Journal of Chromatography B, 878 (2010) 3432–3436

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Rapid and sensitive LC–MS/MS method for determination of felbamate in mouse plasma and tissues and human plasma

Ryan J. Hansen, Bradley J. Samber, Daniel L. Gustafson*

Pharmacology Core Laboratory, Department of Clinical Sciences, Animal Cancer Center, Colorado State University, Fort Collins, CO 80523-1678, United States

ARTICLE INFO

Article history:
Received 11 March 2010
Accepted 8 October 2010
Available online 16 October 2010

Keywords:
Felbamate
LC–MS/MS
Human plasma
Antiepileptic drug
Liquid chromatography tandem mass spectrometry

ABSTRACT

Felbamate (2-phenyl-1,3-propanediol dicarbamate) is a second generation antiepileptic drug used to treat seizures refractory to other antiepileptic drugs. With approximately 3500 new patients exposed annually, several important pharmacologic interaction questions remain unanswered necessitating the need for rapid and accurate methods of felbamate analysis in biological matrices. To this end, a rapid liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed for the measurement of felbamate in mouse plasma and tissues and human plasma. Plasma (100 μL) and tissues homogenates (100 μL of 100 mg/mL) were spiked with internal standard (carisoprodol) prior to protein precipitation with acetonitrile. Samples were chromatographed on a XBridge Phenyl, 2.5 μm, 4.6 mm × 50 mm column with quantitation by internal standard reference monitoring of the ion transitions *m/z* 239 → 117 for felbamate and *m/z* 261 → 176 for carisoprodol. Calibration curves were linear from 2.5 to 500 ng/mL in mouse or human plasma and 25–5000 pg/mg in tissue homogenates. Recoveries were greater than 97% for plasma and homogenates with accuracies >92% in any of the mouse matrices and >88% in human plasma. Comparable accuracies and precision were found with and without the use of the internal standard in preparation of the calibration curves and suggest that the internal standard may not be required.

1. Introduction

Felbamate (2-phenyl-1,3-propanediol dicarbamate) was approved in 1993 for the treatment of epilepsy. Felbamate was the first 'second-generation' antiepileptic drug approved for use to treat partial seizures in adults [1] and partial and generalized seizures associated with Lennox–Gastaut syndrome [2] in children since approval of valproic acid in 1978 [3]; however, shortly after it was black-labeled and only approved for treating seizures refractory to other antiepileptic drugs due to idiosyncratic side effects including aplastic anemia [4] and hepatotoxicity [5]. Upon further examination of the clinical data, it is currently reserved for use in patients with severe epilepsy for whom the benefits outweigh the risks and when administered in this situation, approximately 3500 new patients are exposed annually [6].

Questions as to efficacy and pharmacologic interaction of felbamate with other epileptic drugs and medications remain to be answered. Recently, it appears that use of antiepileptic drugs, including felbamate, nearly double the risk of suicidal thoughts or actions [7] and studies are underway to understand the mechanism. Another unanswered issue is how felbamate induces CYP3A expression as indicated in a pharmacokinetic interaction study [8]. In accordance with this, possible reduced contraceptive efficacy (many being CYP3A4 substrates) cannot be ruled out as altered hormone levels following concomitant treatment of felbamate with oral contraceptives has been observed [8]. In addition, it is unknown what the pharmacokinetics of felbamate are in infants or pregnant women and only a few studies have investigated felbamate pharmacokinetics in children [9–11], indicating an average increase of 20–65% for the apparent clearance in children compared to adults. Therapeutic drug monitoring has become important for the proper treatment of felbamate in patients taking the drug [12] and, if done in a prospective manner, links between steady state felbamate levels and treatment outcome may be possible.

While several groups have published HPLC-UV methods for the analysis of felbamate [13–15], there are none that measure felbamate via LC–MS/MS. A previously published GC–MS method [16] used liquid–liquid extraction, larger sample sizes (1 mL) and had a run time greater than 12 min. No LLOQ was published for this assay and blood was the only biological matrix utilized where the method was described. An LC–MS method was also previously published [17] utilizing LC and a mass analyzer. This method utilized SPE sample preparation and had a run time of 12 min. Urine was the only matrix utilized and no LLOQ was reported. It was our goal to develop a fast (analytical run time of 4 min), simple (acetonitrile drop) and sensitive (LLOQ of 2.5 ng/mL in plasma and 25 pg/mg of

