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High-performance liquid chromatographic method for the simultaneous measurement of remacemide (a novel anticonvulsant and cerebroprotectant) and an active metabolite in human plasma

Janet W. Flynn and James E. O'Brien

Department of Chemistry, Divisional Research and Development, Fisons Pharmaceuticals, Rochester, NY 14603-1710 (USA)

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of both remacemide (a novel anticonvulsant and cerebroprotectant) and an active, major metabolite in human plasma. After the addition of an internal standard, the analytes were extracted from the plasma by ion-exchange solid-phase extraction and measured by an isocratic HPLC system with ultraviolet detection at 210 nm. The recovery of the analytes was >90%. The standard curves were linear over the range of quantitation of approximately 10–500 ng/ml for remacemide itself and 15–250 ng/ml for the metabolite. Both intra-day and inter-day accuracy and precision data were excellent. Remacemide and its metabolite were shown to be stable in human plasma for at least a year when stored at −20°C.

INTRODUCTION

Remacemide hydrochloride (I, Fig. 1), (±)-2-amino-N-(1-methyl-1,2-diphenylethyl)acetamide hydrochloride, is a novel water-soluble anticonvulsant and cerebroprotectant. Compound I is currently in Phase II clinical trials for use in epileptics with generalized tonic/clonic (grand mal) and complex partial seizures. In addition it is being investigated for cerebroprotection in patients undergoing coronary by-pass surgery.

When administered orally to rats and mice, I and its respective desglycine metabolite (II, Fig. 1), (±)-1-methyl-1,2-diphenylethylamine, are active against maximal electroshock (MES)-induced seizures [1–3] and thus, in this respect, have a preclinical profile similar to phenytoin and carbamazepine. Recently compound I was shown to prevent loss of hippocampal CA1 neurons following global ischemia in the rat and dog [4]. This cerebroprotective property could be a result of its conversion to the more active species (compound II). Moreover, compound II possesses non-competitive inhibition of N-methyl-*d*-aspartic acid receptors in the brain, a condition linked to efficacy in animal models of stroke [3–5].

As part of preclinical investigations, methods were developed for plasma level measurement of compound I in rat and dog as well as protein binding in rat, dog and human plasma [6].

Prior to the start of the clinical studies, it was necesary to develop and validate methodology to measure compounds I and II in human plasma. This methodology would have to provide not on-

Correspondence to: J. E. O'Brien, Department of Chemistry, Divisional Research and Development, Fisons Pharmaceuticals, Rochester, NY 14603-1710, USA.

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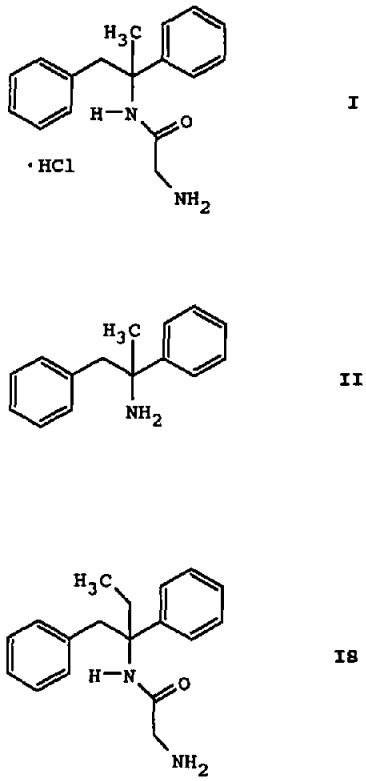

Fig. 1. Structures of I, II and internal standard (IS).

ly enough sensitivity and reliability for a proper understanding of the clinical pharmacokinetics of I but also sufficient selectivity so that co-administered drugs would not constitute an interference to the measurement.

The method presented herein consists of a relatively simple ion-exchange solid-phase extraction procedure followed by the high-performance liquid chromatographic (HPLC) measurement utilizing ultraviolet detection at 210 nm. The methodology provides for the accurate, selective and reliable measurement of I and II in human plasma.

