DETERMINATION OF DIMETHYL SULFOXIDE IN SERUM AND OTHER BODY FLUIDS BY GAS CHROMATOGRAPHY

By
Susan Garretson, M.T. (ASCP)

A THESIS

Presented to the Department of Clinical Pathology
and the Graduate Division of
the Oregon Health Sciences University
in partial fulfillment of
the requirements for the degree of
Master of Science
April 1982

A P P R O V E D

[redacted]

Professor in Charge of Thesis

[redacted]

Chairman, Graduate Council

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to my advisor, Dr. John P. Aitchison, who has given guidance and support throughout my graduate studies, this research project and the preparation of both the journal article and this thesis. I also want to thank Dr. J. Robert Swanson and Dr. Margaret Berroth, as well as the rest of the faculty and staff of the Department of Clinical Pathology for their help and interest. I especially wish to express appreciation to the staff of the toxicology department for their patience and assistance.

Finally, I want to express my deepest thanks to my husband, Stan and son, Anthony, without whose patience and emotional support I could not have completed these studies. It is to them that I dedicate this work.

TABLE OF CONTENTS

LIST OF TABLES

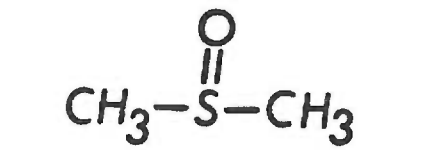
LIST OF ILLUSTRATIONS

I. INTRODUCTION

History and Chemistry

David (1) reviewed the history and chemistry of dimethyl sulfoxide (DMSO). DMSO was originally synthesized by Alexander Saytzeff in 1867 by oxidation of dimethyl sulfide, but it wasn't until the 1940's that its excellent solvent properties were recognized and it came to be used in insecticides, fungicides and herbicides, as well as for other industrial processes. In the late fifties and early sixties cryoprotective properties in a number of tissues were recognized. Dr. Stanley Jacob and Robert Herschler early recognized DMSO's unique ability to rapidly penetrate skin and the potential benefit for treatment of acute musculoskeletal disorders. Since then DMSO has been used extensively in many areas of medical research.

In his review David (1) describes DMSO's chemical classification as that of a dipolar, aprotic solvent. It generally does not donate labile hydrogen atoms to form hydrogen bonds but DMSO does accept protons. The marked polarity of the sulfur-oxygen bond results in a high dielectric constant (greater than 45). Figure 1 demonstrates the tetrahedron-like structure of DMSO. Dipole-dipole attraction of the sulfur-oxygen terminals result in a chainlike molecular arrangement in the liquid state. Aprotic solvents such as DMSO generally lack acidic or basic properties.



Dimethyl Sulfoxide

Figure 1. Chemical structure of DMSO.

DMSO is very hygroscopic forming strong hydrogen bonds with water molecules. It acts as a hydrogen acceptor and actually forms stronger bonds than found in water-water interaction. A study by Szmant (2) indicates that DMSO tends to organize water molecules into ice-like clusters. The effects exerted on biological systems may be related to these molecular changes in water. Szmant found that this ordering was greatest at a 6:2 ratio of water to DMSO.

When mixed with many substances which have hydrogen bond donor groups, DMSO acts as a better solvent than water. The fact that polarizable nonionic substances (i.e. proteins and steroids) tend to be soluble in DMSO may relate to its ability to rapidly penetrate tissue. Hydrogen bond acceptors on the other hand, tend to be more soluble in water than in DMSO.

The physical properties of DMSO are as follows: boiling point 189^{o}C, freezing point 18.45^{o}C, specific gravity 1.1014 and molecular weight 78.13 (3).

Absorption, Distribution, Metabolism And Excretion

The fate of DMSO in man as well as certain mammals has been studied extensively by a number of investigators. It has long been recognized that DMSO is rapidly absorbed through the skin. Kolb *et al.* (4) was able to detect radioactively labelled DMSO in blood 5 minutes after cutaneous administration. This absorption takes place with reversible damage to the tissue.

Denko *et al.* (5) and Mallinin *et al.* (6) studied the distribution of radioactively labelled DMSO in animals. They

found that DMSO tends to be widely distributed in all tissues. The concentrations were higher in soft tissues than in hard tissues. Figure 2 represents the distribution found in various tissues by Denko. Malinin's study (6) indicated that DMSO is confined to interstitial fluid and is localized along cell membranes. There appear to be conflicting reports concerning serum protein binding of DMSO. Gerhards and Gibian (7) report 30% binding to serum protein. Denko et al. (5) reported association with the albumin and globulin fractions, 96% and 4%, respectively. On the other hand Malinin et al. (6) reported no evidence of protein binding by DMSO.

Several investigators have studied the metabolism and excretion of DMSO in man and animals. Figure 3 represents the pathway DMSO takes. Studies by Wong et al. (8) and Kolb et al. (4) indicated that DMSO is reduced to dimethyl sulfide which is excreted in expired air. Kolb found that dimethyl sulfide represented approximately 3% of the dose administered. In addition to Wong and Kolb, Hucker et al. (9) studied the metabolism of DMSO and all three found that DMSO is oxidized to dimethyl sulfone, with both DMSO and dimethyl sulfone excreted in the urine. Gerhards and Gibian (7) in their study found that DMSO was oxidized in vitro to dimethyl sulfone by rat liver microsomes in the presence of $NADPH_2$ or $NADH_2$ and molecular oxygen.

Hucker et al. (9) administered DMSO orally and dermally to humans. The maximum serum level was reached 4 to 8 hours after dermal administration and 1 to 4 hours after oral administration. Urinary excretion of DMSO began very shortly after both forms of

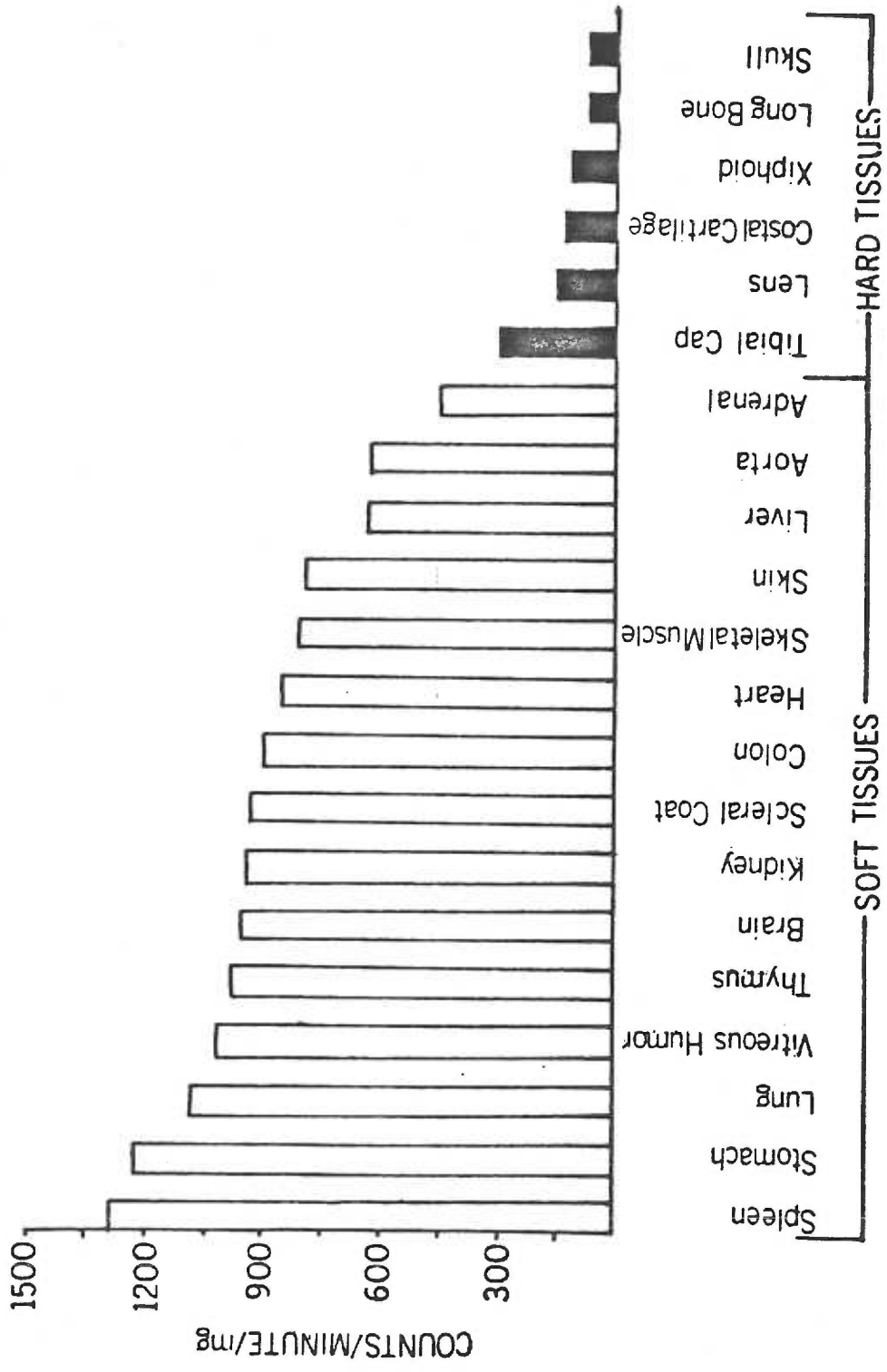


Figure 2. Distribution of ^{35}S-DMSO in the rat. Average of three rats two hr. after receiving 50 uc i.p. From Denko, et al. (5)

