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Fully validated method for rapid and simultaneous measurement of six antiepileptic drugs in serum and plasma using ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

Therapeutic drug monitoring (TDM) may be very useful in the clinical management of antiepileptic drug therapy for multiple reasons, such as individual variability, metabolism, genetic factors or drug–drug or drug–food interactions. In addition, TDM is helpful to study the variation in pharmacokinetics that occurs between individuals. Here, we describe a rapid assay using ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometry to measure the antiepileptic drugs lacosamide, lamotrigine, levetiracetam, primidone, topiramate, and zonisamide. After the addition of internal standards (ISs) and protein precipitation of serum or plasma, 1 µl of sample was separated on a 2.1 × 50 mm reverse phase column (Waters, Acquity UPLC BEH Phenyl, 1.7 µm). Analytes were then ionized and detected by electrospray ionization mass spectrometry with multiple reaction monitoring. Runtime was 2.5 min per injection. Matrix effects were investigated by systematical ion suppression and in-source fragmentation experiments. The calibration curves of the 6 antiepileptic drugs were linear over the working range between 0.05 and 50 mg/L ($r > 0.99$). The limit of detection (LOD) was < 0.05 mg/L, whereas the limit of quantification (LLOQ) was 0.10 mg/L of all drugs measured in the assay. The intraassay and interassay coefficients of variation for all compounds were $< 15\%$ for very low concentration (0.1 mg/L) and $< 8\%$ in the clinically relevant concentration range (> 1.0 mg/L). Mean recoveries were between 87.8 and 98.6% for all drugs. There were no significant ion suppressions detected at the elution times of the analytes. The mean differences between serum and heparinized plasma values were less than 6% for the 6 antiepileptic drugs. All drugs were stable in serum at −20 °C, 4 °C, and even at RT for at least 1 month. In summary, a specific and sensitive stable isotope dilution UPLC-MS/MS method was developed and validated for routine clinical monitoring of lacosamide, lamotrigine, levetiracetam, primidone, topiramate, and zonisamide.

1. Introduction

In the recent years, an increasing number of antiepileptic drugs have been developed. Antiepileptic drugs are very important drugs in the treatment of epilepsy, bipolar disorder and other psychological conditions, including depression, Tourette syndrome, Lennox-Gastaut syndrome, autism, anxiety disorder, and post-traumatic stress disorder. However, for optimal medications of patients to avoid adverse side effects including itching, dizziness, headache, fever, nausea, anorexia, upper respiratory tract infections, diarrhea, double vision, psychomotor slowing, depression, hallucinations and suicidal thoughts, therapeutic drug monitoring (TDM) of antiepileptic drugs is necessary. Furthermore, the therapeutic and most toxic effects of antiepileptic drugs are better correlated with the plasma concentration than with dose. The drug concentration in extracellular fluid around the receptor sites at which most antiepileptic drugs act determines the drug effect at these sites. Drug molecules in extracellular fluid are in equilibrium with molecules of the drug that are simultaneously present in plasma [1]. Many antiepileptic drugs have a narrow therapeutic range; hence relatively modest alterations of drug concentration in plasma can result in loss response or toxic effects. Furthermore, antiepileptic drugs are more or less able to induce or inhibit the activity of cytochrome P450 enzymes influencing the pharmacokinetics or pharmacodynamics of other drugs [2]. Therefore, there is a need to develop solid analytical

Abbreviations: BEH, bridged ethyl hybrid; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring mode; Ref. int., reference interval; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LLOQ, lower limit of quantification; RT, room temperature; TDM, therapeutic drug monitoring

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methods for the measurement of these drugs in biological fluids. In the past, several analytical procedures have been described for the measurement of antiepileptic drugs with immune assays [3–5], high-performance liquid chromatography (HPLC) [6–10], gas chromatography [9–12] and capillary electrophoresis [13,14]. However, high sensitivity of the method is often necessary. For example, topiramate, a broad-spectrum second generation antiepileptic drug with multiple mechanisms of action, is administrated in oral doses of only a few milligrams per day as adjunctive therapy in pediatric patients with epilepsy and, thus, the concentration of topiramate in the plasma of these patients can be low [15]. To increase sensitivity and specificity relative to other chromatographic methods, HPLC was coupled with tandem mass spectrometry (MS/MS). However, most of these methods either required time-consuming sample preparation [6–10] or extended chromatography [10]. A further development of HPLC technology resulted in ultra-performance liquid chromatography (UPLC) which, especially in combination with MS/MS (UPLC-MS/MS), permits reduction of sample volume and often simplification of sample preparations [16–19]. Recently, an UPLC-MS/MS method with a simple sample preparation for detection of antiepileptic drugs was developed [20]. However, measurement of the antiepileptic drugs in this method was performed without any internal standards (ISs) which could avoid quantitative errors resulting from deleterious matrix effect. In addition, the run time was relatively long, at 10 min.

Here, we describe a simple and specific UPLC-MS/MS method to measure the antiepileptic drugs lacosamide, lamotrigine, levetiracetam, primidone, topiramate, and zonisamide in plasma and serum using deuterium-labeled isotopes of the compounds as IS. The use of an IS during the measurement of the antiepileptic drugs is very important because undetected matrix compounds may reduce or enhance the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. Ignoring this effect may adversely affect the reliability of measurement of drug concentrations and the integrity of TDM data generated [21]. Stable isotope-labeled compounds which are very similar to the target analyte are ideal internal standards because they have almost identical overall physicochemical properties compared to their unlabeled counterpart, the target analyte [22]. Sample preparation was performed by a fast and unsophisticated precipitation step using a mixture of zinc sulfate solution, organic solvent (methanol) and ISs. This approach has the advantage of a more homogeneous mixture, as well as a good protein precipitation [23,24]. The method was fully validated and the run time of the measurement was only 2.5 min.

