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Determination of 1,3-di(4-imidazolino-2-methoxyphenoxy)propane in rat, dog and human plasma and urine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic (HPLC) method was developed for the determination of 1,3-di(4-imidazolino-2-methoxyphenoxy)propane (DMP) in rat, dog and human plasma (50–5000 ng/ml) and urine (0.1–10 μg/ml). DMP and DMPent (dimethoxyimidizolinopentamidine, the internal standard), are extracted from alkanized plasma with *n*-butyl chloride–*n*-butanol (9:1, v/v). The organic phase is dried under nitrogen, reconstituted in mobile phase, and washed with hexane. Separation is achieved by ion-pair chromatography on a Zorbax Rx C_8 column with fluorescence detection. The analysis of pooled plasma (80, 400, and 4000 ng/ml) and urine controls (0.3, 1.6, and 8 μg/ml) demonstrated excellent precision and accuracy over a three-day period. The recovery of DMP is >90% from rat, dog, and human plasma and >85% from rat and human urine, and 60–70% from dog urine. The limit of quantitation (LOQ) of the assay is 50 ng/ml in rat, dog and human plasma. Using the high-sensitivity assay, the limit of quantitation was decreased to 5, 2 and 0.6 ng/ml in rat, dog and human plasma, respectively. The LOQ of the assay is 0.1 μg/ml in rat, dog and human urine. The assay was used to determine plasma and urine concentrations of DMP in pharmacokinetic studies in rat and dog.

INTRODUCTION

Pentamidine is an antiprotozoal drug used in the treatment of *Pneumocystis carinii* pneumonia (PCP) associated with acquired immune deficiency syndrome (AIDS) [1]. The usefulness of pentamidine is limited by its systemic toxicity. Dimethoxyimidizolinopropamidine (DMP), 1,3-di(4 - imidazolino - 2 - methoxyphenoxy)propane (DMPent) (Fig. 1) is a synthetic analogue of pentamidine with anti-*P. carinii* activity and a lower incidence of toxic side effects [2–4].

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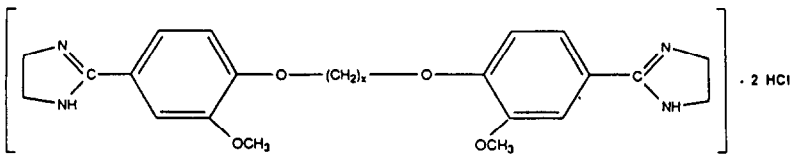

Fig. 1. Structures of dimethoxyimidizolinopropamidine (DMP) and dimethoxyimidizolinopentamidine (DMPent), internal standard.

A sensitive and selective method for the determination of DMP in plasma was necessary to support proposed animal and human pharmacokinetic/biopharmaceutic studies. The utilization of a validated assay provides concentration data on drug disposition from the large number of plasma samples generated by preclinical toxicology, toxicokinetic and drug disposition studies, as well as the initial pharmacokinetic evaluations in man. In this context, pentamidine and its analogues have been extracted from physiological fluids using solid-phase extraction (SPE) [5–7], solvent extraction [8], solvent extraction/back extraction [9], and ion-pair extraction [10] followed by ion-pair HPLC with ultraviolet (UV) [5,9,10] or fluorescence detection [5,7,8].

An HPLC assay for DMP with a limit of quantitation (LOQ) of 8.9 μg/ml from spiked rat urine has been reported [5]. This method was also used to quantitate pentamidine and DMPent in urine of rats injected with pentamidine and DMPent. The method described in the present report uses a simple solvent extraction followed by HPLC analysis with fluorescence detection for the quantitation of DMP in rat, dog and human plasma and urine.

EXPERIMENTAL

Reagents and test materials

Dimethoxyimidizolinopropamidine (DMP) dihydrochloride (99.8% purity) and 1,5-di(4-imidazolino-2-methoxyphenoxy)pentane (DMPent) dihydrochloride, the internal standard (99.0% purity), were synthesized as previously described [11]. Heparinized rat and dog plasma and rat urine was purchased from Rockland (Gilbertsville, PA, USA). Heparinized human plasma was purchased from Interstate Blood Bank (Memphis, TN, USA) and was obtained in-house for the high-sensitivity method. Dog and human urine was collected in-house. Phosphoric acid (85%) (Fisher, Fairlawn, NJ, USA), heptanesulfonic acid sodium salt, 1-hydrate, and octane sulfonic acid, sodium salt (Kodak, Rochester, NY, USA) and all solvents were HPLC grade. Other reagents were prepared from analytical-reagent grade chemicals and Milli-Q water (Millipore, Bedford, MA, USA).

