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Determination of the anticonvulsant felbamate and its three metabolites in brain and heart tissue of rats

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ABSTRACT

An automated, internal standard high-performance liquid chromatographic method for the simultaneous quantitation of felbamate and its three metabolites in adult and neonatal rat brain and heart tissue homogenates was developed and validated. The homogenates prepared from one part of the tissue and four parts of water were extracted with ethyl acetate, and the extract was evaporated to dryness and redissolved in mobile phase. Separation was accomplished on a Waters Resolve C_{18}, 5 μm, 300 mm × 3.9 mm I.D. column with a mobile phase consisting of 0.01 *M* phosphate buffer, pH 6.8–acetonitrile–methanol (800:150:50, v/v/v). Eluting peaks were monitored with an ultraviolet detector at 210 nm. The linear range of the assay for felbamate and the metabolites was 0.20–50.00 μg/ml of homogenate or 1–250 μg/g of brain or heart tissue. The lower limit of quantitation for all four analytes was 0.20 μg/ml of homogenate or 1.00 μg/g of tissue.

INTRODUCTION

Felbamate (FBM), 2-phenyl-1,3-propanediol dicarbamate, is under clinical development as a relatively non-toxic anticonvulsant [1,2]. Studies with laboratory animals have also shown significant neuroprotectant properties of felbamate *in vitro* [3] as well as *in vivo* [4]. Mechanism of action studies of anticonvulsant and neuroprotective effects of FBM required the measurement of drug levels in prospective target tissues, namely the brain and heart. It has previously been observed in a pharmacokinetic study of [^{14}C]FBM in the rat [5] that the brain concentrations of ^{14}C are about half of those in plasma at all times, but actual concentrations of FBM and its metabolites in the brain have not been determined.

Methods for the determination of FBM in human plasma [6] and dog plasma [7] have been published, but a method for the determination of drug and its major metabolites in rat tissues has not been available. This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of FBM and its three major metabolites [8] [the *p*-hydroxyphenyl (pOHF), the 2-hydroxy (2OHF) and the monocarbamate (MCF) metabolites (for structures see Fig. 1)] in rat brain and heart tissue homogenates over the concentration range 0.20–50.00 μg/ml, equivalent to 1–250 μg/g of tissue, and its application to a rat tissue distribution study [9]. The method involves the extraction of FBM and its metabolites from the brain and heart tissue homogenates with ethyl acetate and the HPLC assay of the extracts.

EXPERIMENTAL

Chemicals

FBM, 2OHF, pOHF, MCF and the internal

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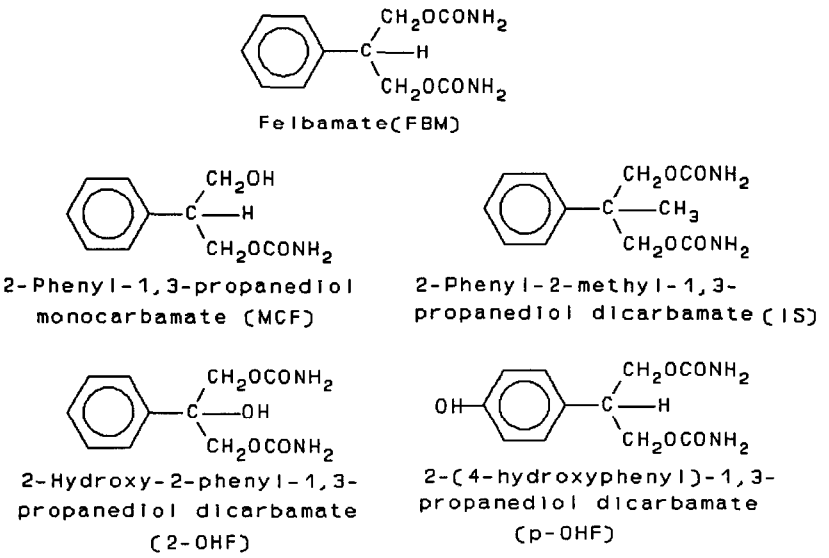

Fig. 1. Structures of FBM and its three metabolites.

standard (I.S.), 2-methyl-2-phenyl-1,3-propanediol dicarbamate, were all from Wallace Labs. (Cranbury, NJ, USA). HPLC-grade methanol, acetonitrile and ethyl acetate, glass distilled, were obtained from Burdick & Jackson (Muskegon, MI, USA). Phosphoric acid, 85%, was from Fischer Scientific (Fairlawn, NJ, USA). Reagent-grade dibasic potassium phosphate from Aldrich (Milwaukee, WI, USA) and 85% orthophosphoric acid from Fischer Scientific were used without further purification. Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Blank rat brain and heart tissues were obtained from normal, untreated Sprague–Dawley rats (Charles River Labs., Kingston, NY, USA).

