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Quantitation of the novel anticonvulsant remacemide in rat and dog plasma and urine: application of the plasma methodology to measure the plasma protein binding of remacemide

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ABSTRACT

Sensitive and selective methods have been developed for quantitation of the novel anticonvulsant remacemide in rat and dog plasma and urine. The methods employed liquid–liquid extraction (urine) or ion-exchange solid-phase extraction (plasma), with an internal standard, followed by high-performance liquid chromatography with ultraviolet detection. The detection limit for both methods was 10 ng/ml. Overall accuracy was 0.00% for plasma and −1.4% for urine with a precision of 6.04 and 3.87% for plasma and urine, respectively. The standard curves were linear for both plasma and urine over a wide concentration range (9.96–2490 ng/ml). The plasma method was also applied to measurement of *in vitro* plasma protein binding of remacemide in rat, dog and human plasma.

INTRODUCTION

Remacemide, prepared as the hydrochloride salt of 2-amino-N-(1-methyl-1,2-diphenylethyl) acetamide (I, Fig. 1) is a novel water-soluble anticonvulsant. When administered orally to rats and mice, I · HCl is potent against maximal electroshock (MES)-induced seizures, but has little activity against chemically induced seizures [1,2] and thus, in this respect, has a similar preclinical activity profile to phenytoin and carbamazepine. I · HCl is currently in phase II clinical trials as an agent for use in generalized tonic/clonic (grand mal) seizures. For preclinical work, a rapid, sensitive and selective method has been developed for the quantitation of I in plasma and urine of beagle dogs and Sprague–Dawley rats. This report describes the methodology for plasma and urine and the application of the plasma proce-

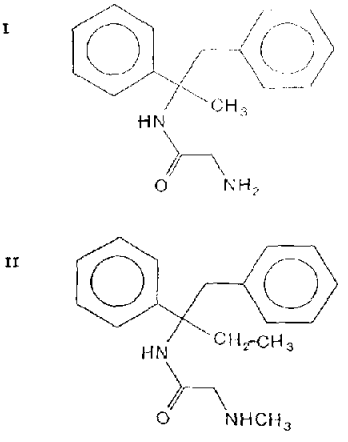

Fig. 1. Structures of remacemide (I) and internal standard (II).

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dure to determine the *in vitro* protein binding of I in rat, dog and human plasma.

EXPERIMENTAL

Chemicals

The analyte, remacemide (I) hydrochloride (FPL 12924), and the internal standard, II (FPL 13667AA, 2-(methylamino)-N-[1-phenyl-1-(phenylmethyl)propyl]acetamide hydrochloride), were synthesized in the Department of Chemistry, Fisons Pharmaceuticals (Rochester, NY, USA). Acetonitrile, methanol, *n*-butanol and water were HPLC grade and obtained from J. T. Baker (Phillipsburg, NJ, USA). Hexane (UV grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). Potassium phosphate (monobasic), phosphoric acid (85%) (HPLC grade), sodium hydroxide (1 *M* solution, Fisher-certified), hydrochloric acid (0.1 *M* solution, Fisher-certified) and sodium bicarbonate (certified A.C.S.) were all obtained from Fisher Scientific (Fairlawn, NJ, USA).

Equipment

Carboxylic acid and benzenesulfonic acid solid-phase extraction columns (100-mg, 1-ml columns) were obtained from Analytichem International (Harbor City, CA, USA). A Vac Elut processing station (Analytichem) was used for extracting plasma samples.