Chromatographic system

The HPLC system consisted of an ABI/Kratos 400 pump (Foster City, CA, USA), a Perkin-Elmer ISS-100 autoinjector (Norwalk, CT, USA), and an ABI/Kratos 980 fluorescence detector with excitation at 275 nm and a UG-11 emission filter (Corion, Holliston, MA, USA). Data collection and calculations were on an HP1000 Model A900 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, PA, USA). The analytical column was a Zorbax Rx C_8 (250 mm × 4.6 mm I.D., 5 μm particle size, Mac-Mod, Chadds Ford, PA, USA) with a Brownlee RP-18 precolumn (15 mm × 3.2 mm I.D., 7 μm particle size, ABI, San Jose, CA, USA). The mobile phase for the plasma assays consisted of 5 m*M* heptanesulfonic acid–acetonitrile-phosphoric acid (71.5:28.5:0.1, v/v/v) with a flow-rate of 1.0 ml/min; the mobile phase proportions were changed to (72:28:0.1) for the high-sensitivity method. For the urine assays, the mobile phase was 5 m*M* octane sulfonic acid–acetonitrile–phosphoric acid (71:29:0.1, v/v/v) with a flow-rate of 1.5 ml/min.

Standard solutions

A stock solution of DMP (100 μg/ml, free base) was prepared by dissolving 11.7 mg of the hydrochloride salt (DMP-HCl) in 10 ml of methanol and diluting to 100 ml with 0.1% (v/v in water) phosphoric acid. A stock solution of the internal standard (DMPent) (100 μg/ml) was prepared by dissolving 10.0 mg of DMPent-HCl in 100 ml of 0.1% phosphoric acid. Working solutions of DMP (0.1 to 20 μg/ml) and DMPent (2.5 μg/ml) were prepared by diluting the stock solutions with 0.1% phosphoric acid. These solutions were stable for at least one month when stored in polypropylene bottles at 5°C. For the high-sensitivity method the working solutions concentrations were 3.0 to 1000 ng/ml for DMP and 1.0 μg/ml for DMPent.

Control samples

Pooled quality control samples (controls) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. Plasma control pools (80, 400, and 4000 ng/ml DMP) were prepared by diluting 200 μl of 10 μg/ml DMP, 1.0 ml of 10 μg/ml DMP, and 1.0 ml of 100 μg/ml DMP, respectively, to 25 ml with blank rat, dog or human plasma. Pooled controls for the high-sensitivity human plasma method were prepared in polypropylene containers at concentrations of 1.2, 10, and 100 ng/ml DMP by diluting 60 μl of 1 μg/ml DMP, 50 μl of 10 μg/ml DMP, and 50 μl of 100 μg/ml DMP to 50 ml with freshly drawn human plasma. Urine control pools (0.3, 1.6 and 8 μg/ml DMP) were prepared by diluting the appropriate amount of DMP working solution to 25 ml with rat, dog or human urine. All control pools were aliquoted into polypropylene vials and stored at −20°C.

Sample preparation

Samples were prepared by adding 200 μl of rat, dog or human plasma (or urine), 50 μl of DMPent (2.5 μg/ml), 100 μl of 0.1% (v/v) phosphoric acid (to compensate for the volume of the DMP working solution added to the calibration standards), 100 μl of 0.5 *M* sodium hydroxide, and 6 ml of *n*-butyl chloride–*n*-butanol (9:1, v/v) to glass test tubes with PTFE-lined screw caps. Calibrations standards (50 to 5000 ng/ml) for plasma, 0.1 to 10 μg/ml for urine, were prepared by using 200 μl of blank plasma or urine and 100 μl of the appropriate DMP working solutions (0.1 to 10 μg/ml) for plasma, 0.2 to 20 μg/ml for urine. The tubes were capped, mixed on a horizontal shaker for 15 min, and centrifuged at 700 *g* for 5 min. The aqueous layer was frozen in a dry ice–acetone bath, the organic layer was transferred to a clean test tube, and the samples were evaporated to dryness under nitrogen at 40°C. For plasma, the samples were reconstituted in 500 μl of mobile phase and washed with 1 ml of hexane. Aliquots of 50 μl were injected into the HPLC system. For urine, the organic layer was transferred to a clean conical tube, and back-extracted with 1 ml of 0.1% (v/v) phosphoric acid. The organic layer was aspirated and the remaining solvent in the aqueous layer allowed to evaporate; 50 μl aliquots were injected into the HPLC system.

