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Short communication

Simultaneous HPLC–UV analysis of rufinamide, zonisamide, lamotrigine, oxcarbazepine monohydroxy derivative and felbamate in deproteinized plasma of patients with epilepsy

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ABSTRACT

We present an implementation of a method we previously reported allowing the newer antiepileptic drugs (AEDs) rufinamide (RFN) and zonisamide (ZNS) to be simultaneously determined with lamotrigine (LTG), oxcarbazepine's (OXC) main active metabolite monohydroxycarbamazepine (MHD) and felbamate (FBM) in plasma of patients with epilepsy using high performance liquid chromatography (HPLC) with UV detection. Plasma samples (250 μL) were deproteinized by 1 mL acetonitrile spiked with citalopram as internal standard (I.S.). HPLC analysis was carried out on a Synergi 4 μm Hydro-RP, 250 mm × 4.6 mm I.D. column. The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5), acetonitrile and methanol (65:26.2:8.8, v/v/v) at an isocratic flow rate of 0.8 mL/min. The UV detector was set at 210 nm. The chromatographic run lasted 19 min. Commonly coprescribed AEDs did not interfere with the assay. Calibration curves were linear for both AEDs over a range of 2–40 μg/mL for RFN and 2–80 μg/mL for ZNS. The limit of quantitation was 2 μg/mL for both analytes and the absolute recovery ranged from 97% to 103% for RFN, ZNS and the I.S. Intra- and interassay precision and accuracy were lower than 10% at all tested concentrations. The present study describes the first simple and validated method for RFN determination in plasma of patients with epilepsy. By grouping different new AEDs in the same assay the method can be advantageous for therapeutic drug monitoring (TDM).

1. Introduction

Rufinamide (RFN) {1-[(2,6-difluorophenyl)methyl]-1H-1,2,3-triazole-4 carboxamide} (Inovelon®, Eisai Co., Ltd., Tokyo, Japan) is a triazole derivative structurally unrelated to current antiepileptic drugs (AEDs). RFN has received Orphan Drug status in both the European Union and the United States for adjunctive therapy in patients with partial seizures and drop attacks associated with the Lennox–Gastaut syndrome [1]. Two HPLC–UV methods coupled with automated procedures utilizing laboratory robotics have been reported for the determination of RFN in body fluids during early pharmaceutical development [2,3]: after plasma dilution with water RFN was automatically extracted on reversed-phase extraction columns and injected into the HPLC system. Neither of these methods has been validated in plasma of patients with epilepsy. The peculiar pharmacokinetics (slow and "dose dependent" absorption) of RFN and its remarkable pharmacokinetic interaction potential with concomitant AEDs [1] suggest that therapeutic drug monitoring (TDM) may be helpful in drug handling in patients with epilepsy.

Zonisamide (ZNS) (1,2-benzisoxazole-3-methanesulfonamide) (Zonegran®, Eisai Co., Ltd., Tokyo, Japan) is another relatively new AED licensed for adjunctive treatment of partial seizures with or without generalization in adults [4]. Several HPLC methods have been published for the determination of ZNS in human plasma, coupled with UV [5–11], diode array [12], or mass spectrometry [13] detection, with different sample pre-treatment procedures: liquid–liquid extraction [5–8,11], solid phase extraction [12,13], or deproteinization [9,10]. Some of these methods allow for the simultaneous determination of ZNS with other AEDs, including the new generation AED levetiracetam (LEV) [8], lamotrigine (LTG) [10–13], oxcarbazepine's (OXC) main active metabolite monohydroxycarbamazepine (MHD) [10,12,13] and topiramate (TPM) [13]. As a result of the reported pharmacokinetic variability and interaction potential, the TDM of ZNS can be considered in guiding dosage adjustment [14].