2. Materials and methods

2.1. Reagents, internal standards, calibrators, and quality-control samples

HPLC-grade water and methanol were purchased from Fisher Scientific GmbH (Schwerte, Germany). Ammonium acetate, formic acid, and primidone were obtained from Sigma-Aldrich (Deisenhofen, Germany). Lacosamide, lacosamide-d_3, lamotrigine, lamotrigine-$^{13}C,d_3$, levetiracetam, levetiracetam-d_3, primidone-d_5, topiramate, topiramate-d_{12}, zonisamide, and zonisamide-d_4 were purchased from Toronto Research Chemicals (North York, Canada).

Primary stock solution of lacosamide, lacosamide-d_3, lamotrigine, lamotrigine-$^{13}C,d_3$, levetiracetam, levetiracetam-d_3, primidone, primidone-d_5, topiramate, topiramate-d_{12}, zonisamide, and zonisamide-d_4, each at a concentration of 100 mg/L, were prepared separately in methanol/water (50:50) and stored at −20 °C. A protein precipitation solution that contained the IS (IS precipitation solution) lacosamide-d_3, lamotrigine-$^{13}C,d_3$, levetiracetam-d_3, primidone-d_5, topiramate-d_{12}, and zonisamide-d_4, each at a concentration of 1.5 mg/L, was prepared by mixing acetonitrile with the appropriate primary stock solutions. Using drug-free serum or plasma from blood donors, we prepared several calibrators (0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 mg/L of all drugs mention above) for the assay. In addition, commercially available calibrators and quality-control samples of the drugs mentioned above purchased from Recipe Chemicals and Instruments GmbH (Munich, Germany), were utilized.

2.2. Serum samples

Venous blood samples from blood donors and patients were collected for routine analysis in heparin sample tubes, as well as in serum monovettes from KABE Labortechnik GmbH (Nümbrecht-Elsenroth, Germany). Anonymized samples were left over from routine analysis; hence, no medical-ethical approval was necessary for this study.

2.3. Sample preparation for UPLC-MS/MS

Sample preparation was performed in a 1.5-ml polypropylene microcentrifuge tube. 50 µl each of plasma, serum, calibrator or quality-control sample were added to 200 µl of a 0.1 mol/L zinc sulfate solution. For protein precipitation 500 µl of IS precipitation solution (see above) was added. After vortex-mixing for 10 s, the mixture was centrifuged at 13.000 g for 5 min, the clear organic supernatant was transferred to the autosampler vessel and 1.0 µl was injected into the UPLC-MS/MS system.

2.4. UPLC-MS/MS analysis

For measurement of the antiepileptic drugs, a 2.1 X 50-mm reverse phase column (Waters, Acquity UPLC BEH Phenyl, 1.7 µm) maintained at 60 °C was used for separation by an UPLC system (Waters Acquity UPLC Basissystem) directly coupled to a Waters TQ tandem mass spectrometer fitted with Z Spray ion source. A 1.0-µl sample was injected at a flow rate of 0.50 ml/min. The gradient program is shown in Table 1. The mass spectrometer was operated in electrospray positive ionization mode, and the system control and data acquisition were performed using MassLynx NT 4.1 software, with automated data processing by the MassLynx QuanLynx program provided with the instrument. Nitrogen was used as the nebulizing gas. Argon was used as the collision gas. Instrument settings were as follows: capillary voltage, 0.6 kV; source temperature, 130 °C; desolvation temperature, 450 °C; collision gas pressure, 3.0×10^{-3} mbar. Sample analysis was performed in the multiple reaction monitoring mode (MRM) of the instrument, with a dwell time of 0.005 s for all compounds.

Table 1
UPLC gradient elution program for the analysis of the six antiepileptic drugs.

Step	Time (min)	Flow rate (mL min^{-1})	Solvent A (%)	Solvent B (%)	Gradient
1	0.0	0.5	90	10	Isocratic
2	1.0	0.5	90	10	Isocratic
3	1.8	0.5	5	95	Linear
4	2.3	0.5	5	95	Isocratic
5	2.5	0.5	90	10	Isocratic

Solvent A, 0.1% formic acid in water containing 2 mmol L^{-1} ammonium acetate; Solvent B, 0.1% formic acid in methanol containing 2 mmol L^{-1} ammonium acetate.

Sample cone energy, collision energy, and mass transitions for all compounds are listed in Table 2.

2.5. Ion suppression

Ion suppression and ion enhancement effects were investigated by a post column infusion experiment as described previously for mycophenolic acid (MPA) [25].

Table 2
Multiple Reaction Monitoring (MRM) transitions monitored (m/z) with cone and collision energy.

Analyte	MRM (m/z)	Dwell (s)	Cone (V)	Collision energy(eV)
Lacosamide	**251.0→ 108.0**	0.005	17	9
	251.0→ 90.9	0.005	17	24
Lacosamide-d_3	**254.0→ 108.0**	0.005	22	10
	254.0→ 91.0	0.005	22	22
Lamotrigine	**255.9→ 144.9**	0.005	52	38
	255.9→ 211.0	0.005	52	26
Lamotrigine-$^{13}C_3$,d_3	**262.0→ 147.9**	0.005	52	40
	262.0→ 217.0	0.005	52	26
Levetiracetam	**171.0→ 126.1**	0.005	17	14
	171.0→ 154.0	0.005	17	8
Levetiracetam-d_3	**174.0→ 129.1**	0.005	16	12
	174.0→ 157.0	0.005	16	6
Primidone	**219.0→ 162.0**	0.005	30	12
	219.0→ 118.9	0.005	30	18
Primidone-d_5	**224.1→ 167.0**	0.005	28	12
	224.1→ 124.0	0.005	28	16
Topiramate-NH_3-Adduct	**357.1→ 263.9**	0.005	25	14
	357.1→ 184.0	0.005	25	14
Topiramate-d_{12}-NH_3-Adduct	**369.1→ 270.0**	0.005	24	14
	369.1→ 190.0	0.005	24	14
Zonisamide	**213.0→ 131.9**	0.005	30	14
	213.0→ 149.0	0.005	30	14
Zonisamide-d_4	**217.0→ 136.0**	0.005	30	16
	217.0→ 153.0	0.005	30	16
Desmethyllacosamide	237.0→ 108.0	0.005	19	9
Lamotrigine N2-glucuronide	431.8→ 255.9	0.005	32	20
Levetiracetam carboxylic acid	171.9→ 126.0	0.005	18	14
2-Ethyl-2-phenyl-malonamide	206.9→ 162.0	0.005	20	12
R-Hydroxy topiramate	356.0→ 201.0	0.005	27	11
N-Acetyl zonisamide	255.0→ 213.0	0.005	22	9