EXPERIMENTAL

Standards and reagents

The analytes, compounds I and II, as well as the internal standard, (±)-2-amino-N-[1-phenyl-1-(phenylmethyl)propyl]acetamide (I.S., Fig. 1) were synthesized in the Department of Chemistry, Fisons Pharmaceuticals (Rochester, NY, USA). Acetazolamide, caffeine, carbamazepine, diazepam, dimethadione, phenobarbital and phenytoin were obtained from Sigma (St. Louis, MO, USA). Ethosuximide and methsuximide were obtained from Parke-Davis (Morris Plains, NJ, USA). Mephenytoin was obtained from Sandoz (East Hanover, NJ, USA), primidone from Wyeth-Ayerst (Philadelphia, PA, USA) and trimethadione from Abbott Labs. (North Chicago, IL, USA). Acetonitrile, methanol and water were HPLC grade and obtained from J. T. Baker (Phillipsburg, NJ, USA). Toluene was high purity grade and from Burdick and Jackson (Muskegon, MI, USA). Sylon-CT was obtained from Supelco (Bellefonte, PA, USA). Potassium phosphate (monobasic, HPLC grade), phosphoric acid (85%, HPLC grade) and sodium bicarbonate (ACS certified) were all obtained from Fisher Scientific (Rochester, NY, USA). The drug-free human plasma used was heparinized and obtained from Biological Specialty (Lansdale, PA, USA).

Solid-phase extraction apparatus

Solid-phase extraction was performed on 1-ml Bond Elut benzenesulfonic (SCX) columns (No. 617101, Analytichem Intenational, Harbor City, CA, USA). The extractions were performed on a Baker-10 SPE System vacuum manifold box (J. T. Baker).

Chromatographic system

The chromatographic system consisted of a Waters Model 510 pump operated at a flow-rate of 1.2 ml/min and a Waters WISP Model 710B autoinjector with a 50-μl injection volume (Waters Assoc., Millipore, Milford, MA, USA). The UV detector was a Kratos Analytical Spectroflow 757 (Kratos, Ramsey, NJ, USA) set at a 210 nm wavelength. The column was a 100 mm × 4.6 mm I.D. hexyl Spherisorb with 3-μm packing (Keystone Scientific, Bellefonte, PA, USA) maintained at 40°C with a FIAtron Model CH-30 column heater (FIAtron Systems, Oconomowoc, WI, USA).

The mobile phase was a 29:71 mixture of acetonitrile–0.05 *M* potassium phosphate (monobasic) adjusted to pH 3.3 using a 1:3 dilution of HPLC-grade 85% phosphoric acid in HPLC-grade water. The mobile phase was filtered through an 0.45-μm Nylon-66 Millipore filter and degassed prior to use.

Autosampler vials, low-volume inserts, Teflon-lined septums and screw caps were obtained from Sunbrokers (Wilmington, NC, USA). The chromatographic performance was monitored on a conventional strip-chart recorder set to 10 mV input. The data were generated using a Hewlett-Packard 3357 laboratory data system (Hewlett-Packard, Palo Alto, CA, USA).

Glassware preparation and handling

It was determined that the method is especially sensitive to contaminants from glassware and adsorptive sites on the glass surfaces. Therefore it was found necessary to carefully wash and rinse all the glassware used in the assay and to deactivate, by silanization, the glassware involved in the final elution and injection steps.

Any glassware that will contain solvents was carefully washed and rinsed; it was then rinsed with the solvent that it will contain. Similarly, glassware that will contain aqueous solutions were carefully rinsed with HPLC-grade water prior to use. The 75 mm × 10 mm disposable borosilicate glass tubes that collect the final column elution and the low-volume glass inserts for the autosampler vials were silanized with a pasteur pipet application of Sylon-CT and left standing a few minutes. The Sylon-CT was removed with a pasteur pipet aspirator and the glassware was rinsed first with the high-purity toluene followed by HPLC-grade methanol. The glassware was then placed in a 60°C oven for 2 h to complete the silanization.

Preparation of standard solutions

A standard stock solution of compound I was prepared by weighing approximately 5 mg of the salt (88.04% base) placing it in a 50-ml volumetric flask and dissolving and bringing to volume in HPLC-grade water. Dilutions of 1:5, 1:25 and 1:100 were made of this stock solution to provide solutions approximating 20, 4 and 1 ng drug base per μl of water.