Sample preparation for high-sensitivity assay

Sample preparation for the high-sensitivity method in human plasma has the following modifications. The sample size was 500 μl, the DMPent concentration was 1.0 μg/ml, 10 ml of extraction solvent was used, the reconstitution volume was 250 μl, the hexane wash volume was 500 μl, and the injection volume was 100 μl. Calibrations standards (0.6 to 200 ng/ml) were prepared by using 500 μl of blank plasma and 100 μl of the appropriate DMP working solutions (3.0 to 1000 ng/ml). The same modification was used for rat and dog plasma with a lower calibration standard of 5 and 2 ng/ml, respectively.

Validation

Duplicate calibration curves were analyzed on each of three days. Triplicate controls at each concentration were analyzed with each calibration curve. Calculations of the calibration curves were by weighted (1/concentration) least squares linear regression analysis of the peak-height ratios of DMP/DMPent *versus* the concentration of DMP.

RESULTS AND DISCUSSION

The chromatographic system for the plasma assay used a Zorbax Rx C_8 collum with an ion-pair mobile phase containing heptanesulfonic acid (HSA). There was very little retention of DMP without the addition of HSA. Representative chromatograms of the calibrations standards and controls demonstrate excellent peak shape and separation between DMP and DMPent (see Fig. 2). The addition of mobile phase modifiers (*e.g.* triethylamine or tetramethylammonium chloride) [4–6,8] was not necessary with the Zorbax Rx C_8 column, although tailing was observed with a Zorbax C_8 column. The mean retention time of DMP and DMPent in the rat, dog and human plasma validations were 4.13, 4.10 and 4.01 min and 6.47, 6.37, and 6.20 min, respectively. The mobile phase composition of the high-sensitivity method in human plasma was