2.6. Validation

The checklist of STARD (Standard for Reporting of Diagnostic Accuracy) [26,27] and the validation report [28] were used as the basis for validating the UPLC–MS/MS assay for the antiepileptic drugs to determine the most important test characteristics like linearity, LOD, LLOQ, precision, and recovery.

2.6.1. Linearity studies

A matrix-based calibration curve for all compounds using drug-free serum was constructed as described for MPA elsewhere [25]. Serum-based commercial available calibrators and controls for all compounds were utilized.

2.6.2. Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The minimum of detectable concentration was assessed as the mean of a drug-free sample plus $3SD_0$, where SD_0 is the value of the standard deviation as the concentration of the analyte approaches 0. The LOD was determined by performing 20 replicate measurements in a single UPLC-MS/MS assay with drug-free serum as a sample. For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the LLOQ, provided that for this value the precision was at least 20%.

2.6.3. Precision

The intraassay precision was determined by analyzing 20 replicates of a very low, low, medium, high, and very high analyte concentration for all compounds on the same day (see Table 3). The interassay precision was also obtained by measurement of a very low, low, medium, high, and very high analyte concentration for all compounds, but on 20 different days over 1 month (see Table 3).

2.6.4. Stability

The stability of all antiepileptic drugs in serum was investigated by measurement of these compounds in a low, medium, and highly concentrated sample stored at $-20\,^{\circ}C$, $4\,^{\circ}C$, and RT after 1 day, 1 week and 1 month, respectively.

2.6.5. Recovery

The recovery efficiency of the assay was determined as described elsewhere [21,29].

2.6.6. Comparison of antiepileptic drugs concentrations in plasma and serum

We enriched drug-free serum, as well as drug-free plasma samples with the same amount of antiepileptic drugs. For a comparative analysis of the drugs in the different materials, 60 samples of the same preparation were used.

3. Results and discussion

3.1. General approaches of the UPLC-MS/MS method

Sample preparation by a simple protein precipitation procedure using IS precipitation solution produced, after a short centrifugation step, a clear supernatant that gave a clear chromatogram for all compounds (Fig. 1). Chromatographic conditions were optimized though several trials to achieve good sensitivity, as well as short run time. All compounds were clearly separated from the void volume (retention time <0.3 min) and elute in <2.0 min, permitting an injection-to-injection cycle time of 2.5 min. The presence of 0.1% formic acid and 2 mmol/L

Table 3
Validation results of LOD, LLOQ, precision, recovery and accuracy (Recovery was performed in the Ref. int.; accuracy was performed using quality control sample).

Analyte	Level							Ref. int. (mg/L)	Recovery (range %)[a]	Accuracy (mg/L), $n=5$ [b]	
	LOD	LLOQ	1	2	3	4	5			Expected	Observed (mean ± SD)
Lacosamide											
Concentration 1 (mg/L)	0.004	0.10	0.1	2.6	10.0	18.0	48.4	3.65–10.7	96.5 (93.5–103.7)	3.65	3.70 ± 0.07
Intraassay (CV, %)			4.9	1.8	1.3	0.9	1.3				
Concentration 2 (mg/L)			0.1	2.7	10.1	18.3	49.6			10.7	9.93 ± 0.21
Interassay (CV, %)			6.8	3.6	2.5	3.9	4.9				
Lamotrigine											
Concentration 1 (mg/L)	0.004	0.10	0.1	3.8	8.7	13.1	42.4	2.87–14.2	98.6 (91.5–112.0)	2.87	2.92 ± 0.04
Intraassay (CV, %)			13.8	3.7	3.7	2.1	2.3				
Concentration 2 (mg/L)			0.1	4.0	9.0	13.4	43.6			14.2	12.94 ± 0.46
Interassay (CV, %)			14.3	6.1	4.1	5.6	4.7				
Levetiracetam											
Concentration 1 (mg/L)	0.047	0.10	0.2	6.9	13.6	22.4	54.8	9.73–23.2	91.6 (87.9–109.1)	9.73	10.3 ± 0.29
Intraassay (CV, %)			6.2	1.6	1.3	1.4	0.8				
Concentration 2 (mg/L)			0.1	6.9	13.6	22.4	54.7			23.2	23.5 ± 0.12
Interassay (CV, %)			9.2	2.6	2.1	2.7	2.6				
Primidone											
Concentration 1 (mg/L)	0.042	0.10	0.1	4.9	8.6	16.4	53.6	4.1–25.3	91.0 (62.7–107.8)	4.10	4.39 ± 0.19
Intraassay (CV, %)			13.9	5.4	5.2	3.8	3.3				
Concentration 2 (mg/L)			0.1	4.6	8.1	15.4	50.8			25.3	25.2 ± 0.20
Interassay (CV, %)			14.5	7.3	5.9	6.0	6.4				
Topiramate											
Concentration 1 (mg/L)	0.032	0.10	0.1	3.8	11.0	18.5	46.6	2.75–8.75	87.8 (49.7–112.4)	2.75	2.72 ± 0.13
Intraassay (CV, %)			14.7	6.1	5.2	6.2	4.4				
Concentration 2 (mg/L)			0.1	3.8	11.1	18.3	46.4				8.39 ± 0.11
Interassay (CV, %)			14.3	5.8	6.7	6.8	6.6				
Zonisamide											
Concentration 1 (mg/L)	0.029	0.10	0.1	8.2	18.7	22.6	80.3	4.68–23.4	93.0 (84.8–103.5)	4.68	4.93 ± 0.21
Intraassay (CV, %)			14.8	4.2	2.5	3.0	3.4				
Concentration 2 (mg/L)			0.1	8.3	18.2	22.1	79.0			23.4	23.5 ± 0.44
Interassay (CV, %)			14.0	5.4	4.5	5.9	4.7				