A stock solution of the metabolite, compound II, was likewise prepared by weighing approximately 7 mg of the hydrochloride salt (84.03% base). Dilutions were made to provide solutions approximating 10, 2 and 1 ng base per μl of water.

The I.S. solution was prepared by weighing approximately 3 mg of the hydrochloride salt (88.56% base) into a 50-ml volumetric flask, dissolving it and bringing to volume in HPLC-grade water. This solution was diluted 1:10 to provide a solution approximating 6 ng base per μl of water.

These dilutions were added to 1 ml of human plasma at appropriate concentrations and volumes (50 μl or less) to provide the method data.

Plasma extraction procedure

Plasma samples (1 ml) were placed in 75 mm × 12 mm glass, disposable culture tubes and 35 μl of the I.S. solution, approximating 200 ng of the I.S., were added. The sample tubes were gently vortex-mixed. Then 1.5 ml of 0.05 *M* potassium phosphate (monobasic, pH 2.5) were added to each sample and gently vortex-mixed. The samples were then applied to the benzenesulfonic acid (SCX) solid-phase extraction columns which had been previously conditioned with 1 ml of methanol, 1 ml of elution fluid (acetonitrile–0.05 *M* sodium bicarbonate, 60:40) and 2 ml of 0.05 *M* potassium phosphate (monobasic). The columns were not allowed to dry before the addition of the plasma samples. The samples were placed on the columns and subsequently washed with 1 ml of 0.05 *M* potassium phosphate which was first placed in the empty sample tube to effect quantitative transfer of the sample. The columns were further rinsed with 3 × 0.5 ml of methanol and finally with 2 ml of water. The columns were then allowed to dry.

The analytes and I.S. were then eluted from the columns into the 75 mm × 10 mm silanized culture tubes with 3 × 0.25 ml of the elution fluid: acetonitrile–0.05 *M* sodium bicarbonate (60:40, v/v).

The samples were then transferred to the silanized glass autosampler vial inserts, and 50 μl were injected into the chromatographic system.

Determination of recovery

The recovery or extraction efficiency of the plasma extraction procedure for both analytes and the I.S. was determined in triplicate. The data of extracted plasma samples spiked with the analytes at 25 and 75% of the range of quantitation and the I.S. at its concentration was compared to absolute standards. The absoltue standards were prepared by direct solution to account for any loss, dilution or concentration effects of the extraction procedure.

Determination of accuracy and precision

Intra-day accuracy and precision were determined by the analysis of an eight-point standard curve in triplicate. The standard curves were prepared by adding known amounts of each analyte to blank heparinized human plasma. The plasma concentrations were selected to span the desired range of quantitation: 9.36–510 ng/ml for I and 14.6–246 ng/ml for II. The twenty-four samples were then taken through the extraction procedure and followed immediately by injection into the chromatographic system. Accuracy was assessed by re-fitting each individual area ratio (area analyte/area I.S.) back into a power regression equation to obtain its predicted concentration value. This value was then used to compute the percentage difference between the amount found and the amount added [(amount found − amount added)/amount added × 100]. The accuracy value was obtained by taking the absolute mean of the percentage difference values and was evaluated at each concentration and as the mean over the entire concentration range.

Precision, percentage relative standard deviation, was also evaluated at each concentration and as the mean over the entire concentration range.

Inter-day accuracy and precision were determined by repeating the intra-day evaluation with duplicate standards analyzed on each of three separate days. These standards were prepared from plasma pools containing each analyte at each concentration level. Six 1.0-ml aliquots were taken at each concentration level and stored frozen until analysis. The overall inter-day accuracy and precision data were then calculated exactly as the intra-day except that each concentration level is represented by six data points.