Here we propose an implementation of a HPLC–UV method we previously reported for the simultaneous measurement of LTG, MHD and felbamate (FBM) in an AED TDM setting [15], which describes the first simple and validated method for RFN determination in plasma of patients with epilepsy and allows RFN

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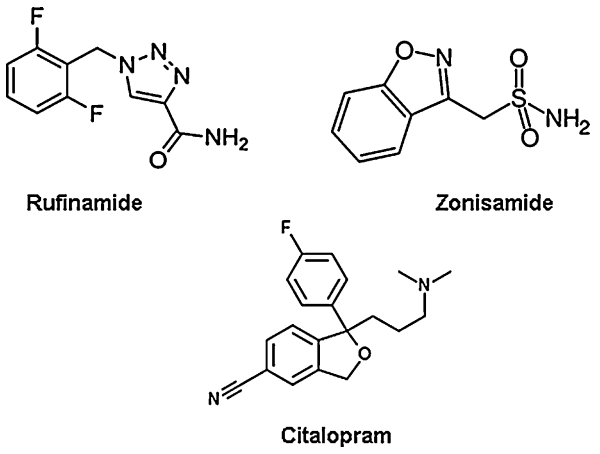

Fig. 1. The chemical structures of rufinamide, zonisamide and citalopram (I.S.).

and ZNS to be concomitantly determined with LTG, MHD and FBM.

2. Experimental

2.1. Reagents and standards

RFN was kindly provided by Eisai Co., Ltd. (Tokyo, Japan); ZNS (sodium salt) and the internal standard (I.S.) citalopram (hydrobromide salt) were purchased from Sigma–Aldrich (St. Louis, MO, USA) (Fig. 1). Acetonitrile, methanol and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a MilliQ Gradient A10 apparatus (Millipore, Billerica, MA, USA). External quality control (VEQ) samples (lyophilized human serum) were obtained from Cardiff Bioanalytical Services Ltd. (Cardiff, UK) according to the annual heath control external quality assessment scheme for therapeutic AED assays (UKNEQAS).

Frozen, drug-free plasma (blank plasma) was obtained from the blood bank of the Maggiore Hospital of Bologna, stored at −20 °C and thawed at room temperature before use.

Stock solutions (1 mg/mL) and subsequent dilutions (100 μg/mL, working solution) of RFN and ZNS were prepared by dissolving the drugs in methanol. Internal standard stock solution (1 mg/mL) was prepared by dissolving 10 mg of citalopram hydrobromide in 8 mL methanol (1 mg citalopram hydrobromide = 0.8 mg citalopram). From this stock solution, 1 mL was diluted with 99 mL acetonitrile to obtain a working solution of 10 μg/mL. All solutions were prepared monthly and stored at 4 °C.

Calibrators (calib, 1–5) of 2.0, 4.0, 10.0, 20.0 and 40.0 μg/mL for RFN and 2.0, 8.0, 20.0, 40.0 and 80.0 μg/mL for ZNS were prepared by pipetting the following volumes of working or stock solutions of the two analytes to 250 μL aliquots of blank pooled plasma: 5, 10, 25 μL of working RFN solution for calibs 1, 2, 3, respectively; 5 and 10 μL of RFN stock solution for calibs 4 and 5. 5 and 20 μL of working ZNS solution were pipetted for calibs 1 and 2; 5, 10, 20 μL of ZNS stock solution were pipetted for calibs 3, 4 and 5, respectively. Plasma calibrators were prepared fresh for each batch and then treated exactly as patients' specimens.

2.2. Chromatographic conditions

The HPLC system consisted of a Series 200 liquid chromatograph, a Series 200 UV/VIS spectrophotometric detector, set at 210 nm, and a Series 200 autosampler connected by a model 600 link chromatography interface to the TotalChrom chromatography workstation (PerkinElmer, Norwalk, CA, USA).

Table 1
List of drugs checked for rufinamide and zonisamide assay interference.

