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Simultaneous determination of felbamate and three metabolites in rat and dog plasmas by high-performance liquid chromatography

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ABSTRACT

An isocratic liquid chromatographic method employing one extraction step and a 150 mm × 4.6 mm I.D. Spherisorb ODS2, 3-μm HPLC column using UV-absorbance detection at 210 nm has been developed for the quantitation of felbamate and three felbamate metabolites in 0.100-ml aliquots of rat and dog plasmas. The linear quantitation range in rat plasma is 0.195–200 μg/ml for felbamate; 1.563–200 μg/ml for the *p*-hydroxy metabolite; 0.391–200 μg/ml for the 2-hydroxy metabolite; and 0.098–200 μg/ml for the monocarbamate metabolite. The linear quantitation range in dog plasma is 0.195–200 μg/ml for felbamate; 0.781–200 μg/ml for the *p*-hydroxy metabolite; 0.195–200 μg/ml for the 2-hydroxy metabolite; and 0.098–200 μg/ml for the monocarbamate metabolite.

INTRODUCTION

Methods for the determination of felbamate (FBM) in conjunction with other antiepileptics in human plasma [1,2], FBM and metabolites in human plasma [3], and FBM in dog plasma [4] have been published. This paper describes an automated HPLC method for the determination of FBM, and the *p*OHF, 2OHF and MCF metabolites in low-volume (0.100 ml aliquot) samples of rat and dog plasmas. In rat plasma this procedure is linear over the concentration range 0.195–200 μg/ml for FBM; 1.563–200 μg/ml for 2-(4-hydroxy-phenyl)-1,3-propanediol dicarbamate (*p*OHF); 0.391–200 μg/ml for 2-hydroxy-2-phenyl-1,3-propanediol dicarbamate (2OHF); and 0.098–200 μg/ml for 2-phenyl-1,3-propanediol monocarbamate (MCF). In dog plasma this procedure is linear over the concentration range 0.195–200 μg/ml for FBM; 0.781–200 μg/ml for *p*OHF; 0.195–200 μg/ml for 2OHF; and 0.098–200 μg/ml for MCF.

EXPERIMENTAL

Chemicals

The standard reference compounds were the same as described previously [5]. Acetonitrile, methanol, methyl-*tert.*-butyl ether, and chloroform (glass distilled) were from Burdick & Jackson (Muskegon, MI, USA); all other chemicals used were of reagent grade. Ultrapure water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA); heparinized rat and

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dog plasmas were purchased from Pel-Freeze (Rogers, AR, USA).

Preparation of standard solutions and plasma calibration samples

Standards were prepared by diluting a methanol solution containing 1.0 mg/ml of FBM and each metabolite to 200.0 μg/ml with the I.S. solution (40.0 μg/ml in 4% methanol in water). Serial twofold dilutions of the 200.0 μg/ml standard with the I.S. solution gave a series of twelve standard solutions containing 200.0, 100.0, 50.00, 25.00, 12.50, 6.250, 3.125, 1.563, 0.7813, 0.3906, 0.1953, and 0.0977 μg/ml of each analyte. All standard solutions contained 40.0 μg/ml of I.S. The I.S. solution was used as the 0.0000 μg/ml standard.

Plasma calibration standards were prepared by pipetting a 0.100-ml aliquot of thawed and vortex-mixed animal control plasma into a 16 × 125 mm extraction tube and adding 0.100 ml of the appropriate standard solution.

Extraction procedure

Sodium hydroxide (1 ml of a 1 *M* solution saturated with ammonium sulfate) was added to the spiked plasma samples. The samples were vortex-mixed and 8 ml of chloroform–methyl-*tert.*-butyl ether (25:75, v/v) was added. The tubes were capped and rotated (Model RD-350 rotator equipped with Model RD-10 head, Glass-Col, Terre Haute, IN, USA) for 45 min at maximum speed. Samples were then centrifuged at 2000 *g* at 0°C for 15 min. A 7.0–7.5-ml aliquot of the organic phase was transferred to a 16 × 100 mm disposable glass culture tube for evaporation. The organic phase was evaporated in a Savant Speed-Vac Concentrator, Model A-290 (Savant Instruments, Farmingdale, NY, USA) under vacuum at a constant chamber temperature of 50°C. The dry samples were reconstituted with 0.200 ml of mobile phase and vortex-mixed. The reconstituted sample was transferred to an HPLC autosampler microvial and capped.

High-performance liquid chromatography

The HPLC equipment consisted of Model 590 or 600E pumps, Model TCM column ovens and controllers and Model 712 WISP autoinjectors (Waters-Millipore, Milford, MA, USA) and Model 783 ultraviolet absorbance detectors (ABI, Foster City, CA, USA). Chromatographic data was acquired on a PE-Nelson 2600 chromatography data system (PE-Nelson, Cupertino, CA, USA).