TABLE I

DRUGS USED IN SELECTIVITY TEST

Drug	Plasma concentration tested (μg/ml)
Acetazolamide	14.6
Caffeine	10.4
Carbamazepine	12.1
Diazepam	0.868
Dimethadione	446
Ethosuximide	90.2
Mephenytoin	20.2
Methsuximide	0.0816
Phenobarbital	40.0
Phenytoin	20.2
Primidone	19.9
Trimethadione	30.0

Selectivity

Selectivity of the assay was assessed not only for heparinized human plasma, the reagents and equipment used during the analysis but also against caffeine and possible co-administered drugs and their metabolites at anticipated concentrations that may be encountered during the analysis of samples from clinical studies. Table I shows the drugs and their highest anticipated therapeutic concentrations that were used in this evaluation [7–9].

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms for the analysis of I and II in human plasma from subjects on concomitant medications are shown in Fig. 2. Control (blank) human plasma was free of interfering endogenous substances at the retention times of

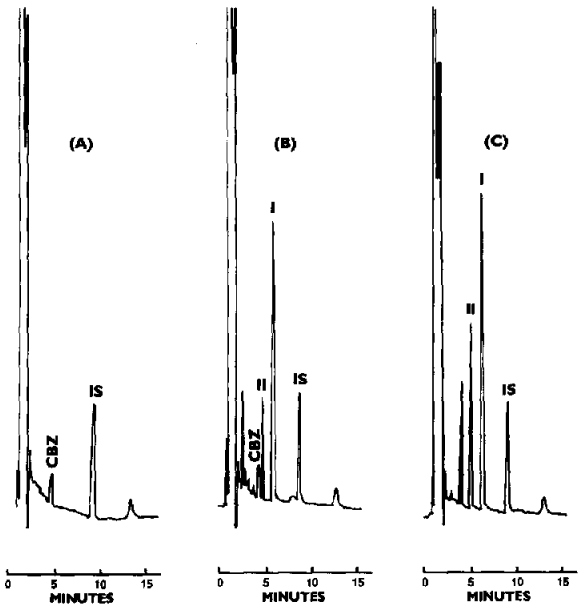

Fig. 2. Representative chromatograms for (A) a subject on concomitant carbamazepine (CBZ) before start of remacemide therapy, (B) the same subject on remacemide therapy showing 102 ng of II per ml plasma and 340 ng of I per ml plasma and (C) a plasma standard of 187 ng of II per ml plasma and 382 ng of I per ml plasma.

the analytes and I.S. Good resolution and peak symmetry were obtained for the analytes and I.S.

The standard curves (n = 9) demonstrated excelent linearity over the concentration range 9.36–510 ng/ml for I and 14.6–246 ng/ml for II with regression coefficients (b) averaging 0.989 and correlation coefficcients (r) averaging 0.999.

TABLE II

RECOVERY OF ANALYTES AND INTERNAL STANDARD

Analyte	n	Added (ng/ml)	Recovery (mean ± S.D.) (%)
Remacemide (I)	3	126	94.4 ± 4.41
		382	92.8 ± 2.01
Metabolite (II)	3	65.6	90.4 ± 6.41
		186	93.4 ± 2.17
(I.S.)	4	227	93.4 ± 0.462

Recovery

The recovery or extraction efficiency of the analytes was assessed at two concentrations approximating 25 and 75% of the range of the standard curve. The I.S. recovery was determined at the concentration used in the assay procedure. In all cases the recovery was >90% (Table II).

Accuracy and precision

Both intra-day and inter-day accuracy and precision were determined as described over the plasma concentration range 9.36–510 ng/ml for I and 14.6–246 ng/ml for II. Eight concentration points in triplicate for each analyte were evaluated for intra-day accuracy and precision (n = 24). The same eight points in duplicate were evaluated over three separate analysis days for the inter-day accuracy and precision data (n = 48). A summary of the observed data is shown in Tables III and IV. The minimum quantifiable level (MQL) was 9.36 ng/ml for I and 14.6 ng/ml for II.