[a] Recovery was performed in the Ref. int.
[b] Accuracy was performed using quality control sample.

ammonium acetate in the mobile phase improved the detection of the analytes, especially for topiramate and topiramate-d_{12} for which the ammonium adducts were used for measurement (Table 2). Maximum sensitivity was achieved by monitoring the fragmentation of single positive charged molecule ions $[lacosamide+H]^+$, $[lacosamide\text{-}d_3+H]^+$, $[lamotrigine+H]^+$, $[lamotrigine\text{-}^{13}C_3,d_3+H]^+$, $[levetiracetam+H]^+$, $[levetiracetam\text{-}d_3+H]^+$, $[primidone+H]^+$, $[primidone\text{-}d_5+H]^+$, $[topiramate+NH_3]^+$, $[topiramate\text{-}d_{12}+NH_3]^+$, $[zonisamide+H]^+$, and $[zonisamide\text{-}d_4+H]^+$, respectively (Table 2). Quantitative errors resulting from potential ion suppression are compensated via IS (deuterium isotope of the corresponding drug), which eluted at the same retention time as the corresponding drug. Besides the easy sample preparation, the short analysis time of 2.5 min, as well as the ability to measure multiple analytes in one run, makes the method attractive for routine drug monitoring or high-throughput screening in clinical studies.

3.2. Validation

Carryover from the 50 mg/L matrix-based calibrator to the drug-free serum or plasma sample was less than 0.1% for all compounds. The calibration curves of all compounds were linear over the working range between 0.05 and 50 mg/L ($r > 0.99$). The limit of detection (LOD) was < 0.05 mg/L (Table 3) whereas the limit of quantification (LLOQ) was 0.10 mg/L of all drugs measured in the assay, which is clearly lower than the typical therapeutic level (see Table 4) and indicates that our method was more sensitive for several drugs in comparison to other assays [7,10,11,30–32]. Accuracy, recovery, intraassay and interassay precision for all compounds were good, as presented in Table 3.

To increase specificity of the assay, we detected two different mass transitions for every drug and for the corresponding IS, respectively (Fig. 1 and Fig. 2). The first mass transition was used for quantification of the drug, whereas the second mass transition was used for verification (Table 2). The chromatogram of zonisamide shows 2 additional peaks in the second transition at a retention time of 2.19 and 2.38 min, which are clearly separate from the zonisamide peak at 1.72 min. In the chromatogram of zonisamide in the first transition, these two additional peaks were not detected, indicating that these peaks resulted from a contamination with the same transition as the zonisamide in the second transition. The mean differences between serum and heparinized plasma values were less than 6% for the 6 antiepileptic drugs (Table 4 and Fig. 6).

Ion suppression is one of the most undesirable processes that can occur during electrospray mass spectrometric measurement as a result of a nonlinear decrease in ionization by sample or mobile phase [25,33]. We investigated ion suppression by analyzing drug-free samples injected by UPLC, while the mass spectrometer was infused by syringe containing drug sample, each at a concentration of 10 mg/L at a rate of 10 μl/min. Furthermore, sample matrix effects from 6 different sources (2 serum samples, 3 plasma samples and 1 urine sample) were investigated. As shown in Figs. 3 and 4, ion suppression was especially observed at 0.2 to 1.0 min, whereas all compounds eluted at a retention time > 1.0 min. Hence, the matrix of the samples did not significantly reduce the sensitivity of the measurement. To investigate the mass spectrometer contamination we measured

