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Simultaneous determination of felbamate and four metabolites in rat cerebrospinal fluid by high-performance liquid chromatography

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ABSTRACT

An isocratic liquid chromatographic method for direct sample injection has been developed for the quantitation of felbamate and four metabolites in rat cerebrospinal fluid. The method uses 0.050- or 0.025-ml aliquots of cerebrospinal fluid diluted with equal volumes of internal standard. Chromatography is performed on a 150 mm × 4.6 mm I.D. Spherisorb ODS2, 3-μm HPLC column eluted with a phosphate buffer–acetonitrile–methanol (820:120:60, v/v/v) mobile phase and ultraviolet absorbance detection at 210 nm. The linear quantitation ranges are: felbamate and the 2-hydroxy metabolite 0.195–200 μg/ml, the propionic acid metabolite 0.195–50.0 μg/ml, the *p*-hydroxy metabolite 0.781 to 50.0 μg/ml, and the monocarbamate metabolite 0.098–50.0 μg/ml.

INTRODUCTION

Felbamate (FBM) is a novel orally active and relatively nontoxic antiepileptic drug (AED). Its unique profile of activity has been demonstrated in both laboratory animals [1–3] and humans [4,5]. In addition, studies with laboratory animals have also shown significant neuroprotectant properties of FBM *in vitro* [6] and *in vivo* [7]. FBM is a lipophilic, water-insoluble, non-ionic compound. In animals, it is rapidly absorbed from an oral dose, distributed into tissues and excreted mostly in the urine [8]. The drug is also metabolized in animals and humans to two hydroxylated metabolites, a monocarbamate metabolite [9,10], and a propionic acid metabolite [11] (see Fig. 1 for structures).

Methods for the simultaneous determination of FBM and AEDs in human plasma [12,13] and FBM and metabolites in human plasma [14] have been reported. Methods for the determination of FBM and metabolites in rat and dog plasma [15],

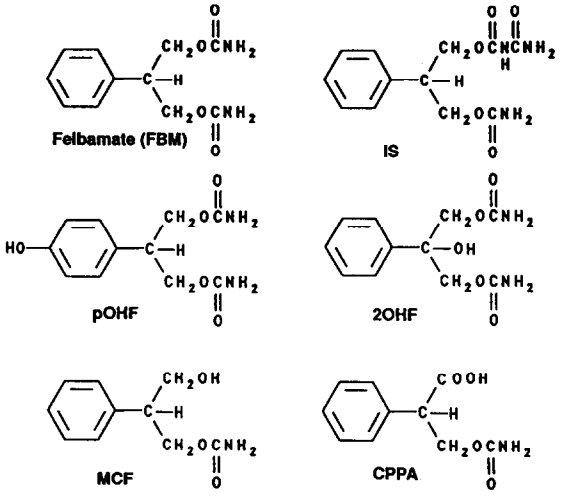

Fig. 1. Structure of felbamate, metabolites, and internal standard.

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and FBM in dog plasma [16] have also been published. A procedure for determination of FBM and its metabolites in rat brain and heart tissue has recently been published [17].

Determination of the distribution characteristics of FBM and metabolites into the central nervous system (CNS) were necessary to support *in vivo* neuroprotective studies of FBM in rats. Other reported methods for the analysis of AEDs in low volume cerebrospinal fluid (CSF) samples by HPLC involve either liquid extraction of a 0.020-ml sample [18], or direct injection from a 0.080-ml sample [19]. This paper describes a direct injection HPLC method for the quantitation of felbamate and four metabolites in low volume, 0.050 to 0.025 ml, rat CSF samples.

EXPERIMENTAL

Chemicals

Felbamate (FBM, 2-phenyl-1,3-propanediol dicarbarmate), its metabolites, 2-(4-hydroxyphenyl)-1,3-propanediol dicarbamate (*p*OHF), 2-hydroxy-2-phenyl-1,3-propanediol dicarbamate (2OHF), 2-phenyl-1,3-propanediol monocarbamate (MCF), 3-carbamoyloxy-2-phenylpropionic acid (CPPA), and the internal standard (I.S.), 2-phenyl-3-carbamoyl-3-oxypropyl allophanate, were from Wallace Laboratories (Cranbury, NJ, USA).