Compound	Concentration (μg/mL)	Retention time (min)
AEDs		
Levetiracetam	20	3.68
Carbamazepine-diol	2	5.66
Primidone	10	5.91
Ethosuximide	40	6.08
Lamotrigine	10	6.38
Felbamate	10	6.84
Monohydroxycarbamazepine	20	7.62
Carbamazepine-epoxide	5	11.09
Phenorbarbital	15	11.51
Oxcarbazepine	10	13.60
Carbamazepine	10	23.53
Phenytoin	10	23.81
Gabapentin	10	n.d.
Pregabalin	10	n.d
Topiramate	5	n.d.
Valproic acid	50	n.d.
Vigabatrin	2	n.d.
Benzodiazepines		
Clobazam	1	n.d.
Clonazepam	1	n.d.
Nitrazepam	1	n.d.
Norclobazam	2	n.d.

n.d., not detectable.

The chromatographic separation was performed with a Synergi 4 μm Hydro-RP, 250 mm × 4.6 mm I.D. column (Phenomenex, Torrance, CA, USA). A C_{18} Securityguard precolumn (Phenomenex) was incorporated into the system and a graphite filter (ESA, Chelmsford, MA, USA) was fitted between the autosampler and precolumn.

The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5), filtered through a 0.22 μm membrane filter (GS type, Millipore), acetonitrile and methanol (65:26.2:8.8, v/v/v). The flow rate was set at 0.8 mL/min.

Column cleaning procedures between batches included a first rinse with 10 column volumes of water and acetonitrile (95:5, v/v), followed by a second flush with 10 column volumes of a mixture of acetonitrile and water (95:5, v/v). Twenty-five injections in a batch were performed on average without the appearance of any memory peaks.

2.3. Blood sampling and plasma processing

Patients' plasma specimens were obtained from morning (8 am), pre-AED dose intake venous blood samples (5 mL), as previously described [15]. To 250 μL of plasma aliquots (calibrators or patient samples), 1.0 mL of I.S. working solution was added. The samples were vortexed for 30 s and then centrifuged at 1500 × *g* at 4 °C for 10 min. Five microliters of the clean upper layer were injected into the chromatographic system.

2.4. Method specificity

Standard solutions of several AEDs and their metabolites were injected to check for possible interferences (Table 1). Blank plasma from six pools was tested for endogenous interferences. In addition, a series of 12 plasma samples from 12 patients with epilepsy not taking RFN and ZNS and treated with commonly prescribed AED and non-AED cotherapies (including hypnotics, antidepressants, different types of antibiotics, anti-inflammatory and cardiac drugs) were analysed to check for drugs which could potentially interfere with the two AEDs determination.

