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Simple and rapid micro-analytical procedures for the estimation of milacemide and its metabolite glycinamide in rat plasma and cerebrospinal fluid by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic technique is described for the determination of milacemide and its primary metabolite glycinamide in rat plasma and cerebrospinal fluid. Milacemide and glycinamide are derivatized with fluorescamine to form a chromophore and a fluorophore and subsequent analysis using ultraviolet and fluorescence detectors, respectively. The extraction procedures are simple with a limit of detection 2 and 0.5 μg/ml for milacemide in plasma and cerebrospinal fluid, respectively, and 0.5 μg/ml for glycinamide in plasma or cerebrospinal fluid. The within-batch coefficients of variation for both analytes were less than 3%. Since only a small amount of sample is required, these techniques are well suited for the study of milacemide pharmacokinetics in the rat.

INTRODUCTION

Milacemide (2-*n*-pentylaminoacetamide), a new drug which has been shown to have significant anticonvulsant [1,2] and antimyoclonic [3] activity and to enhance cognitive function [4,5], is presently undergoing extensive clinical evaluation. Milacemide has a unique metabolic pathway in that it readily crosses the blood–brain barrier (BBB) and is subsequently transformed in the brain by the enzyme monoamine oxidase-B to glycinamide and then to the inhibitory amino acid neurotransmitter glycine [6–9]. Glycinamide and glycine derived from the periphery do not transgress the BBB. As glycine is thought to be responsible for the actions of milacemide in the brain, milacimide can be considered a prodrug.

We have developed rapid high-performance liquid chromatographic (HPLC) analytical procedures for the measurement of milacemide and glycinamide by UV and spectrofluorometric detection and simple extraction procedures, which are suited for the determination of the pharmacokinetics of milacemide and glycinamide in blood and cerebrospinal fluid (CSF) of the rat.

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EXPERIMENTAL

Materials

Milacemide, CP2081AG (2-*n*-pentylaminobutyramide, I) and gabamide (4-aminobutyramide) were obtained from Continental Pharma (Mont-Saint-Guibert, Belgium). Glycinamide and fluorescamine were purchased from Sigma (Poole, U.K.) and Aldrich (Gillingham, U.K.), respectively. Methanol (FSA, Loughborough, U.K.) and acetonitrile (BDH, Poole, U.K.) were of HPLC grade and all other chemicals were of analytical grade.

Stock solutions of milacemide and glycinamide were constituted in water to a concentration of 1.0 mg/ml. These solutions were observed to be stable for at least three months at 4°C. Working standards were made by diluting the stock solutions with drug-free CSF or plasma on the day of analysis.

Extraction and derivatization procedures

Milacemide assay in CSF. To 0.5-ml polypropylene tubes, 10 μl of CSF and 10 μl of internal standard (50 μg/ml I) in 0.5 *M* borate buffer (pH 9.5) and 10 μl of fluorescamine in acetonitrile (0.5 mg/ml) were added. After mixing thoroughly, 5–10 μl were injected into the chromatograph.

Milacemide assay in plasma. Plasma (10 μl) was first deproteinized by mixing with 20 μl of acetonitrile containing 25 μg/ml internal standard (I) in a polypropylene tube. After centrifugation for 3 min at 10 500 *g* in a TDX centrifuge (Abbott, Maidenhead, U.K.), 20 μl of supernatant were removed. To this supernatant, 20 μl of 0.5 *M* borate buffer (pH 9.5) and 20 μl of fluorescamine in acetonitrile (0.5 mg/ml) were added and mixed thoroughly. A 5–10 μl aliquot of the mixture was injected into the chromatograph.

Glycinamide assay. The extraction and derivatization procedure for glycinamide is similar to that of milacemide except that 0.1 *M* phosphate buffer (pH 9.5) and a fluorescamine concentration of 0.1 mg/ml were used. Gabamide was used as the internal standard; 20 μg/ml were used for the CSF assay and 50 μg/ml for the plasma assay.

Milacemide and glycinamide concentrations were determined by the ratio of the peak areas of each drug to the peak area of their respective internal standard.