Acetonitrile and methanol glass distilled were from Burdick & Jackson (Muskegon, MI, USA). All other chemicals were reagent grade. Ultrapure water was obtained from a Barnstead NANOpure II Water System (Barnstead/Thermolyne, Dubuque, IA, USA). Control rat CSF was from Wallace Laboratories.

Preparation of standard solutions

Standards were prepared by diluting a methanol stock solution containing 2.0 mg/ml FBM and 2OHF, and 1.0 mg/ml *p*OHF, MCF, and CPPA to the highest concentration with I.S. solution (10.0 μg/ml I.S. in 20% acetonitrile in 30 m*M* phosphate buffer pH 3.0). Serial two-fold or four-fold dilutions of the highest standard concentration solution containing 200.0 μg/ml FBM and 2OHF, and 50.00 μg/ml *p*OHF, MCF, and CPPA with the I.S. solution gave the solvent standard series of the ten concentrations used. The standard solution series concentrations of FBM and 2OHF were 200.0, 50.00, 12.50, 3.125, 1.563, 0.7813, 0.3906, 0.1953, 0.0977, and 0.0000 μg/ml; concentration of *p*OHF, MCF, and CPPA were 50.00, 12.50, 3.125, 0.7813, 0.3906, 0.1953, 0.0977, 0.0488, 0.0244, and 0.0000 μg/ml. All solutions contained 10.00 μg/ml of I.S. I.S. solution was used as the 0.0000 μg/ml standard.

Preparation of the cerebrospinal fluid samples for analysis

The assay used a 0.050- to 0.025-ml aliquot of rat control CSF which was pipetted into a 0.5-ml Sarstedt centrifuge tube and spiked with an equal volume of the appropriate standard solution. The tubes were capped and vortex-mixed. Samples were then centrifuged at 2000 *g* at 0°C for 10 min. The CSF sample supernatant was transferred to an autosampler microvial and the vial was capped for analysis.

High-performance liquid chromatography

The HPLC equipment consisted of a Model 590 pump, Model TCM column oven and controller, and Model 715 WISP autoinjector (Waters, Division of Millipore, Milford, MA, USA). The ultraviolet absorbance detector was a Model 783 from ABI (Foster City, CA, USA). A PE-Nelson 2600 chromatography data system (PE-Nelson, Cupertino, CA, USA) was used for all data acquisition and quantitation.

The analytical columns used were Spherisorb ODS2, 3 μm, 150 mm × 4.6 mm I.D. (Keystone Scientific, Bellefonte, PA, USA).

The mobile phase was 30 m*M* aqueous monobasic potassium phosphate (pH 3.0)–acetonitrile–methanol (82:12:6, v/v/v). The flow-rate was 1.0 ml/min. The temperature was 40°C, the pressure 76–100 bar, and the injection volume 20 μl for CSF standards and 10 μl for solvent standards. Ultraviolet absorbance detector settings were: wavelength 210 nm; range, 0.01 AUFS; rise time 2.0 s; output, 10 mV; programmed for autozero, and range reset at each injection.

PE-Nelson 2600 system method parameters were: sampling rate, 0.4 s; peak detection thres-

hold, 0.1 μV/s; area threshold 50 μV s minimum peak width, 15 s; delay, 2.5 min; run time 17.0 min. Typical retention times were: *p*OHF, 3.3 min; 2OHF, 4.5 min; MCF, 6.8 min; FBM, 10.5 min; I.S., 14.5 min.

Calculations

The availability of control CSF for calibration standard preparation is extremely limited. Solvent standards were analyzed in conjunction with CSF standards for the determination of calibration standard equivalency. A total of three individual validation sets comprised of triplicate analyses of both the ten concentration series of solvent and CSF were analyzed for method validation. The individual run sets were analyzed on three different days. The three total run sets used one analytical HPLC column and one HPLC system. Peak heights determined by the PE-Nelson 2600 system were used for all quantitative calculations.