Instrumentation

The HPLC system consisted of a 6000A pump, a WISP 710B autoinjector, a column temperature control module (Waters, Milford, MA, USA) and a 783 UV detector (ABI-Kratos, Ramsey, NJ, USA). All peak-height data were collected and analyzed using the Turbochrom II, 2700 Series chromatographic system (PE-Nelson, Cupertino, CA, USA).

Standard solutions

A phosphate buffer was prepared by dissolving 2.3 of dibasic potassium phosphate in 950 ml of water. The pH of the solution was adjusted to 6.8 with phosphoric acid and the final volume was made up to 1000 ml with water. The I.S. solution, 12.5 μg/ml, was prepared in the mobile phase. Stock solutions of 0.500 mg/ml each of pOHF, 2OHF, MCF and FBM were prepared in the I.S. solution. The working solvent standard, 50 μg/ml of each of the analytes, was prepared by diluting the stock solution with the I.S. solution. The 50 μg/ml standard was then serially diluted with the I.S. solution to prepare 25.00, 12.50, 6.250, 3.125, 1.563, 0.7813, 0.3906 and 0.1953 μg/ml solvent standards.

Control rat brain and heart tissue homogenates were prepared from untreated animals (blank) by homogenization with four volumes of water (w/v) for 1 min in an Ultra-Turrax Tissuemizer, Model T25 (IKA-Works, Cincinnati, OH, USA). Standard brain and heart homogenates were prepared by spiking 100 μl of the homogenates with 100 μl of the solvent standards. A blank brain and heart homogenate with 100 μl of the I.S. solution was also included as the 0.0000

μg/ml standard. Three separate sets of standards were prepared and each of the sets was analyzed in triplicate on three different days.

Sample preparation

Each brain or heart homogenate standard, prepared in a separate glass centrifuge tube as described above, was diluted with 2.00 ml of deionized water. The contents of the tubes were mixed for 1 min using a vortex-mixer and centrifuged at 600 *g* for 10 min. A 2.00-ml aliquot of the supernatant was transferred to a 15-ml glass centrifuge tube, and 8 ml of ethyl acetate were added. The tubes were mixed for 1 min using a vortex-mixer and centrifuged at 600 *g* for 10 min. The ethyl acetate extract, 7.5 ml, was transferred to a 100 mm × 16 mm borosilicate glass tube and evaporated to dryness in a Savant Speed-Vac concentrator (Savant Instrument, Farmingdale, NY, USA) at about 50°C. The residue was redissolved in 100 μl of the mobile phase, and 80 μl of the solution were injected onto the HPLC column.

Chromatographic conditions

The mobile phase consisted of 0.01 *M* phosphate buffer, pH 6.8–acetonitrile–methanol (800:150:50, v/v/v). Chromatography was performed at 50°C on a Waters Resolve C_{18} column, 300 mm × 3.9 mm I.D., 5 μm particle size, equipped with a Waters Resolve C_{18} guard column. The flow-rate was 1.0 ml/min, and the eluent was monitored at 210 nm with attenuation 0.01 a.u.f.s. The PE-Nelson method parameters were: sampling rate: 1 pt/s; noise threshold: 1 μV; area threshold: 5 μV/s; delay: 0 min; area reject: 0; peak width: 5 s + 5%; run time: 20 min.

