Pergamon

0731-7085(94)00087-5

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 11, pp. 1443–1451, 1994

0731-7085/94 $7.00 + 0.00

An automated analytical method for the determination of felbamate in human plasma by robotic sample preparation and reversed-phase high performance liquid chromatography

J. HEMPENIUS,* G. HENDRIKS, J. HINGSTMAN, C.K. MENSINK, J.H.G. JONKMAN and C.C. LIN†

Pharma Bio-Research International B.V., P.O. Box 200, 9740 AE Zuidlaren, The Netherlands
†Schering-Plough Research Institute, Kennilworth, NJ 07033-0539, USA

Abstract: An automated analytical method for the determination of felbamate in human plasma is described. Sample cleanup and preparation was performed by means of a Zymate II laboratory robot and consisted of a liquid–liquid extraction of felbamate and the internal standard, primidone, from human plasma to dichloromethane. The dichloromethane was evaporated and reconstituted in a phosphate buffer. Separation was performed by reversed-phase high performance liquid chromatography using a 5 μm Hypersil® ODS column (150 × 4.6 mm) and a mobile phase consisting of a mixture of phosphate buffer (pH = 6.5, 0.015 M) and acetonitrile (79:21, v/v). Quantitation was performed by measurement of the UV absorbance at a wavelength of 210 nm. The lower limit of quantitation was 0.100 μg ml^{-1} using 200 μl of plasma. The mean absolute analytical recovery of felbamate was 75.2% (n = 28). The recovery of the internal standard, primidone was 74.7% (n = 10). The within-day precision was below 3.8% at all concentration levels, except at the lower limit of quantitation (18.3%). The within-day accuracy varied between −3.7 and +7.4%. The between-day precision was below 5.0% at all concentration levels. The between-day accuracy of the method varied between −5.7 and +1.6%. The selectivity of the method towards several other anti-epileptic drugs has been demonstrated.

Keywords: *Felbamate; bioanalysis; robotic sample preparation; HPLC.*

Introduction

Felbamate (2-phenyl-1,3-propanediol-dicarbamate) (Fig. 1) is a chemically unique relatively non-toxic anti-epileptic drug, currently under investigation in clinical trials. Felbamate has demonstrated anticonvulsant activity against electrically and chemically induced seizures in animal models.

So far, two methods have been published for the analysis of felbamate in biological fluids. The method published by Clark *et al.* [1] is very straightforward. The method consists of deproteinization of the plasma sample with acetonitrile followed by reversed-phase HPLC. The method was developed for the analysis of felbamate concentrations in plasma samples from beagle dogs and cannot be used for the analysis in human plasma samples because of interferences of endogenous compounds. The method of Remmel *et al.* [2] consists of liquid–liquid extraction, evaporation of the extraction solvent, reconstitution into the mobile phase followed by reversed-phase HPLC. Although the procedure is rather straightforward it is time-consuming. The use of a laboratory robot may increase the sample throughput by more than a factor of two [3].

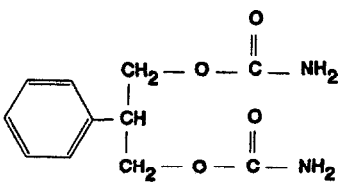

Figure 1
Structural formula of felbamate.

In view of the number of samples generated in pharmacokinetic studies and clinical trials, it was desirable to develop an automated analytical method for the analysis of felbamate. The automated analytical method was validated

*Author to whom correspondence should be addressed.

with regard to selectivity, sensitivity, linearity, accuracy, precision and stability according to the recommendations of Shah *et al.* [4].

Materials and Methods

Robotic system

The sample preparation and the subsequent injection of the sample extracts was performed by a Zymate II Laboratory robot consisting of the following components (Zymark Corp., Hopkinton, MA 01748, USA): a Zymate Z 110 robot (with EasyLab Plus™ Software and PyTechnology Software™) consisting of a robot arm, a Zymate robot controller with user memory, and a 5.25 inch floppy disk drive for storing and retrieving sample preparation methods; a Zymark Z 905 dual function hand was used as a gripper hand and as a syringe hand for pipetting. The hand is parked in a Hand Parking Station; and a Zymark Z 510 Master Lab Station (MLS) equipped with three gas tight syringes (Hamilton, Reno, NE 89510, USA) of 1.0, 1.0 and 5.0 ml, respectively. The fixed nozzle containing four outlet tubes of the MLS was modified [3].

For the extractions a Zymark Z 620 single tube vortexing unit was used. The vortex was pulsed with alternating speeds, 15 s at speed 80 and 2 s at speed 0 (= no rotation).

The Zymark Z 710 Centrifuge Station was used at 1300*g* (3000 rpm). The centrifugation time was 10 min for each sample.