The analytical columns, from two sources, used for method validation were: Hichrom Spherisorb ODS2, 3 μm, 150 mm × 4.6 mm I.D. (YMC, Morris Plains, NJ, USA) and Spherisorb ODS2, 3 μm, 150 mm × 4.6 mm I.D. (Thomson Instrument, Springfield, VA, USA).

The mobile phase was 20 m*M* dibasic ammonium phosphate (in water) pH 6.8 acetonitrile (85:15, v/v). The flow-rate was 1.0 ml/min. The temperature was 40°C and the pressure was 76–100 bar. The injection volume was 100 μl. UV absorbance detector settings were: wavelength 210 nm; range, 0.05 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, 0.8 s; peak detection threshold, 0.1 μV/s; area threshold 5; minimum peak width, 15 s; delay, 2.6 min; bunch rate double, 8.0–10.0 min; run time 21.0–23.0 min. Typical retention times (min): *p*OHF 3.3, 2OHF 4.5, MCF 6.5–7.4, FBM 10.1–11.8 and I.S. 16.9–20.7 min.

Calculations

A total of three individual run sets were analyzed using each plasma matrix. Each run set, comprised of triplicate analyses of each of the twelve plasma standard concentrations, were analyzed on four different days using four HPLC columns and two HPLC systems. Peak heights determined by the PE-Nelson 2600 system were used for all calculations that were described previously [5].

The absolute recoveries of each analyte and the I.S. were calculated by direct comparison of the peak heights of the extracted plasma standards with the peak heights of standards in solvent (4% methanol in water). Individual daily run recoveries of each analyte were averaged for each concentration. The overall average recovery of each analyte was calculated using only the concentration points yielding total error

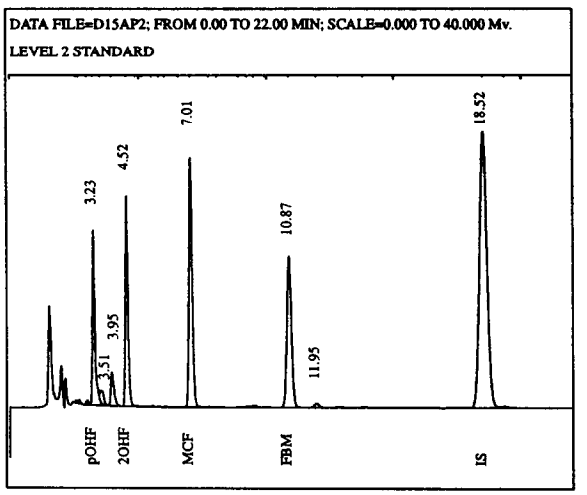

Fig. 1. Chromatogram of a rat plasma calibration standard containing 12.50 μg/ml each of FBM, *p*OHF, 2OHF, MCF and 40.00 μg/ml I.S.

(T.E.) values less than 50% from the plasma data analysis. Recovery of the I.S. was determined from all twelve standards.

RESULTS

Analyte recoveries

The overall mean recoveries in both rat and dog plasmas (±S.D.) were: 85.95% ± 1.31 for FBM, 66.16% ± 2.84 for *p*OHF, 67.34 ± 3.28 for

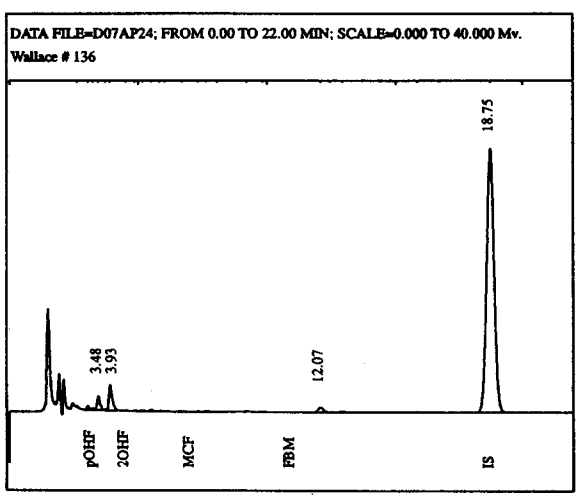

Fig. 2. Chromatogram of a predose (blank) rat plasma study sample containing 40.00 μg/ml I.S.