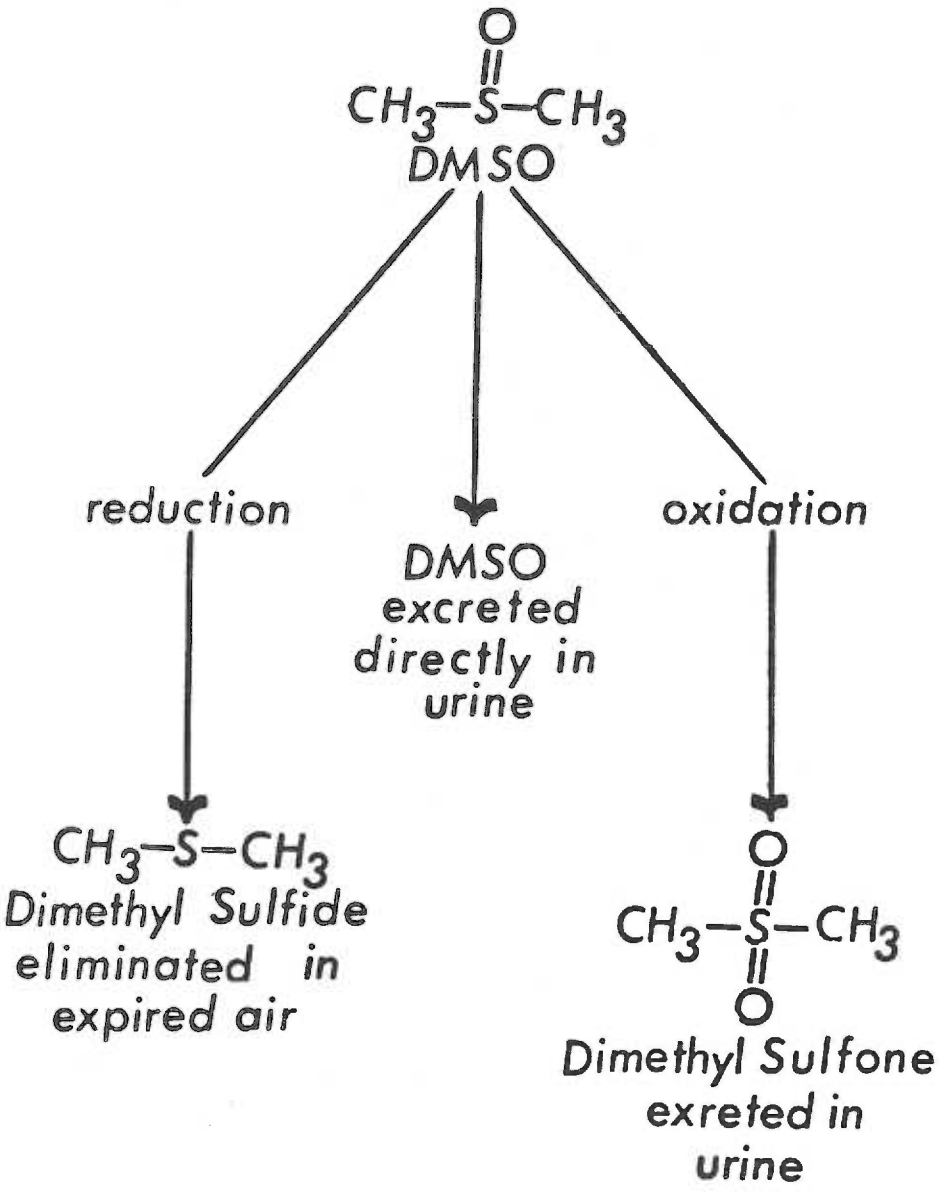


Figure 3. Metabolism and excretion of DMSO in humans.

administration and continued for 36 to 48 hours after dermal administration and 120 hours after oral administration. On the other hand, dimethyl sulfone excretion became significant at 8 hours after dermal administration and 20 hours after oral administration and continued for up to 456 and 480 hours respectively. The $t_{1/2}$ for DMSO was 11 to 14 hours after dermal administration and 20 hours after oral administration. The $t_{1/2}$ for dimethyl sulfone was around 70 hours regardless of the route of administration. About 31% of the dermal dose and 72% of the oral dose was accounted for as DMSO and dimethyl sulfone in the urine. Kolb et al. (4), Wong et al. (8) and Hucker et al. (9) all found that excretion of DMSO or dimethyl sulfone in feces in man is negligible. Hucker suggested that the reason for prolonged excretion of dimethyl sulfone may lie in a lower renal clearance or extensive tissue binding. Another possible explanation is the binding of DMSO to tissue with its slow conversion to dimethyl sulfone.

Williams et al. (10) studied normal human urine and found that it contains a small amount of dimethyl sulfone. He found a range of 4 to 11 mg per 24 hours in 8 subjects. It was suggested that dimethyl sulfone may be derived from dietary sources or possibly may be the product of sulfur containing amino acids.

Pharmacologic Effects

The wide range of pharmacological effects of DMSO have been studied extensively. Perhaps one of its more unique properties is that of rapid reversible membrane penetration without permanent damage. According to Szmant (2) this rapid penetration is probably due to DMSO's polar nature, capacity to accept hydrogen bonds and its compact structure. He further suggests it is the interaction of DMSO with water molecules within the biological system, both directly and indirectly, that results in the ease of penetration. David (1), in his review of the pharmacology of DMSO, suggests that DMSO replaces water molecules in the "ice-like sheath" covering proteins, nucleic acids and polysaccharides of the cell membrane. Proteins are held in their native structure by means of hydrogen and hydrophobic bonding. By replacing water molecules as a hydrogen bond acceptor, DMSO may cause a change in protein configuration thereby allowing rapid penetration. According to David these changes are reversible.

A number of studies have documented antiinflammatory effects of DMSO. According to the review by David (1) a number studies, with animals and tissue cultures, have shown that treatment with DMSO after various forms of trauma have resulted in decreased edema, granuloma formation and fibroblast growth. Wood and Wood (11) in their review of the pharmacological actions of DMSO reported that when DMSO is used as a vehicle for cortisone, a drug known to stabilize lysosomes against lysis by many agents, the concentration of cortisone required could be decreased by 100 fold.

Vasodilation is another pharmacological property discussed by Wood and Wood (11) and David (1). According to these reviews a number of studies have documented vasodilation. Both reported on potent histamine-like properties in the area of application. It was proposed that DMSO, known to react with the glutathione-SH group, may react with receptor SH groups with a resultant blocking of the receptor (11).

A number of studies have reported on bacteriostatic properties of DMSO. The review by Wood and Wood (11) cites a number of studies that indicate that growth of Staphylococcus aureus, Escherichia coli and Pseudomonas in media containg DMSO was inhibited. Other studies indicated that the sensitivity of certain microorganisms to antibiotics was increased after treatment with DMSO. DMSO has also been used as a potent and effective vehicle for transport of antifungal agents.

Diuresis is another pharmacologic property of DMSO which has been documented. David (1) in his review cited a study in which an increase in urine output paralleled the total amount of DMSO administered to cats. Other studies discussed by David also found an increase in urine volume as well as increased sodium and potassium excretion with DMSO administration.

DMSO's effect on collagen deposition, especially pathological collagen, has been studied by a number of investigators. The review by Wood and Wood (12) cited studies which indicated dissolution of pathological collagen and improvement in the form of reduced pain, greater flexibility of diseased skin and enhanced healing of ulcers in scleroderma patients. David (1) discussed

one study in which induced intestinal adhesions in rats were greatly reduced with DMSO treatment.

Both reviews by Wood and Wood (11) and David (1) cite studies to support one of the more well known pharmacologic actions of DMSO, that of analgesia. Several *in vitro* studies report decreased nerve conduction velocity after exposure to DMSO. Another study cited by David indicates that pain relief may occur not only at the application site but also at sites distant from the point of application. A number of clinical trials have demonstrated analgesic action related to acute traumatic injuries as well as nontraumatic pain (12).

A number of other pharmacological properties were reported on in the two reviews mentioned (1, 11). DMSO has been shown to enhance the action and therefore toxicity of many compounds. Cholinesterase inhibition, sedation, muscle relaxation, antagonism to platelet aggregation and radioprotective properties are some of the other effects noted by various investigators. Görög and Kovacs (12) in their study of antithrombic effects using rat mesoappendix found "that topically applied DMSO promotes restoration of the flow after occlusive thrombosis in the venules." Runckel and Swanson (13) in the course of investigating a case of a patient receiving high doses of intravenous DMSO found that DMSO increases the osmolality of serum.

Uses

As a result of the many and often unique pharmacological effects of DMSO, it has been used with greater or lessor success in a wide range of clinical trials as well as other forms of research. In his review David (1) covers many of the areas of clinical research. A number of studies have utilized dermal application in the treatment of acute musculoskeletal disorders as well as various forms of pain, including post operative and post traumatic intractable pain. Görög and Kovaks (12) in an experimental model of adjuvant induced arthritis in rats found that DMSO alone inhibited the arthritic reaction with minimal systemic effect. On the other hand, hydrocortisone, a common arthritis treatment, is known to have systemic effects even when applied dermally. They also found that the antiarthritic effect of hydrocortisone is increased tenfold when DMSO is used as a carrier.

DMSO has also been used to give symptomatic relief in treatment of scleroderma. Engel (14) reported beneficial effects in the areas of "increased mobility, rapid relief of pain and healing of persistant ulcers, arrest of spread of cutaneous diseases, regrowth of hair and return of sensation and sweating." He suggests that these effects are a result of blocking of conduction in nerves, increased blood flow due to dilation of surface vessels and antiinflammatory actions of DMSO. David (1) further discusses the use of DMSO for treatment of skin ulcers, post thrombotic syndromes, Raynaud's disease and brachialgia.

Ek et al. (15) used DMSO in the treatment of interstitial cystitis and found it beneficial in relieving severe pain in patients who had not responded to other forms of treatment. This is the only use for which DMSO is approved for general use.

Ashwood-Smith (16) presented a review of DMSO's use as a radioprotective and cryoprotective agent. As a radioprotective agent it operates "in systems varying from bacterial cells and enzymes to tissue culture cells and whole animals." It was suggested that DMSO may react with radiolysis products of water to form radicals which are less reactive. According to the above review a 5-10% solution of DMSO is able to protect a number of cellular systems from deleterious effects of freezing and thawing.

Another major use of DMSO is the treatment of severe head trauma and spinal cord injury. A number of experimental animal models have demonstrated the benefits of DMSO in this type of trauma. de la Torre et al. (17) demonstrated the effectiveness of treatment with DMSO after experimental brain compression in rhesus monkeys. Kajihara et al. (18) compared DMSO, DMSO plus decadron, urea and saline controls in experimental spinal cord injury to dogs. de la Torre (19) in a later study, compared DMSO to mannitol and decadron as well as saline controls in experimental spinal cord injury in dogs. Brown et al. (20) compared DMSO, mannitol and saline controls in rhesus monkeys after a controlled gunshot wound. In all of these studies, animals treated with DMSO showed significantly higher survival and recovery rate than traditional treatments or the controls.

The basis for success with DMSO in cases of severe head trauma has been postulated by several of these investigators. de la Torre et al. (19) summarized the sequence of events occcurring in central nervous system injury: (1) hemorrhage progressing from gray matter to white matter (2) decreased vascular perfusin and oxygen tension (3) edema (4) cellular and subcellular changes and (5) necrosis with "neuronal shrinkage, cytolysis and gliosis of cord tissue." Brown et al. (20) found DMSO treatment resulted in an increase in mean arterial blood pressure, cerebral perfusion pressure, cerebral blood flow and cerebral metabolic rate of oxygen consumption to near baseline levels. Brown suggested that DMSO's antiedemic and diuretic properties may be responsible for its ability to decrease intracranial pressure. He further suggested that DMSO may cause an increase in cardiac output which may be the basis for improvement in a number of the other parameters. Brown proposed that DMSO's histamine-releasing actions resulting in vasodilation, would increase blood flow and decrease cerebral vascular resistance. Finney et al. (21) suggested that DMSO may be involved in more effective oxygenation of tissue, an action which would have an important implication with nerve tissue. Kajihara et al. (18) postulated hemodilution by DMSO may play some role and offset some of the vascular changes which take place with central nervous system injury. It can be seen that many of the pharmacologic actions of DMSO may be involved in the dramatic improvement in DMSO treated animals after various forms of central nervous system injury.

It is this last use of DMSO that instigated this project. The neurosurgery department at this institution is utilizing DMSO to help control intracranial pressure in cases of severe head trauma with cerebral edema. DMSO was initially administered 1 g/kg every 8 hours until intracranial pressure was under control. With the ability to continuously monitor intracranial pressure, DMSO is now administered 1 g/kg when there is a dangerous increase in intracranial pressure. With administration of DMSO a rapid diuresis occurs with a subsequent, rapid decrease in intracranial pressure.