On-line injections into the HPLC were done by using a Zymark ZP 311 HPLC Injection Station which was equipped with a 20 µl sample loop. The injection station is a part of the Analytical Instrument Interface Module.

The Analytical Interface Module is used for monitoring problems with the HPLC system by reading the analogue signal of the detector and to check whether the internal standard is of the usual height and if its retention time is correct [5]. The bench-layout of the robotic system is given in Fig. 2.

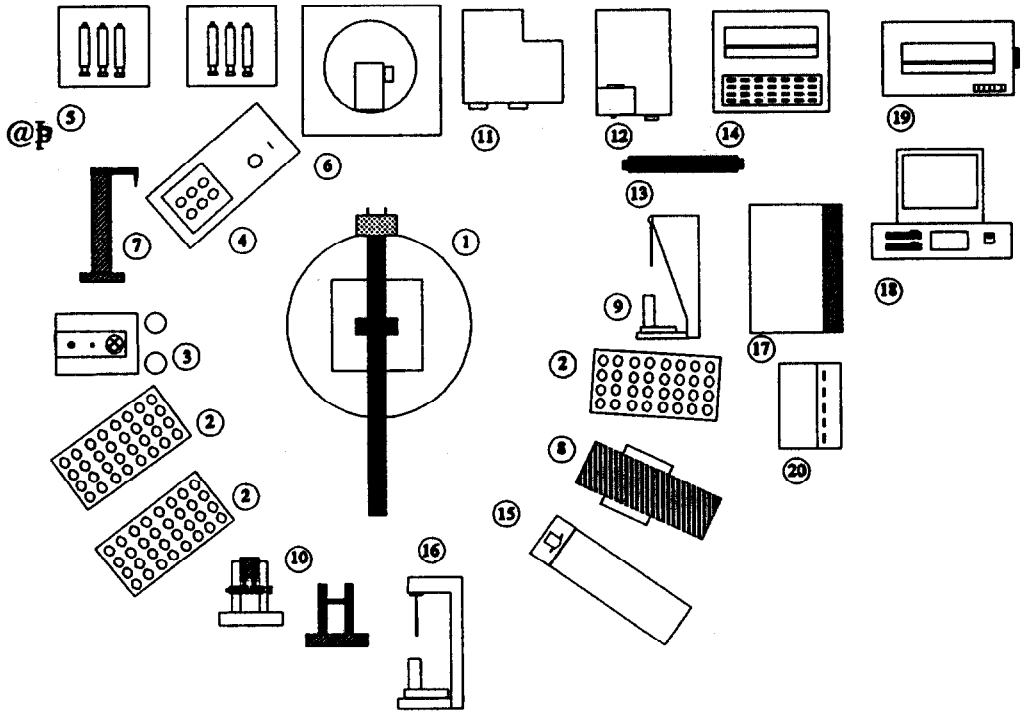

Figure 2
Bench-layout of the robotic system.

1. Robot with arm.
2. Racks for 16 × 100 mm tubes.
3. Vortex station.
4. Evaporation station.
5. Master lab station.
6. Centrifuge.
7. Dispensing nozzle.
8. Tube dispenser.
9. HPLC-injector.
10. Parking station for dual function hand.
11. HPLC-pump.
12. HPLC-detector.
13. HPLC-column.
14. Integrator.
15. Disposal.
16. Liquid–liquid extractor.
17. Controller.
18. Keyboard, monitor and diskdrive.
19. Printer.
20. Analytical instrument interface.

HPLC system

The assay was performed with an HPLC system consisting of the following components: a Waters Model M 510 solvent delivery system at a flow rate of 1.0 ml min^{-1} (Waters Associates, Milford, MA 01757, USA) was used. The analytical column was a 5 μm Hypersil® ODS (150 × 4.6 mm), cartridge column (Chrompack, 4330 EA Middelburg, The Netherlands) thermostated at a temperature of 30°C by means of a Julabo thermostatic waterbath RS-6A (Julabo Labor Technik GmbH, D-7633 Seelbach, Germany).

Detection was performed using a 785A Programmable Absorbance Detector (Applied Biosystems Inc, Foster City, CA 94404, USA). The detector was set at a wavelength of 210 nm, a range of 0.005 AUFS and a rise time of 2 s. Data analysis was done with an SP 4270 computing integrator (Spectra–Physics, San Jose, CA 95134, USA).