Four concentration points were selected for each analyte to perform concentration-weighted linear regression for each run set using TableCurve [20]. The calibration standards were 50.00, 12.50, 3.125 and 0.000 μg/ml for FBM and 2OHF; and 12.50, 3.125, 0.7813, and 0.000 μg/ml for *p*OHF, MCF, and CPPA. Regression lines were determined for solvent and CSF calibration standards. All regression lines were forced through zero. The concentrations of each analyte in all analyses of CSF standards were back-calculated using the standard regression line coefficients obtained from both the solvent and the CSF calibration standards.

The calculated concentrations were then used to determine the mean concentrations (Mean), relative standard deviations (R.S.D.), relative mean error (R.M.E.), and the total error (T.E.) [21] using the equations:

$$\text{R.S.D.} = 100 \times \text{S.D.}/\text{Mean} \quad (1)$$

$$\text{R.M.E.} = 100 \times [\text{abs}(\text{Mean} - \text{ST}_{\text{conc}})]/\text{ST}_{\text{conc}} \quad (2)$$

$$\text{T.E.} = 100 \times [2 \times \text{S.D.} + \text{abs}(\text{Mean} - \text{ST}_{\text{conc}})]/\text{ST}_{\text{conc}} \quad (3)$$

where S.D. is the standard deviation of the mean and ST_{conc} is the concentration of the analyte in the standard dilution series.

The lower limit of quantitation (LOQ) was set at the lowest standard concentration that had an R.S.D. < 20%, R.M.E. < 15%, and T.E. < 50%. This is in line with recently published guidelines [22].

The absolute recoveries for the CSF standards were calculated by direct comparison of the peak heights of CSF standards with the peak heights of standards in solvent (20% acetonitrile in 30 m*M* phosphate buffer pH 3.0). Individual daily run recoveries were averaged for each concentration. Average recovery was calculated using only the concentration points yielding T.E. values less than 50% from the CSF data analysis.

RESULTS AND DISCUSSION

Analyte recoveries

The mean recoveries (±S.D.) were 101.65% ± 1.22 for FBM, 107.57% ± 2.18 for *p*OHF, 102.89 ± 1.11 for 2OHF, 102.24% ± 0.70 for MCF, 97.85% ± 1.78 for CPPA and 100.69% ± 1.48 for the I.S.

Chromatography

The chromatographic resolution of all five analytes and the I.S. was adequate as illustrated by the chromatogram of a solvent calibration standard containing 12.50 μg/ml FBM and 2OHF, 3.125 μg/ml *p*OHF, MCF, CPPA, and 10.00 μg/ml I.S. (Fig. 2). Baseline resolution of all analytes was observed at all concentrations. A pre-dose CSF sample containing 10.00 μg/ml I.S. is shown in Fig. 3. Minor peaks occurred near the retention time of *p*OHF. These peaks were present at less than one-fourth the lower LOQ and did not significantly interfere with the quantitation of *p*OHF. The chromatogram of a rat CSF sample obtained 36 h after a single oral dose of 100 mg/kg FBM is shown in Fig. 4. This sample contained 16.411 μg/ml 2OHF, 18.408 μg/ml FBM, and 10.00 μg/ml I.S. The determined concentrations of *p*OHF, MCF, and CPPA are less than the lower limit of quantitation in this study sample.

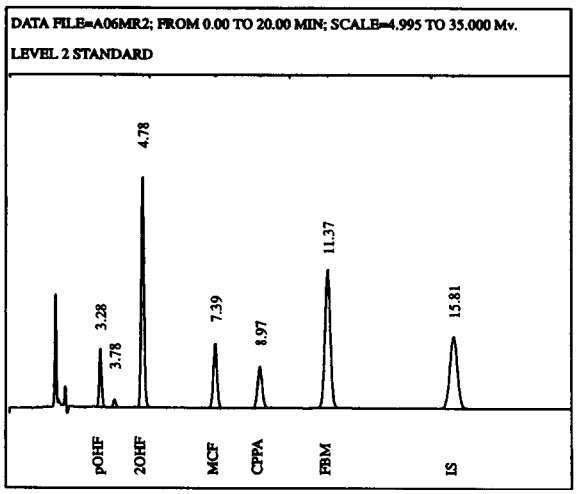

Fig. 2. Chromatogram of solvent calibration standard containing 12.50 μg/ml FBM and 2OHF; 3.125 μg/ml *p*OHF, MCF, CPPA; and 10.00 μg/ml I.S.