TABLE III

ACCURACY AND PRECISION DATA FOR I IN PLASMA

Concentration (ng/ml)		Accuracy (mean %) difference)	Precision (% R.S.D.)
Added	Mean found		
Intra-day (n = 24)			
9.36	9.30	7.36	10.0
25.5	25.9	13.7	19.0
51.0	49.6	7.64	8.95
102	104	3.40	4.58
153	147	3.92	2.96
221	224	5.13	7.14
374	395	5.70	2.71
510	491	3.79	2.65
Mean		6.33	7.25
Inter-day (n = 48)			
9.36	9.93	17.6	19.3
25.5	24.9	7.26	10.6
51.0	50.0	4.67	7.44
102	98.5	5.10	6.88
153	148	3.59	3.36
221	220	5.35	6.91
374	385	3.48	3.06
510	528	4.64	3.75
Mean		6.46	7.66

TABLE IV

ACCURACY AND PRECISION DATA FOR II IN PLASMA

Concentration (ng/ml)		Accuracy (mean % difference)	Precision (% R.S.D.)
Added	Mean found		
Intra-day (n = 24)			
14.6	14.7	5.48	7.96
20.1	20.5	3.48	5.36
40.1	37.5	6.40	2.00
59.3	61.8	5.46	5.82
91.2	89.4	2.38	3.32
146	144	5.25	6.74
201	207	2.82	1.48
246	244	8.01	10.8
Mean		4.91	5.44
Inter-day (n = 48)			
14.6	15.2	9.58	10.4
20.1	19.4	9.45	13.4
40.1	38.7	5.15	4.91
59.3	61.0	4.02	4.34
91.2	91.9	4.31	5.22
146	150	6.40	8.00
201	194	5.47	5.31
246	248	2.23	2.64
Mean		5.83	6.78

In addition, samples containing I and II that had been stored frozen up to a year were analyzed and the resulting data fell within the documented accuracy and precision data.

Selectivity

Selectivity of the assay was assessed not only for the reagents and equipment used during the analysis but also against a large number of samples of control heparinized plasma. In all cases, the peaks of interest were well resolved from any background peaks or endogenous materials in the matrix.

It was imperative to challenge the selectivity of the assay against possible co-administered anticonvulsant-type drugs. The following eleven possible concomitant therapeutic agents (or metabolites) as well as caffeine were checked for interference in the assay: acetazolamide, carbamazepine, diazepam, dimethadione, ethosuximide, mephenytoin, methsuximide, phenobarbital, phenytoin, primidone, trimethadione. This evaluation included not only adding these substances to control plasma at the highest anticipated concentration but also the analysis of plasma from patients on some of these drugs. Valproate, another common therapeutic agent in this area, was not tested as such but plasma samples from several subjects on a regimen of valproate were analyzed and showed no interferences to the assay.

It was obvious that many of the twelve substances tested had completely different acid–base characteristics and thereby extraction profiles from the analytes. Therefore, of the twelve substances tested, only three were partially extracted and appeared in the chromatographic window of the assay and only one, phenytoin, constituted a significant interference. Table V provides a summary of the results.

Since only phenytoin presented problems with the selectivity of the assay, it was found that substituting a 100 mm × 4.6 mm I.D., 5-μm ODS Partisil (Keystone Scientific) column provided the necessary selectivity. However, this column did not provide enough selectivity in other respects, particularly against carbamazepine and its metabolites, and therefore would only be used to analyze the plasma of subjects on concomitant phenytoin. If a subject is on both phenytoin and carbamazepine as concomitant medications, it is necessary to analyze the extracted sample on both columns; the hexyl column is used to measure II where phenytoin and I coelute (Table V)

TABLE V

RETENTION TIMES OF REMACEMIDE AND SOME OTHER COMPOUNDS ON HEXYL SPHERISORB

Analyte/compound	Retention time (min)
I	6.0
II	5.2
IS	9.0
Carbamazepine	4.5
Phenytoin	6.1
Diazepam	17

and the ODS column to measure I where carbamazepine/metabolite and II coelute.

Verification of the integrity of the analyte peaks by liquid chromatography–mass spectrometry was performed initially on standards and eventually on the samples from clinical studies.

CONCLUSIONS

The procedure reported here for the measurement of I and II in human plasma is selective, reproducible, accurate and precise. Solid-phase extraction of the plasma followed by HPLC–UV measurement provided a convenient method to simultaneously measure I and II in human plasma. Plasma samples containing I and II and stored frozen at −20°C for one year and then analyzed were found to be stable. The assay procedure described herein has successfully been utilized in the analysis of hundreds of samples from clinical studies.

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