M. Okouneff, G. P. Klein, and S. C. Ngo. 1981. Lack of specificity of cortisol determination in human urine. *J. Clin. Endocr. Metab.* 53:91-98.
- Peterson, C. G., and R. G. Robertson. 1967. A pharmacodynamic study of dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 141:273-276.
- Peterson, M. E., S. R. Gilbertson, and W. D. Drucker. 1982. Plasma cortisol response to exogenous ACTH in 22 dogs with hyperadrenocorticism caused by adrenocortical neoplasia. *Am. Vet. Med. Assoc.* 180(5):542-544.
- Peterson, M. E., D. N. Orth, N. S. Halmi, A. C. Zielinski, D. R. Davis, F. T. Chavez, and W. D. Drucker. 1986. Plasma immunoreactive proopiomelanocortin peptides and cortisol in normal dogs and dogs with addison's disease and cushing's syndrome: basal concentrations. *Endocrinology.* 119(2):720-730.
- Rado, J. P., L. Vegh, and I. Sawinsky. 1974. Interference of triamterene with cortisol determination in urine. *Horm. Metab. Res.* 6:429-430.
- Raff, H., M. M. Skelton, and A. W. Cowley, Jr. 1990. Cortisol inhibition of vasopressin and ACTH responses to arterial hypotension in conscious dogs. *Am. J. Physio.* 254(1):R64-69.
- Ralston, J. M., A. M. Stenhouse, N. S. Stenhouse, G. J. Buck, S.F. Lucks, J. A. Reynoldson, and J. R. Bolton. 1988. Cortisol concentration in blood and urine of horses. *Australian. Vet. J.* 65(1):1-5.

- Ralston, J. M., A. M. Stenhouse, C. I. Russo. 1990. Cortisol levels in blood and urine of horses. Page 181-186 in J. P. Rodgers, and T. Toms, eds., Proceedings of the 8th international conference of racing analysts and veterinarians. Harare, Zimbabwe.
- Rammler, D. H., and A. Zaffaroni. 1967. Biological implications of DMSO based on a review of its chemical properties. *Ann. N. Y. Acad. Sci.* 141:13-23.
- Ratliff, C. R., and F. F. Hall. 1973. Fluorometric measurement of urinary free hydrocortisone (cortisol). *Clin. Chem.* 19(12):1400-1402
- Richardson, J. 1973. Topical use of dimethyl sulfoxide (DMSO). *The southwestern veterinarian.* Spring:223-226.
- Richkind, M., and L. E. Edqvist. 1973. Peripheral plasma levels of corticosteroids in normal beagles and greyhounds measured by a rapid competitive protein binding technique. *Acta. Vet. Scand.* 14:745-757.
- Rijnberk, A., P. J. derKinderen, and J. H. Thijssen. 1968. Spontaneous hyperadrenocorticism in the dog. *J. Endocr.* 41:397-406.
- Rose, J. Q., and W. J. Jusko. 1979. Corticosteroid analysis in biological fluids by high-performance liquid chromatography. *J. Chromatogr.* 162:273-280.
- Rowed, D. W., and J. C. De La Torre. 1973. Dimethyl sulfoxide in experimental hypoxic respiratory depression. *Arch. int. Pharmacodyn.* 204:105-109.
- Rubin, L. F. 1983. Toxicologic update of Dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 411:6-10.
- Rucker, N. C., N. V. Lumb, and R. J. Scott. 1983. Combined pharmacological and surgical treatments for acute spinal cord trauma. *Ann. N. Y. Acad. Sci.* 411:191-199.
- Sayegh, J. F., and P. Vestergaard. 1978. Multi-column chromatography and the use of isotopes in the study of steroid metabolism. *ACTA Endocrinologica.* 217(9):196-200.
- Sams, W. M., Jr. 1967. The effects of dimethyl sulfoxide on nerve conduction. *Ann. N. Y. Acad. Sci.* 243:337-357.