The HPLC system consisted of a Waters Model M-45 pump operated at a flow-rate of 1 ml/min, a Waters WISP Model 710B autosampler with 50-μl injection volume (Waters Assoc., Milford, MA, USA), a 100 mm × 4.6 mm column packed on site with 3 μm particle size Spherisorb hexyl (Phase Sep, Norwalk, CT, USA), maintained at room temperature, an LDC Spectromonitor III Model 1204A variable-wavelength UV detector (Riviera Beach, FL, USA) operated at 218.6 nm and a Berthold (Linear Instruments, Reno, NV, USA) 10-mV input chart recorder operated at 5 mm/min. The data generated by the UV detector were integrated on a Hewlett Packard 3357 laboratory automation system (Palo Alto, CA, USA). For plasma samples, the mobile phase consisted of 30% acetonitrile in 0.05 *M* KH_2PO_4 (final pH of 4.5 ± 0.02 for rat and human plasma and 4.0 ± 0.02 for dog plasma). For urine samples, the pH of the mobile phase was adjusted to 3.5 ± 0.02. The mobile phase was filtered through an 0.45-μm Ultipor Nylon-66 filter (Pall Trinity Micro, Cortland, NY, USA) prior to use.

Standard solutions

An aqueous stock solution of I · HCl was prepared to provide a concentration of 249 μg/ml of I. Aqueous dilutions of stock I were prepared to provide concentrations ranging from 99.6 to 24 900 ng/ml. An aqueous stock solution of the internal standard, II, was prepared at a concentration of 249 μg/ml and diluted to provide a spiking solution at a concentration of 4980 ng/ml.

Extraction of rat and dog urine

To a 1-ml urine sample, 100 μl of internal standard solution were added followed by 100 μl of 1 *M* NaOH and the sample was mixed. *n*-Butanol (10%) in hexane (5 ml) was then added and the sample mixed for 20 min on a rotator and then centrifuged for 10 min at 900 *g*. The upper organic layer was then transferred and 300 μl of 0.1 *M* hydrochloric acid were added followed by mixing and centrifugation for 5 min at 900 *g*. The upper organic layer was discarded and the remaining aqueous layer transferred to a WISP microvial for HPLC analysis. Standard curves were prepared (in duplicate) in the same manner, using blank (control) urine spiked with 100 μl of a standard solution of I.

Plasma sample extraction

The methodology was originally developed and validated for 1-ml samples of rat plasma using carboxylic acid solid-phase extraction columns. The methodology was subsequently modified for dog plasma (using benzenesulfonic acid columns) and for analysis of smaller-volume (0.5 ml) plasma samples.

Rat plasma

To a 1-ml plasma sample, 100 μl of internal standard solution were added followed by 1 ml of 0.05 *M* KH_2PO_4 (pH 2.5) and the sample was mixed thoroughly. The carboxylic acid solid-phase extraction columns were conditioned with 2 ml of acetonitrile followed by 2 ml of 0.05 *M* KH_2PO_4 (pH 2.5) and were not allowed to dry. The plasma sample was then added and aspirated through the column. Endogenous components in plasma were removed by washing the columns with 2 ml of 0.05 *M* KH_2PO_4 (pH 2.5) and the columns allowed to air dry for 3 min. The analyte was then eluted with 1 ml of 50% methanol in 0.05 *M* KH_2PO_4 and the eluent was placed in a WISP vial for HPLC analysis. Standard curves were prepared (in duplicate) in the same manner, using blank (control) plasma spiked with 100 μl of a standard solution of I.

Dog plasma

Using the methodology described above in dog plasma, additional endogenous components were found to interfere with the quantitation of I. Hence, for dog plasma, benzenesulfonic acid solid-phase extraction columns were used instead of carboxylic acid columns and a 5-min centrifugation step (900 *g*) was included after addition of the internal standard and the buffer. The rat methodology was then followed up to and including the 3-min drying step after which the following procedure was used. The column was washed with 2 ml of 0.05 *M* KH_2PO_4 (pH 2.5)–methanol (85:15 v/v) and then dried briefly followed by addition of 2 ml of 0.1 *M* sodium bicarbonate ($NaHCO_3$) solution. The analyte was then eluted with 1 ml of acetonitrile – 0.1 *M* $NaHCO_3$ (50:50, v/v).

Reduced volume samples (0.5 ml)

To 0.5-ml plasma samples, 50 μl of internal standard solution were added. The methodology was then the same as described above for dog plasma with the exception of reducing the column wash volumes and elution volume by one half.