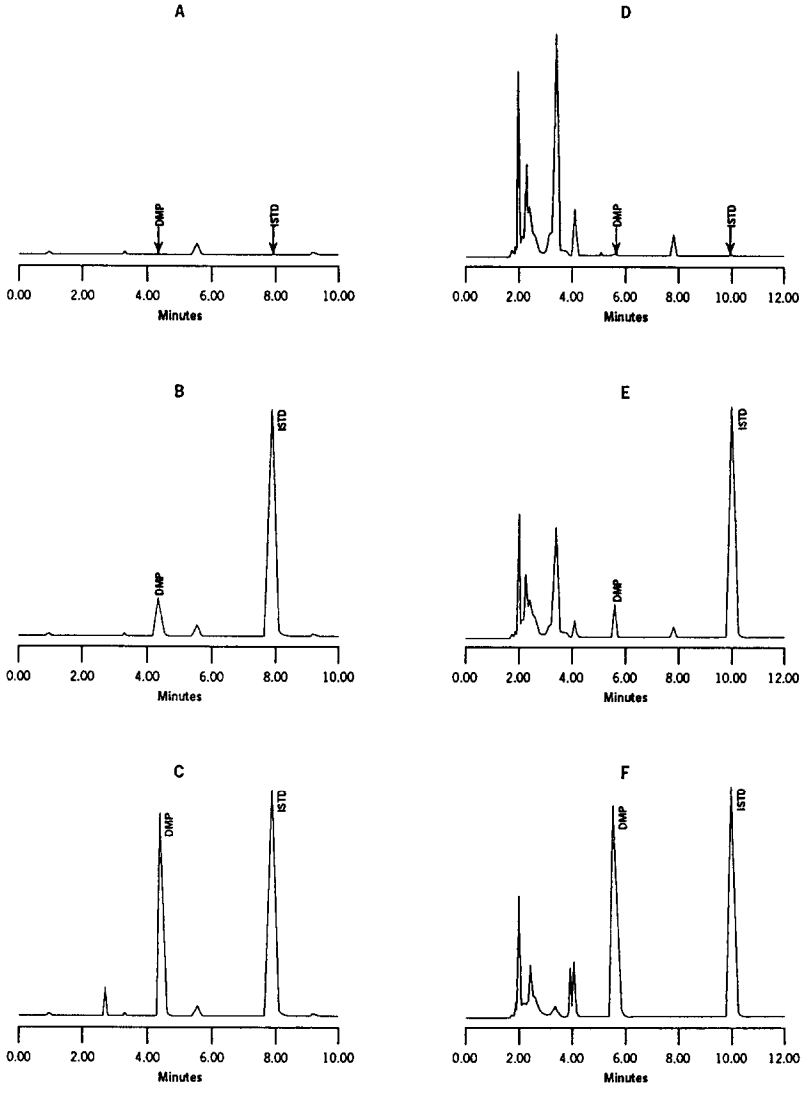

Fig. 2. Chromatograms of control rat plasma (A), 50 ng/ml calibration standard in rat plasma (B), plasma from one rat taken 1 h after a 2.5 mg/kg i.v. dose of DMP-lactate (C), control rat urine (D), 0.2 μg/ml calibration standard in rat urine (E), and urine collected 0–12 h from one rat administered 10 mg/kg DMP-lactate orally (F).

modified slightly to provide better separation from endogenous peaks, and the mean retention times for DMP and DMPent were 6.41 and 11.4 min, respectively.

In the urine assay, optimal chromatography was achieved by using a Zorbax Rx C_8 column and an ion-pair mobile phase containing octanesulfonic acid (OSA). Using OSA as the ion-pair reagent resulted in greater retention than heptanesulfonic acid, which was used for the plasma method. This was necessary to move the DMP and DMPent peaks into clean regions of the chromatograms. The mean retention time of DMP and the DMPent in the rat, dog, and human urine validations were 6.08, 5.88 and 6.07 min and 11.3, 10.7 and 11.2 min, respectively.

Fluorescence detection was used because it provided a greater specificity than UV. Endogenous interferences were not observed in blank rat, dog or human plasma or urine. The freshly-drawn human plasma for the high-sensitivity method was obtained in-house to avoid the potential matrix interferences sometimes encountered with out-dated plasma. Representative chromatograms of processed rat plasma and urine, including samples from animals administered DMP, are presented in Fig. 2.

Recovery, accuracy and precision

Stock and working standard solutions of DMPs in 0.1% phosphoric acid were stable for at least 1 month when stored at 5°C. Working solutions must be prepared and stored in polypropylene containers because the concentration of DMP decreased over time when stored in glass, probably due to absorption.

Excellent recovery and specificity was achieved in the plasma assay by using a simple solvent extraction procedure. *n*-Butanol was added to *n*-butyl chloride to increase the polarity of the extraction solvent and thus increase recoveries. The recoveries for DMP and DMPent were greater than 90% in all three plasma matrices. Recoveries were calculated by comparing the peak heights of extracted calibration standards with the peak heights of unextracted recovery standard at the same nominal concentrations.

The recoveries for DMP and DMPent were greater than 85% in rat and human urine, but were only 60–70% in dog urine. The lower recoveries in dog urine appear to be matrix related.

Calibration curves were linear over concentration ranges of 50 to 5000 ng/ml DMP in rat, dog and human plasma; 0.6 to 200 ng/ml DMP in human plasma; and 0.1 to 10 μg/ml DMP in rat, dog and human urine with mean ($n = 6$) correlation coefficients ≥ 0.9997 (Table I). Calculated values for the calibration standards of the high-sensitivity method in human plasma are presented in Table II.

The within-day precision of the plasma method as measured by the relative standard devia-

TABLE I

CALIBRATION CURVE PARAMETERS FOR DMP IN RAT, DOG AND HUMAN PLASMA AND URINE

Results of validation studies over three-day periods.

Matrix	*n*	Slope (mean ± S.D.)	R.S.D.	Mean intercept	Mean correlation coefficient
Rat plasma	6	0.0031 ± 0.00010	3.2	0.0032	0.9999
Dog plasma	6	0.0031 ± 0.00008	2.6	0.0002	0.9998
Human plasma	6	0.0028 ± 0.00021	7.5	0.0050	0.9999
Human plasma (high-sensitivity)	6	0.0219 ± 0.00097	4.4	−0.0006	0.9997
Rat urine	6	0.8879 ± 0.01608	1.8	0.0087	1.0000
Dog urine	6	0.7844 ± 0.03003	3.8	0.0119	0.9997
Human urine	6	0.8972 ± 0.00753	0.8	0.0066	1.0000

TABLE II

CALIBRATION CURVE DATA FOR DMP IN HUMAN PLASMA (HIGH-SENSITIVITY METHOD)

Results of validation study over a three-day period.