Toxicity

Rubin (22) reviewed a number of experiments concerning toxicity of DMSO. Table 1 represents the LD_{50} for a single dose administered by various routes in several animals. It can be seen from this table that the acute toxicity of DMSO is extremely low. Work by a number of investigators tends to indicate that in general DMSO has low systemic toxicity, although the forms and degree as well as the presence or absence of toxic effects vary from study to study and from specie to specie. When applied dermally DMSO produces a reversible skin irritation which ranges from burning or stinging to development of mild lesions with chronic application (22, 23). Vogin et al. (24) reported scaling and flaking of skin at the site of application, while oral administration resulted in emesis, ptalism and anorexia.

Table 1. Single-Dose Toxicity of DMSO*

Species	LD_{50}DMSO				
	Applied to Skin	Taken by Mouth	Into Blood Stream	Beneath Skin	Into Body Cavity
	mg/kg body weight				
Mouse	50,000	16,500-24,600	3,800-8,900	13,900-20,500	14,700-17,700
Rat	40,000	17,400-28,300	5,200-8,100	12,000-20,500	13,000
Dog	11,000	10,000	2,500	---	---
Monkey	11,000	4,000	4,000	---	---

*From Rubin (22).

Wood et al. (25) studied the effect of DMSO on enzymes. He found mild increases in LDH, SGPT and CPK under various conditions of DMSO administration, with LDH being the most commonly elevated. Hemoglobin, bilirubin and glucose showed transient elevation after 5g/kg/day intraperitoneal injection. Mild hemolysis occurred after intraveneous injection. Norred et al. (26) studied the mechanism of hemolysis by DMSO and concluded that part of this effect was due to "its initial ability to gain entrance into the red blood cell (due to its hygroscopicity and small size) and its affinity for proteins, resulting in their disfiguration, denaturation or dissolution, and thus loss in cellular integrity." He further suggests that DMSO's ability to remove lipid from the red blood cell membrane may be involved in its hemolytic effect.

Brobyn (27) performed two extensive studies on the effects of DMSO on male prisoners, one for 2 weeks and the other for 90 days (1g/kg/day). Extensive physical and ophthalmologic examin-

ations including hematological, chemistry and urine tests were conducted before, during and after the DMSO administration. There appeared to be no significant toxicity in either study. In both studies skin reaction, breath odor, headache, dizziness and sedation appeared to be common side effects.

Toxic change within the eye is an area of major concern and controversy with DMSO administration. Rubin (22) in his review of the literature, reported that some animal studies found cortical fibers of the lens became less relucent than normal resulting in a myopia which was dose related. This effect has been reported in rabbits, dogs, swine and rats. Both positive and negative results have been reported in monkeys. Brobyn (27), in his studies with prisoners, found no evidence of ocular changes in humans.

One of the unique pharmacological effects of DMSO is its ability to transport other substances across membranes (23). It can be used beneficially as a vehicle for medicinal agents but may also result in increased toxicity. Penetration of toxic and carcinogenic substances may also be increased resulting in "enhanced" toxicity.

Methodology

Since the recognition of the unique pharmacological properties of DMSO a number of investigators have developed methods for measuring DMSO concentration. Many of the methods were set up to study the absorption, metabolism and excretion of DMSO in animals and man. All of the methods proposed for the

measurement of DMSO in concentrations found in biological fluids, involved detection and quantitation by gas liquid chromatography. These methods generally vary in sample preparation and the type of column packing used. Wallace and Mahon (28) in 1964 presented a chromatographic system for separating pure compounds of dimethyl sulfide, dimethyl sulfoxide and dimethyl sulfone. They used a stainless steel column packed with 20% Carbowax (20 MM) on Chromosorb W and a temperature program of 25^{o}C to 205^{o} for separation of all three compounds and the internal standard. For quantitation they recommended the use of the internal standard diphenylmethane, with use of response factors for actual quantitation. Williams et al. (10, 29) developed a method for determining dimethyl sulfone in human urine and later used the same method for determination of DMSO and dimethyl sulfone in rabbit urine. His method involved a methylene chloride or chloroform extraction of urine with evaporation of extract and redissolution of residue in methanol. An aliquot of the methanol was injected onto a stainless steel column packed with 30% butanediol succinate on Chromosorb W. The gas chromatograph was equipped with a flame ionization detector. Quantitation was by comparison of peak heights of unknown concentrations to peak heights of known amounts. Paulin et al. (30) quantitated DMSO levels in plasma, whole blood and CSF. His method involved injection of sample directly onto a copper column packed with 25% Carbowax 20 M on Chromosorb P. The gas chromatograph was equipped with a flame ionization detector. Quantitation was by

comparison of peak areas to a calibration curve. The lower limit of sensitivity was 22 ug/ml, with linearity extending from 22 ug/ml to 110 ug/ml for CSF and plasma. Recovery for plasma ranged from 87.3 to 100%, with recovery decreasing as concentration increased. Recovery from whole blood samples was inconsistant. Hucker et al. (9, 31) investigated the absorption metabolism and excretion of DMSO in two studies and used gas chromatography to determine urine levels in man and animals and also human serum levels. His method of sample preparation (serum and urine) was an initial heptane/isoamyl alcohol "clean up" extraction. After addition of NaCl, the aqueous layer was extracted with chloroform which was then evaporated to dryness and redissolved in acetone. The acetone containing the DMSO was then injected into a glass column packed with 3% Carbowax 20 M on Chromosorb G. The gas chromatrograph was equipped with an argon ionization detector. Quantitation was by means of a daily standard curve of peak heights vs. DMSO concentration. Hucker's recovery was $76 \pm 11\%$. Wong et al. (8) studied the fate of DMSO by quantitating levels in urine, and plasma of humans and miniature pigs and expired air in the pigs. The samples were prepared by methanol dilution and precipitation, with injection of the supernatant into a stainless steel column packed with 20% Carbowax (20 MM) on Chromosorb W. The gas chromatograph was equipped with a flame ionization detector. Quantitation was by comparison of peak area to concentration. The lower limit of sensitivity was 10 ug/ml in urine and plasma. Tiews et al. (32) studied the metabolism and excretion in cows and calves

after DMSO administration. Direct injections of urine were made onto a Teflon column packed with 3% Carbowax 15 M on Teflon. The gas chromatograph was equipped with a flame ionization detector. Ogata and Fujii (33) determined DMSO and dimethyl sulfone concentrations in rat urine. Sample preparation consisted of a double chloroform extraction with direct injection after the addition of the internal standard, methyl disulfide. A glass column was packed with 5% polyethylene glycol (20M) on Shimalite. The gas chromatograph was equipped with a flame photometric detector. Quantitation was by means of a standard curve (concentration vs. $\sqrt{\text{DMSO/Methyldisulfide}}$). Turkevich et al. (34, 35) presented two diferrent methods for quantitating DMSO. In his first paper (34) the biological specimen was passed through an ion exchange medium and then used Hucker's (9) chromatographic system for final isolation and quantitation. In a subsequent paper Turkevich et al. (35) presented a chromatographic system for determination of aqueous DMSO solutions. The column was packed with 15% Carbowax 20M on Chromosorb (NAW DMCS) and detection was by plasma ionization. The range studied was 1.078 to 10.132 mg/ml, with an average recovery of 100%, and coefficient of variation of $\pm$ 2.26%. Pearson et al. (36) studied occurence and levels of DMSO, dimethyl sulfone, and dimethyl sulfide in fruits, vegetables, grains, and beverages. The sample preparation was similar to Hucker's (9) extraction of biological specimens. The final methanol solution was injected onto a teflon column packed with 15% FFAP on Chromosorb-T. The gas chromatograph was equipped

with a flame photometric detector. Concentrations were calculated by comparison of unknowns to standards using the ratio of the log of the amount injected to the log of peak area or height. Linearity extended over a range of 6 to 50 ng injected using 1, 10 or 100 ug/ml DMSO solutions. Recovery was 90 to 100% and reproducibility was 10 to 15%. The lower detection limit was 1.2 ng.

As can be seen many of the previous procedures were set up in order to study the fate of DMSO after various forms of administration and statistical analysis of the methods was limited. A number of other shortcomings with previous methods are evident in an initial review of the literature. Only Turkevich et al. (35), Wallace and Manon (28), and Ogata and Fujii (33) suggested using an internal standard, a method now considered to be the most accurate, precise method of quantitation. Hucker et al. (9) and Ogata and Fujii (33), were the only researchers to present actual data on linearity and Hucker's standard curve was nonlinear. Hucker et al. (9) and Paulin et al. (30) were the only researchers to present chromatograms and in both cases the peaks for DMSO came off the solvent front. Solvent extractions, as proposed by a number of investigators (9, 29, 31, 33, 36) tend to be time consuming requiring an evaporation step. Recovery with solvent extractions tends to be considerably less than 100%. Paulin's direct injection technique (30) results in variable recovery depending on concentration and would result in considerable protein buildup in the injection port area. None of the previous work presented

sufficient studies and statistical analysis to be set up as routine analysis in a clinical laboratory.

II. STATEMENT OF THE PROBLEM

As stated earlier the Oregon Health Sciences University neurosurgery department is using intravenous DMSO administration to control cerebral edema following severe head trauma. They requested that the clinical laboratory develop a method for monitoring the DMSO concentration. In response to this request, the overall aim for this project was to develop a procedure that would be practical for the clinical laboratory. It was determined that, as in the previous work, gas-liquid chromatography would be the method of choice. The approach to this technique required the evaluation of a number of parameters including: column material, stationary phase and solid support, and temperatures. In addition, with gas chromatography the method of sample preparation and choice of internal standard also needed to be determined. Once the most effective sample preparation, internal standard, and chromatographic system were found, the traditional studies to establish the method as acceptable and reliable for clinical use were performed. These studies included between run and within run precision, recovery, linearity, interference and comparison to a reference method.

III. APPROACH TO THE PROBLEM

Sample Preparation

It was the goal of this project to establish the most direct method of sample preparation with as little manipulation as possible. Initially it was hoped that a direct injection technique similar to the one commonly used for blood alcohols would work. The sample (plasma) was diluted 1:2 with internal standard solution and 1 ul injected into the gas chromatograph. There were two problems with this approach. The protein resulted in a buildup in the injection port area and a resultant tailing of both DMSO and the internal standard peaks leading to a decrease in peak area ratio.

The next sample preparation tried was a trichloroacetic acid precipitation with neutralization by means of dry sodium bicarbonate. Neutralization was required before injection into the gas chromatograph in order to protect the stationary phase. This worked fairly well except for elution of a large initial peak, probably resulting from volatilization of the trichloroacetic acid. The DMSO peak eluted on the tail of this front causing problems with reproducibility.

Another form of acid protein precipitation and neutralization was tried in an attempt to eliminate the interference from the salt peak. 200 ul of 1.8 M perchloric acid was used for precipitation of 1 ml of plasma or serum. Using cold $HClO_4$ and placing the plasma-acid mixture in an ice bath for 10 minutes enhanced the protein precipitation. The base used to

neutralize the supernatant from the above precipitation was potassium hydroxide. The reaction is as follows:

$$HClO_4 + KOH \xrightarrow[cold]{} KClO_4 + H_2O$$

By placing the tubes in ice for several minutes the precipitation of the $KClO_4$ is maximized. The supernatant is then injected onto the gas chromatograph. This last step eliminated the large initial peak and improved the reproducibility. This was the sample preparation chosen and is presented in the materials and method section.