Other instruments

All analytical weighing was performed with a Mettler AT 261 analytical balance equipped with a Mettler GA 45 printer (Mettler Instruments AG, CH-8606 Greifensee, Switzerland). The pipetting was done by using a Finnpipette (Labsystems, Oy 00810 Helsinki, Finland). The polypropylene pipette-tips were obtained from Sarstedt (Sarstedt, D-5223 Nürmbrecht, Germany). Standard solutions and spiked plasma samples were prepared using Hamilton Micro-liter® syringes (Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland).

The mobile phase was degassed by ultrasonication in a Bransonic 3200 ultrasonic bath (Branson Europa B.V., 3700 AA Soest, The Netherlands). Borosilicate culture tubes 16 × 100 mm (Baxter Healthcare Corp., McGaw Park, IL 60085-6787, USA) were used for the extraction procedure.

Chemicals and reagents

The chemicals used in this analysis were obtained from the following companies: Felbamate was supplied by Schering–Plough Research Institute (Schering–Plough Research Institute, USA). Primidone (Batch Number: 50826-KM-HV) was supplied by Aldrich (Aldrich Chemie Benelux S.A., B-2880 Bornem, Belgium). Phosphoric acid, hydrochloric acid and disodium hydrogen phosphate dihydrate were of analytical grade and supplied by Merck (Merck, D-6100 Darmstadt, Germany). Dichloromethane of HPLC-grade and acetonitrile of far UV-grade were supplied by Labscan (Unit T26 Stillorgan Industrial Park Co., Dublin, Ireland). Water was purified by using a Milli-RO-15 and a Milli-Q water purification system (Millipore Corp., Bedford, MA 01730, USA). The mobile phase was prepared by mixing 420 ml of acetonitrile and 1580 ml of phosphate buffer (pH = 6.5, 0.015 M). The phosphate buffer (pH = 6.5, 0.015 M) was prepared by dissolving 5.3 g of disodium hydrogen phosphate dihydrate in 2000 ml of water. The pH of this solution was adjusted to 6.5 using phosphoric acid (85%). The mobile phase was degassed by ultrasonication for 30 min before use. Stock solutions of felbamate were prepared by dissolving 10.0 mg of felbamate, accurately weighed, in 10.0 ml of methanol. If necessary, 1000 μl of this stock solution was diluted to 10.0 ml with water to obtain standard solutions of felbamate.

The stock solution of primidone, the internal standard, was prepared by dissolving 10.0 mg of primidone in 10.0 ml of methanol. The stock solutions of felbamate and primidone were stored at +4°C for a maximum period of 3 months.

The internal standard solution was prepared by diluting 500 μl of the stock solution of primidone to 500 ml with water. This solution was stored at room temperature. Calibration samples and validation samples were made, in a concentration range of 0.100–50.0 μg ml^{-1}, by properly diluting the stock or standard solutions of felbamate with drug free human plasma. The spiked plasma pools were stored at −20°C and thawed just before use.

Extraction procedure

Plasma samples to be analysed were thawed in a waterbath at a temperature of 37°C and centrifuged for 2 min at 1500*g* (2000 rpm). Aliquots of 200 μl of the plasma samples were transferred into borosilicate tubes. These tubes were placed in the sample racks of the laboratory robot system.

Subsequent sample preparation was done by the robotic system and consisted of the following steps:

— simultaneous addition of 200 μl of the internal standard solution, 100 μl of 0.001 M HCl and 3.0 ml dichloromethane,
— extraction of the analytes on the vortex station for 30 s,

— centrifugation for 10 min at 1300*g* (3000 rpm),
— collection of the organic layer in a clean borosilicate tube and evaporation of the extraction solvent for 20 min at 50°C under a gentle stream of nitrogen,
— reconstitution of the residue into 500 µl phosphate buffer with the vortex station for 30 s,
— on-line injection of 20 µl of the sample extract into the HPLC system.

Results and Discussion

Extraction procedure

As felbamate is a dicarbamate, quantitative extraction may be expected under basic conditions. It was seen, however, that there was no effect of small pH changes on the absolute recovery. Even after addition of 0.001 M HCl instead of basic solutions no effects on the recovery were seen. The advantage of the addition of 0.001 M HCl over the addition of basic solutions was that endogenous compounds, which interfered with felbamate in the chromatogram system, were not extracted.