Data acquisition and calculations

Peak heights for the analytes and the I.S. in the three intra-day calibration sets were measured and the corresponding peak-height ratios (analyte/I.S.) generated by the PE-Nelson Turbochrom II chromatographic system [10]. The three sets of intra-day triplicate ratio values per concentration were then transposed by computer in such a way that three inter-day triplicate sets were generated. Each of the three intra-day sets contained only measurements at one particular calibration day. Each of the three inter-day sets contained only measurements obtained at three different calibration days. Both the intra- and inter-day sets had the same number of calibration points. For each calibration day a fresh set of standards was prepared. The concentration (x) *versus* peak-height ratios (y) for the standards were subjected to unweighted and weighted (weight = $1/x$) least-squares (linear regression) analysis. The model selected to fit calibration data was $y = A + Bx + \varepsilon$, using a curve-fitting program for PCs' TableCurve [11]. It generated the coefficient values A and B and their 95% confidence limits, the y-residuals (in %), the F values as goodness-of-fit parameter, the variance σ^2, representing an estimate of the residual error ε, and the 95% prediction band around the regression line as an indicator of the overall variability in the calibration data within a set. The F and σ^2 values were obtained from the program-generated ANOVA table of the regression as described by Draper and Smith [12].

The regression parameters A and B for the weighted linear-fit of concentration *versus* peak-height ratio data, generated by triplicate analyses of each of the intra-day set, were used to calculate the analyte concentrations. Statistical analysis to evaluate the assay precision (R.S.D.), accuracy (R.M.E.) and total error (T.E.) was performed using the standard formulae given below:

$$\text{R.S.D.} = \frac{100 \times \text{S.D.}}{\bar{x}}$$

$$\text{R.M.E.} = \frac{100 \times \text{abs}(\bar{x} - x)}{x}$$

$$\text{T.E.} = \frac{100 \times [2\ \text{S.D.} + \text{abs(M.E.)}]}{x}$$

where $\bar{x}$ is the mean value of set and S.D. and M.E. are the standard deviation of the mean and mean error.

Recoveries of FBM, the three metabolites and

the I.S. from the spiked brain and heart homogenates were obtained from peak heights after extraction from homogenate standards and peak heights measured for standards prepared in mobile phase.

RESULTS

Baseline resolution of analytes and the I.S. was accomplished for both tissue matrices as is evident from Figs. 2 and 3. Shown are typical chromatograms for a control adult rat brain or heart homogenate (A), a spiked homogenate standard containing 12.50 μg/ml FBM and all of its three metabolites (B) and a brain or heart homogenate sample taken at 1 h post-dose from an adult rat (C) or from a neonatal rat both dosed orally with 500 mg/kg FBM (D). As can be seen there were no detectable backgound peaks at the retention time of the four analytes in the control brain and heart tissue homogenates. The retention times for pOHF, 2OHF, MCF, FBM and I.S. varied less than 7% and were in the range 3.8–4.0, 5.1–5.5, 7.3–8.3, 10.6–11.4 and 17.1–18.9 min, respectively, for both the brain and heart tissues.

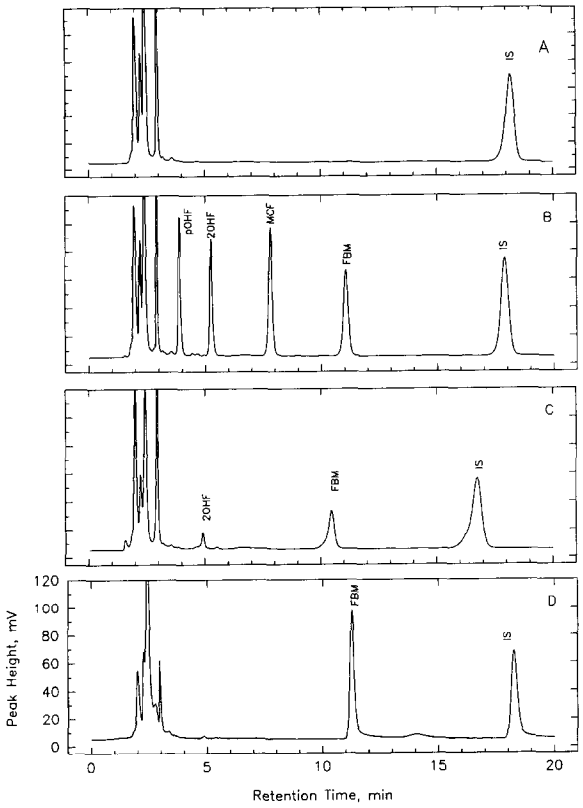

Fig. 2. Chromatograms of extracts from control rat brain homogenates with I.S. (A), an adult rat control homogenate spiked with 12.5 μg/ml FBM, pOHF, 2OHF, MCF and I.S. (B) and a brain homogenate taken 1 h after oral dosing with 500 mg/kg FBM from an adult (C) or neonatal rat (D).