Packing and Column

In choosing an appropriate stationary phase for any gas chromatographic assay there are certain guidelines which are generally followed. A certain amount of trial and error as well as guesswork is involved. MacNair and Bonelli (37) outlined some of the basic principles involved. According to them the stationary phase is "probably the most important parameter in G.L.C." There are five requirements for a stationary phase: (1) the solvent should have high solubility for the sample components of interest, (2) sample components should have variable partition coefficients in the solvent chosen, (3) the solvent should have low vapor pressure at operating temperatures, (4) the solvent should have thermal stability at the operating temperature, (5) the solvent should not react with sample components and (6) stationary phase should have similar chemical

properties, i.e. polar components require polar stationary phases. In addition to these basic considerations McReynolds constants (38) were used to determine polarity of various stationary phases. By choosing n-butanol and 2-pentanone, compounds with similar polarity to DMSO, and examining their McReynolds constants it was possible to determine which stationary phases might be appropriate. Since an aim of this project was to use as direct an approach as possible, it was desired that the stationary phase would stand up to aqueous based injections. DMSO, being a highly polar compound would require a relatively polar stationary phase. Due to its high boiling point, 189^{o}C, it would require a phase which would tolerate a temperature in the range of 140^{o}C to 190^{o}. Initially due to the similarity in polarity to alcohol, the alcohol packing, Porapack QS, was tried. The peaks were wide and showed adsorption and tailing, two aspects which cause problems with quantitation. A complete list of packings tried is given in Table 2. Many of these were tried because of their high polarity while others were tried due to their general purpose use. Both coated as well as uncoated and bonded packings were tried. The only two stationary phases to give acceptable peaks were 40% Castorwax on Chromosorb W and 20% Carbowax 20M on Supelcoport. Most of the other packings were rejected for either inappropriate retention time or poor peak shape and efficiency, (i.e. adsorption and tailing on the chromatograms). These problems were most likely associated with interaction between DMSO and exposed solid support due to low

percent loading. A considerable amount of work was done with the castorwax column and it was found to give good peak shape and fair resolution. The major problem with castorwax was that it is not commercially available and attempts to prepare it were not acceptably successful. 20% Carbowax 20M on Supelcoport packing is commercially available and resulted in better peak shape, efficiency and resolution than the Castorwax packing. The efficiency of the 20% Carbowax 20M as measured in theoretical plates ($\bar{N}$) and HETP is as follows: HETP = 0.050, $\bar{N}$ = 615. The Carbowax 20M phase is one often used for alcohol determination and stands up very well to aqueous based injections. The heavy coating assures complete coverage of the solid support eliminating the problems of adsorption and tailing.

Silanized glass columns are considered to be the most inert type of column. Silanization eliminates adsorption of DMSO to the oxide and hydroxyl groups on the inner surface of the column. It is for this reason a silanized glass column was chosen for this procedure.

Table 2. Packings evaluated for suitablility of DMSO determination

Porapak QS
2% Carbowax 1500 on Carbopack C
5% Carbowax 20M on Carbopack B
3% Carbowax SP 2100 DB on Supelcoport
3% OV - 225 on Chromosorb W (HP)
3% OV - 210 on Chromosorb W (HP)
Ultrabond
Carbopack C-HT (uncoated)
N-Octane Poracil C
40% Castorwax on Chromosorb WAW
10% SP 2340 on Supelcoport
10% SP 216 PS on Supelcoport
10% SP 2310 on Supelcoport
20% Carbowax 20M on Supelcoport

Internal Standard.

Internal standardization was chosen as the method of quantitation due to its well recognized superiority. The peak area of DMSO of unknown concentration was divided by the peak area of the internal standard and this ratio compared to a peak area ratio for a known concentration of DMSO. This method of quantitation eliminates many possible errors associated with other methods. With this technique, internal standard is added to the sample at the initial step and anything affecting the component to be measured will, theoretically, also affect the internal standard and the final ratio will remain the same. This eliminates errors in processing and injection from one sample to the next due to such things as pipetting, temperature and gas flow changes, as well as the amount injected into the gas chromatograph.

MacNair and Bonelli (37) present several guidelines for choosing the internal standard:

(1) Must have good resolution from other component peaks.

(2) Should elute relatively close to the peaks it will be used to quantitate.

(3) The concentration of the internal standard should be similar to the concentration of component to be measured.

(4) Should be of a similar chemical structure to the component to be measured.

The similarity in chemical structure and chemical reactivity are especially important. The closer the chemical structure of the

component and the internal standard the more likely both will be affected to the same degree and in the same manner by factors affecting the system.

These were the guidelines followed in choosing the internal standard for the DMSO assay. Initially it was thought that an alcohol might work due to the high degree of polarity of DMSO and alcohols. Other compounds of similar polarity were tried as well as a number of sulfur compounds of similar structure. Table 3 presents a complete list of the compounds tried. Most of these were rejected on the basis of inappropriate retention times. Early in the development of the procedure, tertiary amyl alcohol was used relatively successfully. It eluted earlier than DMSO. It is generally preferable for the internal standard to elute after the component to be measured. It was also felt that an internal standard with a more similar chemical structure should be tried. Of the other compounds tried only diethyl sulfoxide, used by Turkevich (35), and diethyl sulfone were satisfactory. Diethyl sulfoxide worked very well but is not commercially available and therefore not practical. Diethyl sulfone also worked very well and was the final choice. The one drawback to diethyl sulfone is that it requires a temperature program to achieve reasonable retention times. The retention time of DMSO is 1.84 minutes and 5.73 minutes for diethyl sulfone. Diethyl sulfone has very good peak shape and results in very reproducible quantitation. The chemical similarity of diethyl sulfone to DMSO can be seen in Figure 4.

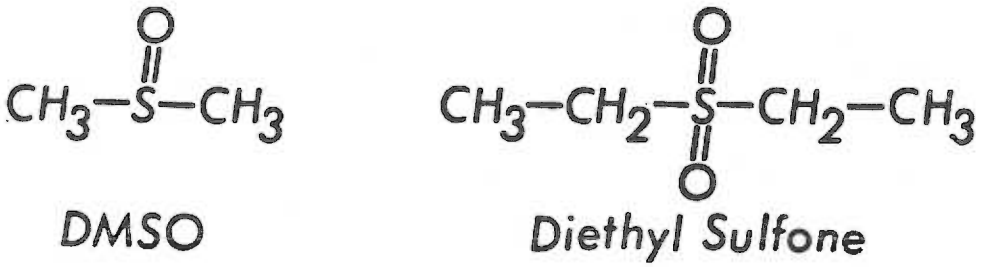


Figure 4. Comparison of chemical similarity of DMSO and diethyl sulfone.

Table 3. Compounds tried as possible internal standards for DMSO

tertiary butyl alcohol	n-octyl alcohol
isopentyl alcohol	n-propanol
tertiary amyl alcohol	n-butanol
n-amyl alcohol	N,N-dimethyl acetamide
p-dioxane	N,N-dimethyl formamide
methyl isobutyl ketone	diethyl sulfoxide
isoamyl alcohol	dimethylaminopropionitrile
hexyl alcohol	tetramethylene sulfoxide
	diethyl sulfone

Temperatures/Gas Flow

The proper temperature for the injection port is important to insure complete and rapid vaporazation of the sample components. The optimal temperature was determined by varying the temperature from 200°C up to 280°C in 10°C increments and recording peak areas of DMSO and diethyl sulfone. The temperature resulting in the maximum peak area, 260°C, was determined to be the optimal operating temperature.

The optimal oven temperature is important to assure appropriate retention times and the best peak shape. In the case of DMSO, diethyl sulfone and dimethyl sulfone, the metabolite of DMSO, it was found that a temperature program from 155°C to 170°C was necessary to optimize both of these parameters.

Quantitation

After the many aspects of sample preparation and chromatographic system had been determined, the choice of which data (peak height or peak area) and which means of processing (calculation or plotted standard curve) would yield the most accurate and precise results had to be determined. The day to day precision study was used to evaluate these aspects. Both aqueous and plasma standards were set up at 1, 5 and 10 g/L. Standard curves were plotted, peak area ratios vs. concentration and peak height ratios vs. concentration. The concentrations for the split plasma samples (2, 6, and 9 g/L) were determined from each of these curves. In addition the 5 g/L aqueous and plasma based standards were used to calculate the concentrations of the samples. Table 4 presents the means and coefficient of variation for each method of quantitation. This data represents 13 runs on 13 different days for those methods involving plasma standards and 20 runs on 20 different days for those methods involving aqueous standards. An evaluation of the data in terms of accuracy and precision, from all of these analyses indicated that the best results were obtained by using peak area ratios and calculation of concentration from the 5 g/L aqueous based standard.

TABLE 4. Comparison of various methods of quantitation.

	1	2	3	4	5	6	7	8
$\bar{x}_{g/l}$	2.05[a]	2.05[a]	2.15	2.09	2.08	2.11	2.14	2.15
$cv_{\%}$	2.7[b]	3.3	4.4	4.2	3.3	3.3	3.7	4.7
$\bar{x}_{g/l}$	6.01[a]	6.02	6.17	5.99[a]	6.07	6.14	6.17	6.22
$cv_{\%}$	1.7[b]	2.0	2.9	2.8	2.2	2.4	2.9	3.4
$\bar{x}_{g/l}$	8.86	8.88	8.97[a]	8.74	8.91	9.03[a]	8.83	9.07
$cv_{\%}$	1.2[b]	1.5	2.6	2.4	1.7	1.8	2.4	2.9

[a] value closest to actual supplemented value for each concentration
[b] smallest CV for each concentration

1-aqueous standard - calculated value - peak area ratio (N=20)

2-aqueous standard - graph value - peak area ratio (N=20)

3-aqueous standard - calculated value - peak height ratio (N=20)

4-aqueous standard - graph value - peak height ratio (N=20)

5-plasma standard - calculated value - peak area ratio (N=13)

6-plasma standard - graph value - peak area ratio (N=13)

7-plasma standard - calculated value - peak height ratio (N=13)

8-plasma standard - graph value - peak height ratio (N=13)

Reference Method

No established reference method exists for the measurement of DMSO at this time. Because of this fact, isotope dilution - mass spectrometry (selective ion monitoring), ID-MS (SIM), was chosen as the alternate reference method with which to compare results obtained by GC-FID. ID-MS (SIM) is generally accepted as one of the most accurate and precise analytical techniques available. It has been utilized extensively in the field of pharmacology research for quantitative applications (39). The National Bureau of Standards uses ID-MS as a definitive method for measuring inorganic substances at trace levels and electrolytes (except sodium) in serum (40). The NBS has proposed this technique as a potential definitive methodology for organic compounds with accuracies of up to 1% under optimum conditions. Björkhem et al. (41, 42, 43) has done a considerable amount of work using ID-MS for evaluation of the accuracy of routine clinical chemistry methods. They have developed methods for quantitating urea, uric acid, creatinine, cholesterol, triglyceride, glucose, cortisol, progesterone and testosterone.