* Corresponding author at: Department of Clinical Sciences, Animal Cancer Center, Colorado State University, 1678 Campus Delivery, Fort Collins, CO 80523-1678, United States. Tel.: +1 970 297 1278; fax: +1 970 297 1254.
E-mail address: Daniel.Gustafson@colostate.edu (D.L. Gustafson).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.006

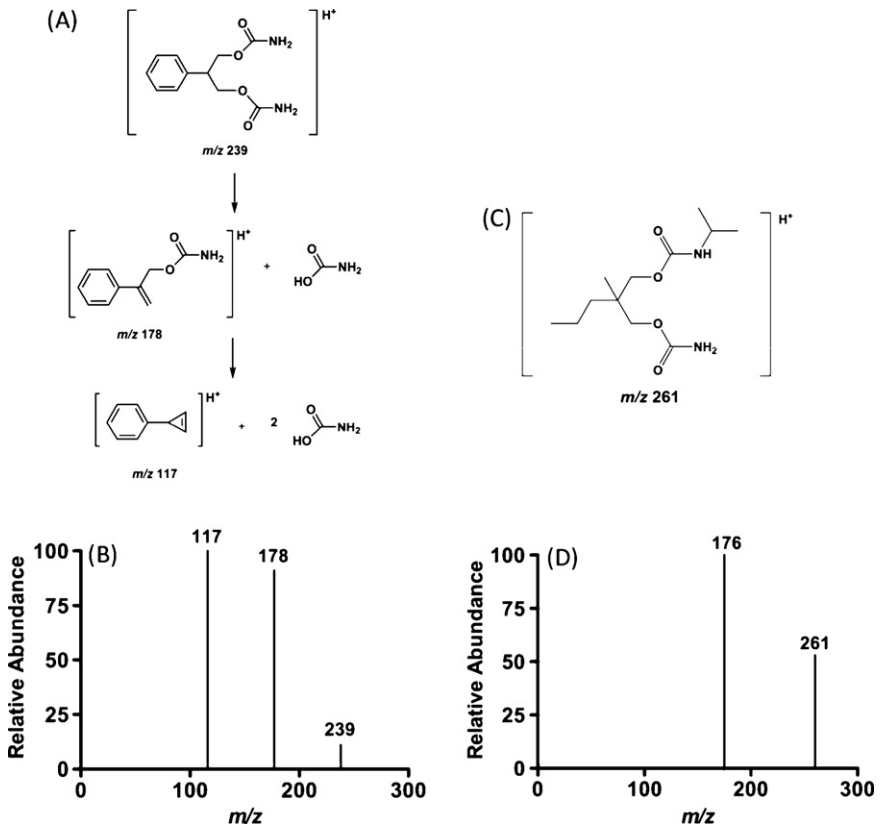

Fig. 1. Proposed structures of felbamate $[MH]^+$ ion and product ions, and the product ion spectra for felbamate and carisoprodol. (A) The proposed structures *m/z* 178 and 117 and (B) the product ion spectrum for felbamate. (C) The reported $[MH]^+$ parent ion for carisoprodol and (D) the product ion spectrum for the internal standard, carisoprodol, under the described mass spectrometric conditions noted in text.

tissue) LC–MS/MS method to facilitate measurement of felbamate to aid in preclinical and clinical studies.

2. Experimental

2.1. Chemicals and reagents

Felbamate and carisoprodol were purchased from Sigma–Aldrich Corporation (St. Louis, MO). Pooled blank sodium heparin collected mouse and human plasma was purchased from Innovative Research (Novi, MI). Mouse tissues were obtained from untreated mice obtained from lab animal resources (LAR) at Colorado State University. The LAR facilities are specific pathogen free and are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All other reagents were of analytical grade and were purchased from commercial suppliers.

2.2. Standards preparation

Standard dilutions of felbamate and carisoprodol were prepared in methanol:water (1:1). For the analysis in plasma, felbamate was added to naïve pooled mouse or human plasma (2.5–500 ng/mL). To analyze felbamate in tissues, felbamate (25–5000 pg/mg) was added to control tissue homogenate (100 mg/mL in water) and the samples prepared as described in Section 2.3.

2.3. Sample preparation

One hundred microliters (100 μL) of plasma or tissue homogenate (100 mg/mL in water) was used for the sample preparation. Each sample was spiked with 1 ng (10 μL of 100 ng/mL) carisoprodol, vortexed briefly, and then 300 μL acetonitrile was added. Samples were vortex mixed continuously for 10 min followed by centrifugation for 10 min at 21,000 × *g* at RT. The supernatant was collected then analyzed via LC–MS/MS.