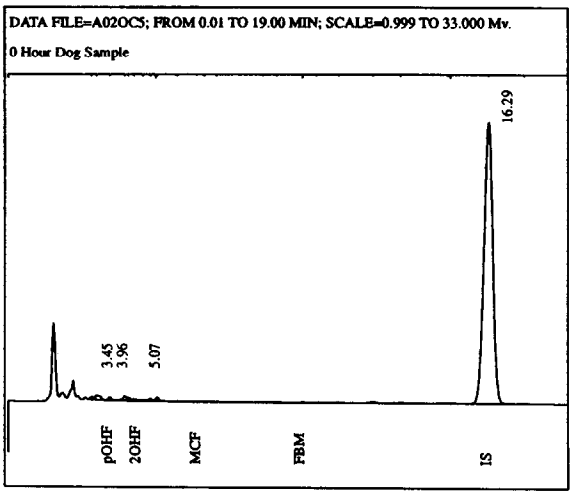

Fig. 3. Chromatogram of a predose (blank) dog plasma study sample containing 40.00 μg/ml I.S.

2OHF, 79.80% ± 2.96 for MCF and 89.86% ± 0.62 for the I.S.

Chromatography

The chromatographic resolution of all four analytes and the I.S. is adequate as illustrated by the 12.50 μg/ml rat plasma standard shown in Fig. 1. Baseline resolution of all analytes was observed at all concentrations. Minor peaks

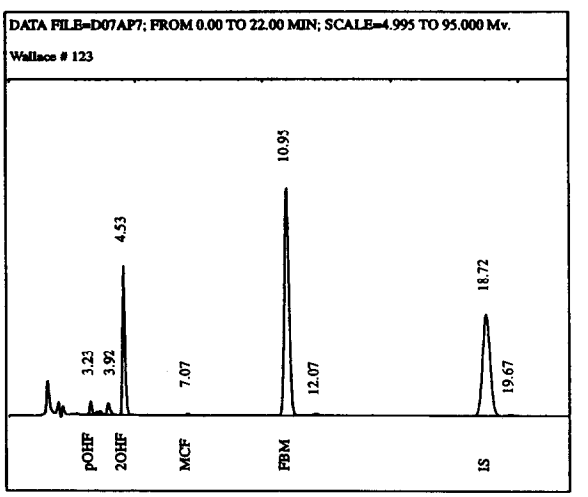

Fig. 4. Chromatogram of a rat plasma study sample containing 51.78 μg/ml of FBM, 2.46 μg/ml of *p*OHF, 24.350 μg/ml of 2OHF, 0.25 μg/ml of MCF, and 40.00 μg/ml of I.S.

TABLE I

STATISTICAL ANALYSIS OF FELBAMATE AND METABOLITES IN RAT PLASMA: PRECISION, ACCURACY, AND TOTAL ERROR

Standard concentration (μg/ml)	n	Determined			
		Mean (μg/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)
FBM					
0.000	9	0.000	–	–	–
0.195	9	0.204	3.40	4.48	11.57
1.563	9	1.614	1.18	3.26	5.69
12.500	9	12.842	0.56	2.73	3.89
100.000	8	99.558	0.67	0.44	1.78
200.000	9	189.830	1.14	5.08	7.25
pOHF					
0.000	9	0.375	121.54	–	–
1.563	9	1.751	12.81	12.09	40.80
12.500	9	12.643	4.74	1.14	10.73
100.000	8	99.627	3.81	0.37	7.96
200.000	9	195.122	3.97	2.44	10.19
2OHF					
0.000	9	0.021	166.93	–	–
0.391	9	0.447	7.54	14.35	31.59
1.563	9	1.653	3.76	5.82	13.77
12.500	9	12.947	2.69	3.57	9.16
100.000	8	99.395	2.18	0.60	4.94
200.000	9	187.273	2.75	6.36	11.51
MCF					
0.000	9	0.025	125.31	–	–
0.098	9	0.112	14.50	14.72	47.99
1.563	9	1.671	3.73	6.97	14.95
12.500	9	12.930	2.46	3.44	8.52
100.000	8	99.394	1.78	0.61	4.13
200.000	9	185.474	2.46	7.26	11.82

occur near the retention time of most analytes. Chromatograms of predose rat and dog samples containing 40.00 μg/ml of I.S. are shown in Figs. 2 and 3, respectively. A typical rat study sample containing 51.78, 2.46, 24.35, and 0.25 μg/ml of FBM, *p*OHF, 2OH and MCF, respectively, and 40.00 μg/ml of I.S. is shown in Fig. 4. A typical dog study sample containing 48.22, 2.11, 3.25 and 0.12 μg/ml of FBM, *p*OHF, 2OH and MCF, respectively, and 40.00 μg/ml of I.S. is shown in Fig. 5.

Linearity

The linearity for all analytes in rat and dog plasma indicated by the correlation coefficients (r^2), was greater than 0.9956. All *y*-intercepts were less than $1.530 \cdot 10^{-10}$ as determined by TableCurve linear regression forced through zero.