The general principle involved in ID-MS (SIM) is the ability of the mass spectrometer to consistantly fragment molecules and selectively quantitate specific ions with a high degree of precision. A specific quantity of stable isotope (labelled with ^{2}H, ^{13}C ^{14}C or ^{15}N) of the same molecule to be measured, is added to a specific quantity of the specimen to be measured. In this procedure ^{2}H labelled DMSO was used. This mixture is

then processed (in the DMSO procedure, by acid precipitation), chromatographed and subjected to mass fragmentation. The mass spectrometer is programed to quantitate the mass ion peaks for the molecule being measured and its isotope and print out a ratio of the two. This ratio can then be used for quantitation in the same manner as the peak area ratio which is discussed under the internal standard section. In addition, if a second peak for each compound is quantitated, the ratio of this peak to the mass peak for each should remain constant from specimen to specimen. If there is a change in this ratio it indicates the presence of an interfering substance.

The accuracy and precision of the technique lie in several aspects of the system. The stable isotope is an internal standard, the merits of which were discussed earlier. As an internal standard, the stable isotope of a compound is ideal. It varies by only a very small mass number and will react chemically and chromatographically very much like its analogue. Another aspect which supports this technique is the consistancy with which the molecules are fragmented into ions and the precision with which the mass spectrometer is able to quantitate the ion flow. The high degree of specificity comes from monitoring the ratio of two ions of each component. It is highly unlikely that an interfering compound would affect both ion peaks of a particular compound to the same degree. It is due to these aspects that ID-MS (SIM) represents one of the most sophisticated techniques available for quantitating substances and is the reason it was chosen as a reference method for the GC-FID results.

IV. MATERIALS AND METHODS

Reagents and Standards

Stock DMSO Standard (500 g/L). Weigh 50.00 g of Methyl Sulfoxide, 99.9% purity, Gold Label, Spectrophotometric grade (Aldrich Chemical Co., Milwaukie, WI, 53233) into a 100 ml volumetric flask and dilute almost to volume with deionized water. After allowing the DMSO-water solution to cool to room temperature, dilute to volume. Store tightly capped at $4^{o}C$.

Working DMSO Standard (5 g/L). Dilute 0.50 ml of the stock standard (500 g/L) to 50 ml with deionized water. Divide this solution into 1.5 ml aliquots and store at $-20^{o}C$.

Diethyl Sulfone Solution (Saturated, filtered). Dilute 3.5 g of diethyl sulfone (ICN Pharmaceuticals, Inc., Life Sciences Group, Plainville, NY, 11803) to 100 ml in a volumetric flask with deionized water. After mixing for 15 minutes, filter the solution through Whatman No. 2 filter paper and store tightly capped at room temperature. This is the internal standard for the GC-FID procedure.

$^{2}H_{6}$ Labelled DMSO Solution (30.0 g/L). Weigh 1.50 g of (Methyl sulfoxide) $-d_{6}$, isotopic purity 99.96% D, Gold Label (Aldrich Chemical Co., Milwaukie, WI, 53233) into a 50 ml volumetric flask and dilute to volume with deionized water. The diluted DMSO-d_{6} is stored at $-20^{o}C$ to inhibit exchange of the deuterium in labelled DMSO with the hydrogen of water. This is the internal standard for the ID-MS (SIM) method.

Perchloric Acid Solution (1.8 mol/L). Add 14.3 ml of 70-72% perchloric acid to a 100 ml volumetric flask and slowly add deionized

water to bring the solution to volume. This solution is used for precipitating serum, plasma and spinal fluid proteins.

Potassium Hydroxide Solution (1.0 mol/L). Dissolve 6.60 g of potassium hydroxide pellets (85% purity) in deionized water and dilute to volume in a 100 ml volumetric flask with deionized water. This solution is used to neutralize the perchloric acid supernatant and concomitantly precipitate out the perchlorate ion.

Materials

pH Paper -pHydrion Papers - range 4.5 to 7.5.

Filter paper - Whatman No. 2, 11.0 cm diameter.

13 x 100 mm Disposable glass test tubes

Pasteur pipettes.

Glass funnel.

Instrumentation

GC-FID Method - A Hewlett Packard gas chromatograph, Model 5840A equipped with a 5840A GC Terminal and flame ionization detector (Hewlett Packard, Avondale, PA, 19311) was used for these studies. The injection port was fitted with a removable glass liner. The column was silanized glass (64 cm x 0.2 cm, i.d.) packed with 20% Carbowax 20 M on Supelcoport (80/100 mesh), (Supelco Park, Bellfonte, PA, 16823). It was conditioned in the chromatograph by holding the oven temperature at 50^{o}C

for 20 minutes, increasing at 2°C per minute up to 190°C and holding at this temperature at least 15 hours. During conditioning, the detector was disconnected and nitrogen flow (carrier gas) was 30 ml per minute. The operating conditions for the assay were as follows: injector temperature - 260°C, oven temperature - initial 155°C, hold for 3.0 minutes; temperature program - 30°C per minute to 170°C and hold for 3.5 minutes, detector temperature 250°C, hydrogen flow 30 ml per minute, nitrogen flow 30 ml per minute and air flow 240 ml per minute. The attenuation was set at 2^{11}. The 5840A GC terminal was programmed to integrate and print out peak area and retention time.

ID-MS (SIM) Method - A Finnigan 3200 Gas Chromatograph/Mass Spectrometer (electron impact) with a 6100 MS Data System and Silent 700 Electronic Data Terminal (Finnigan Corporation, Sunnyvale, CA 94086) was used. The gas chromatograph was fitted with a 107 cm x 0.2 cm (i.d.) glass column packed with 20% Carbowax 20 M on 80/100 Supelcoport and conditioned under the same conditions as in the GC-FID procedure. For the assay the gas chromatograph conditions were set as follows: injector temperature 250°C and oven temperature 170°C (isothermal). Electron impact ionization mass spectra were recorded at an ionizing energy of 70 eV. Sensitivity was set at 10^{-5} A/V. The Selective Ion Monitoring program acquisition parameters were set as follows: mass range - (63, 66, 78, 84), integration time - M, seconds per scan-1, threshold-3, and maximum run time-6 minutes. The Auto Area Program was set according to the

Finnigan software manual (44) to determine the area under the peaks for the four monitored ions. This program gave the peak area ratio for the ions, 78/84 (DMSO/DMSO-d_6) which was used for calculation of concentrations. Also printed were peak heights and retention times. The area ratios of the ion peaks, 63/78 (DMSO) and 66/84 (DMSO-d_6), were quantitated to rule out interference.

Procedure

GC-FID Method - Add 200 ul of internal standard (diethyl sulfone solution) to 1.0 ul of aqueous standard, controls and specimen to be measured, in 13 x 100 mm disposable tubes. Vortex briefly. To each tube add 200 ul of cold 1.8 mol/L $HClO_4$. Vortex 15 seconds and place in an ice bath for 10 minutes. Centrifuge for 5 minutes at 750 x G. Transfer the supernatant to a clean tube. The supernatant may be cloudy at this step, but will clear with the final centrifugation step. Adjust the pH of the supernatant to between 5 and 7 with 1 mol/L KOH solution (150 to 175 ul for serum or plasma and 300 to 325 ul for aqueous standards, urine or CSF.) A 0.1 mol/L KOH solution and 0.18 mol/L $HClO_4$ solution may help with this final adjustment. Place the tubes in an ice bath for 5 minutes, centrifuge at 330 x G for 3 minutes and immediately transfer each supernatant to a clean tube. Inject 1 ul into the gas chromatograph using the parameters stated earlier and begin the temperature program. Prior to beginning a series of analyses, inject the 5 g/L aqueous standard. Duplicate injections are

made and the peak area ratios are averaged if they agree within 5% for samples, and 3% for the standard. The glass liner should be removed and cleaned after every 10 to 12 injections of biologically based specimens.

Calculation - The peak area for the DMSO peak is divided by the peak area for the internal standard, to give a peak area ratio for each injection. The concentration is calculated from the aqueous 5 g/L standard and corrected with the appropriate factor for the matrix being tested as follows:

$$\text{Unk. Conc.} = \frac{(\text{Unk. Peak Area Ratio}) \times (\text{Std. Conc.}) (\text{Correction Factor})}{(\text{Std. Peak Area Ratio})}$$

$$\text{Unk. Peak Area Ratio} = \frac{\text{Unk. Peak Area}}{\text{Unk.-Int. Std. Peak Area}}$$

$$\text{Std. Peak Area Ratio} = \frac{\text{Std. Peak Area}}{\text{Std.-Int. Std. Peak Area}}$$

The correction factors for the various biological matrices are 0.974 for plasma and serum and 1.000 for urine and CSF. If the concentration is greater than 10 g/L the specimen should be diluted with blank matrix and rerun.

ID-MS (SIM) Method - Specimen preparation was the same as the GC-FID procedure except 200 ul DMSO-d_6 solution was added in the initial step in place of the diethyl sulfone solution. Duplicate 1 ul injections were made at the parameters described earlier, and as before, peak area ratios agreeing within 5% were averaged for quantitation purposes.

Calculation - A linear regression was run on the peak area ratios (DMSO/DMSO-d_6) for standard solutions and then, using the resultant formula, Y = -.03379 + 8.05078X (X = peak area ratio, Y = DMSO concentration), the average peak area ratios for the various concentrations were substituted for X.

Linearity, Precision, Recovery and Accuracy Studies

Linearity - Two sets of specimens were run for the linearity study. One set consisted of plasma specimens supplemented with DMSO at 0.05, 0.10, 0.50, 1.00, 5.00 and 10.00 g/L. The other set consisted of specimens supplemented with DMSO at 5.0, 10.0, 20.0 and 30.0 g/L. Three separate specimen preparations were made at each level with the average peak area ratio of duplicate injections used for statistical and graphical analysis.

Precision - Specimens for day to day precision consisted of plasma supplemented with DMSO at 2, 6, and 9 g/L and dimethyl sulfone, the metabolite, at a level of 5% of the DMSO level (0.1, 0.3 and 0.45 g/L respectively). Aliquots of 1.5 ml were frozen and analyzed on 20 different days over a period of 7 weeks. The specimen for the within day precision study was plasma supplemented with 6 g/L DMSO and 0.30 g/L dimethyl sulfone. Twenty aliquots were analyzed, 10 on each of two consecutive days.

Analytical Recovery - Plasma, serum, urine and cerebral spinal fluid were supplemented with DMSO at 5.00 g/L. Three preparations of each matrix were processed and the concentration calculated from the average of the injections for a processed

5.00 g/L aqueous standard. In addition, the same aqueous standard after addition of internal standard was diluted with deionized water and injected directly onto the gas chromatograph.

Accuracy - Drugs commonly coadministered with DMSO in severe head trauma cases, preservatives used in the injectable forms of these drugs and other commonly prescribed drugs were tested at high therapeutic levels or elevated levels for possible interference with DMSO. The effect of hemolysis, a common occurrence with intraveneous DMSO therapy, was tested by dividing a blood sample and hemolyzing one aliquot. Both aliquots were centrifuged and the serum supplemented with DMSO at 5.00 g/L. The hemoglobin concentration of the hemolyzed aliquot was 5 g/L. The specimens for the reference method comparison consisted of aliquots of the linearity study standards, 0.10, 0.50, 1.00, 5.00 and 10.00 g/L. The values obtained by the reference method were statistically compared to those found in the linearity study.