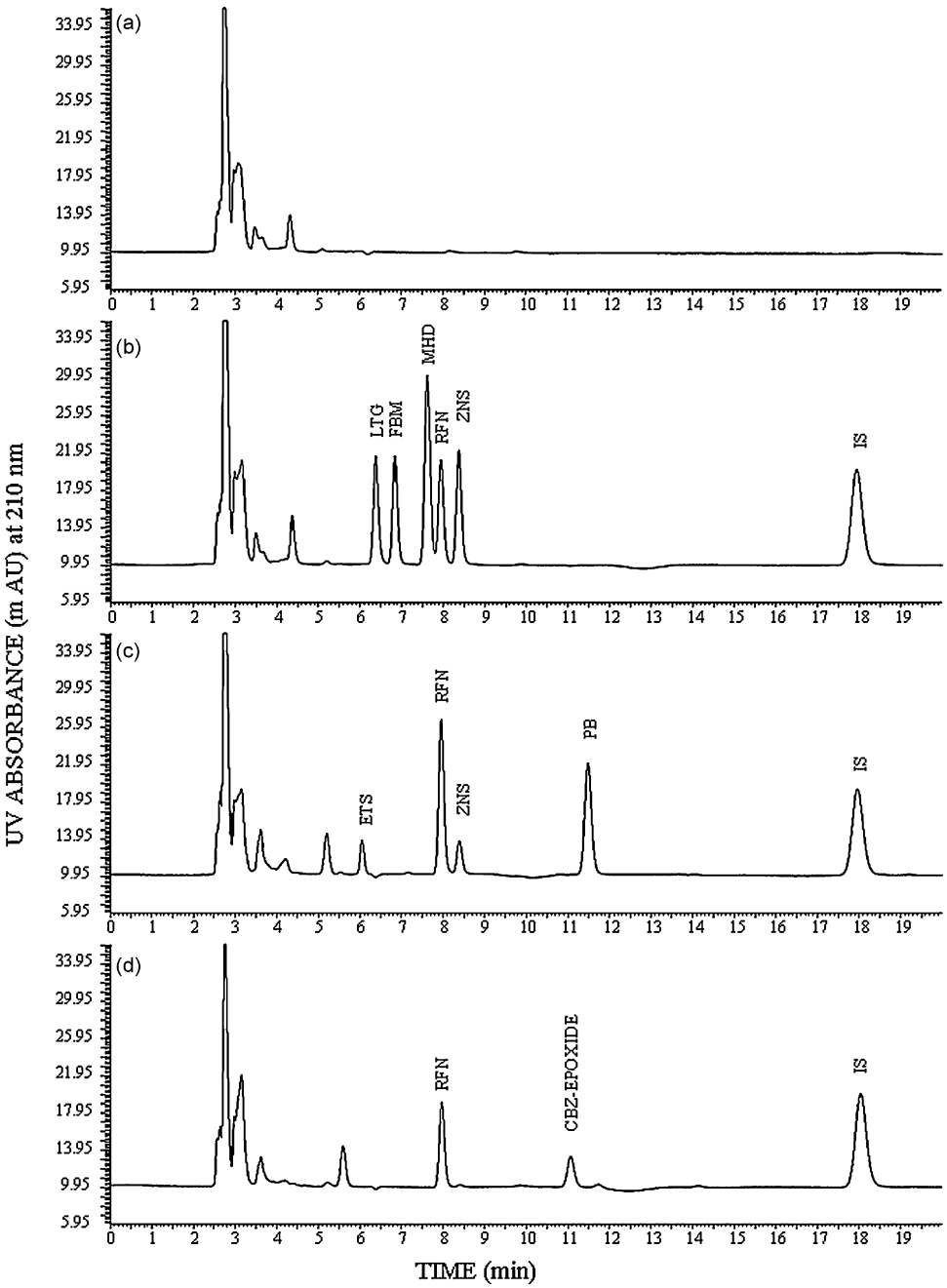

Fig. 2. Chromatograms obtained by injecting 5 μL of (a) deproteinized blank plasma; (b) deproteinized blank plasma spiked with LTG: 10.0 μg/mL, FBM: 40.0 μg/mL, MHD: 20.0 μg/mL, RFN: 10.0 μg/mL, ZNS: 10.0 μg/mL and I.S.: 40.0 μg/mL; (c) plasma specimen of a patient treated with RFN (1600 mg/day), ZNS (100 mg/day), ethosuximide (450 mg/day), phenobarbital (75 mg/day) and clobazam (30 mg/day): RFN, 21.1 μg/mL, ZNS, 6.2 μg/mL; (d) plasma specimen of a patient treated with RFN (1600 mg/day), carbamazepine (600 mg/day) and gabapentin (1300 mg/day): RFN, 10.8 μg/mL. RFN, rufinamide; ZNS, zonisamide; I.S., internal standard; LTG, lamotrigine; FBM, felbamate; MHD, monohydroxycarbamazepine; ETS, ethosuximide; PB, phenobarbital; CBZ-epox, carbamazepine-epoxide.

2.5. Method validation

Calibration curves for RFN and ZNS were run on each analysis day ($n = 13$) over 4 months. The analyte to I.S. peak area ratios were plotted against RFN and ZNS matched concentration added to the blank plasma. The calibration curves were calculated by the least square method. Linearity was assessed by determining the coefficient of correlation (r) of the points of the curves. Plasma concentrations of RFN and ZNS were expressed in μg/mL (conversion factors to μmol/L, 4.20 for RFN and 4.71 for ZNS).