Recovery

Extraction efficiency was assessed (in triplicate) for I at two concentrations (at approximately 25 and 75% of the highest concentration used in the standard curve) and for the internal standard at the concentration used in the assay. Recovery was calculated in the following way: % recovery = (peak height of extracted standard/peak height of non-extracted standard) × 100.

Accuracy and precision

Replicate analyses of rat plasma samples, spiked with a known amount of I, were prepared to determine the accuracy and precision of the method. The samples to be analyzed were prepared as five replicates of three different concentrations to be analyzed on three separate occasions. These replicate samples were quantitated against a standard curve (duplicate) prepared from a separate gravimetric weighing. The accuracy of the method was calculated from a comparison of calculated I concentration and the known added concentration and is expressed as the mean percent difference: accuracy = mean % found − 100%. The precision of the method is expressed as the percent relative standard deviation: precision = (standard deviation of mean × 100%)/mean.

Protein binding

The extent to which I was bound to plasma proteins was determined using the method of ultrafiltration [3]. Plasma (1.8 ml) was spiked with 200 μl of a standard solution of I, mixed gently, and incubated at 25°C for 15 min. An aliquot of plasma (0.5 ml) was then removed for extraction for determination of plasma total I concentration. A 1-ml volume of the remaining plasma was transferred to an Amicon micropartition cell and centrifuged at 25°C for 30 min in an IEC B-20A centrifuge with an 870 fixed-angle rotor at 4000 rpm (2000 *g*). Approximately 650 μl of plasma ultrafiltrate were obtained under these conditions. An aliquot (0.5 ml) of plasma ultrafiltrate was extracted for determination of free (unbound) I. Binding determinations were carried out in duplicate. The percent plasma protein

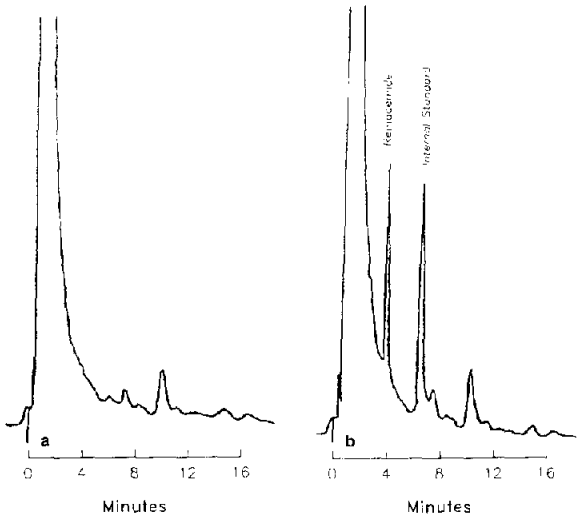

Fig. 2. Typical chromatograms of rat plasma (1 ml). (a) Blank rat plasma; (b) blank rat plasma spiked with 249 ng/ml I and 498 ng/ml internal standard.

binding was calculated as [(plasma total I − ultrafiltrate I)/plasma total I] × 100.

RESULTS

Plasma

Rat plasma. Typical chromatograms for analysis of I in rat plasma are shown in Fig. 2. Control (blank) plasma was free of interfering endogenous components at the retention times of I and the internal standard. The standard curve was essentially linear over the anticipated plasma I concentration range of 10 – 2500 ng/ml, however, the standard curve was divided into overlapping low and high standard curves. The low curve consisted of standards 9.96 – 498 ng/ml (r^2 = 0.9999; y-intercept of -0.0112; slope of 0.0038) and the high curve consisted of standards 99.6 – 2490 ng/ml (r^2 = 0.9994; y-intercept of 0.0733; slope of 0.0036).

Good recovery of I (> 80%) and the internal standard (> 75%) from rat, dog and human plasma was obtained using the solid-phase extraction procedure described for rat plasma and these data are presented in Table I. The sensitivity was defined as the lowest concentration that was detectable and measured consistently in 1 ml of plasma. This concentration, 9.96 ng/ml, became the minimum quantifiable level (MQL) for I in plasma.

