Proceedings of the 17th International Conference of Racing Analysts and Veterinarians, Antalya, Turkey

EFFECTS OF DIMETHYLSULPHOXIDE ON ORAL BIOAVAILABILITY OF A TRIAZINE-BASED ANTIPROTOZOAL AGENT TOLTRAZURIL SULFONE IN THE HORSE

L. Dirikolu, W. Karpiesiuk*, A. F. Lehner*, C. Hughes* and T. Tobin*

*Department of Veterinary Biosciences, University of Illinois, College of Veterinary Medicine, 3830 VMBSB, 2001 South Lincoln Avenue. Urbana, Illinois 61822; *Department of Veterinary Science, The Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky, 40546, USA*

ABSTRACT

Having identified triazine-based antiprotozoal agents for the treatment and prophylaxis of equine protozoal myeloencephalitis (EPM) in the horse, subsequent *in vitro* studies confirmed that the EPM-causative agent *Sarcocystis neurona* is sensitive to the triazine-based antiprotozoal agent, toltrazuril sulfone. Triazine-based antiprotozoal agents are lipophylic and they may be expected to be well absorbed following oral administration. However, although an increase in lipid solubility generally increases the absorption of chemicals, extremely lipid-soluble chemicals may dissolve poorly in GI fluids, and their corresponding absorption and bioavailability would be low. Also, if the compound is administered in solid form and is relatively insoluble in GI fluids, it is likely to have only limited contact with the GI mucosa, and therefore its rate of absorption will be low.

Based on the above considerations, we sought a solvent with low or no toxicity that would maintain triazine agents in solution. Because the oral route is preferred for continuing drug therapy, such a solvent would allow an increased rate of absorption following oral administration. We designed a cross-over study in which each horse served as its own control. In this study, we demonstrated that dimethylsulphoxide (DMSO) increased the oral bioavailability of toltrazuril sulfone (Ponazuril) 3-fold, relative to oral administrations of aqueous solutions of toltrazuril sulfone. Our cross-over study indicated that toltrazuril sulfone has high oral bioavailability in DMSO (71%). The high bioavailability of the DMSO-preparation suggests that its daily oral administration will routinely yield higher and more effective plasma and CSF concentrations in treated horses. Also, this improved formulation would allow clinicians to administer loading doses of toltrazuril sulfone in acute cases of EPM. Another option would involve administration of toltrazuril sulfone in DMSO mixed with feed (1.23 kg daily dose) meeting FDA regulatory requirements for the levels of DMSO permissible in pharmaceutical preparations.

INTRODUCTION

Triazine-based antiprotozoal agents are known for their lipophylic characteristics and they may be expected to be well absorbed following oral administration. Additionally, the absorption of chemicals from the gastrointestinal (GI) tract depends on physiochemical properties of compounds, such as lipid solubility, and dissociation rate (Houston *et al.* 1974). Although it is often generalised that an increase in lipid solubility increases the absorption of chemicals, extremely lipid-soluble chemicals may poorly dissolve in GI fluids, and their absorption and bioavailability is low (Houston *et al.* 1974). If the compound administered is a solid and relatively insoluble in GI fluids, it will have limited contact with the GI mucosa, and therefore, its rate of absorption will be low (Gorringe and Sproston 1964; Bates and Gibaldi 1970).

Based on the above considerations we sought a solvent that would maintain triazine agents in solution, thus allowing an increased rate of absorption following oral administration, as the oral route is preferred for continuing drug therapy. Bioavailability is an important parameter in clinical trials because the majority of a drug's

therapeutic and toxic effects are proportional to both dose and bioavailability. Additionally, poor oral bioavailabiliy results in more variable and poorly controlled plasma drug concentrations and therefore therapeutic effects. When bioavailability is low, inter- and intra-subject variability in bioavailability are magnified and incomplete bioavailability becomes a major concern. Another problem associated with the poor and variable bioavailability is that it is generally difficult to predict and control plasma drug concentration of any given dose. It was therefore important for us to try to maximise oral bioavailability of triazine-based agents with the goal of maximising our ability to control plasma drug concentrations and therefore the clinical efficacy of these agents.