Calibration standard concentration (ng/ml)	Calculated concentration (mean ± S.D., $n = 6$) (ng/ml)	R.S.D. (%)	Deviation (%)
0.6	0.63 ± 0.036	5.7	5.0
1	0.99 ± 0.067	6.8	−1.0
2	2.04 ± 0.092	4.5	2.0
6	5.94 ± 0.132	2.2	−1.0
20	19.2 ± 1.20	6.3	−4.0
60	59.4 ± 1.0	1.7	−1.0
200	202 ± 0.8	0.4	1.0

tion (R.S.D.) of the daily mean ($n = 6$) of the rat, dog and human plasma controls was ≤5.5% at all control concentrations. The overall ($n = 18$) precision of the method over three days was ≤4.3% at all concentrations (Table III).

The accuracy of the method was measured by comparing the means of the measured concentrations of the controls with their theoretical concentration. All of the daily ($n = 6$) and overall ($n = 18$) mean values for the plasma method were within 6.5% and 4.8% of their theoretical values, respectively (Table III).

Calculated values for the calibration standards in rat, dog, and human urine are presented in (Table IV). The within-day precision of the method as measured by the relative standard deviation (R.S.D.) of the daily mean ($n = 6$) of the rat, dog and human urine controls was ≤4.4% at all control concentrations. The overall ($n = 18$) precision and accuracy of the method over three days was ≤4.3% and 6.3%, respectively, at all concentrations (Table V).

Stability

The stability of DMP in plasma was determined by measuring the concentration changes in the control samples over time. The concentration of DMP in plasma controls was observed to decrease over time when stored in glass containers, so all control pools were aliquoted into polypropylene vials. The concentration of DMP in rat plasma controls stored in poly-

TABLE III

PRECISION AND ACCURACY OF THE ASSAY FOR DMP IN RAT, DOG AND HUMAN PLASMA

Results of validation studies over a three-day period with six determinations per day.

Control concentration (ng/ml)	Calculated concentration (Overall mean ± S.D., $n = 18$) (ng/ml)	R.S.D. (%)	Deviation (%)
Rat plasma			
80	80.3 ± 3.04	3.8	0.4
400	395 ± 8.7	2.2	−1.3
4000	3960 ± 101	2.6	−1.0
Dog plasma			
80	80.8 ± 1.81	2.2	1.0
400	397 ± 10.9	2.7	−0.8
4000	4020 ± 93	2.3	0.5
Human plasma			
1.2	1.15 ± 0.49	4.3	−4.2
10	9.52 ± 0.318	3.3	−4.8
100	97.3 ± 1.78	1.8	−2.7
400	395 ± 5.9	1.5	−1.3
4000	3920 ± 130	3.3	−2.0

TABLE IV

CALIBRATION CURVE DATA FOR DMP IN RAT, DOG AND HUMAN URINE

Results of validation study over a three-day period.

Calibration standard concentration (μg/ml)	Calculated concentration (Mean ± S.D., $n = 6$) (μg/ml)	R.S.D. (%)	Deviation (%)
Rat			
0.1	0.10 ± 0.000	0.0	0.0
0.2	0.20 ± 0.004	2.0	0.0
0.5	0.49 ± 0.008	1.6	−0.2
1	1.00 ± 0.008	0.8	0.0
2	2.00 ± 0.014	0.7	0.0
5	4.99 ± 0.044	0.9	−0.2
10	10.0 ± 0.05	0.5	0.0
Dog			
0.1	0.10 ± 0.004	4.0	0.0
0.2	0.20 ± 0.008	4.0	0.0
0.5	0.48 ± 0.012	2.5	−4.0
1	0.99 ± 0.026	2.6	−1.0
2	2.01 ± 0.062	3.1	0.5
5	5.06 ± 0.130	2.6	1.2
10	9.94 ± 0.148	1.5	−0.6
Human			
0.1	0.10 ± 0.000	0.0	0.0
0.2	0.20 ± 0.004	2.0	0.0
0.5	0.50 ± 0.005	1.0	0.0
1	0.99 ± 0.004	0.4	−1.0
2	2.00 ± 0.021	1.1	0.0
5	5.00 ± 0.022	0.4	0.0
10	10.0 ± 0.05	0.5	0.0