Dog plasma. Recovery of I and the internal standard from dog plasma (1 ml, Table II) using the modified method was very similar to recovery using the rat method (see Table I), but the chromatography was improved with the inclusion of the additional wash steps (Fig. 3).

Reduced volume method. Recoveries of I and the internal standard from rat and human plasma using the reduced volume modification of the dog plasma methodology are presented in Table II. For rat and human plasma, a comparison of the rat method (1 ml, Table I) and this reduced vol-

TABLE I

RECOVERY OF I (n = 3) AND INTERNAL STANDARD (n = 6) FROM 1 ml OF RAT, DOG OR HUMAN PLASMA USING THE ORIGINAL RAT PLASMA METHODOLOGY

Compound	Concentration (ng/ml)	Recovery (%)		
		Rat	Dog	Human
Remacemide	652	85.5 ± 1.5	89.3 ± 4.8	83.1 ± 1.2
	1630	83.5 ± 0.9	89.0[a]	81.6 ± 1.6
Internal standard	498	75.6 ± 3.3	86.1 ± 2.4	83.2 ± 1.0

[a] n = 2.

TABLE II

RECOVERY OF I (n = 3) AND INTERNAL STANDARD FROM 1 ml OF DOG PLASMA, USING THE EXTRACTION PROCEDURE MODIFIED FOR DOG PLASMA, AND FROM 0.5 ml OF RAT AND HUMAN PLASMA USING THE 0.5-ml METHODOLOGY

Compound	Concentration (ng/ml)	Recovery (%)		
		Dog	Rat	Human
Remacemide	698	90.1 ± 1.4	81.2 ± 0.9	87.1 ± 2.0
	1740	88.4 ± 3.7	78.0 ± 2.8	85.4 ± 1.4
Internal standard	498	87.1 ± 2.4[a]	92.6 ± 1.4	85.6 + 0.8

[a] n = 6.

ume dog method (0.5 ml) shows that there is essentially no change in recovery, and hence no loss of sensitivity, with the smaller volume samples.

Accuracy and precision. Using the rat plasma method, the percentage of I found at three different concentrations is presented in Table III for freshly spiked samples and for samples spiked and then frozen at −20°C for 7 or 26 days. The mean percentage found was 100 ± 6.04% (n = 44) resulting in an overall method accuracy of 0.00% and a precision of 6.04%. These results demonstrate that I is stable in plasma samples for at least 26 days. The extracted standard curve was also found to be stable for up to 7 days when stored in a refrigerator at 4°C.

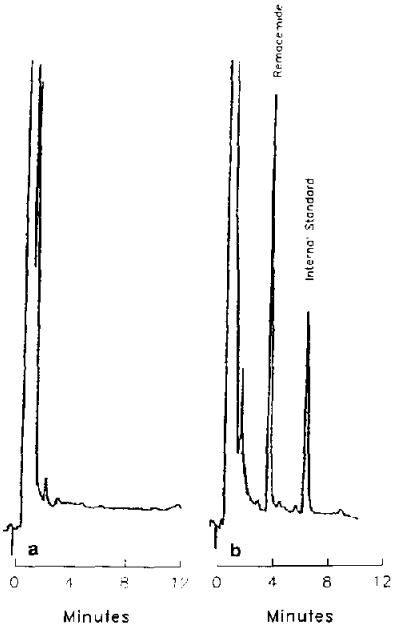

Fig. 3. Typical chromatograms of dog plasma (1 ml). (a) Blank dog plasma; (b) blank dog plasma spiked with 498 ng/ml I and 498 ng/ml internal standard.

Urine

Typical chromatograms for rat urine are presented in Fig. 4. The standard curve was linear for both rat and dog urine over the concentration range studied. For regression analysis the curve was truncated at 498 ng/ml. For rat urine, the low curve (9.96–498 ng/ml) y-intercept was −0.0112, and the slope was 0.0038 (r^2 = 0.9997); the high curve (99.6–2490 ng/ml) y-intercept was

TABLE III

MEAN (± S.D.) PERCENTAGE FOUND (n = 5) FOR I IN RAT PLASMA AND STABILITY OF SPIKED AND FROZEN SAMPLES

Concentration (ng/ml)	Fresh	Frozen for 7 days	Frozen for 26 days
21.3	108.8 ± 6.9	101.4 ± 8.9[a]	102.2 ± 6.6
1190	97.0 ± 4.0	96.6 ± 4.8	98.8 ± 3.8
2130	99.1 ± 2.9	101.7 ± 3.9	96.3 + 3.2

[a] n = 4.