Because DMSO is relatively safe to use and its parenteral administration enhances the ability of high molecular-weight substances to be absorbed, we chose DMSO as the solvent to keep our triazine-based agents in solution. There are many reports in the literature indicating an increase in the absorption characteristics of various compounds, mainly skin penetration, in the presence of DMSO (Rubin 1975; Yellowlees *et al.* 1980; Jimenez and Wilkens 1982; Brayton 1986; Elzinga *et al.* 1989; Gyrd-Hansen *et al.* 1993; Ehninger *et al.* 1995; Winker *et al.* 1995; Schuler *et al.* 1998; Watanabe *et al.* 2000). Therefore, it is widely accepted that DMSO enhances the penetration of non-polar compounds through the biological membrane, but the extent of this penetration depends on both the chemical's properties and route of administration. Additionally, it has been shown that DMSO does not potentiate absorption and tissue distributions of various compounds (Egorin *et al.* 1982; Rubinstein and Lev-El 1980). In one of these studies (Egonin *et al.* 1982), it was shown that the pharmacokinetic parameters of cyclophosphamide are not altered when administered either orally or iv with and without DMSO. DMSO did not alter peak plasma and CSF concentrations or plasma and CSF half-lives of cyclophosphamide in 10 human subjects.

Therefore, the enhancement of absorption and distribution for some compounds could be significant ranging from 1- to 100-fold, while for others the effects might be negligible. We therefore wanted to determine if DMSO enhanced the oral bioavailability of triazine-based agents in a clinically significant manner.

Materials and Methods

Horses and sample collection

The animals used were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. Horses were not fed for at least 1 h after oral administration of compound. The animals were vaccinated annually for tetanus and were de-wormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). Horses were kept in a 20-acre field until they were placed in box stalls where they were provided water and hay *ad libitum*. Animals used in these experiments were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care Use Committee, which also approved the experimental protocol.

We used a randomised cross-over study with a 2 x 2 latin square design in order to determine absolute bioavailability and pharmacokinetic characteristics of toltrazuril sulfone in the horse. Four mature Thoroughbred mares weighing 453–526 kg were used for the toltrazuril sulfone study. Toltrazuril sulfone (150 mg/mL in DMSO) was administered either orally or iv to horses at a single dose of 2.2 mg/kg or 1 mg/kg, respectively. Horses were allowed a 3 week interval between subsequent dosing regimens after the last sample collection. Blood samples were obtained for analyses at 0, 0.16, 0.33, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h into heparinised tubes that were centrifuged at 4°C 2,000 rpm x g for 15 min, and the plasma stored at -20°C until assayed.

In a second experiment, toltrazuril sulfone (2.2 mg/kg body weight in DMSO) was administered daily for 28 days to 2 horses either iv or orally. Blood samples were collected daily as described above. A licensed veterinarian collected CSF samples at the lumbosacral space at Days 0, 7, 14, 21 and 28. The CSF was retained for analysis if there was no visible evidence of blood contamination and stored in test tubes at -20°C until analysed.

In a third experiment, toltrazuril sulfone was administered to 2 mature horses weighing 500–545 kg at a single oral dose of 2.2 mg/kg of toltrazuril sulfone suspended in 0.5 L water, by nasogastric intubation. Blood samples were collected at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h as described above. In a fourth series of experiments, toltrazuril sulfone was administered to 4 horses weighing 559–591 kg at a single oral dose of 2.2 mg/kg in DMSO combined with 0.5 oz beet pulp added to 1 lb sweet feed. Blood samples were collected at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h as described above.

Toltrazuril sulfone measurements by HPLC

Sample preparation

A standard solution of 1 mg toltrazuril sulfone was prepared in 1 mL HPLC grade methanol.

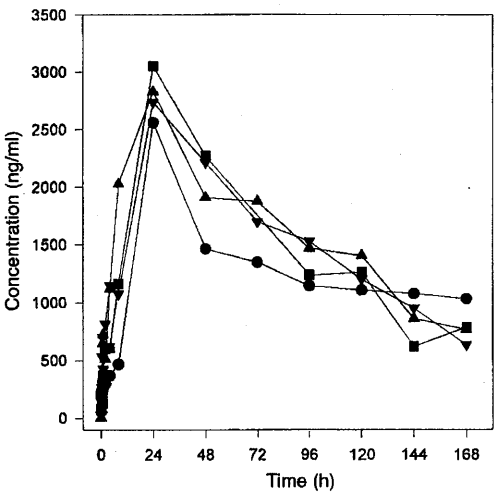

Fig 1: Plasma concentrations of toltrazuril sulfone from 4 horses following single oral administration (Dose: 2.2 mg/kg in DMSO).