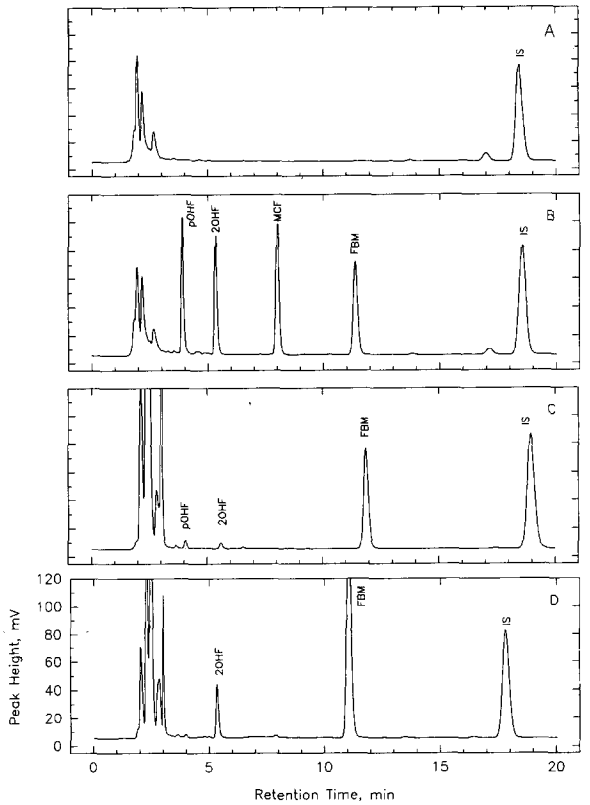

Fig. 3. Chromatograms of extracts from control rat heart homogenates with I.S. (A), an adult rat control homogenate spiked with 12.5 μg/ml FBM, pOHF, 2OHF, MCF and I.S. (B), a heart homogenate taken 1 h after oral dosing with 500 mg/kg FBM from an adult (C) or neonatal rat (D).

The mean absolute recoveries of pOHF, 2OHF, MCF, FBM and I.S. are listed in Table I for the spiked brain and heart homogenate. The mean absolute recoveries of the four analytes and the I.S. from neonatal rat brain and heart tissue homogenates were very similar to those from the adult rat.

The linear relationship $y = A + Bx + \varepsilon$ between standard concentration (x) and detector response

TABLE I

ABSOLUTE RECOVERIES OF ANALYTES (%) FROM RAT BRAIN AND HEART TISSUE HOMOGENATES FOR THE WHOLE CALIBRATION RANGE

	FBM	pOHF	2OHF	MCF	I.S.
Brain					
Mean[a]	91.3	97.1	83.9	87.5	88.0
Min	87.1	91.0	80.8	85.6	–
Max	102.4	104.9	87.3	91.3	–
Heart					
Mean[a]	96.3	98.5	90.9	90.0	88.0
Min	90.9	95.2	87.4	87.7	–
Max	101.9	102.5	92.7	93.8	–

[a] Mean value over all concentrations, excluding blank.

expressed as peak-height ratio (y) was used as model. The validity of the model within the range 0.20–50.00 μg/ml was confirmed by the large F ratio values for all of the weighted sets, by far exceeding the tabulated value ($F_{1,31,0.95} = 4.16$). In addition, the y-residuals after weighting were evenly and randomly distributed around the zero line. Generally, weighting by the reciprocal of the concentration ($1/x$) considerably improved the results of regression at the low end of the range. The resulting precision (R.S.D.), accuracy (R.M.E.) and total error (T.E.) for all four analytes calculated based on the coefficients A and B from weighted linear regression of the three intra-day calibration sets are given in Table II for the brain and in Table III for heart homogenates

TABLE II

PRECISION (R.S.D.,%), ACCURACY (R.M.E.,%) AND TOTAL ERROR (T.E.,%) FOR THREE INTRA-DAY CALIBRATION SETS IN RAT BRAIN HOMOGENATES

Standard concentration	FBM			pOHF			2OHF			MCF		
(μg/ml)	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.
0.195	8.5	8.5	23.5	5.4	5.3	15.3	9.4	17.7	31.1	3.5	1.7	8.8
0.781	3.7	0.9	8.1	5.3	2.6	13.6	2.3	1.2	5.9	1.7	6.2	10.3
3.125	1.6	4.2	7.8	2.7	4.0	9.7	1.1	4.7	7.2	0.8	6.7	8.9
12.500	0.4	6.6	8.0	2.1	6.4	11.2	1.0	6.5	9.0	0.7	7.3	9.3
50.000	0.5	7.8	9.5	1.7	8.8	13.2	1.4	7.7	11.5	0.8	8.0	10.5