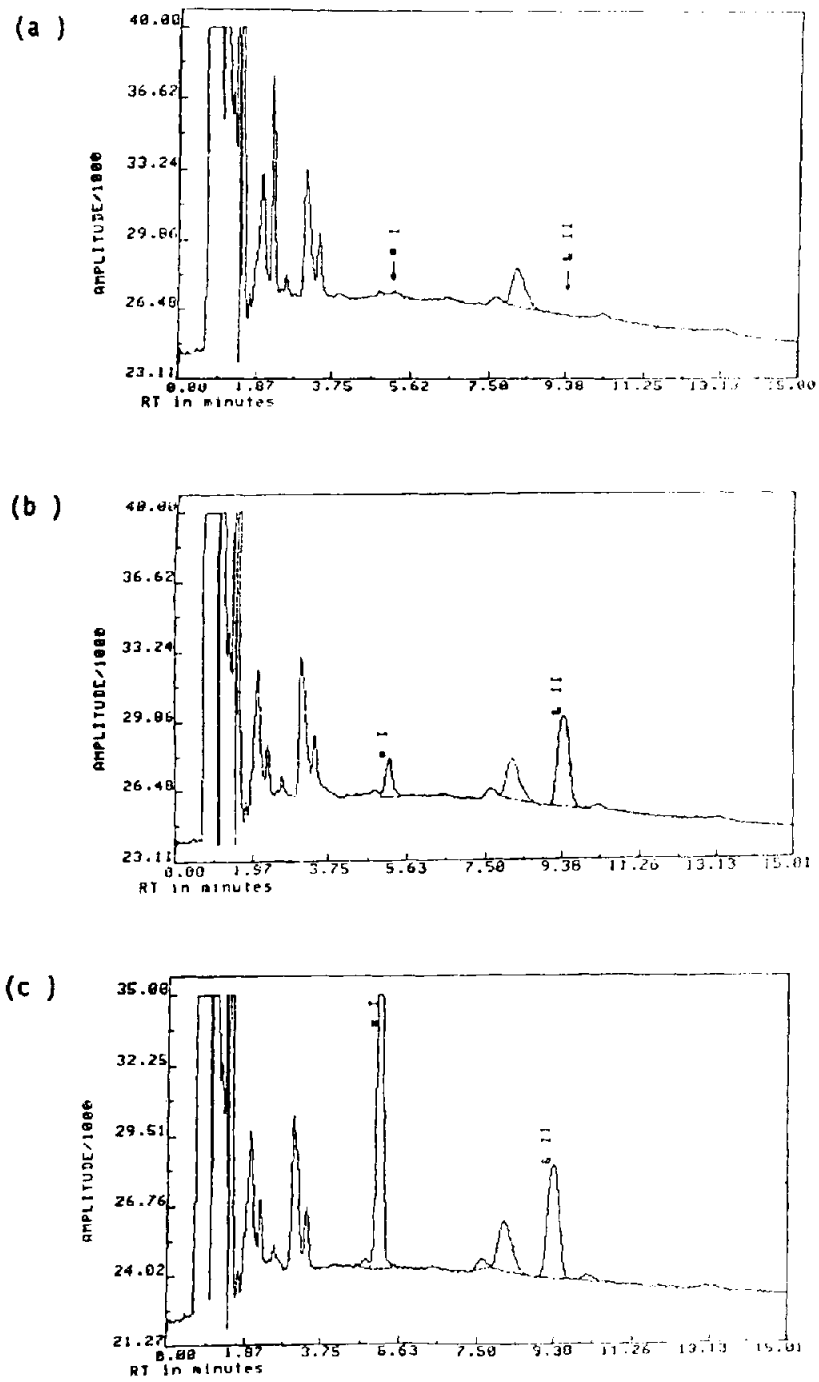

Fig. 4. Typical chromatograms of rat urine extracts (1 ml). (a) Blank rat urine; (b) blank rat urine spiked with 49 ng/ml I and 498 ng/ml internal standard; (c) blank rat urine spiked 498 ng/ml I and 498 ng/ml internal standard.