Selectivity

The selectivity of the analytical procedure was determined by analysing six independent blank plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analysing a test solution of the pure compounds and with

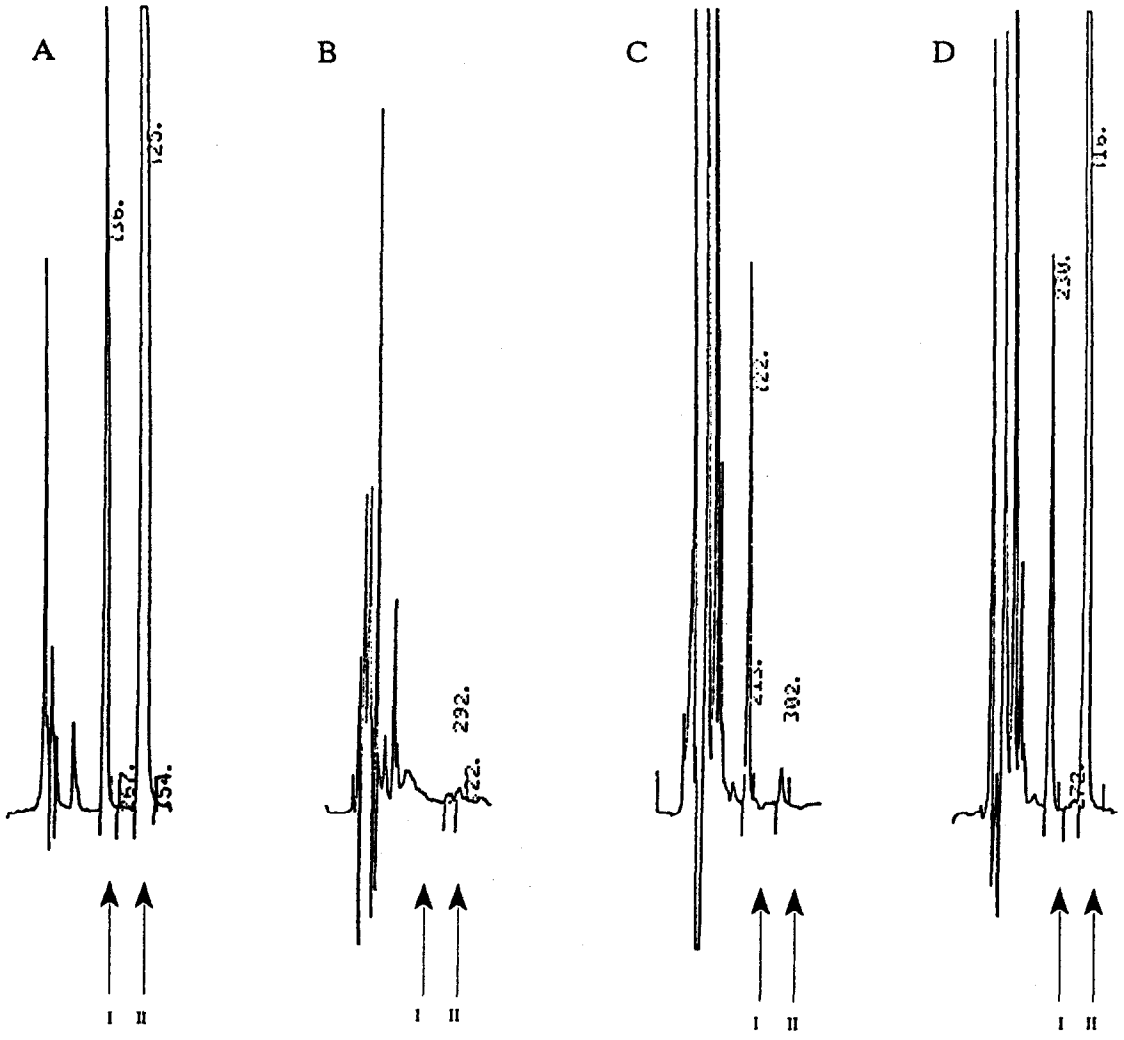

Figure 3
Chromatograms obtained by analysing a test solution containing the pure compounds of primidone (I) and felbamate (II) (A), a blank plasma sample (B), a plasma sample spiked with felbamate showing the lower limit of quantitation (0.100 µg ml^{-1}) (C) and a plasma sample from a subject taken after oral administration of felbamate (1200 mg) concentration 9.33 µg ml^{-1} (D), retention times: primidone (internal standard) ≈ 236 s and felbamate ≈325 s.

chromatograms obtained by analysing plasma samples taken from subjects after oral administration of a felbamate. No interfering peaks were detected at the retention times of the compounds of interest, as can be seen in Fig. 3. The method was also tested for the selectivity towards several commonly used anti-epileptic drugs and their metabolites. A chromatogram obtained by analysing a mixture containing 4-hydroxyphenobarbital, primidone, felbamate, 7-aminoclonazepam, 7-acetamidoclonazepam, phenobarbital, carbamazepine-10,11-epoxide, oxcarbazepine, carbamazepine, phenytoin and clonazepam is given in Fig. 4.