For assay precision and accuracy assessment, blank plasma pools were spiked using suitable volumes of the same RFN and ZNS working solutions used as for the calibrators, to yield three concentrations (i.e., 2.0, 10.0, 40.0 μg/mL for RFN and 2.0, 20.0, 80.0 μg/mL for ZNS) corresponding to the lower, middle and upper points of the calibration curve, separated into 250 μL aliquots and stored frozen at −20 °C, up to a maximum of 3 weeks [10]. The precision of the method was assessed by determining the relative standard deviation (R.S.D. = 100 × S.D./mean) at the three plasma RFN and ZNS concentrations within the same analysis ($n = 6$, intraday precision) and in triplicate over a series of six analyses ($n = 18$, interday precision).

The accuracy of the method was determined by comparing the means of the calculated concentrations at the three plasma

Table 2
Precision and accuracy of rufinamide and zonisamide assay.

Amount added to blank plasma (μg/mL)	Intraday (n = 6)			Interday (n = 18)		
	Calculated concentration (mean ± S.D.) (μg/mL)	Precision (R.S.D.%)	Accuracy (%)	Calculated concentration (mean ± S.D.) (μg/mL)	Precision (R.S.D.%)	Accuracy (%)
RFN 2.0 (LOQ)	2.00 ± 0.11	5.5	0.0	1.94 ± 0.19	9.8	−3.0
10.0	10.23 ± 0.45	4.4	2.3	10.40 ± 0.59	5.7	4.0
40.0	39.97 ± 2.41	6.0	−0.1	40.95 ± 2.45	6.0	2.4
ZNS 2.0 (LOQ)	2.06 ± 0.20	9.7	3.0	2.16 ± 0.20	9.3	8.0
20.0	20.32 ± 0.31	1.5	1.6	20.07 ± 0.58	2.9	0.3
80.0	82.80 ± 3.54	4.3	3.5	80.86 ± 2.83	3.5	1.1

Precision (R.S.D.%) = 100 × S.D./mean; accuracy (%) = 100 × (mean concentration found − known concentration)/known concentration; interday (n = 18) = triplicate samples, over a series of six analyses on different days; LOQ, limit of quantitation.

concentrations for RFN and ZNS with the nominal concentrations (percentage differences), within the same analysis (n = 6, intraday accuracy) and in triplicate over a series of six analyses (n = 18, interday accuracy).

The absolute recovery of RFN, ZNS and I.S. was calculated as the ratio of the drug peak area from deproteinized blank plasma spiked with RFN and ZNS, at the three concentrations above, or with the I.S. (40 μg/mL) to the peak area obtained from the injection of RFN, ZNS and I.S. standard solutions, at the same theoretical concentrations, reconstituted in methanol, over a series of six analyses.

The lower limit of quantitation (LOQ) was defined as the lowest concentration of the calibration curves matched to a RFN and ZNS signal-to-noise ratio of 10:1, with an associated R.S.D. and inaccuracy <20% [16]. The precision and accuracy at the LOQ were determined both intraday (n = 6) and interday (triplicate samples over six analyses, n = 18) [16].

The lower limit of detection (LOD) was determined in triplicate by comparing measured signals from plasma samples with known low concentrations of RFN and ZNS with those of blank plasma samples and calculated as 3 times the baseline noise [16].