2.4. Mass spectrometry

Positive ion electrospray ionization (ESI) mass spectra were obtained with a MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA) with a turbo ionspray source interfaced to an Agilent 1200 Series Binary Pump SL HPLC system (Santa Clara, CA). Samples were chromatographed with an XBridge Phenyl, 2.5 μm, 4.6 mm × 50 mm column (Waters Corporation, Milford, MA) protected by a C18 guard cartridge, 4.0 mm × 2.0 mm (Phenomenex, Torrance, CA). An LC gradient was employed with mobile phase A consisting of 10 mM ammonium acetate and mobile phase B consisting of acetonitrile. Chromatographic resolution was achieved by increasing mobile phase B linearly from 10% to 85% from 0 to 1.5 min, maintaining at 85% from 1.5 to 3 min, decreasing linearly from 85% to 10% from 3 to 3.25 min, followed by re-equilibration of the column at 10% mobile phase B from 3.25 to 4 min. The LC flow rate was 1 mL/min, the sample injection volume was 20 μL, and the analysis run time was 4 min.

The mass spectrometer settings were optimized as follows: turbo ionspray temperature, 600 °C; ionspray voltage, 5500 V; declustering potential (DP), 11 V (felbamate) and 16 V (carisoprodol); entrance potential (EP), 8.5 V; collision energy (CE), 25 V (felbamate) and 13 V (carisoprodol); collision cell entrance potential (CEP), 10 V; collision cell exit potential (CXP), 2 V; curtain gas, N_2, (CUR), 35 units; collision gas, N_2, (CAD), medium; nebulizer gas, N_2, 65 units; and auxiliary gas, N_2, 65 units. The product ion scan obtained on infusion of felbamate is shown in Fig. 1. The pre-

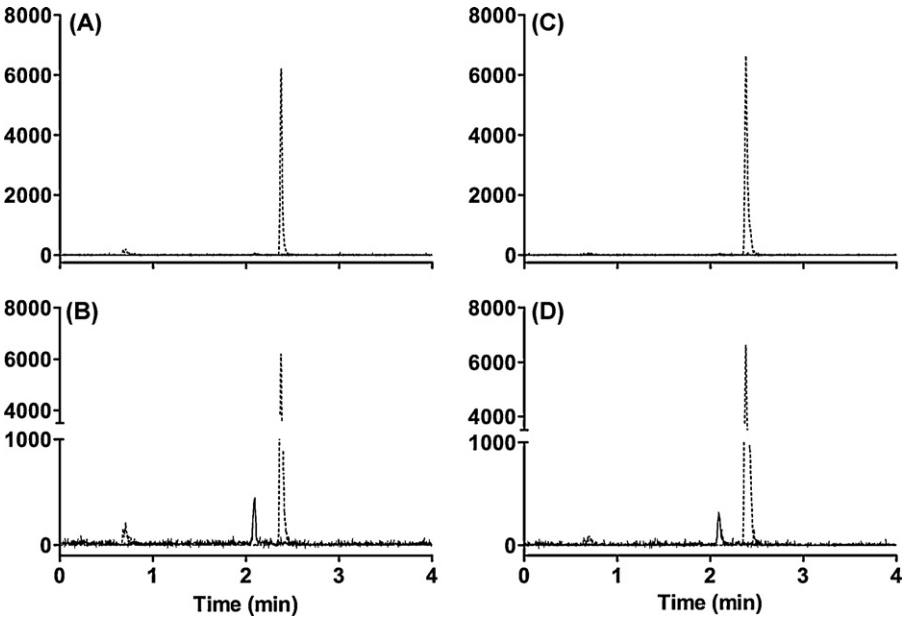

Fig. 2. MRM chromatograms of spiked blank mouse plasma and brain homogenate. Blank mouse plasma spiked with 1 ng carisoprodol (A) and a felbamate plasma standard at 2.5 ng/mL (LLOQ) spiked with 1 ng carisoprodol (B). Blank mouse brain homogenate spiked with 1 ng carisoprodol (C) and a felbamate brain homogenate standard at 25 pg/mg (LLOQ) spiked with 1 ng carisoprodol (D). The solid line represents *m/z* transition from 239 → 117 (felbamate, R_t = 2.1 min) and the dashed line represents *m/z* transition from 261 → 176.3 (carisoprodol, R_t = 2.4 min).

dominant product ion was *m/z* 117. Samples were quantified by internal standard reference method in the MRM mode monitoring ion transitions *m/z* 239 → 117 for felbamate and *m/z* 261 → 176 for the internal standard, carisoprodol (product ion scan is shown in Fig. 1). The dwell times for each ion transition were 100 ms. Q_1 and Q_3 were both operated in unit resolution mode.