TABLE III

PRECISION (R.S.D.,%), ACCURACY (R.M.E.,%) AND TOTAL ERROR (T.E.,%) FOR THREE INTRA-DAY CALIBRATION SETS IN RAT HEART HOMOGENATES

Standard concentration	FBM			pOHF			2OHF			MCF		
(μg/ml)	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.	R.S.D.	R.M.E.	T.E.
0.195	7.1	5.0	20.0	11.8	8.2	29.8	5.5	9.9	21.9	15.4	9.6	37.4
0.781	2.8	6.3	12.1	5.5	1.1	12.0	2.6	7.2	12.8	4.4	0.6	9.5
3.125	6.9	11.2	26.5	3.2	1.6	8.1	1.5	6.7	9.9	2.6	4.4	9.7
12.500	0.5	5.8	6.9	3.3	3.3	10.2	1.6	5.4	8.8	3.2	5.9	12.7
50.000	3.6	13.8	21.9	5.6	12.6	25.2	2.5	13.3	19.0	4.3	14.4	24.4

TABLE IV

COMPARISON OF CALIBRATION LINE COEFFICIENTS *A* AND *B* 95% CONFIDENCE LIMITS, GOODNESS-OF-FIT *F* RATIO AND σ^2 VALUES FOR INTRA-DAY *VERSUS* INTER-DAY CALIBRATION SETS IN RAT BRAIN HOMOGENATES

	Range[a] (95% confidence limits)		Mean (n = 3)	
	$A \times 10^4$	B	F	$\sigma^2 \times 10^5$
FBM				
Intra-day	18.99–75.33	0.069–0.072	55 370	1.002
Inter-day	19.42–76.59	0.070–0.071	54 388	1.063
pOHF				
Intra-day	−0.25–89.55	0.115–0.133	116 394	2.336
Inter-day	−7.17–100.10	0.119–0.128	9801	16.891
2OHF				
Intra-day	0.51–68.86	0.094–0.102	43 164	2.465
Inter-day	7.81–64.38	0.096–0.101	23 171	3.217
MCF				
Intra-day	−4.26–43.19	0.094–0.111	53 702	2.383
Inter-day	−32.72–66.77	0.101–0.108	6680	17.387

[a] The highest and lowest values among the three sets are given.

TABLE V

COMPARISON OF CALIBRATION LINE COEFFICIENTS *A* AND *B* 95% CONFIDENCE LIMITS, GOODNESS-OF-FIT *F* RATIO AND σ^2 VALUES FOR INTRA-DAY *VERSUS* INTER-DAY CALIBRATION SETS IN RAT BRAIN HOMOGENATES

	Range (95% confidence limits)		Mean (n = 3)	
	$A \times 10^4$	B	F	$\sigma^2 \times 10^5$
FBM				
Intra-day	2.73–48.85	0.080–0.082	89 211	1.347
Inter-day	5.48–44.35	0.080–0.082	90 457	1.296
pOHF				
Intra-day	−61.33–66.27	0.118–0.131	18 847	17.255
Inter-day	−67.56–90.71	0.118–0.131	17 606	20.444
2OHF				
Intra-day	−16.37–52.71	0.101–0.109	38 930	4.832
Inter-day	−12.01–61.86	0.102–0.109	34 882	5.457
MCF				
Intra-day	−21.25–34.44	0.114–0.121	58 424	3.824
Inter-day	−24.32–49.07	0.114–0.121	45 395	4.645

(only five out of eleven standard concentrations covering the whole range are listed).

For assessment of the size of the intra-day *versus* the inter-day precision the parameters used were the 95% confidence limits for coefficients A and B, the F ratio values and the residual error values represented by σ^2. For the three intra-day calibration sets these parameters are listed in Table IV and V for the brain and heart tissue, respectively, in comparison with the values obtained from the inter-day sets formed by transposition.