Linearity

Each day a calibration curve was prepared by analysing the spiked calibration samples with felbamate concentrations of 0.100, 0.500, 2.50, 10.0, 20.0, 30.0, 40.0 and 50.0 μg ml^{-1}. Felbamate concentrations were calculated by using weighted least squares linear regression ($W = Y^{-1}$). The linearity of the calibration curve was investigated and was shown to be good over the concentration range of 0.100–50.0 μg ml^{-1}. For a typical calibration curve the regression equation for peak height ratio (y) against concentrations (x) was: $y = 0.5176x + 0.0195$. In general, coefficients of correlation of at least 0.9993 were observed.

Sensitivity

The practical lower limit of quantitation was 0.100 μg ml^{-1}, which was also the lowest concentration of the calibration curve with a relative standard deviation and bias of equal or less than 20% (see Fig. 3).

Recovery

The absolute analytical recovery was determined at three different concentrations for felbamate (0.500, 20.0 and 40.0 μg ml^{-1}) and for the internal standard, primidone, at the concentration which was used during the analysis of the plasma samples (1.0 μg ml^{-1}).

For the determination of the absolute analytical recovery of felbamate and the internal standard, primidone, spiked samples were

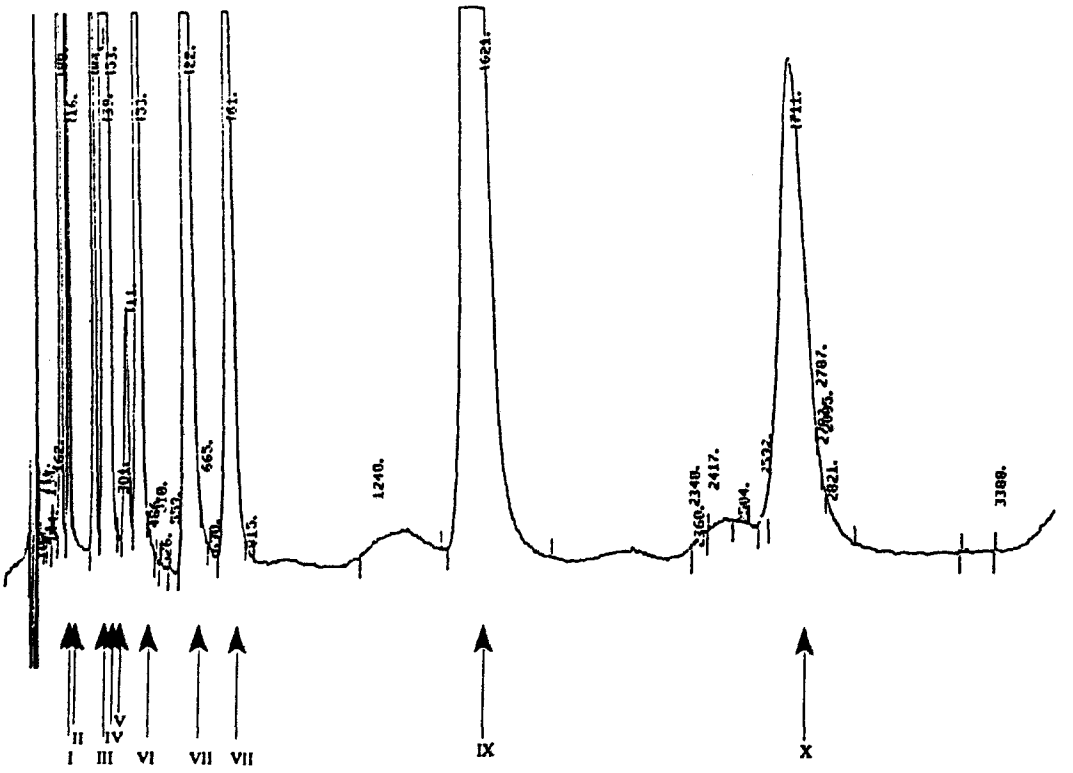

Figure 4
Chromatogram showing the selectivity towards other anti-epileptic drugs and metabolites: retention times I, t = 188 s 4-hydroxyphenobarbital; II, t = 216 s primidone (internal standard); III, t = 304 s felbamate; IV, t = 339 s 7-aminoclonazepam; V, t = 353 7-acetamido-clonazepam; VI, t = 453 s phenobarbital; VII, t = 622 s carbamazepine-10,11-epoxide; VIII, t = 761 s oxcarbazepine; IX, t = 1621 s carbamazepine + phenytoin; X, t = 2711 s clonazepam (concentration of all compounds: 2.5 μg ml^{-1}).

analysed 10 times as described above. The peak heights of these injections were compared to peak heights measured after injecting 10 times an aqueous solution of felbamate and the internal standard, primidone, of the same concentration.