3. Results and discussion

During the development of the assay different mobile phases were tested. The mixture already described in our previous work [15] of potassium dihydrogen phosphate buffer (50 mM, pH 4.5), acetonitrile and methanol (65:26.2:8.8, v/v/v), combined with a longer (250 mm instead of 150 mm) Synergi 4 μm Hydro-RP column, at a lower isocratic flow rate (0.8 mL/min instead of 1.0 mL/min) maintained a good separation and reasonable retention times of LTG, FBM and MHD (Table 1) and incorporated the two new analytes in the same assay, with retention times (mean ± S.D., n = 12) of 7.90 ± 0.05 min for RFN and 8.35 ± 0.05 min for ZNS (Fig. 2). We had to search for a different I.S., as 4-methylprimidone previously used as I.S. for the simultaneous determination of LTG, FBM and MHD overlapped with ZNS in the new chromatographic conditions. Among the several compounds tested citalopram proved the most suitable, as it did not interfere with any of the numerous possibly co-eluted AEDs and showed an acceptable retention time (17.93 ± 0.11 min, mean ± S.D., n = 12) (Fig. 2). Citalopram is an antidepressant unfrequently coadministered with AEDs; moreover, from our checks, citalopram was not detectable under the described analytical conditions in deproteinized plasma specimens of patients with depression receiving the drug at therapeutic doses and no other potentially interfering peaks were present in the chromatographic run over 30 min.

No endogenous interferences were found in any of the six blood donors' blank specimens analysed. None of the possibly coprescribed AEDs and benzodiazepines tested interfered in the analysis: elution times of the drugs checked over a 30-min run are reported in Table 1. Moreover, analyses of plasma samples from different patients with epilepsy not taking RFN and ZNS and treated with commonly prescribed AED and non-AED cotherapies showed no interfering peak.

Calibration curves showed a linear and reproducible correlation between RFN and ZNS plasma concentrations and matched analyte to I.S. peak area ratios. Equations (mean ± S.D., n = 13) of the regression lines were $y = 0.0107\ (\pm 0.00291) + 0.0371\ (\pm 0.00712)x$, $r = 0.9995\ (\pm 0.0005)$ for RFN and $y = 0.0110\ (\pm 0.00425) + 0.0216\ (\pm 0.00608)x$, $r = 0.9990\ (\pm 0.0008)$ for ZNS, where x is RFN or ZNS concentration, expressed in μg/mL, y is the analyte to I.S. peak area ratio, expressed in arbitrary area units and r is the correlation coefficient.

The results of precision and accuracy analyses for RFN and ZNS are reported in Table 2. Both intra- and interassay precision and accuracy were <10% for the whole concentration range. The LOQ was set at 2.0 μg/mL for RFN and ZNS, a limit considered suitable for TDM application [1,17]. The LOD was 0.5 μg/mL for both analytes. Mean ± S.D. absolute recovery for RFN was 96.5 ± 4.6% at a plasma drug concentration of 2.0 μg/mL, 97.9 ± 2.4% at 10 μg/mL and 102.9 ± 2.6% at 40 μg/mL. Recovery for ZNS was 97.5 ± 1.3% at a plasma drug concentration of 2.0 μg/mL, 97.7 ± 2.1% at 20 μg/mL and 99.9 ± 3.6% at 80 μg/mL; recovery for the I.S. (40 μg/mL) was 103.4 ± 4.5%.

The assay was applied to the plasma determination of RFN (29 samples) and ZNS (34 samples) in patients with epilepsy referred to our laboratory for TDM over 3 months. Eighteen patients were treated with RFN (dosage range, 300–1800 mg/day) and 28 with ZNS (75–500 mg/day) combined with different AED cotherapy. We compared measured plasma concentrations of ZNS with those routinely obtained in our laboratory by a commercially available kit for the HPLC–UV determination of AEDs (order no. 28005, Chromsystems, Munchen, Germany). Steady-state plasma trough concentrations ranged from <LOQ in 1 patient (around 1.5 μg/mL) to 21.1 μg/mL for RFN and from 2.1 to 38.4 μg/mL for ZNS. Results for ZNS obtained by our method *vs.* reference Chromsystems' assay were highly correlated: the linear regression equation for correlation was $y = 0.996x - 0.681$, $r = 0.986$, with a standard error of 1.97, where y is the ZNS plasma concentration by our assay and x is the ZNS concentration by reference method.