2.5. Data analysis

Quantitation of felbamate was based on linear standard curves in spiked matrix using the ratio of felbamate peak area to carisoprodol peak area and $1/x^2$ weighting of linear regression. Additionally, quantitation was determined using the felbamate peak area alone with $1/x^2$ weighting to determine the necessity of the internal standard. Parameters for the assessment of assay performance were calculated as follows:

$$\text{Accuracy}\,(\%) = \left(1 - \left|\frac{\text{Theoretical} - \text{Measured}}{\text{Theoretical}}\right|\right) \times 100$$

$$\text{Precision}\,(\text{RSD}\%) = \frac{\text{Standard deviation calculated values}}{\text{Mean calculated values}} \times 100$$

$$\text{Matrix factor}\,(\text{MF}) = \frac{(\text{Analyte peak area in post extraction spiked sample})}{(\text{Analyte peak area in solvent sample})}$$

3. Results and discussion

3.1. Chromatography

Felbamate and carisoprodol eluted at 2.1 and 2.4 min, respectively. Peaks were detected by monitoring ion transitions *m/z* 239 → 117 for the analyte felbamate and *m/z* 261 → 176.3 for carisoprodol. No interfering peaks were detected at the monitored ion transitions in extracted matrix (plasmas or tissue homogenates). Chromatographic conditions were optimized for peak shape. Representative chromatographs observed in mouse plasma (Fig. 2A and B) and mouse brain homogenate (Fig. 2C and D) standards are shown.

3.2. Use of carisoprodol as an internal standard

Carisoprodol, a structurally similar dicarbamate (Fig. 1C), was selected as an internal standard due to similar retention times during the elution portion of the chromatography and its robust (the average coefficient of variation (%CV) of the area under the curve for the transition used to identify carisoprodol was 3.9% (±0.4%) for all standard and quality control samples) and consistent fragmentation [18]. Accuracy and precision calculations in determining felbamate concentrations within batch and between batches were found to be slightly better when the internal standard was not used; however, the fact that both accuracy and precision were within acceptance for biological assay [19] using the felbamate/internal standard ratio, inclusion of an internal standard in the assay can be used to confirm injection of unknown samples with low amounts or no felbamate.

3.3. Assessment of ion suppression and matrix effects

Ion suppression and matrix effects were assessed with this method by the comparison of responses obtained from solvent standards (standards added to water prior to an acetonitrile drop) and extracted standards (mouse plasma and mouse brain tissue homogenate) with compounds added post extraction, where the amount of analyte was normalized to the extraction volume. For felbamate, a slight ion enhancement was observed in mouse plasma samples (average matrix factor (MF)) was 1.07) and no ion suppression was observed in mouse brain homogenate (average MF = 1.00). For the internal standard, slight ion enhancement was observed in mouse plasma samples (average MF = 1.12) and mouse brain homogenate (average MF = 1.10). The use of small sample volumes (100 μL) and subsequent final sample dilution with acetonitrile is possible due to the high instrument sensitivity of the LC–MS/MS assay.

Table 1
Precision and accuracy for felbamate assay in mouse and human plasma within and between runs.

Concentration[a] (ng/mL)	Within representative run ($n=4$)			Between runs ($n=3$)		
	Observed	Accuracy	Precision (RSD%)	Observed	Accuracy	Precision (RSD%)
Mouse						
2.5				2.30 ± 0.22	92.0	9.5
5	5.12 ± 0.33	97.6	6.5	5.00 ± 0.32	100	6.3
10				10.2 ± 0.4	98.0	3.7
25				26.2 ± 0.3	95.2	1.3
50	50.7 ± 2.5	98.6	4.9	53.2 ± 1.9	93.6	3.6
100				104 ± 4	96.0	3.9
250				243 ± 9	97.2	3.7
500	455 ± 10	91.0	2.1	468 ± 13	93.6	2.7
Human						
2.5				2.43 ± 0.10	97.2	4.1
5	5.56 ± 0.18	88.8	3.1	5.16 ± 0.41	96.8	8.0
10				10.3 ± 0.3	97.0	2.5
25				25.7 ± 1.0	97.2	4.1
50	50.7 ± 0.9	98.6	1.7	52.7 ± 2.4	94.6	4.6
100				98.9 ± 2.4	98.9	2.5
250				237 ± 3	94.8	1.1
500	475 ± 3	95.0	0.6	475 ± 12	95.0	2.5

[a] Values are concentration in the plasma (100 μL) that was extracted for analysis.