Data on the absolute analytical recovery of felbamate and the internal standard primidone are given in Table 1. The mean recovery of felbamate was found to be 75.2 ± 2.2% (n = 28). The recovery of the internal standard was found to be 74.7 ± 2.0% (n = 10).

Within-day accuracy and precision

The within-day accuracy and precision were determined by evaluation of the results of the analyses of eight different spiked plasma pools 14 times on the same day. The results are given in Table 2.

The relative standard deviations (RSD) were

Table 1
Absolute analytical recovery of felbamate and primidone

	Analytical recovery (%) Felbamate			
Number	0.500 μg ml^{-1}	20.0 μg ml^{-1}	40.0 μg ml^{-1}	Primidone 1.00 μg ml^{-1}
1	76.2	78.9	72.2	75.9
2	78.1	79.5	73.6	73.9
3	77.4	79.1	71.8	74.0
4	75.3	76.0	76.6	74.0
5	76.3	82.2	69.1	74.6
6	75.5	75.0	73.5	79.4
7	73.9	75.3	74.4	73.3
8	74.8	75.4	71.8	73.1
9	74.6	72.3	71.9	74.9
10	74.8	—*	(91.7)†	74.0
Mean (%)	75.7	77.1	72.8	74.7
SD (%)	1.3	3.0	2.1	1.8
RSD (%)	1.8	3.9	2.9	2.4
n	10	9	9	10

*Sample preparation error.
†Rejected as an outlier by the Grubbs test (α = 0.05).

Table 2
Within-day accuracy and precision

	Plasma concentration of felbamate (μg ml^{-1})							
Number	0.100	0.500	2.50	10.0	20.0	30.0	40.0	50.0
1	0.081	0.492	2.57	10.6	20.9	31.0	41.0	43.9
2	0.108	0.503	2.54	10.6	20.6	29.3	37.9	48.6
3	0.083	0.516	2.53	10.6	20.8	30.1	38.6	47.6
4	0.110	0.502	2.54	10.6	20.7	29.9	38.3	46.9
5	0.109	0.504	2.53	10.5	20.5	30.1	39.0	47.4
6	0.096	0.510	2.55	10.6	21.8	30.4	36.6	47.2
7	0.078	0.507	2.51	10.5	20.7	30.6	39.9	(41.8)†
8	0.134	0.499	2.46	10.3	20.3	30.3	40.3	48.3
9	0.106	0.513	2.54	10.3	20.3	30.5	39.1	50.1
10	0.108	0.516	2.52	10.3	20.4	30.5	40.1	48.4
11	0.111	0.518	2.49	10.3	(17.2)*	30.5	40.2	51.4
12	0.107	0.521	2.50	10.5	20.5	30.3	38.9	48.4
13	0.124	0.533	2.53	10.8	21.0	30.8	39.1	47.9
14	0.149	0.543	2.60	10.6	20.7	30.9	(31.0)†	49.6
$\bar{X}$ (μg ml^{-1})	0.107	0.513	2.53	10.5	20.7	30.4	39.2	48.1
SD (μg ml^{-1})	0.020	0.014	0.03	0.15	0.41	0.43	1.18	1.79
RSD (%)	18.3	2.6	1.4	1.4	2.0	1.4	3.0	3.7
Bias (%)	7.4	2.5	1.2	5.1	3.5	1.3	−2.1	−3.7
n	14	14	14	14	13	14	13	13

*Sample preparation error.
†Rejected as an outlier by the Grubbs test (α = 0.05).

below 3.8% at all concentrations, except at the lowest limit of quantitation (0.100 μg ml^{-1}), where the RSD was 18.3%. The accuracy of the method expressed by the bias ($\bar{x}$ − μ)/μ) varied between −3.7 and +7.4% at all concentrations.

Between-day accuracy and precision

The between-day accuracy and precision were determined by evaluating the results of the analyses of eight independently spiked plasma pools over a period of 8 days. The concentrations of the plasma pools were divided over the entire calibration range. The results are given in Table 3. The RSDs were below 5.0% at all concentrations. The accuracy of the method expressed by the bias (($\bar{x}$ − μ)/μ) varied between −5.7 and 1.6% at all conentrations.

Stability

The stability of felbamate during storage at room temperature was determined by analysing spiked plasma samples at three concentrations (0.500, 20.0 and 40.0 μg ml^{-1}) during a total period of 28 h. Plasma samples containing felbamate showed no signs of deterioration during the analyses stored at room temperature (see Fig. 5).