Statistical Analysis - Data from the linearity study and reference method comparison were analyzed by means of linear regression, according to guidelines presented by Phillips (46). A Hewlett Packard 9815A calculator was used with General Statistics Vol. I Program (46). For the linearity study a linear regression was run on each set of specimens, with peak area ratios (X) compared with theoretical concentration (Y). For the reference method regression, concentrations obtained by ID-MS (SIM) (X) were compared with those obtained by GC-FID (Y). The slope, Y-intercept, standard error of estimate and

correlation coefficient were calculated. Data from the recovery study was analyzed by means of the Student's t test to determine if a significant difference existed between the various biological fluids tested and aqueous based standards. A confidence level of 0.05 was used. The Hewlett-Packard 9815A with General Statistics Vol. I program (46) was used to calculate the Student's t test with Phillips (45) used as a reference. The standard deviations for the slope and intercept for fifteen standard curves (range of 0 to 10 g/L) run on fifteen different days were determined. Data from the precision studies were analyzed to give the mean, standard deviation and coefficient of variation for each concentration.

V. RESULTS

Chromatograms - Figure 5 shows chromatograms of (A) plasma from a patient actually receiving intravenous DMSO and (B) a blank plasma (with 200 ul of deionized water added in place of the internal standard.) The concentration of the DMSO in the patient specimen was 5.59 g/L. Figure 6 is a chromatogram of the 5.00 g/L aqueous standard. The retention times of DMSO, dimethyl sulfone (the metabolite) and diethyl sulfone are 1.77 minutes, 5.17 minutes and 5.74 minutes, respectively.

Linearity - Figure 7 represents graphically the linearity of plasma specimens from 0 to 10.00 g/L. Table 5 presents the results of the linear regression analyses for the two different ranges, 0 to 10 g/L and 5 to 30 g/L.

Table 5. Linear Regression - linearity study

Range g/L	Slope	Intercept	R	Std. Error of Est.
0.05 - 10.00	16.397	-0.018	1.000	0.060
5.00 - 30.00	14.571	-3.535	0.881	4.982

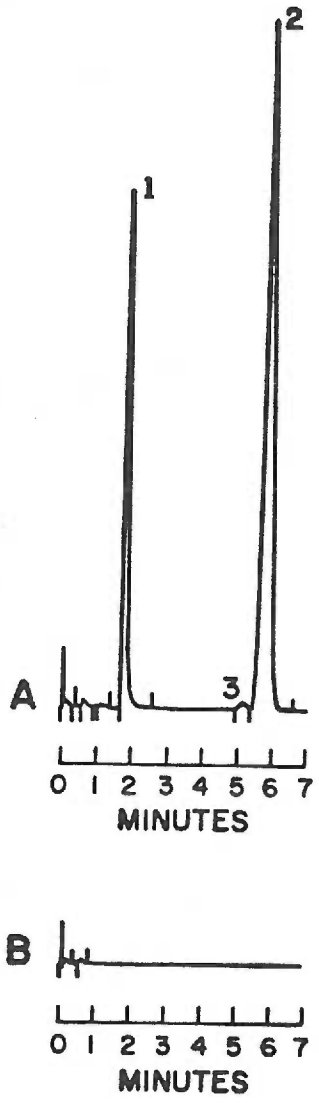


Figure 5. A. Representative chromatogram of a plasma specimen from a patient receiving DMSO therapy (concentration = 5.59 g/L). Peak 1 = DMSO, Peak 2 = diethyl sulfone (internal standard), Peak 3 = dimethyl sulfone (metabolite). B. Chromatogram of blank plasma specimen.

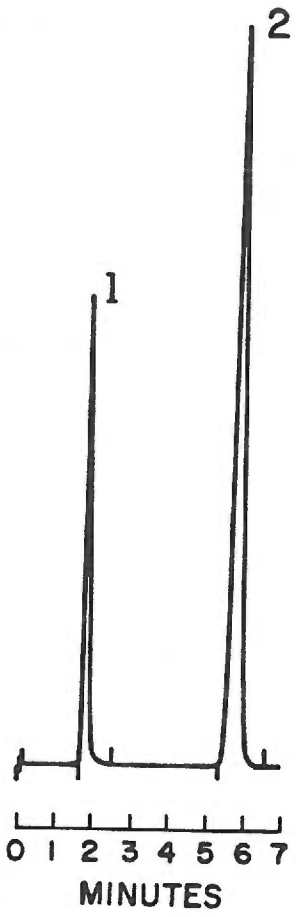


Figure 6. Chromatogram of a 5 g/L aqueous standard.
Peak = 1 DMSO, Peak 2 = diethyl sulfone (internal standard).

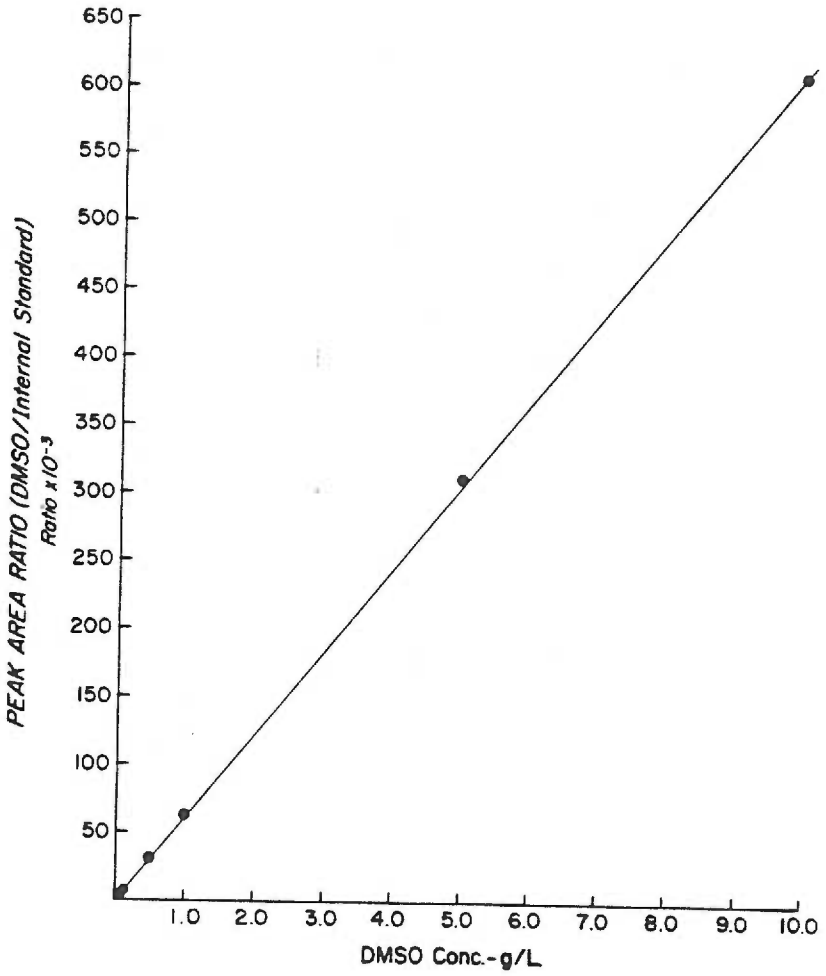


Figure 7. Linearity of the GC-FID method.

Table 6. Statistical analysis of day to day precision study.

Number of Analyses	Mean, g/L	SD, g/L	CV, %
20	2.05	±0.05	2.4
20	6.04	±0.12	2.0
20	8.91	±0.13	1.4

Precision - Table 6 represents the statistical analysis (mean, standard deviation and coefficient of variation) of the day to day precision at each of the three concentrations tested. The within day precision was as follows: n = 20, mean = 6.12 g/L, standard deviation = 0.04 g/L and coefficient of variation = 0.65. The means and standard deviations of the slope and intercept, for aqueous standard curves (1.0 g/L, 5.0 g/L and 10.00 g/L) analyzed on 15 separate days, were 17.704 ± 0.267 (slope) and -0.001 ± 0.01 g/L (intercept). The difference in slope between the linearity study and the average slope of the 15 standard curves was due to a difference in concentration of the diethyl sulfone solutions used.

Recovery - The results of the recovery study are given in Table 7. The correction factor was calculated from the observed recovery to correct for differential recovery of DMSO between an aqueous matrix and plasma or serum matrix. The need for a correction factor was determined by use of the Student's t tests. These showed no significant difference between a processed aqueous matrix and unprocessed aqueous, urine and CSF matrices. Serum and plasma matrices did show a significant

difference from the processed aqueous matrix but were not significantly different from each other.

Table 7. Recovery of DMSO in various matrices and corresponding correction factors.

Matrix	Observed Conc., g/L[a]	% Recovery	Correction Factor[b]
Processed dH_2O	5.00	100.0	---
Unprocessed dH_2O	5.01	100.2	---
Urine	5.04	100.9	1.000
Serum	5.13	102.6	0.974
Plasma	5.14	102.8	0.974
CSF	4.96	99.2	1.000

a. Observed concentrations were calculated from the processed dH_2O standard.

b. Urine and spinal fluid are not significantly different from the process deinionized water matrix and therefore require no correction. The recoveries for serum and plasma are not significantly different from each other, and the averaged recoveries were used for a single correction factor.

Interference - Drugs commonly coadministered with DMSO in severe head trauma as well as other commonly administered drugs were tested for possible co-elution with DMSO or the internal standard. Table 8 represents the drugs tested and found not to interfere. Valproic acid was found to have a retention time of 5.45 minutes. The internal standard has a retention time of 5.74 minutes and therefore would co-elute with valproic acid. At a concentration of 0.140 g/L, valproic acid increases the area of the internal standard peak an average of 0.6%.

Of the substances commonly used as preservatives which we tested (ethyl alcohol, monothioglycerol, methylparaben, propylparaben, benzyl alcohol and propylene glycol), only propylene glycol, a preservative in injectable phenytoin and pentobarbital, has an interfering retention time. In a dilution equivalent to a maximum therapeutic intraveneous dose of pentobarbital, propylene glycol shows up as a shoulder on the front of the DMSO peak with a retention time of 1.57 minutes. The difference between the hemolyzed and unhemolyzed serum specimens was 0.03 g/L, a difference of 0.6%.

Table 8. Drugs tested for possible co-elution with DMSO or the internal standard and found not to interfere.

Carbamazepine	Lidocaine	*Phenytion
Desipramine	Methotrexate	Primidone
*Dexamethasone	*Methyldopa	Procainamide
Digoxin	*Pentobarbital	Theophylline
Ethosuximide	Phenobarbital	Quinidine
Imipramine		

*These drugs are commonly coadministered with DMSO in severe head trauma patients.

Reference Method Comparison - Figure 8 represents graphically a comparison of the split samples processed by GC-FID and ID-MS (SIM). A linear regression analysis of the data, X = ID-MS (SIM) values and Y = GC-FID values, resulted in a slope = 1.033, intercept = -0.003, correlation coefficient = 1.000, and standard error of estimate = 0.033.