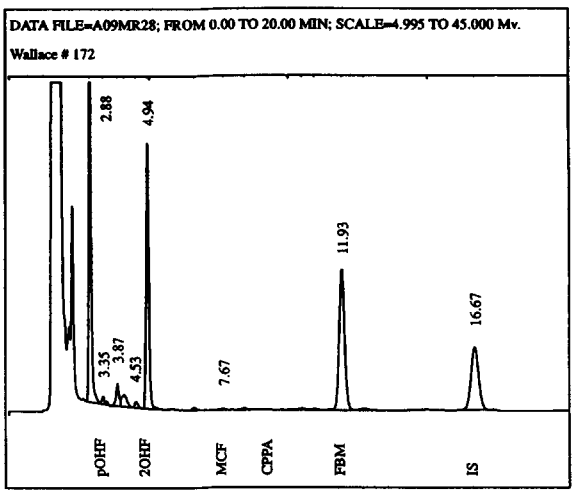

Fig. 4. Chromatogram of a rat CSF study sample containing 16.411 μg/ml 2OHF, 18.408 μg/ml FBM, and 10.00 μg/ml I.S.

Linearity

The solvent and CSF calibration curves were linear as indicated by the correlation coefficients (r^2), which were greater than 0.9999 for FBM, MCF, and CPPA; 0.9981 for *p*OHF; and 0.9998 for 2OHF. All *y*-intercepts, for both solvent and CSF calibration line regressions, were less than $3.607 \cdot 10^{-10}$ as determined by TableCurve linear regression forced through zero. There were no significant differences between the solvent standard and spiked CSF standard calibration line regressions.

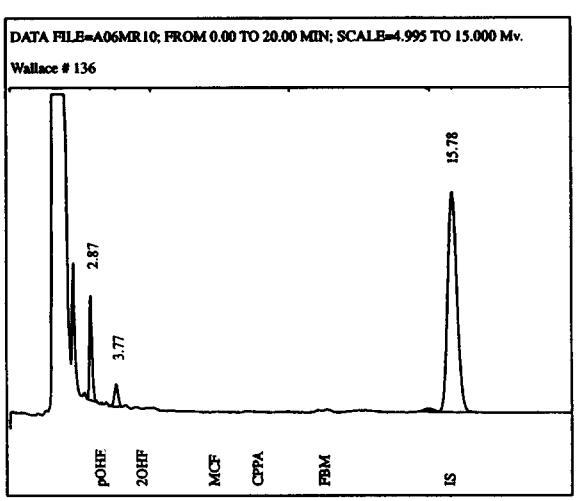

Fig. 3. Chromatogram of a pre-dose rat CSF study sample containing 10.00 μg/ml I.S.

Precision, accuracy and variability

Table I lists the overall precision (R.S.D.), accuracy (R.M.E.) and total error (T.E.) for the limits of quantitation and the calibration concentrations for each analyte calculated by both the solvent and spiked CSF calibration line regressions. There were no significant differences between the determined CSF performance parameters calculated from the solvent calibration or the spiked CSF calibration line regressions. The slightly lower values for accuracy, precision and total error from the CSF calibration for *p*OHF can be attributed to interference from the endogenous background components. The values reflect the combined intra-day and inter-day variability for precision and accuracy.

Routine use experience

Accurate quantitation of felbamate and the four metabolites in CSF can be performed using easily prepared solvent standards. Daily calibration standards do not have to be prepared in CSF.

Routine analysis of samples is performed using the four solvent calibration standards 50.00,

TABLE I

STATISTICAL ANALYSIS OF FELBAMATE AND METABOLITE LEVELS DETERMINED IN SPIKED RAT CSF STANDARDS USING SOLVENT OR CSF CALIBRATION STANDARDS