- Schoneshofer, M., A. Fenner, and H. J. dulce. 1980a. Interferences in the radioimmunological determination of urinary free cortisol. *Clinica Chimica* 101:125-134.
- Schoneshofer, M., A. Fenner, G. Altinok, and H. J. Dulce. 1980b. Specific and practicable assessment of urinary free cortisol by combination of automatic high-pressure liquid chromatography and radioimmunoassay. *Clinica Chimica*. 106:63-73.
- Schoneshofer, M., and A. Fenner. 1981. A convenient and efficient method for the action and fractionation of steroid hormones from serum or urine. *J. Clin. Chem. Clin. Biochem.* 19:71-74.
- Scurry, M. T., and L. Shear. 1969. Stop-flow analysis of the reabsorption of cortisol. *Endocr.* 84:681-682.
- Seki, T., Y. Yamaguchi. 1983. New fluorimetric detection method of corticosteroids after high-performance liquid chromatography using post-column derivatization with glycinamide. *J. Liq. Chromatogr.* 6(6):1131-1138.
- Sellnow, L. 1987. MSM: an aid from nature. *J. Equine. Vet. Sci.* 7:3459-3463.
- Setchell, K. D. R., B. Alme, M. Axelson, and J. Sjoval. 1976. The multicomponent analysis of conjugates of neutral steroids in urine by lipophilic ion exchange chromatography and computerized gas chromatography-mass spectrometry. *J. Steroid. Biochem.* 7:615-629.
- Shalaby, A., and M. Shahjahan. 1991. Improved high performance liquid chromatography method for the determination of some corticosteroids. *J. Liq. Chromatogr.* 14(7):1267-1274.
- Shackleton, C. H. L., and J. O. Whitney. Use of sep-pak cartridges for urinary steroid extraction: evaluation of the method for use prior to gas chromatographic analysis. *Clinica Chimica. ACTA.* 107:231-243.
- Shealy, C. N. 1966. The physiological substrate of pain. *Headache.* 6:101.
- Siegel, E. T. 1968. Assessment of pituitary-adrenal gland function in the dogs. *Am. J. Vet. Res.* 46:173-180.
- Singh, A. K. 1989. Screening of steroids in horse urine and plasma by using electron

impact and chemical ionization gas chromatography-mass spectrometry. *J. Chromatogr.* 479:233-242.

- Skrabalak, D. S., T. R. Covey, and J. D. Henion. 1984. Qualitative detection of corticosteroids in equine biological fluids and the comparison of relative dexamethasone metabolite/dexamethasone concentration in equine urine by micro-liquid chromatography-mass spectrometry. *J. Chromatogr.* 315:359-372.
- Slone, D. E., R. C. Purohit, V. K. Ganjam, and J. L. Lowe. 1983. Sodium retention and cortisol (hydrocortisone) suppression caused by dexamethasone and triamcinolone in equine. *Am. J. Vet. Res.* 44(2):280-283.
- Slone, D. E., V. K. Ganjam, R. C. Purohit, W. R. Ravis. 1983. Cortisol (hydrocortisone) disappearance rate and pathophysiologic changes after bilateral adrenalectomy in equine. *Am. J. Vet. Res.* 44(2):276-279.
- Smith, E. R., Z. Hadidiian, and M. M. Mason. 1967. The single-and repeated-dose toxicity of dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 141:96-109.
- Sowell, J. G., A. A. Hagen, and R. C. Troop. 1971. Metabolism of cortisol-4-¹⁴C by rat lung tissue. *Steroids.* 18:289-301.
- Steinetz, B. G., O. D. Sherwood, M. L. Birkhimer, M. C. Butler, and W. K. Sawyer. 1973. Prolonged elevation of plasma cortisol levels in dogs treated with an 18 amino acid synthetic corticotrophin. *Experientia.* 29:1367-1368.
- Stolp, R., R. J. M. Croughs, J. C. Meijer, and A. Rijnberk. 1982. Plasma cortisol response to thyrotrophin releasing hormone and luteinizing hormone releasing hormone in healthy kennel dogs and in dogs with pituitary-dependent hyperadrenocorticism. *J. Endocrinology Ltd.* 93:365-369.
- Stull, C. L., and A. V. Rodiek. 1988. Responses of blood glucose, insulin and cortisol concentration to common equine diets. *J. Nutrition.* 118:206-213.
- Sulzberger, M. B., T. A. Cortese, L. Fishman, H. S. wiley, and P. S. Pevakovich. 1967. Some effects of DMSO on human skin in vivo. *Ann. N. Y. Acad. Sci.* 141:437.
- Teigland, M. B., and V. R. Saurino. 1967. Clinical evaluation of dimethyl sulfoxide in equine applications. *Ann. N. Y. Acad. Sci.* 141:471-477.