Standards were prepared by the addition of a specified amount of toltrazuril sulfone in 60% Solvent B 40% Solvent A (see instrumentation) to blank plasma samples, 1 mL each, over a range from 25–10,000 ng/mL. Janssen compound R 62646, a structural analogue of diclazuril, was used as the internal standard. The internal standard was prepared in 1 mL methanol (1 mg/mL) and diluted 1–10 in 60% Solvent B/40% Solvent A to yield 100 ng/µL standard solution. To each sample, 20 µL of 100 µg/mL internal standard was added. Then, 2 mL of 0.1 M potassium phosphate buffer (pH 6.0) was added to each sample and the pH was adjusted to 6.0 as necessary.

Extraction method

Varian 'Bond Elut' columns were placed into an SPS24 VacElut vacuum chamber and treated sequentially with 2 mL of HPLC grade methanol and 2 mL of 0.1 M potassium phosphate buffer (pH 6.0). The vacuum was turned off as soon as the buffer reached the top of the sorbent bed to prevent column drying. The specimen was drawn slowly through the column taking at least 2 min to pass the specimen through the Bond Elut column. The column was then rinsed sequentially with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0): methanol, 80:20, 1 mL of 1.0 M acetic acid and 1 mL of hexane. The column was allowed to dry for 5–10 min after each rinse. A labelled silanised glass tube was placed below the column and an eluate was collected by slowly rinsing the column with 4 mL of dichloromethane. The solvent was evaporated under a stream of nitrogen gas at 40°C using silanised taper bottom tubes. The residue was resuspended in 150 µL of 60% Solvent B 40% Solvent A mixture with moderately vigorous vortexing and sonication. This solution was placed into a 300 µL vial for HPLC analysis.

Instrumentation

The HPLC procedure was adapted from that described for diclazuril (Dirikolu *et al.* 1999). The instrument employed was a Beckman System Gold HPLC system with 2 110B solvent delivery pumps, a 168 photodiode array detector and a 502 autosampler. The column was a Beckman Ultrasphere ODS, 5 µm particle size, 4.6 mm x 15 cm column size, protected with an Altech C-18 guard column. The mobile phase consisted of 40% Solvent A and 60% Solvent B run with a flow rate of 1 mL/min. Solvent A was 80% [0.5% ammonium acetate in water] : 20% acetonitrile. Solvent B was 80% methanol, 20% acetonitrile. Acetonitrile (A998-4, Fisher Scientific, New Jersey, USA) and methanol (MX0488-1, EM Science, New Jersey, USA) were HPLC grade. After preparation, Solvents A and B were filtered and degassed with 0.45 µm type HV Millipore filters. The diode array detector was set up for single wavelength acquisition at 255 nm with a 12 nm span. Injections were made with a 20 µL sample loop.

Pharmacokinetic analysis

Pharmacokinetic analyses were performed, using a non-linear regression program (Winnonlin, version 3.1) (Pharsight Corporation, North Carolina, USA).

RESULTS

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) in DMSO to 4 horses, analysis of plasma samples showed good oral absorption (Fig 1), with an observed mean peak plasma concentration of 2,795 ± 102 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration. Observed peak plasma concentrations from 4 horses were closely distributed ranging from the lowest 2,560 ng/mL to highest 3,051 ng/mL (Fig 1). Thereafter, the plasma concentration declined to 803 ± 83 (SEM) ng/mL at 168 h after administration with an apparent average half-life of ~ 82 h. The predicted mean time required to achieve peak plasma concentration (T_{max}) following oral administration was 29 ± 3 (SEM) h.