Standard concentration (μg/ml)	Determined concentration			
	Mean (μg/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)
FBM solvent calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.211	3.93	8.10	16.60
3.125	3.151	0.25	0.83	1.34
12.500	12.554	0.36	0.43	1.15
50.000	49.802	0.19	0.40	0.78
200.000	192.377	0.27	3.81	4.32
FBM CSF calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.211	3.86	8.29	16.64
3.125	3.157	0.20	1.02	1.41
12.500	12.576	0.30	0.61	1.21
50.000	49.892	0.14	0.22	0.49
200.000	192.724	0.20	3.64	4.02
pOHF solvent calibration analysis (n = 9)				
0.000	0.063	198.55	–	–
0.781	0.851	10.37	8.98	31.59
3.125	3.340	4.30	6.88	16.07
12.500	12.927	1.11	3.41	5.70
50.000	50.525	1.47	1.05	4.03
pOHF CSF calibration analysis (n = 9)				
0.000	0.059	198.55	–	–
0.781	0.815	8.62	4.31	22.29
3.125	3.200	2.59	2.40	7.70
12.500	12.391	1.14	0.87	3.13
50.000	48.432	1.41	3.14	5.88
2OHF solvent calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.214	3.32	9.43	16.69
3.125	3.214	0.87	2.86	4.64
12.500	12.809	1.10	2.47	4.73
50.000	50.137	0.41	0.27	1.09
200.000	192.299	0.88	3.85	5.53
2OHF CSF calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.212	3.31	8.55	15.74
3.125	3.188	1.01	2.03	4.09
12.500	12.705	0.92	1.64	3.50
50.000	49.732	0.32	0.54	1.17
200.000	190.740	0.66	4.63	5.88
MCF solvent calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.098	0.105	4.82	7.20	17.53
0.781	0.792	0.68	1.38	2.76
3.125	3.169	0.97	1.40	3.37
12.500	12.543	0.40	0.34	1.15
50.000	49.035	0.66	1.93	3.22
MCF CSF calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.098	0.104	4.81	6.57	16.82
0.781	0.787	0.85	0.78	2.49
3.125	3.150	0.80	0.80	2.41
12.500	12.469	0.29	0.25	0.83
50.000	48.745	0.50	2.51	3.48
CPPA solvent calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.197	6.84	0.65	14.41
0.781	0.760	1.20	2.70	5.03
3.125	3.035	0.79	2.88	4.41
12.500	12.158	0.63	2.74	3.96
50.000	47.043	1.88	5.91	9.45
CPPA CSF calibration analysis (n = 9)				
0.000	0.000	–	–	–
0.195	0.202	6.55	3.50	17.06
0.781	0.782	1.22	0.08	2.51
3.125	3.122	0.87	0.11	1.85
12.500	12.504	0.34	0.03	0.71
50.000	48.386	2.24	3.23	7.56

12.50, 3.125 and 0.000 μg/ml for FBM and 2OHF; and 12.50, 3.125, 0.7813, and 0.000 μg/ml for *p*OHF, MCF, and CPPA. The standard sets are analyzed at the beginning, middle and end of each study sample sequence. Statistical evaluation of the calibration standards analyzed during routine sample analysis is performed by calculating the mean, R.S.D., R.M.E. and T.E. This assures the daily assay performance is equivalent to that established by validation.

During routine use endogeneous CSF constituents may interfere with the determination of low levels of FBM and the metabolites. In order to compensate for variable levels of these endogenates from subject to subject, the lower LOQ

may be adjusted to be greater than four-times the background concentration for each analyte.

This method has been applied to the analysis of approximately 550 adult and neonatal rat CSF samples from a single oral dose felbamate pharmacokinetic study [23]. Based on this analysis, the quantitation range of 0.195–200 μg/ml for FBM appears to be sufficient for pharmacokinetic and disposition studies. The upper limits of quantitation for *p*OHF, 2OHF, MCF exceed the levels required. The linear quantitation range for 2OHF could be narrowed to 0.195–100 μg/ml. The concentrations of 2OHF in the study samples varied from 0.230 to 63.322 g/ml. Only 55% of the samples contained quantifiable levels of MCF. Only 2% of the MCF levels were above 0.50 μg/ml and no sample contained more than 0.70 μg/ml. Quantifiable levels of *p*OHF were present in only 2.8% of the samples. The levels of *p*OHF varied between 0.82 and 1.41 μg/ml. There was no chromatographic evidence of the presence of CPPA in any of the samples analyzed.

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