- Tietz, N. W. 1970. Fundamentals of clinical chemistry. W. B. Saunders. Philadelphia, Pa.
- Tiews, J., E. Scharrer., N. Harre, and L. Flogel. 1975. Metabolism and excretion of dimethyl sulfoxide in cows and calves after topical and parenteral application. *Ann. N. Y. Acad. Sci.* 243:139-150.
- Tobin, T., and W. E. Woods. 1979. Pharmacology review: drug metabolism and elimination in horses. *J. Equine. Med. Surg.* 3:150-156.
- Tobin, T. 1981. Drugs and the performance horse. Charles C. Thomas, Springfield, IL.
- Tobin, T., and J. W. Blake. 1987. Equine drug research report. Department of Veterinary Science, University of Kentucky, Lexington, Kentucky. 15pp.
- Toutain, P. L., R. A. Brandon, H. D. Pomyers, M. Alvinerie, and J. D. Baggot. 1984. Dexamethasone and prednisolone in the horse: pharmacokinetics and action on the adrenal-gland. *Am. J. Vet. Res.* 45(9):1750-1756.
- Ulick, S., C. K. Chan, and J. Z. Wang. 1991. Measurement of 4 urinary C-18 oxygenated corticosteroids by stable isotope dilution mass fragmentography. *J. Steroid Biochem.* 38(1):59-66.
- Wade, S. E., and A. D. Haegele. 1991. Differential measurement of cortisol and cortisone in human saliva by HPLC with UV detection. *J. Liq. chromatogr.* 14(9):1813-1827.
- Waller, F. T., C. T. Tanabe, and H. D. Paxton. 1983. Treatment of elevated intracranial pressure with dimethylsulfoxide. *Ann. N. Y. Acad. Sci.* 411:286-292.
- Watrin, L.R. 1988. The influence of synthetic corticosteroids on racing performance. Page 37-38 in T. Tobin, J. W. Blake, M. Potter, and T. Wood eds., *Proceeding of the 7th International Conference of Racing Analysis and Veterinarians.* Louisville, Kentucky.
- Watson, E. D., and K. Hinrichs. 1989. Adrenal production of sex steroids in the mare. *Theriogenology.* 32(6):913-918.
- Welch, R. D., R. M. DeBowes, and H. W. Liepold. 1989. Evaluation of the effects of intra-articular injection of dimethylsulfoxide on normal equine articular tissues. *Am. J. Vet. Res.* 50(7):1180-1182.

- Willard, M. D., K. Refsal, E. Thacker. 1987. Evaluation of plasma aldosterone concentrations before and after ACTH administration in clinically normal dogs and in dogs with various diseases. *Am. J. Vet. Res.* 48(12):1713-1718.
- Williams, K. J. H., S. H. Burstein, and D. S. Layne. 1966. Metabolism of dimethyl sulfide, dimethyl sulfoxide, and dimethyl sulfone in the rabbit. *Arch. Biochem. Biophys.* 117:84-87.
- Wong, L. K., and E. L. Reinertson. 1984. Clinical considerations of dimethyl sulfoxide. *Iowa state Veterinarian.* 46(2):89-95.
- Wood, D. C., and J. Wood. 1975. Pharmacologic and biochemical considerations of dimethyl sulfoxide. *Ann. N. Y. Acad. Sci.* 243:7-19.
- Yellowlees, P., C. Greenfield, and N. McIntyre. 1980. Dimethylsulfoxide-induced toxicity. *Lancet.* 2(8202):1004-1006.
- Zolovick, A., D. W. Upson, and B. E. Eleftheriou. 1966. Diurnal variation in plasma glucocorticosteroid levels in the horse. *J. Endocr.* 35:249-253.

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APPENDIX**TDxFLx Fluorescence Polarization Immunoassay****Materials Supplied in Kit**

TDx/TDxFLx Cortisol Reagent Pack:

P Cortisol pretreatment solution.

Surfactant in buffer (4 ml).

Preservative: 0.1% sodium azide.

S < 1% cortisol antiserum (mouse, monoclonal and goat) solution. Stabilizers in buffer (3 ml).

Preservative: 0.1% sodium azide.

T < 0.01% cortisol fluorescein tracer solution.

Surfactant and stabilizers in buffer (3 ml).

Preservative: 0.1% sodium azide.

Cortisol calibrators:

Six vials with accurately measured amounts of cortisol in buffer at the following concentrations:

Vial	Cortisol concentration ($\mu\text{g}/\text{dl}$)
A	0.0
B	2.5
C	5.0

D	10.0
E	25.0
F	60.0

Preservative: 0.1% Sodium azide.

Cortisol Controls:

Vial	Cortisol Concentration ($\mu\text{g}/\text{dl}$)
L	2.6-5.4
M	12.2-17.8
H	33.2-46.8

Preservative: 0.1% sodium azide.

Reagents not supplied in kit:

Hydrocortisone standard at 1 $\mu\text{g}/\text{ml}$ (methanol)

Blank urine sample.

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Vortex samples for 5 seconds prior to use.
3. Perform instrument calibration using cortisol calibrators.
4. Add 75 μl of standard, or control/sample to tri-well cartridges on carousel.
5. Put reagent pack into the analyzer.

6. Start run.

7. Get print out of fluorescence intensity of each sample