It has been suggested that a T.E. of less than 50% is an acceptable overall variability for assays [13]. Therefore, the lower limit of quantitation (LOQ) for this method was arbitrarily set at the lowest concentration yielding a total error value less than 50%. Based on this criteria, the LOQ for pOHF, 2OHF, MCF and FBM was set at 0.195 μg/ml of homogenate or about 1.0 μg/g of brain or heart tissue, and the corresponding upper limit of quantitation was set at 50 μg/ml or 250 μg/g. It was established with a separate calibration run that this can be extended to 100 μg/ml with a slight decrease in precision.

DISCUSSION

Good resolution of FBM and its three metabolites from matrix components was accomplished and a clean interference-free background in tissue extracts from untreated animals was obtained under the used conditions within a reasonable run time of 20 min. The expected drug tissue levels within the projected experimental sampling periods ranged between 1 and 150 μg/g, because high oral doses well above the pharmacologic dose (50–100 mg/kg, anticonvulsant ED_{50}) of FBM, up to 2000 mg/kg, were given in this exploratory study [9]. However, metabolite levels were expected to be lower.

Higher precision and accuracy and a lower T.E. resulted for the intra-day sets when concentration-weighted regression was applied to the data. The improvement *versus* the unweighted regression was most pronounced in the y-residuals, which have been suggested to be more reliable quality-of-fit indicators than the correlation coefficient r^2 [14].

Unweighted data could be used for the regression with satisfactory results within a more narrow concentration range of 0.4–25.0 μg/ml.

Actual study samples were scheduled to be analyzed over a period of weeks. The assessment of the inter-day precision variability for all four analytes was important. The precision for assays carried out on the same equipment over an extended period of time has intra- and inter-day components. These can be assessed separately from multiple daily calibration runs by rearrangement of the data leading to one group of sets containing only daily replicates per set and another group containing one of each of the daily replicates per concentration. From the goodness-of-fit F ratio and the residual error σ^2 values, representing the overall random error variability in each of the three intra-day or inter-day sets in Tables IV and V, it is evident that the inter-day component is relatively small. This is true for FBM and 2OHF in brain and for FBM, 2OHF and MCF in heart tissue. The inter-day σ^2 value for pOHF in both tissues and for MCF in brain was relatively large, indicating that data from the two metabolites derived from analyses at different days will have much more variability. The same conclusion can also be drawn from the large decrease in the F values. However, the concentrations of both metabolites in rat tissues are comparably much lower than those of FBM and 2OHF and were not important for the outcome of the kinetic study.

The method described was applied to the determination of FBM and metabolites in brain and heart tissue samples from a rat tissue distribution study [9]. As an illustration of the results obtained with this method the concentration *versus* time curves in neonatal brain and adult heart homogenates are shown in Fig. 4. About six hundred tissue samples of each of the two tissues were analyzed in the course of the study using nineteen separate daily calibrations containing a ten-concentrations standard set. Forty study samples and ten calibration samples were extracted and analyzed in a 24-h period. At least five

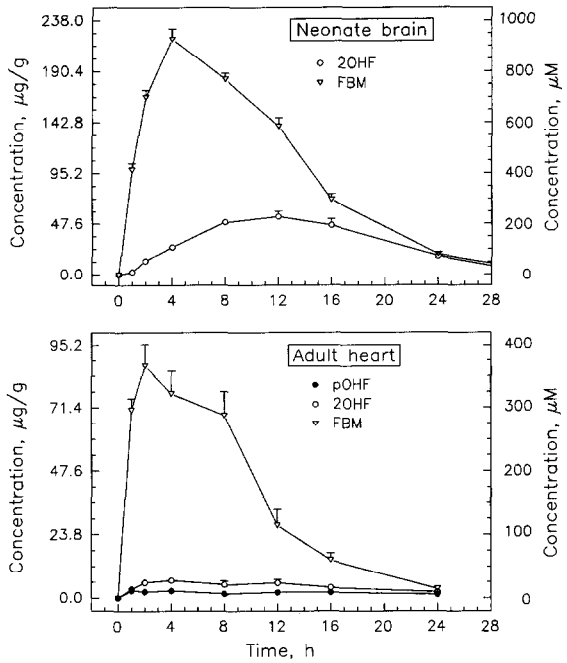

Fig. 4. Mean concentration–time curves for FBM and metabolites in tissues from rats dosed with 500 mg/kg FBM.

hundred homogenate samples were analyzed per column before change was necessitated by the deterioration of separation.

This method was also applied successfully without modification to the determination of drug and metabolites in surgical human brain samples from FBM-treated patients.

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