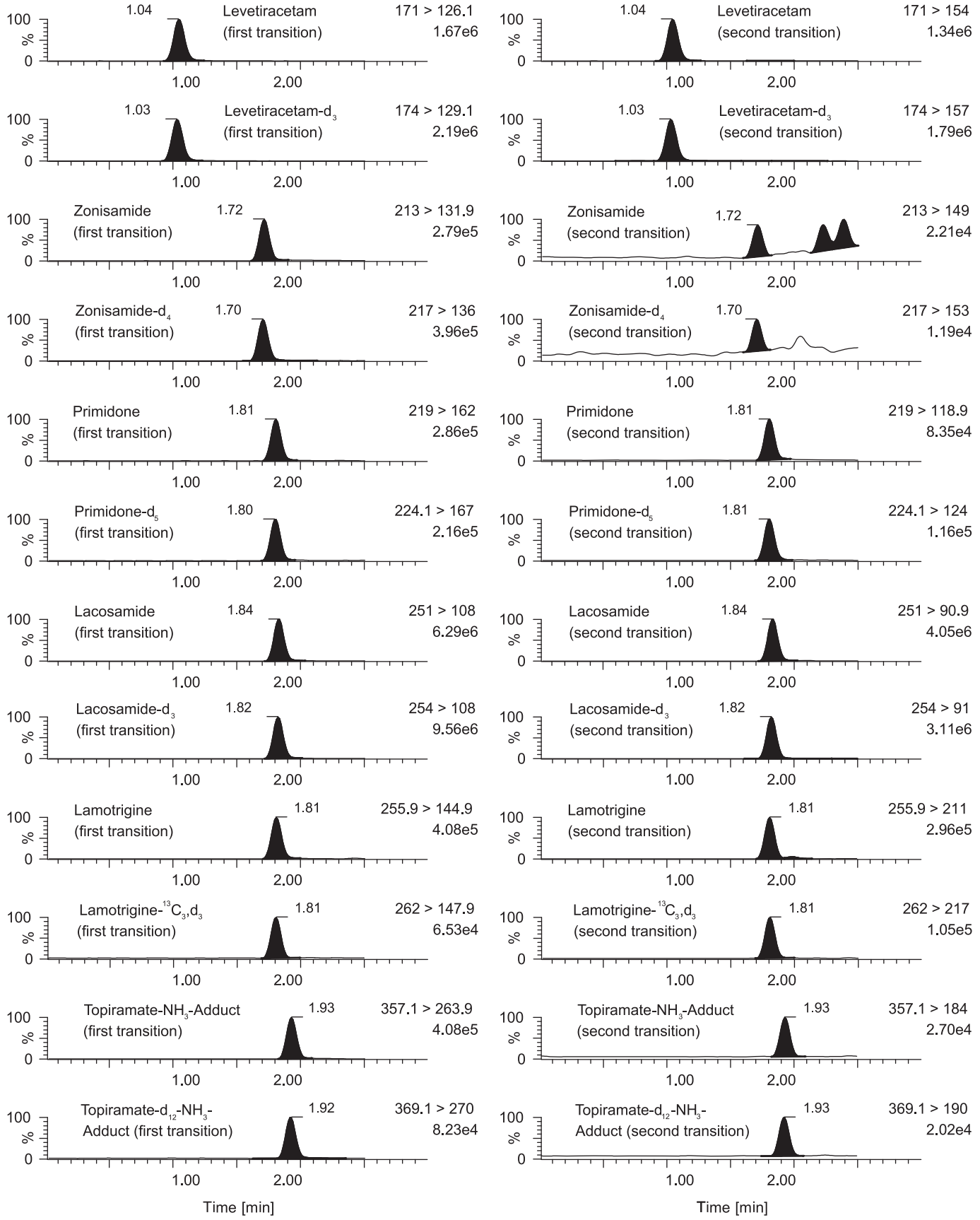

Fig. 1. MRM chromatograms of a serum sample containing each antiepileptic drug (control serum), as well as the internal standards. The first mass transition which was used for quantification of the drug is depicted on the left, whereas the second mass transition which was used for verification is depicted on the right.

Table 4
Comparison of antiepileptic drugs concentration in plasma and serum.

Analyte	n	Range [*Tentative target range*] (mg/L)	Serum Mean (mg/L)	Plasma Mean (mg/L)	Difference between Means (%)	*P*-value
Lacosamide	57	0.54-177.4 *[5.0-10.0]*	41.0	40.1	2.19	0.038[a]
Lamotrigine	60	0.03-44.0 *[3.0-14.0]*	9.20	8.76	4.78	0.030[a]
Levetiracetam	58	0.83-208.1 *[10.0-37.0]*	48.6	49.4	−1.65	0.066
Primidone	59	0.37-127.6 *[8.0-12.0]*	28.5	27.7	2.81	0.162
Topiramate	58	0.39-178.4 *[5.0-25.0]*	38.0	36.2	4.87	0.043[a]
Zonisamide	60	0.03-223.2 *[10.0-38.0]*	45.3	42.9	5.36	0.001[a]

[a] If the calculated *P*-value is less than 0.05, the conclusion is that the mean difference between the paired observations is statistically different from 0.