The stability during repeated freezing and

Table 3
Between-day precision and accuracy

	Plasma concentration of felbamate (μg ml^{-1})							
Date (y-m-d)	0.100	0.500	2.5	10.0	20.0	30.0	40.0	50.0
93-10-05	0.088	0.515	2.50	10.5	20.9	30.9	40.3	47.6
93-10-11	0.096	0.518	2.48	10.1	20.1	30.0	40.1	49.7
93-10-12	0.100	0.502	2.48	10.0	19.9	30.5	40.2	49.5
93-10-13	(0.303)*	0.506	2.44	10.0	20.1	30.8	39.3	49.8
93-10-14	0.088	0.525	2.53	10.2	20.3	30.5	39.4	49.6
93-10-15	0.099	0.491	2.52	10.1	20.2	30.4	39.5	49.9
93-10-20	0.094	0.500	2.54	10.2	20.4	30.3	39.3	49.8
93-10-21	0.095	0.494	2.54	10.2	20.3	30.2	39.6	49.7
$\bar{x}$ (μg ml^{-1})	0.094	0.506	2.50	10.2	20.3	30.4	39.7	49.4
SD (μg ml^{-1})	0.005	0.012	0.04	0.2	0.3	0.3	0.4	0.8
RSD (%)	4.9	2.4	1.4	1.5	1.6	1.0	1.1	1.5
Bias (%)	−5.7	1.3	0.1	1.6	1.4	1.5	−0.7	−1.1
n	7	8	8	8	8	8	8	8

* Rejected as an outlier by the Grubbs test (α = 0.05).

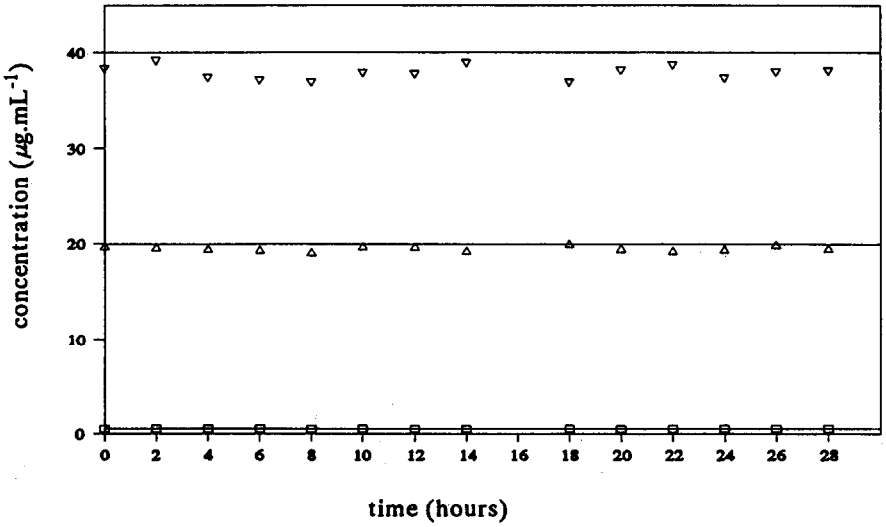

Figure 5
Stability of felmabate in human plasma during storage at room temperature.

thawing was determined by five freeze/thaw cycles at two concentrations (0.500 and 40.0 μg ml^{-1}). After each freeze/thaw cycle the samples were analysed in duplicate. The data are presented in Fig. 6. No significant deterioration was observed.

Dilution test

If a plasma sample has a concentration higher than 50.0 μg ml^{-1}, this sample has to be diluted in such a way that the concentration after dilution will be within the validated calibration range. To validate this dilution a spiked plasma pool was prepared with a concentration of 80.0 μg ml^{-1}. An aliquot of 50 μl was taken and blank human plasma was added to a total volume of 200 μl. These samples were analysed as described in *Extraction procedures*. The mean (±SD) results after dilution were 80.3 ± 2.8 mg l^{-1}. The bias $((\bar{x} - \mu)/\mu)$