3.4. Linearity and LLOQ

Standard curves in spiked mouse plasma and human plasma were linear over the range of 2.5–500 ng/mL. The linearity of standard curves in all matrices was greater than $r^2 = 0.99$ using uniform, $1/x$, or $1/x^2$ weighting. While all three forms of weighting gave linear regression analysis with r^2 values approaching 1, the use of $1/x^2$ weighting for the standard curve resulted in higher accuracy at lower standard values.

As measured in mouse and human plasma, the lower limit of quantitation (LLOQ), based on standard analytical method validation guidelines [19], was determined to be 2.5 ng/mL in plasma. The LLOQ as measured in mouse brain and liver homogenate is 25 pg/mg of tissue. An injection volume of 20 μL allows for quantification of 12.5 pg (~52.5 fmol) per sample injection using the acetonitrile-based sample preparation and analysis described.

3.5. Precision, accuracy and recovery

The precision and accuracy of felbamate measurement were assessed in spiked mouse and human plasma (Table 1), and mouse brain and liver homogenate (Table 2). The accuracy of within-run drug measurements in both mouse and human plasma at concentrations 5, 50 and 500 ng/mL ranged from 88.8 to 98.4% with the precision (as measured by RSD) ranging from 0.6 to 6.5%. The between-run accuracy at concentrations ranging from 2.5 to 500 ng/mL in either mouse or human plasma was from 92.1 to 100% and the precision from 1.1 to 9.5%. The within-run accuracy of the assay at concentrations of 50, 500 and 5000 pg/mg in mouse brain and liver tissue was from 94.1 to 97.1% with the precision ranging from 0.4 to 8.4%. The between-run accuracy at concentrations ranging from 25 to 5000 pg/mg in mouse brain and liver tissue was from 92.5 to 99.7% with the precision ranging from 1.1 to 5.9%. All accuracy and precision measurements were within the minimum accuracy (85%) and precision (±20%) ranges for acceptable analytical method validation [19]. The average recovery of felbamate from spiked mouse plasma and mouse brain tissue was 100% and 97%, respectively. Recovery was determined by the comparison of the ratios of the felbamate peak area to IS peak area of standards added to matrix followed by sample preparation to the peak area ratio in post addition of standards following sample preparation.

Table 2
Precision and accuracy for felbamate assay in mouse brain and liver within and between runs.

Concentration[a] (ng/mL)	Within representative run ($n=4$)			Between runs ($n=3$)		
	Observed	Accuracy	Precision (RSD%)	Observed	Accuracy	Precision (RSD%)
Brain						
25				24.5 ± 0.3	98.0	1.1
50	52.0 ± 4.4	96.0	8.4	52.0 ± 1.7	96.0	3.3
101				99.2 ± 4.4	99.2	4.5
250				252 ± 11	99.2	4.2
500	525 ± 21	95.0	4.0	526 ± 25	94.8	4.8
1000				1010 ± 41	99.0	4.0
2500				2480 ± 147	99.2	5.9
5000	4740 ± 17	94.8	0.4	4620 ± 127	92.4	2.7
Liver						
25				25.2 ± 0.6	99.2	2.2
50	50.2 ± 1.9	99.6	3.8	49.3 ± 2.8	98.6	5.6
100				101 ± 3	99.0	3.4
250				246 ± 6	98.4	2.5
500	518 ± 25	96.4	4.8	498 ± 8	99.6	1.6
1000				1000 ± 21	100	2.1
2500				2480 ± 40	99.2	1.6
5000	5060 ± 181	98.8	3.6	5110 ± 169	97.8	3.3

[a] Values are concentration per unit of mass brain or liver tissue in the spiked homogenate.