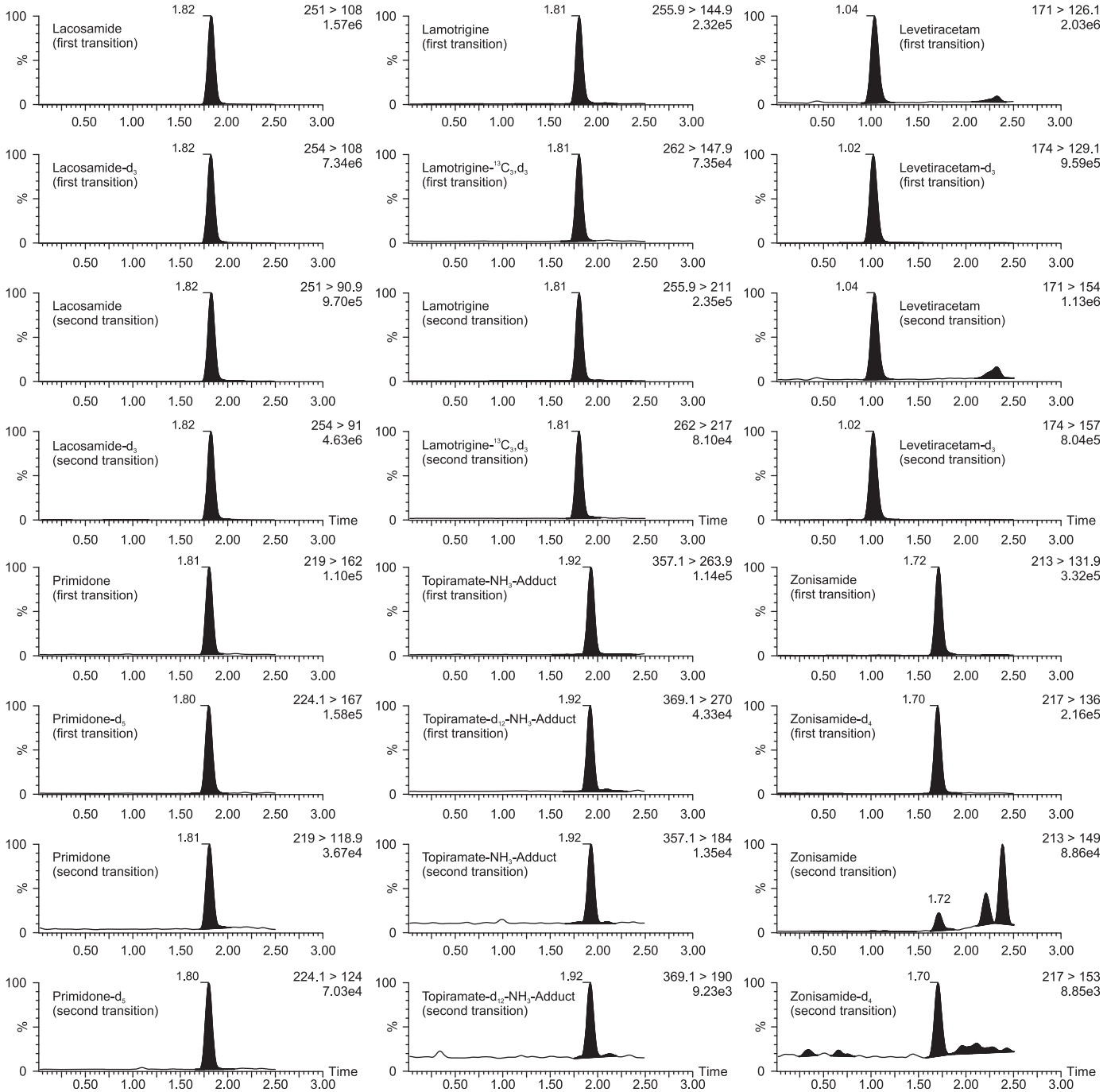

Fig. 2. MRM chromatograms of patient samples. The first mass transition which was used for quantification of the drug and the second transition which was used for verification are depicted. In addition, the mass transitions of the internal standard are shown.

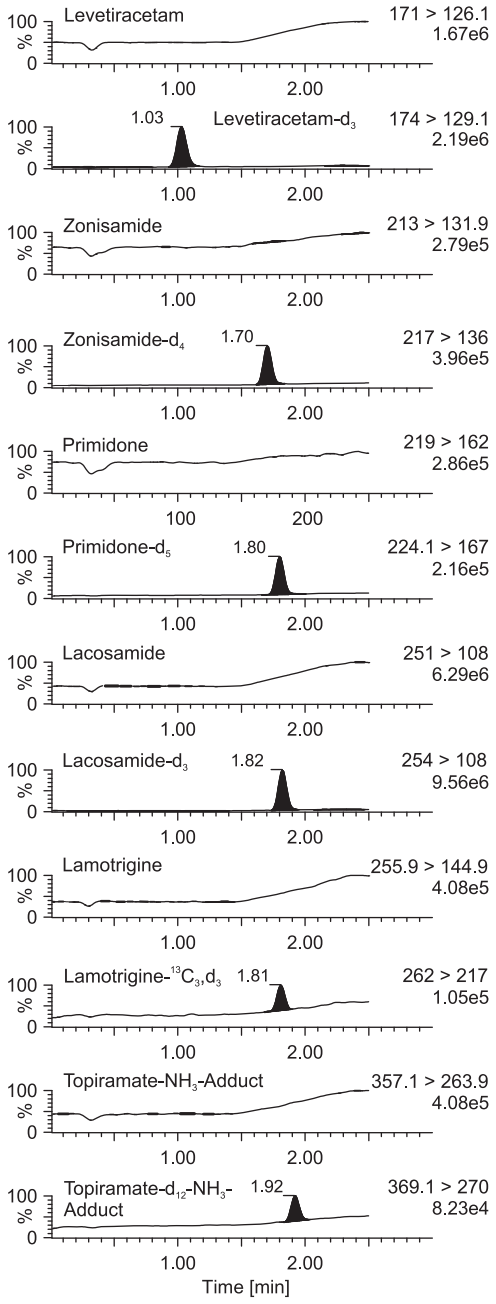

Fig. 3. Ion suppression profile for all antiepileptic drugs, performed with a post-column flow injection of 1 mg/L drug into the UPLC eluate of a drug-free sample. In addition, the mass transitions of the internal standards are shown.

a sample 400-fold and determined the ionization performance by the area under the curve. The sensitivity of the mass spectrometer diminished during this measurement series by about 10%. However, quantitative errors resulting from matrix effects or contamination are compensated via the ISs (deuterium isotopes of the analytes) which are structurally identical to the corresponding analytes.

Drug metabolites can adversely affect the measurement of the antiepileptic drugs, especially if co-eluted with the analyte, because an in-source fragmentation of the metabolite to the parent drug can result in wrong (too high) values. Therefore, we have investigated the major metabolites of lacosamide, lamotrigine, levetiracetam, primidone, topiramate, and zonisamide in our assay. A drug-free sample was enriched with desmethyl lacosamide, lamotrigine