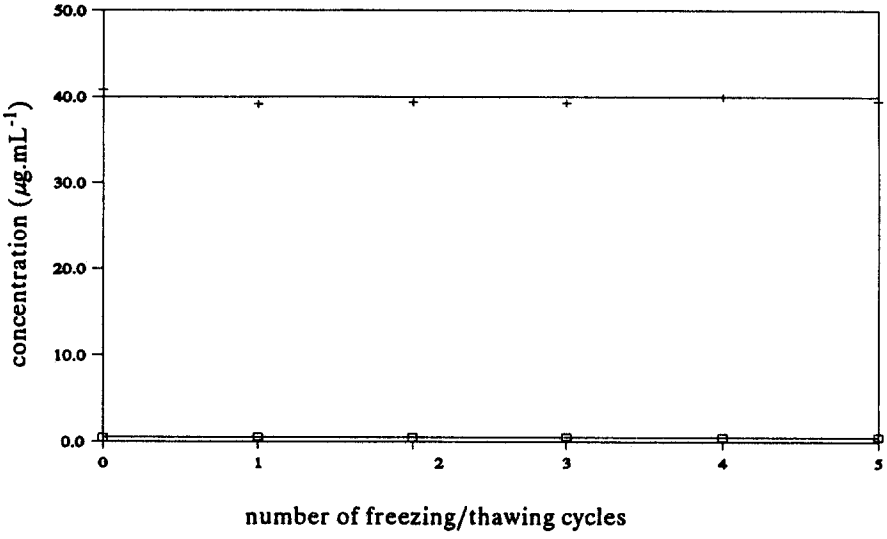

Figure 6
Stability of felbamate during freezing and thawing.

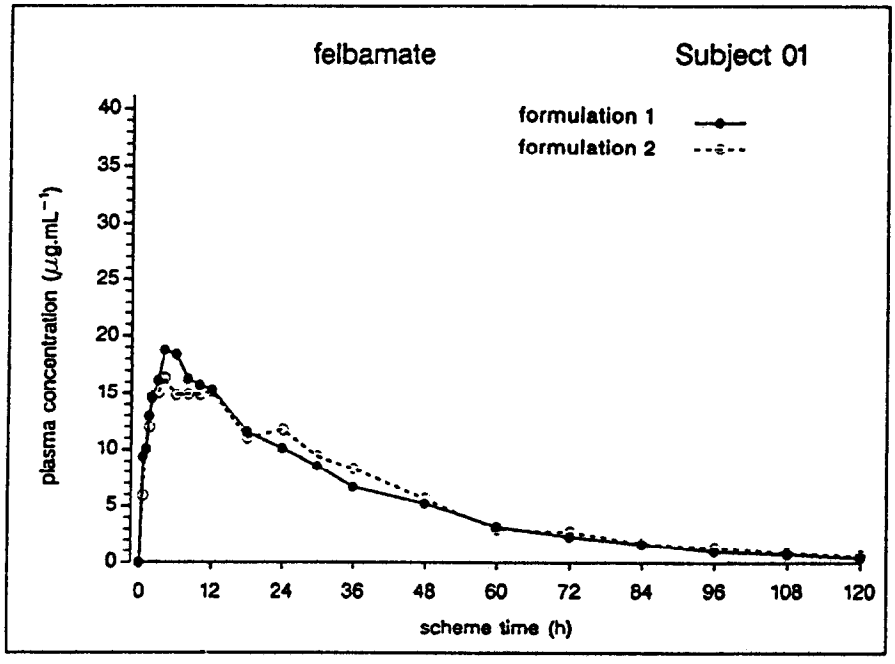

Figure 7
Plasma concentration–time curves. Plasma concentration–time curves after the application of the robotic method in a pharmacokinetic study. Comparison of two 1200 mg formulations of felbamate in a healthy volunteer.

of the mean observed felbamate concentration is 0.4%, which appeared not to be a significant difference according to the students *t*-test (α = 0.05).

Application of the method

The method is currently in use for the analysis of thousands of samples generated from clinical trials, including pharmacokinetic and drug-interaction studies. The sample throughput of the method is about 100 samples per 24 h (calibration curve and quality control samples included). A plasma concentration curve of a subject after the administration of an oral dose of two formulations of 1200 mg of felbamate is given in Fig. 7.

Conclusions

The method described for the determination of felbamate in human plasma has proved to be selective and sensitive. It also shows good precision and accuracy. In view of the number of samples that can be analysed per day this method is suitable for routine analysis of plasma samples in clinical trials.

References

[1] L.A. Clark, J.K. Wichmann, N. Kucharczyk and R.D. Sofia, *J. Chromatogr.* **573**, 113–119 (1992).
[2] R.P. Remmel, S.A. Miller and N.M. Graves, *Therapeutic Drug Monitoring* **12**, 90–96 (1990).
[3] J. Hempenius, J. Wieling, J.H.G. Jonkman, O.E. de Noord, P.M.J. Coenegracht and D.A. Doornbos, *J. Pharm. Biomed. Anal.* **8**, 313–320 (1990).
[4] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, *J. Pharm. Sci.* **81**, 309–312 (1992).
[5] J. Wieling, J. Hempenius, H.J. Jeuring, J.H.G. Jonkman, P.M.J. Coenegracht and D.A. Doornbos, *J. Pharm. Biomed. Anal.* **8**, 577–582 (1990).
[6] F.E. Grubbs and G. Beck, *Technometrics* **14**, 847–854 (1972).

[Received for review 9 February 1994; revised manuscript received 26 April 1994]