3.6. Stability of felbamate samples

Felbamate stability in plasma at 5, 50 and 500 ng/mL was determined for three freeze–thaw cycles and long-term stability in plasma (21-day at −70 °C). At 50 and 500 ng/mL, felbamate was stable under freeze–thaw and long-term conditions, while there was a slight reduction (∼10%) in felbamate concentration at the 5 ng/mL concentration under both stability conditions. Post extraction mouse plasma samples spiked with felbamate proved to be stable after 72 h at room temperature. To assess stability, standards and QC samples were re-injected after 72 h at room temperature. All samples met acceptance criteria [19].

4. Conclusion

Felbamate is an anticonvulsant drug used for the treatment of epilepsy. The current literature does not present an LC–MS/MS based assay for the measurement of felbamate in biological matrices. Therefore, we developed a quick and robust analytical method for the measurement of felbamate in mouse plasma, brain, liver, and human plasma. Carisoprodol was used as an internal standard in this analysis, although data analysis proved that the internal standard was not necessary for accurate determination of felbamate, the internal standard did served as an injection volume consistency. In summary, we have developed and validated a rapid, accurate method for felbamate quantitation in plasma and tissues by LC–MS/MS utilizing a fast and simple sample preparation that provides a format for therapeutic drug monitoring in clinical and preclinical studies in addition to pharmacokinetic and bioavailability studies. While reported levels in adult patients is in the range of 9–60 μg/mL [12], it has previously been shown samples can be diluted with naïve matrix [15].

Acknowledgement

This work was carried out and funded by the Pharmacology Core Laboratory located in the Animal Cancer Center at Colorado State University.

References

[1] P.K. Jensen, Epilepsia 34 (Suppl. 7) (1993) S25.
[2] S.R. Snodgrass, N. Engl. J. Med. 328 (22) (1993) 1641.
[3] F.a.D. Administration, FDA Drug Bull. 8 (2) (1978) 14.
[4] P.B. Pennell, M.S. Ogaily, R.L. Macdonald, Neurology 45 (3 Pt 1) (1995) 456.
[5] M.G. O'Neil, C.S. Perdun, M.B. Wilson, S.T. McGown, S. Patel, Neurology 46 (5) (1996) 1457.
[6] J.M. Pellock, E. Faught, I.E. Leppik, S. Shinnar, M.L. Zupanc, Epilepsy Res. 71 (2–3) (2006) 89.
[7] F.a.D. Administration, FDA Safe. Alert (2008).
[8] V. Saano, P. Glue, C.R. Banfield, P. Reidenberg, R.D. Colucci, J.W. Meehan, P. Haring, E. Radwanski, A. Nomeir, C.C. Lin, et al., Clin. Pharmacol. Ther. 58 (5) (1995) 523.
[9] C.R. Banfield, G.R. Zhu, J.F. Jen, P.K. Jensen, R.C. Schumaker, J.L. Perhach, M.B. Affrime, P. Glue, Ther Drug Monit. 18 (1) (1996) 19.
[10] L. Carmant, G.L. Holmes, S. Sawyer, N. Rifai, J. Anderson, M.A. Mikati, J. Pediatr. 125 (3) (1994) 481.
[11] M.T. Kelley, P.D. Walson, S. Cox, L.J. Dusci, Ther. Drug Monit. 19 (1) (1997) 29.
[12] S.I. Johannessen, T. Tomson, Clin. Pharmacokinet. 45 (11) (2006) 1061.
[13] M. Contin, M. Balboni, E. Callegati, C. Candela, F. Albani, R. Riva, A. Baruzzi, J. Chromatogr. B 828 (1–2) (2005) 113.
[14] J Hempenius, G. Hendriks, J. Hingstman, C.K. Mensink, J.H. Jonkman, C.C. Lin, J. Pharm. Biomed. Anal. 12 (11) (1994) 1443.
[15] P.H. Tang, J. Anal. Toxicol. 32 (5) (2008) 373.
[16] P.N. Friel, B.K. Logan, E.J. Formosco, J. Epilepsy 10 (1997) 283.
[17] C.D. Thompson, M.T. Barthen, D.W. Hopper, T.A. Miller, M. Quigg, C. Hudspeth, G. Montouris, L. Marsh, J.L. Perhach, R.D. Sofia, T.L. Macdonald, Epilepsia 40 (6) (1999) 769.
[18] W. Skinner, D. McKemie, S. Stanley, Chromatographia 59 (Suppl. 1) (2004) S61.
[19] S. Bansal, A. DeStefano, AAPS J. 9 (1) (2007) E109.