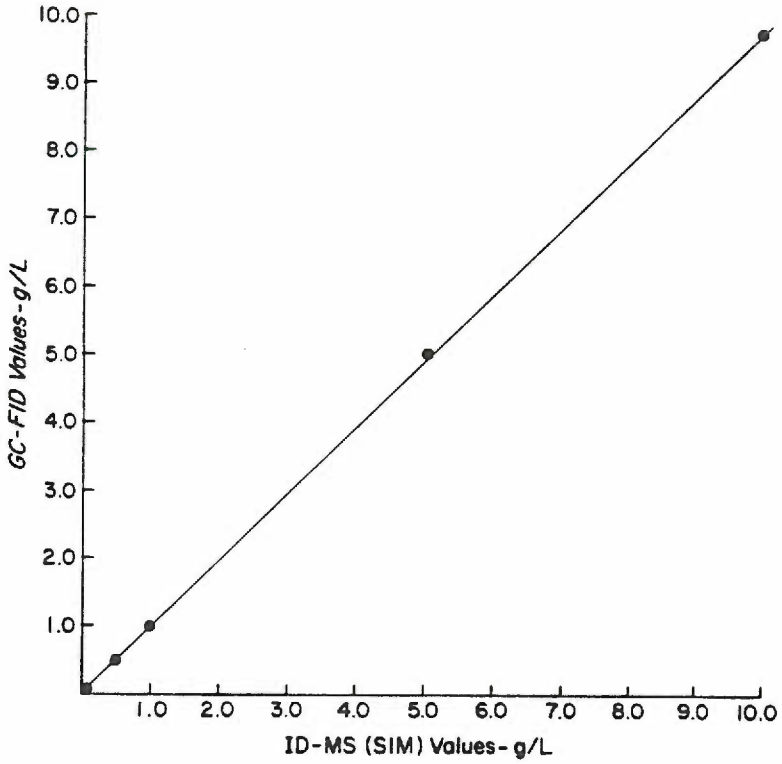


Figure 8. Comparison of results obtained by GC-FID with results obtained by ID-MS (SIM) on split samples.

Reference Range - At present no reference range exists for DMSO. Table 9 represents the concentrations observed in two patients receiving intraveneous DMSO therapy. Patient 1 was receiving 1 g/Kg every 8 hours, while patient 2 was receiving 1 g/Kg according to his intracranial pressure which was continuously monitored. When this pressure increased dangerously he was given another dose. Table 9 also indicates for each specimen how many 1 g/Kg intraveneous doses the patient had received at the time of collection.

Table 9. Representative DMSO concentrations in patients receiving intravenous DMSO therapy.

Patient	Time of Collection	Number of 1g/Kg DMSO Doses	Conc. g/L
1	trough	6	7.21
	post dose[a]	7	10.18
	post final dose[a]	10	10.58
2	post initial dose[a]	1	1.90
	post dose[b]	3	3.24

a. These post dose specimens were all collected within 60 minutes of completion of DMSO infusion.

b. This post specimen was collected 4 hours after completion of DMSO infusion.

VI. DISCUSSION

The method presented here provides an accurate, easy to perform, low cost means of measuring DMSO in biological as well as aqueous matrices.

As discussed earlier, the internal standard chosen initially was diethyl sulfoxide, the same as that used by Turkevich (35). This compound is not commercially available and therefore is not practical for routine clinical use. Diethyl sulfone was tried because of its chemical similarity to DMSO and, as can be seen in Figures 5A and 6, it separates very well from DMSO and gives nearly baseline resolution from dimethyl sulfone. The close vicinity of the internal standard and metabolite did not interfere with quantitation of DMSO. Diethyl sulfone is more polar than DMSO, and is retained longer on the column. A temperature program is required to achieve a reasonable retention time for the analysis.

Several aspects of the procedure require further comment. One step requiring practice is adjustment of the pH after perchloric acid precipitation. The supernatant contains little buffering capacity and the amount of base required varies from specimen to specimen. Use of the more dilute KOH and $HClO_4$ solutions facilitates the adjustment. This neutralization step protects and lengthens the life of the column. By utilizing KOH and an ice bath, potassium perchlorate is precipitated out, resulting in an extremely clean chromatogram. Temperature is important at this step in the procedure. After final centrifugation the supernatant should either be transferred immediately to a clean tube or placed

back in the ice bath until it can be transferred. This precaution provides for maximum preciptation of potassium perchlorate and prevents redissolution of the salt.

A glass liner, which was cleaned after 10 to 12 injections of biologically based specimens, was used in the injection port. There is a buildup of residual protein and salt on the glass liner resulting in a gradual decrease in the peak area ratio for DMSO (6% over 20 injections).

Sample preparation time is relatively short. With practice, a standard and six specimens require 30 minutes of technologist time with an overall time of one hour. The time required for each injection is 12 minutes, 7 minutes for the temperature program and 5 minutes for equilibration of the column to the lower oven temperature.

The sample preparation presented here offers certain advantages over previous methods. With the direct injection technique (30, 32) there is rapid build-up of protein in the injection port with a resultant tailing of peaks and deterioration of reproducibility. The methanol precipitation technique (8) produces a cloudy supernatant which again results in more rapid build-up of protein in the injection port. It also results in a greater dilution of the specimen than in the procedure presented here. By contrast, perchloric acid precipitation gives an extremely clear supernatant. The solvent extraction methods (9, 10, 29, 31, 33, 36) involve the inherent variable of recovery. Solvent extraction involves the transfer of a compound from one solvent to another, while precipitation involves the injection of the aqueous portion of the biological fluid, already containing the compound, directly

onto the column. The extraction methods as well as the methanol precipitation (8) involve the injection of organic solvents such as methanol and acetone onto the column resulting in more rapid deterioration of the stationary phase, whereas injecting an aqueous supernatant "steam cleans" the column.

The column packing chosen, 20% Carbowax 20M on Supelcoport, exhibited the best peak shape, efficiency and resolution. This is similar to the stationary phases suggested initially by Wallace and Mahon (28) and later was used in studies by Paulin et al. (30), Wong et al. (8) and Turkevich et al. (35). Hucker et al. (9, 32) and Tiews (32) used a much lower loading, 3% Carbowax 20M. In the present trials it was found that the lower loaded packings resulted in peaks showing adsorption and tailing. This was probably due to interaction with exposed solid support.

The choice of the internal standard and the superiority of this method of quantitation has already been discussed. Of the previous methods only three (28, 33, 35) recommended the use of an internal standard for quantitation. The other methods (8, 9, 29, 30, 32, 34, 36) used external standardization, which is subject to much greater error and requires extreme precision and reproducible technique of the analyst.

Limited preliminary investigation indicated that a concentration range of 1 to 10 g/L would be appropriate. In this study the range was extended from 0.05 to 30 g/L. Figure 7 demonstrates the excellent linearity of the method in the range of 0.05 to

10.0 g/L. Table 5 gives further evidence by means of linear regression analysis of the linearity in this range. It also compares statistical analysis of the linearity of this range to the 5.0 to 30.00 g/L range. It can be seen from Table 5 that the slope of the upper range is significantly different from the lower range. There is also a deterioration in the correlation coefficient, Y intercept and standard error of estimate in the upper range. It is for this reason that specimens with concentrations greater than 10 g/L should be diluted with blank matrix and rerun.

Linearity data for much of the previous methodology is limited. Paulin et al. (30) claimed linearity in plasma and CSF in the range of 22 to 110 ug/ml, although no actual data was presented. Hucker et al. (9) presented a standard curve in the range of 0.1 to 1 ug DMSO injected onto the column. His curve for DMSO was nonlinear. Ogata and Fujii (33) presented a standard curve of $\sqrt{\text{DMSO/methyldisulfide}}$ peak areas vs. DMSO concentration for the range of 0.039 to 0.49 g/L in rat urine. His curve showed reasonable linearity. Although Turkevich (35) did not present a standard curve, his recovery and CV data indicate good linearity in the range 0.8 to 10 g/L with aqueous standards. The method presented here has excellent linearity not only in lower ranges but up to 10 g/L.

As can be seen in Table 4 the best precision was obtained by calculating the unknown concentration from a 5 g/L aqueous standard. Use of an aqueous matrix for the standard facilitates preparation. When using this method of quantitation a positive

error of 3% occurs in estimating plasma or serum concentration. If the concentrations are corrected by multiplying by 0.974, an accurate result is obtained. The average recovery of serum and plasma in Table 7, after correction, is 100.1%. Student's t tests indicated that at a confidence level of .05, the recovery of DMSO from urine and CSF was not significantly different from the processed aqueous standard and therefore requires no correction factor.

The average observed recovery found in plasma and serum was 102.7% and 100.9% in urine. This is considerably better than the range of 87.3 to 100% found by Paulin et al. (30) with a direct injection or the 76±11% recovery found by Hucker et al. (9) with the chloroform extraction technique. The corrected recovery value for the method presented here is very similar to the recovery of 100.0% found by Turkevich et al. (35) in aqueous solutions of DMSO.

Both between run and within run precision studies showed a CV range well within clinical requirements. As can be seen in Table 6 all CV's are less than 3%. By averaging duplicate injections precision is increased because there is carryover from one injection to the next. The error is greatest when going from a high concentration to a low concentration. When injecting a 2 g/L specimen immediately after a 9 g/L specimen, the carryover resulted in a 1 to 13% increase in concentration with an average of 5%. The CV's presented by Turkevich et al. (35) ranged from 0.45 to 2.26% for aqueous solutions, a range which is very similar to our precision for biological specimens.

Pearson et al. (36), the only other author to present precision data, claimed 10 to 15% reproducibility, a relatively wide range for use in clinical testing.

Of all the drugs tested for possible interference with DMSO quantitation, only valproic acid had a retention time which would pose a potential problem. By co-eluting with and increasing the area of the internal standard, valproic acid interference would cause a decrease in the apparent concentration of DMSO. At the concentration tested, 0.14 g/L (elevated), the increase in area of the internal standard would be less than 1% and at therapeutic levels would therefore be less than 0.5% (for the concentration of internal standard used in this procedure.) The potential for interference is decreased by the fact that valproic acid is not commonly coadministered with DMSO in severe head trauma cases. Of the various preservatives commonly found in injectable drugs, only propylene glycol (a preservative in phenytoin and pentobarbital) had an interfering retention time. It shows up as a detectable shoulder on the front side of the DMSO peak. If a specimen is collected immediately after intraveneous injection of high doses of phenytoin or pentobarbital, the interfering peak may interfere with DMSO quantitation. Caution should be taken therefore to avoid specimen collection for DMSO quantitation immediately after intraveneous injection of these drugs.

By comparing Figure 5 A and 5 B it can be readily seen that there are no interfering endogenous substances in normal plasma. The difference between the hemolyzed and nonhemolyzed serum,

supplemented at 5 g/L with DMSO, was less than 1%. This would indicate that hemolysis, commonly found with intraveneous DMSO therapy, does not interfere with DMSO quantitation.

As discussed earlier, ID-MS (SIM) is considered to be an excellent technique for use as a reference and in some cases definitive method for quantitation of various substances. It was for these reasons that it was chosen for comparison of results obtained by GC-FID. As can be seen in Figure 8 and the linear regression analysis, the two methods agreed very well with a correlation coefficient (r) of 1.000.

There is no established therapeutic or reference range for DMSO. The practice in the case of severe head trauma patients has been to administer DMSO intravenously, 1g/Kg every eight hours or with an increase in intracranial pressure. Table 9 shows that in this type of patient the range extended up to 10.6 g/L with continued therapy. Hucker et al. (9) in his study of the absorption, excretion and metabolism of DMSO administered 1g/Kg both orally and dermally to human subjects. He found the average peak serum concentration after oral administration (single dose) was 2.113 g/L and after dermal administration (single dose), 0.532 g/L. Both of these concentrations are within the range of this method.