VEQ samples at unknown concentrations were processed monthly over 4 months (n = 4).

Measured plasma concentrations of both RFN and ZNS were within 1 S.D. from mean of the results returned by laboratories adhering to UKNEQAS AEDs scheme (11 laboratories for RFN and 27 for ZNS).

This procedure described is the first simple and validated method for the determination of RFN in plasma of patients with epilepsy. By minor changes in the chromatographic conditions and the identification of a new suitable, commercially available I.S. we extended the application of our previously reported method [15], allowing RFN and ZNS to be simultaneously determined with LTG, MHD and FBM in plasma of patients with epilepsy. Data on RFN plasma concentration values in patients on chronic therapy are scanty: median plasma steady-state RFN concentrations around 15 μg/mL have been measured in clinical trials in patients taking 1800 mg/day (40 mg/kg/day); the highest RFN plasma concentration in clinical trials was around 45.0 μg/mL, matched to a dose of 50 mg/kg/day [1]. Suggested therapeutic doses range between 1800 and 3600 mg/day.

For ZNS, a reference range of 10–38 μg/mL has been reported at therapeutic dosages of 300–500 mg/day [17].

4. Conclusion

The HPLC procedure developed for the analysis of RFN and ZNS in deproteinized plasma is simple, sensitive and specific, and can be applied simultaneously for the determination of LTG, FBM and MHD in patients with epilepsy. The rapid, single step, plasma preparation coupled with the simple HPLC–UV isocratic chromatographic apparatus and the incorporation of five new AEDs in the same assay make the method cost-effective and suitable for analysis of a large number of samples in a TDM setting.

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References

[1] E. Perucca, J. Cloyd, D. Critchley, E. Fuseau, Epilepsia 49 (2008) 1123.
[2] L.A. Brunner, M.L. Powell, Biomed. Chromatogr. 6 (1992) 278.
[3] M.C. Rouan, C. Souppart, L. Alif, D. Moes, J.B. Lecaillon, J. Godbillon, J. Chromatogr. B 667 (1995) 307.
[4] H.M. Neels, A.C. Sierens, K. Naelaerts, S.L. Scharpè, G.M. Hatfield, W.E. Lambert, Clin. Chem. Lab. Med. 42 (2004) 1228.
[5] D.J. Berry, J. Chromatogr. 534 (1990) 534.
[6] K. Furuno, R. Oishi, Y. Gomita, K. Eto, J. Chromatogr. B 656 (1994) 456.
[7] M. Nakamura, K. Hirade, T. Sugiyama, Y. Katagiri, J. Chromatogr. B 755 (2001) 337.
[8] J.M. Juenke, P.I. Brown, F.M. Urry, G.A. McMillin, J. Anal. Toxicol. 30 (2006) 27.
[9] T. Yoshida, K. Imai, S. Motohashi, S.I. Hamano, M. Sato, J. Pharm. Biomed. Anal. 41 (2006) 1386.
[10] C.L. Ma, Z. Jiao, Y. Jie, X.J. Shi, Chromatographia 65 (2007) 267.
[11] E. Greiner-Sosanko, D.R. Lower, M.A. Virji, M.D. Krasowski, Biomed. Chromatogr. 21 (2007) 225.
[12] T.A.C. Vermeij, P.M. Edelbroek, J. Chromatogr. B 857 (2007) 40.
[13] M. Subramanian, A.K. Birnbaum, R.P. Remmel, Ther. Drug Monit. 30 (2008) 347.
[14] S.I. Johannessen, T. Tomson, Clin. Pharmacokinet. 45 (2006) 1061.
[15] M. Contin, M. Balboni, E. Callegati, C. Candela, F. Albani, R. Riva, A. Baruzzi, J. Chromatogr. B 828 (2005) 113.
[16] Harmonized Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1), International Conference on Harmonization, November, 2005.
[17] T. Mimaki, Ther. Drug Monit. 20 (1998) 593.