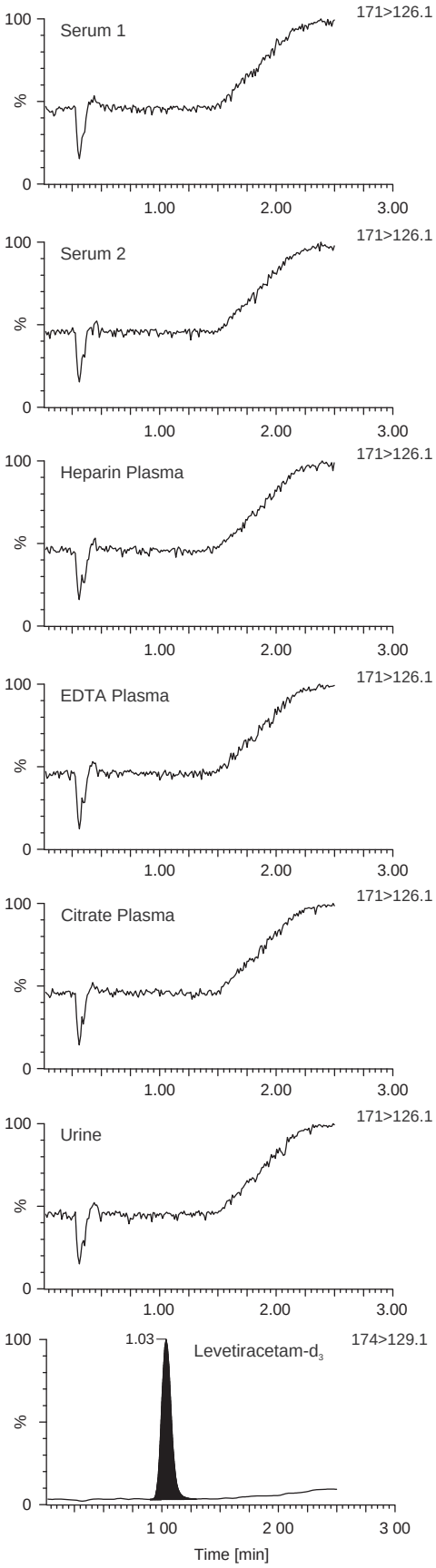

Fig. 4. Ion suppression profile for Levetiracetam, performed with a post-column flow injection of 1 mg/L drug into the UPLC elute of drug-free samples from 6 different sources (Serum 1, Serum 2, Heparin Plasma, EDTA Plasma, Citrate Plasma, and urine). In addition, the mass transition of the internal standard is shown.

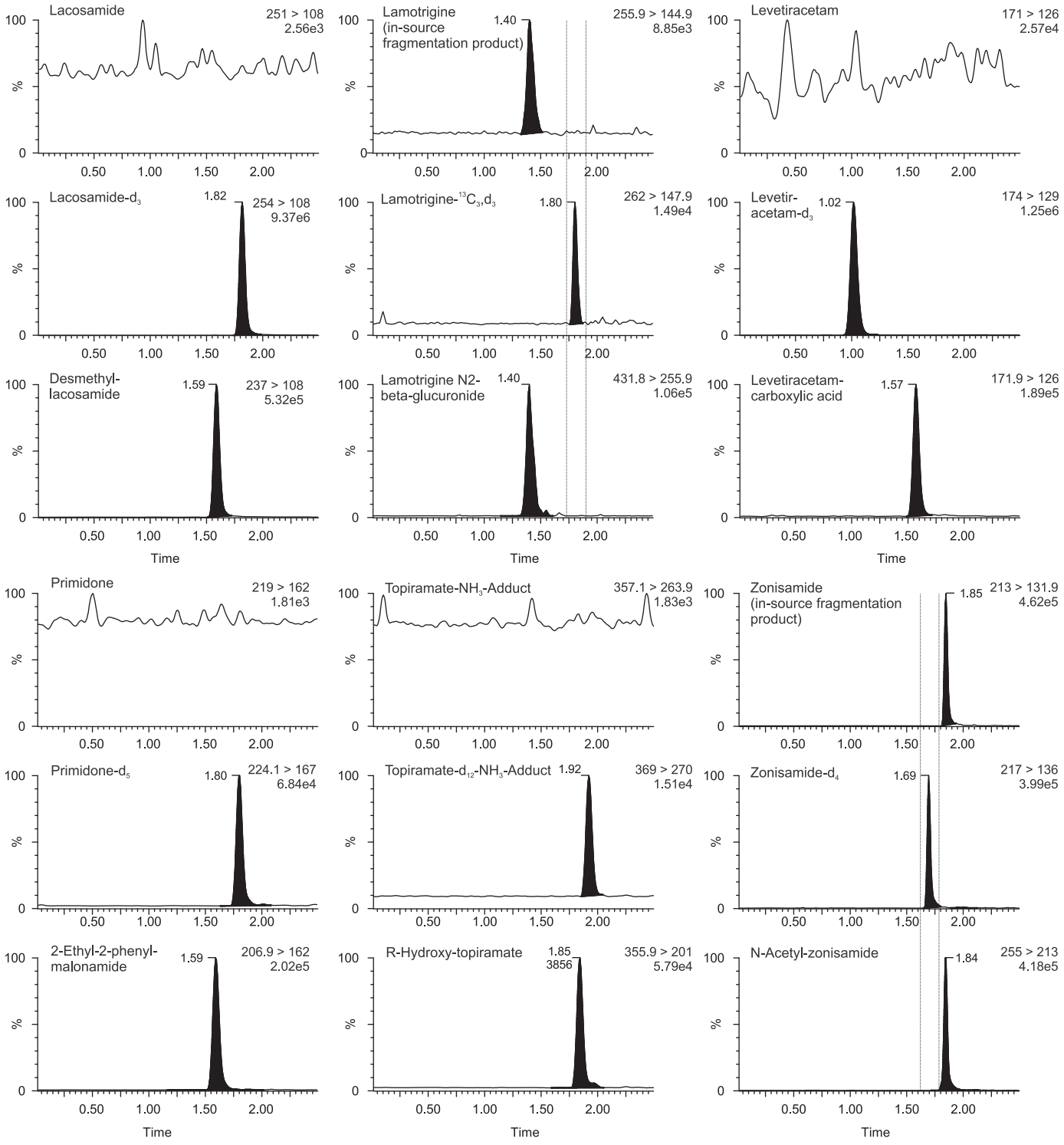

Fig. 5. MRM chromatogram of a serum sample containing the antiepileptic drug metabolites desmethyl lacosamide, lamotrigine N2-beta-glucuronide, levetiracetam carboxylic acid, 2-ethyl-2-phenylmalonamide, R-hydroxy topiramate, and N-Acetyl zonisamide, as well as the internal standards lacosamide-d_3, lamotrigine-$^{13}C_3$,d_3, levetiracetam-d_3, primidone-d_5, topiramate-d_{12}, and zonisamid-d_4 of the antiepileptic drugs.

N2-beta-glucuronide, levetiracetam carboxylic acid, 2-ethyl-2-phenylmalonamide, R-hydroxy topiramate, and N-Acetyl zonisamide, each at a concentration of 10 mg/L and used for measurement in the assay. As shown in Fig. 5, in-source fragmentation of the antiepileptic metabolite to its parent drug was detected by lamotrigine N2-beta-glucuronide and N-Acetyl zonisamide, but the retention times were, in these two cases, different from the retention time of the parent drug, with the result that each metabolite, and thus its in-source fragmentation product were clearly separated from its parent drug.