At the present time no method has been published supported by the studies necessary to establish it as a valid method for quantitating DMSO in biological fluids in a clinical situation. There have been and still are many areas of clinical research involving the use of DMSO. At our institution, the use of DMSO

in severe head trauma cases is of particular concern and interest. In the past due to the fact that there has been no established method for measuring DMSO, no work has been done in comparing levels to effects. The ability to measure DMSO and comparison of concentration to presence, absence, degree or duration of effect will facilitate establishment of the clinical usefulness of this drug. In the future, it is possible that this method can be used for routine therapeutic drug monitoring analysis in those conditions for which an optimal concentration range has been established.

VII. CONCLUSION

The method presented here for measuring DMSO in biological matrices shows excellent linearity precision and accuracy. It has a relatively simple sample preparation, protein precipitation and neutralization, with excellent recovery. The reagents and materials for specimen preparation are readily obtainable and inexpensive. There appear to be no serious interferences with the method. There is a considerable amount of DMSO research going on at our institution as well as other institutions. This procedure will aid in the illucidation of the possible benefits and uses of DMSO and allow correlation of experimental results to serum, plasma, urine or CSF concentration.

VIII. REFERENCES

1. David, N.A., The pharmacology of dimethyl sulfoxide 6544. Ann. Rev. Pharmacol. 12, 353-73 (1972).

2. Szmant, H., Physical properties of dimethyl sulfoxide and its function in biological systems. Ann. N.Y. Acad. Sci. 243, 20-23 (1975).

3. CRC Handbook of Chemistry and Physics, 59th ed., Weast, R.C., Ed. and Astle, M.J., Assoc. Ed., CRC Press Inc., West Palm Beach, FL, 1978-1979, pp C-507.

4. Kolb, K.H., Jaenicke, G., Kramer, M., and Schulze, P.E., Absorption, distribution and elimination of labelled dimethyl sulfoxide in man and animals. Ann. N.Y. Acad. Sci. 243, 20-23 (1975).

5. Denko, C.W., Goodman, R.M., Miller, R. and Donovan, T., Distribution of dimethyl sulfoxide - ^{35}S in the rat. Ann. N.Y. Acad. Sci. 141, 77-84 (1967).

6. Malinin, G.I., Fontana, D.J. and Braungart, D.C., Distribution of C^{14} - labelled dimethyl sulfoxide in tissues of intact animals. Cryobiology, 5, 328-35 (1969).

7. Gerhards, E. and Gibian, H., The metabolism of dimethyl sulfoxide and its metabolic efects in man and animals. Ann. N.Y. Acad. Sci. 141, 65-76 (1967).

8. Wong, K.K., Wang, G.M., Dreyfuss, J., and Schreiber, E.C., Absorption, excretion, and biotransformation of dimethyl sulfoxide in man and miniature pigs after topical application as an 80% gel. J. Invest. Dermatol. 56, 44-48 (1971).

9. Hucker, H.B., Miller, H.K., Hochberg, A., Brobyn, R.D., Riordan, F.H. and Calesnick, B., Studies on the absorption, excretion and metabolism of dimethyl sulfoxide (DMSO) in man. J. Pharmacol. Exp. Ther. 155, 309-17 (1967).

10. Williams, K.I.H., Bernstein, S.H. and Layne, D.S., Dimethyl sulfone: isolation from human urine. Arch. Biochem. Biophys. 113, 251-52 (1966).

11. Wood, D.C. and Wood, J. Pharmacologic and biochemical considerations of dimethyl sulfoxide. Ann. N.Y. Acad. Sci. 243, 7-19 (1975).

12. Görög, P. and Kovács, I.B. Antiarthritic and antithrombotic effects of topically applied dimethyl sulfoxide. Ann. N.Y. Acad. Sci. 243, 91-97 (1975).

13. Runckel, D.N. and Swanson, J.R., Effect of dimethyl sulfoxide on serum osmolality. Clin. Chem. 26, 1745-47 (1980).

14. Engel, M.F., Dimethyl sulfoxide in the treatment of scleroderma. South. Med. J. 65, 71-73 (1972).

15. Ek, A., Engberg, A., Frödin, L. and Jönsson, G., The use of dimethyl-sulfoxide (DMSO) in the treatment of interstitial cystitis. Scand. J. Urol. Nephrol. 12, 129-31 (1978).

16. Ashwood-Smith, M.J., Current concepts concerning radioprotective and cryoprotective properties of dimethyl sulfoxide in cellular systems. Ann. N.Y. Acad. Sci. 243, 246-56 (1975).

17. de la Torre, J.C., Rowed, D.W., Kawanaga, H.M. and Mullan, S., Dimethyl sulfoxide in the treatment of experimental brain compression. J. Neurosurg. 38, 345-54 (1973).

18. Kajihara, K., Kawanaga, H., de la Torre, J.C., and Mullan, S., Dimethyl sulfoxide in the treatment of experimental acute spinal cord injury. Surg. Neruol. 1, 16-23 (1973).

19. de la Torre, J.C., Johnson, C.M., Goode, D.J., and Mullan, S., Pharmacologic treatment and evaluation of permanent experimental spinal cord trauma. Neurology 25, 508-14 (1975).

20. Brown, F.D., Johns, L.M., and Mullan, S., Dimethyl sulfoxide in experimental brain injury, with comparison to mannitol. J. Neurosurg. 53, 58-62 (1980).

21. Finney, J.W., Urschel, H.C. Balla, GA., Race, G.J., Jay, B.E., Pingree, H.P., Dorman, H.L. and Mallams, J.T., Protection of the ischemic heart with DMSO alone or DMSO with hydrogen peroxide. Ann. N.Y. Acad. Sci. 141, 231-41 (1967).

22. Rubin, L.F., Toxicity of dimethyl sulfoxide, alone and in combination. Ann. N.Y. Acad. Sci. 243, 98-103 (1975).

23. Smith, E.R., Hadidian, Z., and Mason, M.M., The toxicity of single and repeated dermal applications of dimethyl sulfoxide. J. Clin. Pharmacol. 8, 315-21 (1968).

24. Vogin, E.E., Carson, S., and Cannon, G., Chronic toxicity of DMSO in primates. Toxicol. Appl. Pharmacol. 16, 606-12 (1970).

25. Wood, D.C., Weber, F.S., and Palmquist, M.A., Continued studies in the toxicology of dimethyl sulfoxide (DMSO). J. Pharmacol. Exp. Ther. 177, 520-27 (1971).

26. Norred, W.P., Ansel, H.C., Roth, I.L., and Peifer, J.J. Mechanism of dimethyl sulfoxide-induced hemolysis. J. Pharm. Sci. 59, 618-22 (1970).

27. Brobyn, R.D., The human toxicology of dimethyl sulfoxide. Ann. N.Y. Acad. Sci. 243, 500-509 (1975)

28. Wallace, T.J. and Mahon, J.J., Gas-liquid chromatographic separation of sulfur compounds. Nature 201, 817-18 (1964).

29. Williams, K.I.H., Burstein, S.H., and Layne, D.S., Metabolism of dimethyl sulfide, dimethyl sulfoxide, and dimethyl sulfone in the rabbit. Arch. Biochem. Biophys. 117, 84-87 (1966).

30. Paulin, H.J., Murphy J.B. and Larson, R.E., Determination of dimethyl sulfoxide in plasma and cerebralspinal fluid by gas-liquid chromatography. Anal. Chem. 38, 651-52 (1966).

31. Hucker, H.B., Ahmad, P.M. and Miller, E.A., Absorption, distribution and metabolism of dimethylsulfoxide in the rat, rabbit and guinea pig. J. Pharmacol. Exp. Ther. 154, 176-84 (1966).

32. Tiews, J., Scharrer, E., Harre, N., and Flögel, L., Metabolism and excretion of dimethyl sulfoxide in cows and calves after topical and parenteral application. Ann. N.Y. Acad. Sci. 243, 139-50 (1975).

33. Ogata, M. and Fujii, T., Quantitative determination of urinary dimethyl sulfoxide and dimethyl sulfone by the gas chromatograph equipped with a flame photometric detector. Ind. Health 17, 73-78 (1979).

34. Turkevich, N.M., Vladzimirskaya, E.V., Tereshchuk, S.I. and Tomashevskaia, M.F., [Determination of dimexide, methisazone, and their metabolites in body fluids.] Farmatsiia 26, 33-35 (1977).

35. Turkevich, N.M., Tershchuk, S.I. and Tereshchuk, O.M., [Determination of dimexide by gas chromatography.] Farmatsiia 28, 33-35 (1979).

36. Pearson, T.W., Dawson, H.J. and Lackey, H.B. Natural occurring levels of dimethyl sulfoxide in selected fruits, vegetables, grains, and beverages. J. Agri. Food Chem. 29, 1089-91 (1981).

37. MacNair, H.M. and Bonelli, E.J., Basic Gas Chromatography, Varian, Palo Alto, CA, 1969, pp 37-48 and 150-51.

38. Rowland, F.W. and Kananen, G.E., Gas Chromatography. In CRC Handbook Series in Clinical Laboratory Science, Section B: Toxicology, Vol. I, I. Sunshine, Section Ed., CRC Press, Inc., West Palm Beach, FL 1978, pp 32-37.

39. Jenden, D.J. and Cho, A.K., Commentary: Selected ion monitoring in pharmacology. Biochem. Pharmacol. 28, 705-13 (1979).

40. Cali, J.P., Rationale for reference methods in clinical chemistry. Pure and Appl. Chem. 45, 64-68 (1976).

41. Björkhem, I., Blomstrand, R., Lantto, O., Svensson, L., and Önman, G., Toward absolute methods in clinical chemistry: application of mass fragmentography to high-accuracy analyses. Clin. Chem. 22, 1789-1801 (1976).

42. Björkhem, I., Blomstrand, R., Eriksson, S., Falk, O., Kallner, A., Svensson, L. and Önman, G., Use of isotope dilution-mass spectrometry for accuracy control of different routine methods used in clinical chemistry. Scand. J. Clin. Lab. Invest. 40, 529-34 (1980).

43. Björkhem, I., Bergman, A., Falk, O., Kallner, A., Lantto, O., Svensson, L., Akerlöf, E., and Blomstrand, R., Accuracy of some routine methods used in clinical chemistry as judged by isotope dilution-mass spectrometry. Clin. Chem. 27, 733-35 (1981).

44. Finnigan 6000 Series GC/MS Data System Operating Manual, Revision I, (Catalogue No. 06100-90190), Finnigan Corp., Sunnyvale, CA, 1978, pp. 3-34 to 3-37.

45. Phillips, D.S., Basic Statistics for Health Science Students, W.H. Freeman and Co., San Francisco, CA, 1978, pp 48-52.

46. Hewlett Packard 9815A Software, General Statistics, Vol. I. (Part No. 09815-15001, Rev. B.), Hewlett Packard Desktop Computer Division, Ft. Collins, CO, 1975, pp. 53-74.

APPENDIX: Article published in the Journal of Analytical Toxicology. Vol. 6, March/April 1982, pp. 76-81.