Instrumentation

The HPLC system used consisted of a Model SP 8700 solvent delivery system and a Model SP 4270 integrator–printer plotter (Spectra-Physics, Maidenhead, U.K.). The analytical column was Hypersil 5ODS (5 μm particle size, 15 cm × 4.6 mm I.D., Hichrom, Reading, U.K.) attached to a Zorbax ODS cartridge guard column (1.25 cm × 4 mm I.D., Du Pont, Wilmington, DE, U.S.A.). A Spectroflow 783 UV detector (Kratos, Ramsey, NJ, U.S.A.), set at an absorption wavelength of 313 nm, was used for the detection of milacemide and a Model FS 970 spectrofluorometer (excitation wavelength 395 nm and emission filter cut-off

470 nm; Kratos) was used for the detection of glycinamide. For milacemide the mobile phase was acetonitrile–borate buffer (0.05 mol/l, pH 7.0) (12:82, v/v) and for glycinamide methanol–phosphate buffer (0.025 mol/l, pH 6.5) (33:67, v/v). In the plasma glycinamide assay, the methanol concentration was 31%. The flow-rate was set at 1.0 ml/min. The mobile phase was filtered through a 0.45-μm Millipore filter and degassed with helium before use.

RESULTS AND DISCUSSION

Because milacemide and glycinamide have no native absorbance or fluorescence properties, their assay in biological samples is only possible after derivatization. Glycinamide, a primary amine, can react with fluorescamine to give a fluorescent product fluorophore [10] and milacemide, a secondary amine, can be transformed by fluorescamine into non-fluorescent aminoenone chromophore with an absorption maximum of 310–330 nm [11]. These derivatizations combined with simple extraction procedures provide the basis for the sensitive HPLC procedure described above.

Derivatized milacemide, glycinamide and their corresponding internal standards were observed to be stable for at least 24 h at room temperature at pH 9.5. Thus overnight analysis using an autosampler is possible. A linear relationship between the peak-area ratios (drug/internal standard) and the concentrations of milacemide and glycinamide was confirmed by adding known amounts of drugs to blank plasma and CSF (2.5–750 μg/ml for milacemide, 1–40 μg/ml for glycinamide) and subjecting these to the derivatization and extraction procedures and chromatography. Quantitation was achieved by the peak-area ratios of the drugs to internal standard. These were linearly related over the ranges studied.

The minimum detectable concentration in injection samples was 2 and 0.5 μg/ml for plasma and CSF milacemide, respectively, and 0.5 μg/ml for plasma and CSF glycinamide.

Within-batch precision was determined from analysis of pooled plasma of CSF samples containing milacemide and glycinamide at two different concentrations. Coefficients of variation were less than 3% (Table I).

The relative analytical recovery (extractability) from plasma (after deproteinization with acetonitrile) and CSF at different concentrations for milacemide and glycinamide are shown in Table II. Recoveries, calculated by comparing the drug-spiked plasma or CSF with the actual added concentrations, ranged from 93 to 100% for milacemide and 88 to 102% for glycinamide.

Figs. 1 and 2 show typical chromatograms of milacemide and glycinamide, respectively. Blank plasma and CSF spiked with commonly prescribed anticonvulsant drugs (phenobarbitone, phenytoin, carbamazepine, ethosuximide and sodium valproate) were analyzed for possible chromatographic interference. These drugs did not interfere with the analysis. Possible interference by metabolites of these drugs has not been assessed. Fig. 3 shows a typical kinetic profile of milace-

TABLE I

WITHIN-BATCH PRECISION FOR THE DETERMINATION OF MILACEMIDE AND GLYCINAMIDE IN SPIKED RAT PLASMA AND CSF (n = 8–10)

Concentration added (μg/ml)	Plasma		CSF	
	Concentration measured (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration measured (mean ± S.D.) (μg/ml)	C.V. (%)
Milacemide				
25	24.3 ± 0.8	3.4	23.9 ± 0.5	2.0
100	95.0 ± 2.3	2.4	97.2 ± 1.7	1.8
Glycinamide				
5.0	4.4 ± 0.1	1.4	–	–
12.5	–	–	12.1 ± 0.2	1.6
20.0	19.2 ± 0.2	0.9	–	–
50.0	–	–	50.1 ± 1.0	2.0

mide and glycinamide in rats after intraperitoneal administration of 400 mg/kg milacemide.

In conclusion, we describe HPLC procedures which are simple and rapid for the assay of milacemide and glycinamide. These procedures require only small amounts of sample and consequently the methods are ideally suited for pharmacokinetic studies in the rat where only small samples would be available.