Analysis of plasma samples indicated rapid absorption characteristics of toltrazuril sulfone

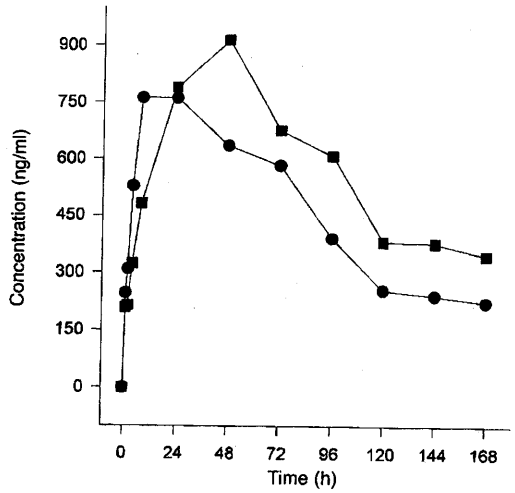

Fig 2: Plasma concentrations of toltrazuril sulfone following single 2.2 mg/kg oral administration in water from 2 horses.

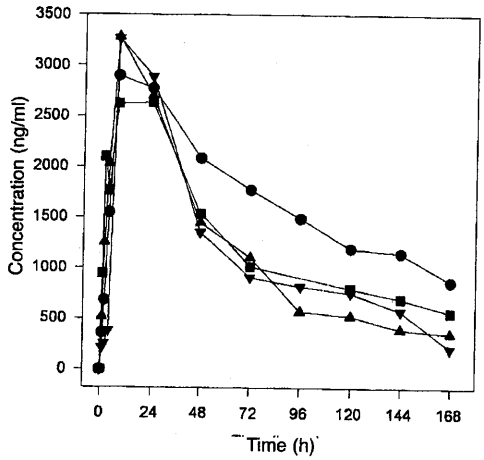

Fig 3: Plasma concentrations of toltrazuril sulfone following 2.2 mg/kg oral administration in DMSO mixed with 0.5 oz. beet pulp added to 1 lb. sweet feed.

administered in DMSO, the mean plasma concentration being 137 ng/mL ± 35 (SEM) at 10 min following oral administration. Analysis of plasma samples following both iv and oral administration indicated high bioavailability of toltrazuril sulfone in horses following oral administration in DMSO. Mean bioavailability of toltrazuril sulfone in DMSO was 71% ± 3.6 (SEM). The bioavailability of toltrazuril sulfone in DMSO in horses included in our study was relatively consistent, and it ranged from 61.3–78.2%. The mean plasma half-life of toltrazuril sulfone in these horses was 81 ± 9 (SEM) h.

Based on these pharmacokinetic parameters, the average steady state concentrations (Cssavg) of toltrazuril sulfone for each horse was calculated. The Cssavg values from 4 horses were estimated to be between 13,572 and 15,690 ng/mL following daily oral administration of 2.2 mg/kg of toltrazuril sulfone in DMSO. To confirm these calculations, 2 horses were dosed daily with 2.2 mg/kg toltrazuril sulfone in DMSO orally. Analysis of plasma samples from these 2 horses indicated steady state plasma concentrations of toltrazuril sulfone of approximately 12–14 µg/mL and 16–18 mg/mL after approximately 10–12 days (data not shown). Steady state concentrations of toltrazuril sulfone in the CSF samples from these 2 horses ranged between 125–130 and 180–220 ng/mL.

The Cssavg values from 4 horses were estimated to be between 19,641 and 22,525 ng/mL following daily iv administration of 2.2 mg/kg toltrazuril sulfone in DMSO. To confirm these results, 2 horses were dosed daily with 2.2 mg/kg toltrazuril sulfone iv in DMSO. Analysis of plasma samples indicated steady state plasma concentrations of toltrazuril sulfone of approximately 19–22 µg/mL after approximately 2–3 weeks (data not shown). Steady state concentrations of toltrazuril sulfone in the CSF samples from these 2 horses ranged between 150–170 ng/mL and 190–220 ng/mL.