TABLE IV

RECOVERY OF I AND INTERNAL STANDARD FROM 1 ml OF RAT OR DOG URINE

Compound	Concentration (ng/ml)	Rat		Dog	
		Recovery (%)	n	Recovery (%)	n
Remacemide	698	57.8	2	64.3 ± 2.3	3
	1740	61.1 ± 0.6	3	66.4 ± 0.5	3
Internal standard	498	54.6 ± 1.2	11	54.5 ± 2.6	6

TABLE V

MEAN (± S.D.) PERCENTAGE FOUND (n = 5) FOR I IN RAT URINE AND STABILITY OF SPIKED AND FROZEN SAMPLES

Concentration (ng/ml)	Fresh	Frozen for 8 days	Frozen for 30 days
21.3	102.9 ± 7.3	100.5 ± 4.9	97.7 ± 0.8[a]
1190	98.1 ± 0.8[a]	98.8 ± 1.4	94.8 ± 0.9
2130	97.7 ± 0.8	100.1 ± 0.9	96.5 ± 2.8

[a] n = 4.

0.0185 and the slope was 0.0039 (r^2 = 0.9999). Recoveries of I and the internal standard from rat and dog urine were adequate and are shown in Table IV. Recovery of both analytes was similar from rat and dog urine. The minimum quantifiable level for I was 9.96 ng/ml. I was stable in frozen (−20°C) rat urine for up to 30 days (Table V) with a mean percentage found of 98.6 ± 3.8% (n = 43) resulting in an overall method accuracy of −1.4% and a precision of 3.87%. The extracted standard curve was stable for up to 4 days at room temperature.

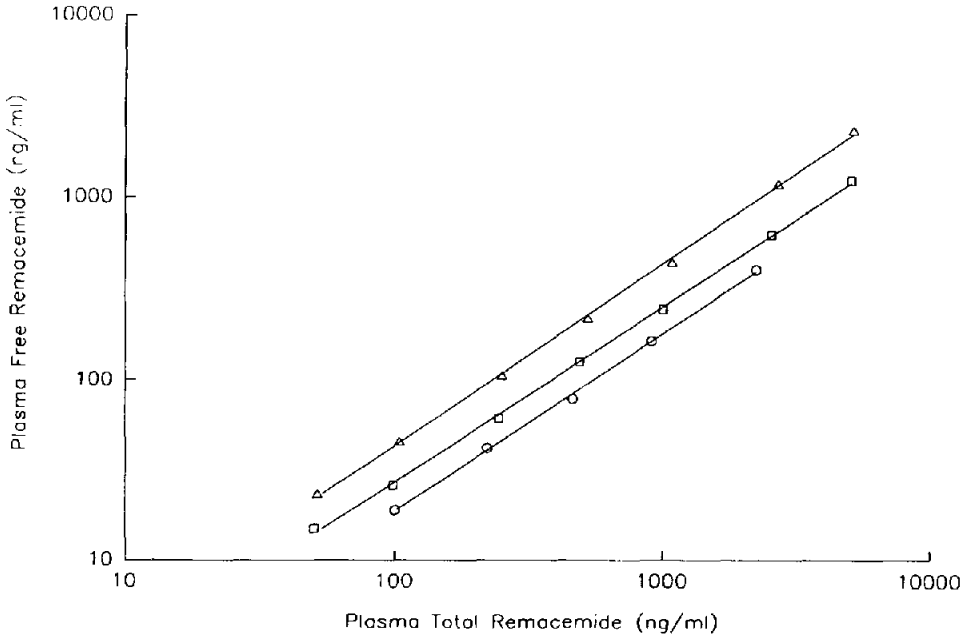

Fig. 5. Plasma free I concentration as a function of plasma total I concentration in rat (○), dog (△) and human (□) plasma.