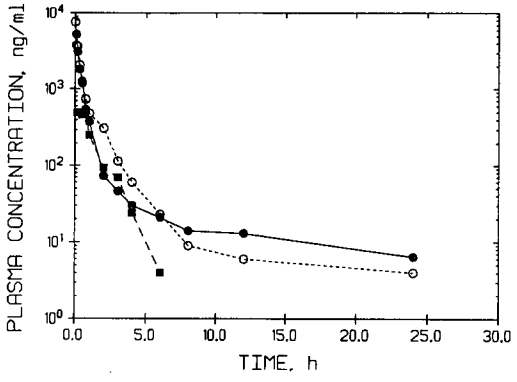

Fig. 3. Plasma concentration–time profiles of DMP in rat (●), and dog (○) after a 2.5-mg/kg i.v. dose of DMP-lactate, and in a dog after a 100-mg/kg oral dose (■).

propylene deviated less than 5% from theoretical after storage for 3 months at −20°C. No stability problems were observed during the validation of the method in rat, dog or human plasma.

The concentration of DMP in urine controls was observed to decrease over time even when the control pools were aliquoted into three polypropylene vials. The concentrations of DMP in rat urine controls stored at −20°C were within 4% of theoretical during the validation, but after 3 months the 0.3- and 1.6-μg/ml controls should be analyzed within 2 weeks of collection if possible.

Application of the method to biological specimens

At present, this method has been used to measure DMP plasma concentrations in preclinical pharmacokinetic studies designed to determine the disposition of DMP in rat and dog.

The plasma concentration–time profile of DMP was determined in rats (one rat sacrificed at each time-point) and in two dogs (Fig. 3). The rats received a single 2.5 mg/kg intravenous dose of DMP-lactate as an aqueous solution; one dog each received the aqueous solution of DMP-lactate as a single 2.5 mg/kg intravenous or 100 oral dose. Using the modification described for the high-sensitivity method for human plasma DMP concentrations as low as 4 to 6 ng/ml in rat and dog plasma were determined. Without the modification and resultant lower LOQ, plasma concentrations of DMP would have been measurable only up to 2 to 4 h post-dose in these preclinical pharmacokinetic studies.

In both rat and dog, the plasma concentration–time profile of DMP declined in an apparent biphasic manner after intravenous administration. After the oral administration to a dog, absorption was rapid as shown by the C_{max} (503 ng/ml) being attained at 20 min. Plasma concentrations declined rapidly thereafter and were non-measurable at 8 h. The extent of absorption was very low relative to the intravenous dose.

In these experiments urine was collected from both rat and dog over set intervals and assayed for DMP. The data (Table VI) indicate that in both species DMP is eliminated in urine follow-

TABLE V

PRECISION AND ACCURACY OF THE ASSAY FOR DMP IN RAT, DOG, AND HUMAN URINE

Results of validation studies over a three-day period with six determinations per day.

Control concentration (μg/ml)	Calculated concentration (Overall mean ± S.D., $n = 18$) (μg/ml)	R.S.D. (%)	Deviation (%)
Rat			
0.3	0.29 ± 0.006	2.1	−3.3
1.6	1.56 ± 0.029	1.9	−2.5
8	7.99 ± 0.124	1.6	−0.1
Dog			
0.3	0.28 ± 0.012	4.3	−6.7
1.6	1.55 ± 0.047	3.0	−3.1
8	7.92 ± 0.276	3.5	−1.0
Human			
0.3	0.28 ± 0.008	2.9	−6.7
1.6	1.50 ± 0.036	2.4	−6.3
8	7.75 ± 0.164	2.1	−3.1

ing i.v. administration with the highest excretion rate occurring 0–12 h post dose. The lower urinary elimination following oral administration probably reflects the low oral bioavailability. It was estimated that approximately 40% and 10% of the dose was eliminated as DMP by rat and dog, respectively, over 24 h following the i.v. administration.

TABLE VI

CONCENTRATIONS OF DMP IN RAT AND DOG URINE FOLLOWING THE INTRAVENOUS AND ORAL ADMINISTRATION OF DMP-LACTATE

Rat data = mean ± S.D. of urine collections from six rats.

Time (h)	Concentration (μg/ml)		
	Rat (2.5 mg/kg i.v.)	Dog 1 (2.5 mg/kg i.v.)	Dog 2 (100 mg/kg p.o.)
0–12	44.4 ± 13.8		
12–24	1.7 ± 0.5		
0–2		18.1	0.22
2–4		12.5	0.95
4–6		8.84	1.31
6–8		3.64	1.64
8–12		1.03	1.40
12–24		2.81	3.46

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