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) in aqueous solution to 2 horses, analysis of plasma samples showed detectable plasma concentrations following oral administration of this aqueous solution (Fig 2), with the observed mean peak plasma concentration of 772 ± 14 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration. Observed plasma concentrations from 2 horses at 24 h post administration were in close agreement with values of 758 ng/mL to 786 ng/mL. The plasma concentrations of toltrazuril sulfone from these 2 horses were 633 ng/mL and 910 ng/mL at 48 h post administration with the mean plasma concentration of 771 ng/mL ± 138.5 (SEM). Thereafter, the plasma concentration declined to 286 ± 61 (SEM) ng/mL at 168 h after administration with an apparent average half-life of ~ 77 ± 3.5 (SEM) h. The predicted mean time required to achieve peak plasma concentration (T_{max}) following oral administration was 24 ± 9 (SEM) h.

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg in DMSO) on 0.5 oz beet pulp added to 1 lb sweet feed to 4 horses, analysis of plasma samples showed relatively rapid absorption of toltrazuril sulfone (Fig 3), with

the observed mean peak plasma concentration of 3,013 ± 157 (SEM) ng/ml of toltrazuril sulfone at 8 h after administration. Observed plasma concentrations from 4 horses at 8 h post administration were in close agreement with values of lowest 2,622 ng/mL to highest 3,276 ng/mL. Thereafter, the plasma concentration declined to 503 ± 142 (SEM) ng/ml at 168 h after administration with an apparent average half-life of ~ 65.4 ± 8 (SEM) hours. The predicted mean time required to achieve peak plasma concentration (T_{max}) following oral administration was 16 ± 3.6 (SEM) h. The relative bioavailability of toltrazuril sulfone in DMSO mixed with feed compared to toltrazuril sulfone in DMSO without feed was 68.4% indicating approximately 31% reduction in bioavailability of toltrazuril sulfone in DMSO when given with feed.

Discussion and Conclusion

Early preliminary experiments suggested that the oral bioavailability of diclazuril as Clinacox may vary between individual horses in a clinically significant manner. For example, in our small sample of 4 horses there was a 2-fold difference between the peak plasma concentrations of diclazuril as Clinacox observed in the high (1.6 μg/mL) and the low (0.75 μg/mL) horses. These differences presumably translate into equivalent differences in steady state concentrations of diclazuril attained in plasma and ultimately in the CSF of treated animals. For example in our clinical efficacy study, when the dosage was adjusted from 5 mg/kg to 5.5 mg/kg and the treatment interval was extended from 21 days to 28 days to allow for longer duration of detectable CSF level (6 horses in our clinical efficacy trial), the rate of post treatment relapse rate was reduced indicating the variability of oral absorption of diclazuril in horses.

Three possible solutions to this problem present themselves. The most practical is the development of a highly bioavailable oral preparation of diclazuril which will routinely yield effective plasma and CSF concentrations of this agent in all horses treated. A secondary approach is to monitor plasma concentrations of diclazuril during therapy and adjust the oral dose to compensate for any deficits in bioavailability in individual animals. A third approach is to develop a highly bioavailable oral preparation of a diclazuril related compound, which will routinely yield effective plasma and CSF concentrations of this agent in all horses treated. With literature review, DMSO was suggested as a candidate solvent which increases the absorption characteristics of these compounds in the horse.

To further investigate the effect of DMSO on the absorption characteristics of triazine agents, we compared the results of oral administration of toltrazuril sulfone in water and DMSO. It was found that the mean peak plasma concentration of toltrazuril sulfone at 24 h following oral administration in DMSO was approximately 4 times higher than following oral administration in water. The relative bioavailability of toltrazuril sulfone in water compared to in DMSO was 33% indicating an approximately 3-fold reduction in the bioavailability of toltrazuril sulfone following oral administration in water versus in DMSO. The mean bioavailability of toltrazuril sulfone in DMSO was 71% indicating low hepatic extraction ratio characteristic of this drug in DMSO following oral administration.

In summary, it is very clear that the toxicity of DMSO is minimal when used in clinically normal doses and concentrations. The toxicity effects are most frequently seen in abnormally high experimental doses and concentrations. Additionally, in our clinical efficacy study, we did not observe any toxic effects related to administration of DMSO for more than 28 days. Therefore, DMSO is very suitable solvent to keep our triazine-based agents in solution and increase the rate and extent of absorption of these agents in clinically significant level. Additionally, these results indicate that DMSO will provide less variable and better-controlled plasma drug concentrations of triazine agents and therefore, drug effects. The ability of DMSO to increase bioavailability of these agents will also allow us to predict and control plasma drug concentration of any given dose with the goal of maximising our ability to control the clinical efficacy of these agents. On the other hand, research on the clazurils clinical efficacy, toxicity and the establishing of an appropriate therapeutic window for these compounds clearly require further study.