Plasma protein binding

To assess potential binding of I to the Amicon micropartition cell membrane, I was spiked into control plasma ultrafiltrate at a concentration of 24.9 ng/ml and centrifuged through the cells at 25°C. The filtrate was then assayed for I. No loss of I was observed indicating that I did not adhere to the membrane at these low concentrations and that ultrafiltration was an appropriate method to use for plasma protein binding measurements. Plasma free (unbound) I concentrations are plotted as a function of plasma total concentrations of I for rat, dog and man in Fig. 5. Although the extent of plasma protein binding differed between species, binding was concentration-independent over the anticipated plasma concentration range of I.

DISCUSSION

Rapid and reliable methods have been developed for quantitation of I in rat and dog plasma and urine. These methods were developed to monitor I concentrations during toxicity studies in both rats and dogs and to obtain additional preclinical information on I such as plasma protein binding data and pharmacokinetics [4] in both species.

The plasma methods were originally developed for analysis of 1-ml samples and were subsequently modified for 0.5-ml samples. This is particularly useful for rat studies in which sample volumes are often limited. In situations when the sample volume was less than 0.5 ml, the sample was brought up to volume with control (blank) plasma from the same species. The use of solid-phase extraction columns enables many samples to be processed simultaneously resulting in accurate and reproducible assay of as many as 40 samples per day. The plasma method described here was applied to human plasma from healthy and drug-free volunteers and used for *in vitro* protein binding studies only; the validated methodology for clinical plasma samples will be presented elsewhere.

The clinical efficacy and toxicity of a drug is often more closely related to the free (unbound) concentration in the plasma or serum than to the total concentration. For anticonvulsants, and other centrally active compounds, cerebrospiral fluid (CSF) concentrations are in rapid equilibration with drug at the site of action [5]; for I, plasma free concentrations and CSF concentrations are related in a linear manner. Therefore, plasma free concentrations of I are useful for predicting concentrations of I at the active site [6]. The plasma protein binding of I is linear over the anticipated therapeutic concentration range; for the rat, the *in vitro* data agree well with data obtained following an oral dose of I [6]. The extent to which I was protein-bound in the plasma differed between species, and was 82, 57 and 74% in rat, dog and human plasma, respectively. Species differences in protein binding are not unusual; for example propanalol, quinidine and chlorpheniramine show large interspecies variation in protein binding [7,8]. This may be attributed to differences in binding protein concentrations or in affinity of the protein for the drug.

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REFERENCES

1 M. L. Stagnitto, G. C. Palmer, J. M. Ordy, R. C. Griffith, C. N. Becker, R. J. Gentile, G. E. Garske, J. M. Frankenheim and J. H. Woodhead, *Epilepsy Res.*, 7 (1990) 11.
2 G. C. Palmer, E. W. Harris, J. J. Napier, M. L. Stagnitto, G. E. Garske, R. C. Griffith and E. A. Swinyard, *Prog. Clin. Biol. Res.*, 361 (1990) 435.
3 J. B. Whittam and K. F. Brown, *J. Pharm. Sci.*, 70 (1981) 146.
4 K. R. Case, M. S. Eisman, G. E. Machulskis and S. J. Moore and T. C. M. Wilson, in preparation.
5 M. Danhof and G. Levy, *J. Pharm. Exp. Ther.*, 229 (1984) 44.
6 T. C. M. Wilson, G. E. Machulskis, G. E. Garske and E. W. Harris, *Soc. Neurosci.*, 14 (1988) 1034.
7 Y. Sawada, M. Hanano, Y. Sugiyama, H. Harashima and T. Iga, *J. Pharmacokin. Biopharm.*, 12 (1984) 587.
8 Y. Sawada, M. Hanano, Y. Sugiyama and T. Iga, *J. Pharmacokin. Biopharm.*, 13 (1985) 477.