TABLE II

RECOVERY OF MILACEMIDE AND GLYCINAMIDE FROM PLASMA AND CSF

Drug	Concentration (μg/ml)	Recovery (mean ± S.D., n = 8–10) (%)	
		Plasma	CSF
Milacemide	25	97.3 ± 3.2	95.4 ± 0.8
	100	95.0 ± 2.3	97.2 ± 1.7
Glycinamide	5.0	87.4 ± 1.2	–
	12.5	–	96.6 ± 1.5
	20.0	95.8 ± 0.9	–
	50.0	–	100.1 ± 2.0

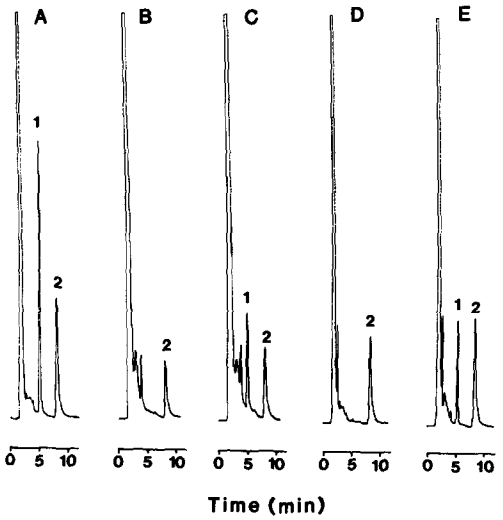

Fig. 1. Chromatograms of milacemide. (A) Standard solution (50 μg/ml milacemide and I); (B) blank plasma; (C) plasma sample 1.5 h after intraperitoneal administration of milacemide (400 mg/kg); (D) blank CSF; (E) CSF sample 1.5 h after intraperitoneal administration of milacemide (400 mg/kg). Peaks: 1 = milacemide; 2 = I. The injection volume was 5 μl.

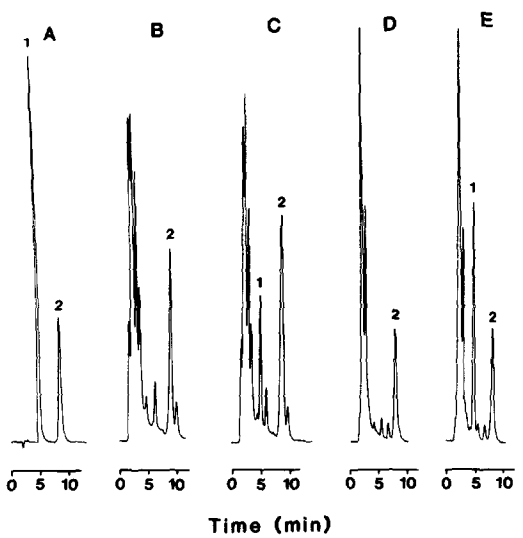

Fig. 2. Chromatograms of glycinamide. (A) Standard solution (20 μg/ml glycinamide and gabamide); (B) blank plasma; (C) plasma sample 0.5 h after intraperitoneal administration of milacemide (400 mg/kg); (D) blank CSF; (E) CSF sample 2.5 h after intraperitoneal administration of milacemide (400 mg/kg). Peaks: 1 = glycinamide; 2 = gabamide. The injection volume was 5 μl.

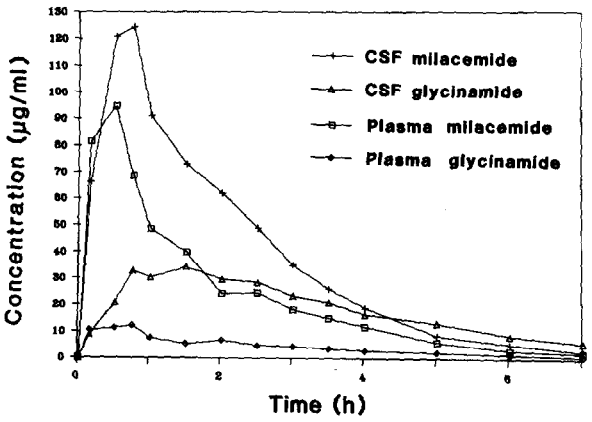

Fig. 3. Time course of milacemide and glycinamide in rat plasma and CSF after intraperitoneal administration of milacemide (400 mg/kg).

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