Matched aliquots of serum and heparinized plasma were analyzed and compared by paired samples *t*-test and were considered clinically significant at 5% (program: MedCalc version 11.6.1.0). No significant differences were observed by measurement of levetiracetam, as well as primidone, whereas lacosamide, lamotrigine, topiramate, and zonisamide measurements in plasma

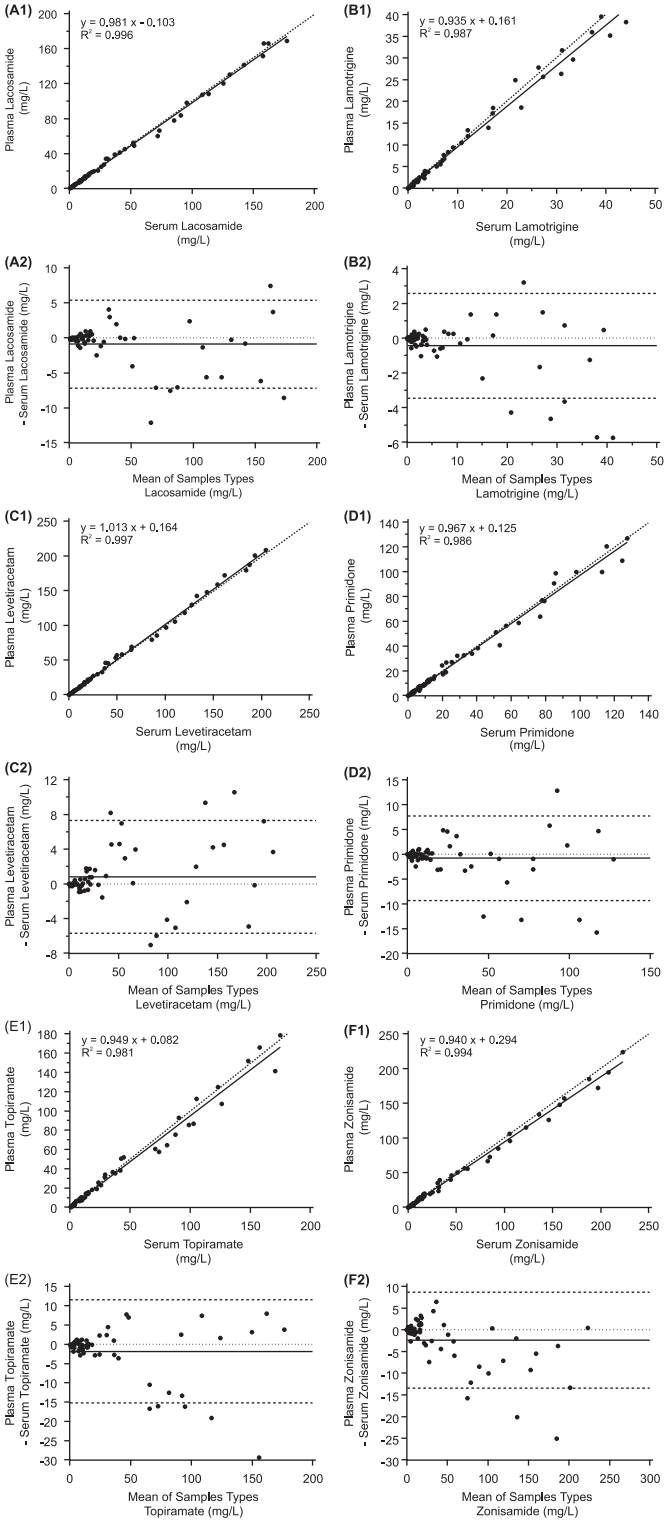

Fig. 6. Comparison of lacosamide, lamotrigine, levetiracetam, primidone, topiramate, and zonisamide in serum and plasma, $n=57$, $n=60$, $n=58$, $n=59$, $n=58$, and $n=60$, respectively. In the scatter diagrams (A1, B1, C1, D1, E1, F1) the regression line (solid line) and the line of identity $X=Y$ (dotted line) are shown. Bland–Altman plots (A2, B2, C2, D2, E2, F2) showing the mean difference between plasma and serum measurement (solid line). The dashed lines indicate two standard deviations (SD) above and below the mean. An ideal mean difference of 0 is indicated by a dotted line.

were significantly lower than in serum. However, the mean differences between serum and heparinized plasma values were less than 6% for all analyzed antiepileptic drugs (Table 4). As shown in Fig. 6, good correlations were found by cross-validation between serum and plasma for all drugs investigated. In stability analysis, the mean percentage changes of peak area were less than 10% for the 6 antiepileptic drugs at −20 °C, 4 °C and even at RT for at least 1 month. Further investigations have shown that all 6 antiepileptic drugs were also stable for at least 3 days in whole blood at −20 °C, 4 °C and RT.

Recently, a fast UPLC-MS/MS method for the measurement of levetiracetam was published with 3-amino-2-naphthoic acid as IS because deuterated levetiracetam was not available at the time of the assay development [32]. However, levetiracetam and 3-amino-2-naphthoic acid are structurally dissimilar and, therefore, 3-amino-2-naphthoic is less suitable as IS than the levetiracetam-d_3 which was used in our assay. A further UPLC-MS/MS method for the detection of several antiepileptic drugs which was also published recently used no internal standards for compensation, but checked only the positional differences to investigate whether time-dependent change of MS detection sensitivity existed [20]. However, compensation via structurally identical isotopes is the most secure method of eliminating potential errors. Therefore, our method generates not only fast, but also very reliable results.

4. Conclusion

We have developed a specific and sensitive UPLC-MS/MS method for fast determination of 6 antiepileptic drugs in human serum or plasma using stable isotopic labeled components as ISs which eliminate undesirable matrix effects. After a quick and simple protein precipitation step, simultaneous and reliable measurement of the 6 drugs was performed in only 2.5 min.

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