Precision, accuracy and variability

Tables I and II list the overall precisions (R.S.D), accuracy (R.M.E.) and total error (T.E.) (as defined previously [5]) for the limits of quantitation and the calibration concentrations for each analyte in rat and dog plasma, respectively. The values reflect the combined intra-day

TABLE II

STATISTICAL ANALYSIS OF FELBAMATE AND METABOLITES IN RAT PLASMA: PRECISION, ACCURACY, AND TOTAL ERROR

Standard concentration (μg/ml)	n	Determined			
		Mean (μg/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)
FBM					
0.000	9	0.022	24.16	–	–
0.195	9	0.228	3.56	16.86	25.18
1.563	8	1.645	0.73	5.29	6.83
12.500	9	12.962	0.40	3.70	4.52
100.000	9	99.464	0.44	0.54	1.41
200.000	9	188.151	1.19	5.92	8.17
pOHF					
0.000	9	0.000	–	–	–
0.781	8	0.790	5.14	1.12	11.52
1.563	8	1.567	4.70	0.26	9.68
12.500	9	12.623	2.94	0.98	6.92
100.000	9	99.874	2.50	0.13	5.13
200.000	9	192.283	6.86	3.86	17.06
2OHF					
0.000	9	0.020	85.52	–	–
0.195	9	0.233	10.97	19.42	45.64
1.563	8	1.657	1.79	6.06	9.85
12.500	9	12.965	1.93	3.72	7.71
100.000	9	99.451	1.76	0.55	4.05
200.000	9	183.288	5.26	8.36	17.99
MCF					
0.000	9	0.014	81.31	–	–
0.098	9	0.116	7.02	18.64	35.29
1.563	8	1.657	1.96	6.02	10.17
12.500	9	12.967	1.41	3.73	6.67
100.000	9	99.450	1.10	0.55	2.74
200.000	9	182.097	3.11	8.95	14.62

and inter-day variability for precision and accuracy.

Specificity of assay

Compounds used for anesthesia or euthanasia such as ketamine, pentobarbital, and phenytoin will cause significant interferences and very late eluting peaks. All lots of animal plasma should be analyzed for the presence of these peaks prior to use in preparing calibration standards. Only plasma lots with interfering peaks less than one-fourth the lower LOQ of all analytes should be used.

Routine use experience

Routine analysis of study samples is performed using the four plasma calibration standards (100.0, 12.50, 1.563 and 0.000 μg/ml) analyzed in triplicate. 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.

Endogeneous plasma constituents may inter-

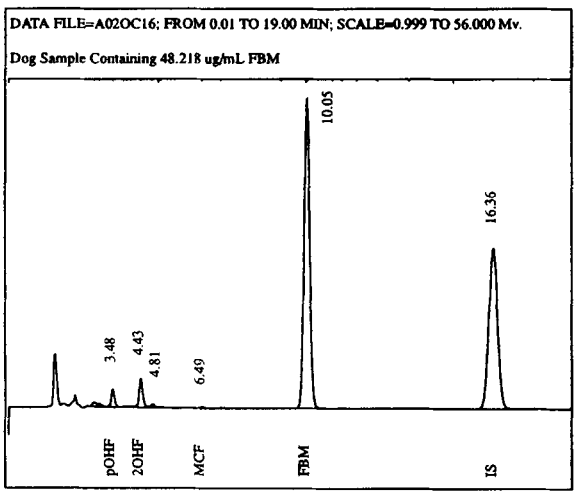

Fig. 5. Chromatogram of a dog plasma study sample containing 48.22 μg/ml of FBM, 2.11 μg/ml *p*OHF, 3.25 μg/ml 2OHF, 0.12 μg/ml MCF, and 40.00 μg/ml of I.S.

fere 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 LOQ for each analyte must be greater than four times the average background concentration.

The retention times of FBM, MCF and the I.S. decreased with column age and may significantly differ from column to column. The retention time shifts and column variability did not affect resolution and no changes in column capacity have been observed.

DISCUSSION

This method has been applied in the analysis of approximately 100 dog plasma samples and 700 rat plasma samples. The quantitation range of 0.195 to 200.000 μg/ml for FBM appears to be sufficient for single dose pharmacokinetic and drug interaction studies. Based on the analysis of plasma samples from a single oral dose FBM distribution study in rats [6], plasma concentrations of FBM varied between 1 and 200 μg/ml, 2OHF varied between 1 and 200 μg/ml, and the concentrations of MCF and *p*OHF varied between 0.1 and 5 μg/ml following single oral 100–2000 mg/kg doses of FBM.

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