Acknowledgements

This research was supported by grants from the New Ace Research Company and Fellowship program from the Ministry of National Education of Turkey.

References

Bates, T.R. and Gibaldi, M. (1970) Gastrointestinal absorption of drugs. In: *Current Concepts in the Pharmaceutical Sciences: Biopharmaceutics.* Eds: J. Swarbrick Philadelphia: Lea & Febiger, pp 57-100.

Brayton, C.F. (1986) Dimethyl Sulfoxide (DMSO): A Review. *Cornell Vet.* **76,** 61.

Dirikolu, L., Woods, W.E., Carter, W., Harkins, J.D.,

Boyles, J., Lehner, A.F., Granstrom, D.E. and Tobin, T. (1999) Diclazuril in the horse: Its identification, detection and preliminary pharmacokinetics. *J. vet. Pharmacol. Therap.* **22,** 374-379.

Egorin, J.M., Kaplan, R.S., Salcman, M., Aisner, J., Colvin, M., Wiernik, P.H., and Bachur, N.R. (1982) Cyclophosphamide plasma and cerebrospinal fluid kinetics with and without dimethyl sulfoxide. *Clin. Pharmacol. Therap.* **32(1),** 122-128.

Ehninger, G., Schuler, U., Renner, U., Ehrsam, M., Zellr, K.P., Blanz, J., Storb, R. and Deeg, J.H. (1995) Use of water-soluable busulfan formulation-pharmacokinetic studies in a canine model. *Blood* **85(11),** 3247-3249.

Elzinga, L.W., Bennett, W.M. and Barry, J.M. (1989) The effect of dimethyl sulfoxide on the absorption of cyclosporine in rats. *Transplantation* **47(2),** 393-395.

Gorringe, J.A.L. and Sproston, E.M. (1964) The influence of particle size upon the absorption of drugs from the gastrointestinal tract. Ed: T.B. Binn In: *Absorption and Distribution of Drugs.* Baltimote: Williams & Wilkins, pp 128-139.

Gyrd-Hansen, N., Brimer, L. and Rasmussen, F. (1993) Percutaneous absorption of organophosphorus insecticides in pigs, the influence of different vehicles. *J. vet. Pharmacol. Ther.* **16(2),** 174-180.

Houston, J.B., Upshall, D.G. and Bridges, J.W. (1974) A re-evaluation of the importance of partition coefficients in the gastrointestinal absorption of nutrients. *J. Pharmacol. exp. Ther.* **189,** 244-254.

Jimenez, R.H. and Wilkens, R.F. (1982) Dimethyl sulfoxide: a perspective of its uses in rheumatic disease. *J. lab. clin. Med.* **100,** 489-500.

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

Rubinstein, E. and Lev-El, A. (1980) The effect of dimethyl sulfoxide on tissue distribution of gentamicin. *Experientia* **36(1),** 92-93.

Schuler, U.S., Ehrsam, M., Schneider, A., Schmidt, H., Deeg, J. and Ehninger, G. (1998) Pharmacokinetics of intravenous busulfan and evaluation of the bioavailability of the oral formulation in conditioning for haematopoietic stem cell transplantation. *Bone Marrow Transplant* **22(3),** 241-244.

Watanabe, E., Sudo, R., Takahashi, M. and Hayashi, M. (2000) Evaluation of absorbability of poorly-water soluable drugs: validity of the use of additives. *Biol. Pharm. Bull* **23(7),** 838-843.

Winker, N., Weniger, P., Klein, W., Ott, E., Kocsis, F., Schoket, B. and Korpert, K. (1995) Detection of polycyclic aromatic hydrocarbon exposure damage using different methods in laboratory animals. *J. appl. Toxicol.* **15(1),** 59-62.

Yellowlees, P., Greenfield, C. and McIntyre, N. (1980) Dimethylsulfoxide induced toxicity. *Lancet* **2(8207),** 1044-1046.