Fast and Sensitive Pharmacokinetic Assessment Using an Agilent LC/MS Triple Quadrupole System

Application Note

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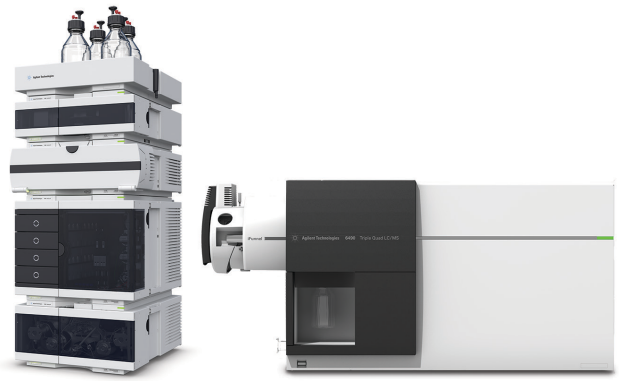
Abstract

A sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS) method has been developed for the simultaneous quantitation of a full panel of 26 antiepileptic drugs (AEDs). Among the 26 AEDs, bioanalytical method parameters including accuracy, precision, linearity, and limit of quantitation (LOQ) were determined for gabapentin (GBP), tiagabine (TGB), and lamotrigine (LTG) in rat plasma. The multiwash feature of the Agilent 1290 Infinity II LC autosampler was evaluated to assess sample carryover. A simple protein precipitation sample preparation method using 50 µL of plasma provided sufficient analytical sensitivity for analyzing low-dose cassette intravenous (IV) administration. The pharmacokinetics (PK) were assessed for the three selected drugs in rats. This Application Note describes a selective and sensitive high-throughput method that demonstrates the suitability of the Agilent 1290 Infinity II LC system and Agilent 6495 Triple Quadrupole system for *in vivo* PK determinations.



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Introduction

Drug metabolism and pharmacokinetic (PK) studies are an integral part of the drug discovery process. Early discovery and optimization demands PK evaluation of hundreds of new chemical entities (NCE), and requires extensive animal and bioanalytical resources[1]. Individual screening of these NCEs is a routine process; to optimize the throughput, researchers have adopted innovative approaches such as cassette dosing PK studies or cassette dosing sample bioanalysis[2]. The cassette dose PK approach involves intravenous (IV) or oral (PO) administration of multiple compounds in a single study, and calculation of PK parameters. Avoid cassette dosing of compounds with the same molecular weights. Typically, dose selection in the cassette approach is such that the combined NCE dose does not interfere with the absorption and metabolism (rate and extent) of the individual drugs, to avoid potential drug-drug interactions. In contrast to individual PK screening, cassette PK studies (n = 3–5 NCEs) use very low doses of compounds (0.5–1 mg/kg). Thus, the cassette dosing approach requires highly efficient separation and sensitive mass spectrometers for rapid and unambiguous PK assessment of target analytes[3]. Moreover, methods that require the determination of both parent drugs and circulating metabolites further add to the total number of analytes to be quantitated in a single run.

To simulate a typical drug screening scenario, we chose a full panel of antiepileptic drugs (AEDs), and developed a generic short LC/MS/MS analytical method for the separation of all AEDs. Three AEDs from the panel were selected for the rat PK assessment to simulate a cassette dosing approach. The bioanalytical method was developed for a full panel of AEDs to analyze samples for a rat study and determine relevant PK parameters.

Experimental Conditions

Standards and chemicals

Chromsystems AED standard mix (Gräfelfing, Germany), containing all 26 AEDs, was used to develop the LC/MS/MS method. Individual standards of gabapentin (GBP), tiagabine (TGB), and lamotrigine (LTG) were purchased from Sigma-Aldrich for PK assessment using a cassette dosing methodology. Methanol, acetonitrile, ammonium acetate, formic acid, acetazolamide internal standard, and all other chemicals used for the experiments were purchased from Sigma-Aldrich (Bangalore, India). Water used in all experiments was demineralized and double-distilled, and obtained from a Milli-Q Integral Water Purification System (Merck, Darmstadt, Germany).

Animals and blank matrices

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Ethics Committee (Syngene International Ltd., Bangalore) and in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC, MD USA). Sprague-Dawley rats were purchased from Vivo Bio Tech Ltd. (Hyderabad, India) and were housed at 21 ±3 °C and relative humidity of 50 ±20 %. Animals were maintained in 12-hour light and 12-hour dark cycles, and had free access to standard laboratory rodent diet (Tetragon Chemie Pvt. Ltd., Bangalore, India) and water. Rat plasma used for method development was purchased from Bioreclamation Inc. (Westbury, NY, USA).

LC/MS/MS Method for separation of 26 AEDs

For the LC method development, the Chromsystems AED standard mix was resuspended using 1 mL of distilled water, and allowed to rest for 15 minutes at room temperature. The concentration of analytes in the standard mix was found to be in a wide range of 0.1–80 mg/L. A fast LC method was developed using an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column, and chromatographic separation of all 26 AEDs was achieved within 6 minutes. MS/MS data were acquired in a sensitive dynamic multiple reaction monitoring (DMRM) mode. The DMRM algorithm in the Agilent Triple Quadrupole LC/MS system automatically constructs DMRM timetables for multiple analytes throughout the LC/MS analysis based on the retention time window for each analyte. It allows the instrument to acquire MRM data only during a stated retention time window, thus reducing the number of concurrent ion transitions and enhancing the efficiency of data acquisition.

Instrumentation

An Agilent 1290 Infinity II LC system coupled to an Agilent 6495 Triple Quadrupole MS system was used for the analytical studies. The chromatographic separation was carried out on an Agilent ZORBAX Phenyl-Hexyl column using a gradient elution with an Agilent 1290 Infinity II LC system. High-analytical sensitivity MS detection was carried out in DMRM mode using an 6495 Triple Quadrupole LC/MS with iFunnel technology. Table 1 lists the the LC instrumentation and gradient parameters, and Table 2 lists the 6495 MS parameters. DMRM detection in positive and negative ionization modes was used to acquire the data. Protonated precursors of the analytes were selected for DMRM-based quantification. Table 3 provides the DMRM details used for data acquisition.

The LC/MS/MS system consisted of the following modules:

- Agilent 1290 Infinity II Binary Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167BA) with multiwash feature enabled
- Agilent 1290 Infinity II Diode array detector (G7116B)
- Agilent 6495 Triple Quadrupole LC/MS with ESI-AJS source

Assessment of bioanalytical method reproducibility using three selected AEDs

The reproducibility of the newly developed generic method was evaluated using GBP, TGB, and LTG for the cassette dosing PK experiments.

Stock solutions and calibrants

We used 10 mM primary stock solutions of GBP, TGB, LTG, and acetazolamide (internal standard) prepared in DMSO. Primary stocks were diluted with acetonitrile:water (1:1 v/v) to prepare a 2 mM secondary stock solution.

A working stock of GBP, TGB, and LTG standards at 400 µm was prepared in acetonitrile:water (1:1 v/v). Working standards in concentrations of: 3.05, 6.10, 12.21, 24.41, 48.83, 97.66, 195.31, 390.63, 781.25, 1,562.50, 3,125, 6,250, 12,500, 25,000, 50,000, 100,000, and 200,000 nM were prepared by serial dilution using acetonitrile:water (1:1 v/v).

To prepare the rat plasma calibration curve, 5-µL aliquots of these neat working standards were spiked into 100 µL rat plasma to achieve 0.15, 0.31, 0.61, 1.22, 2.44, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.50, 625, 1,250, 2,500, 5,000, 10,000, and 20,000 nM concentrations. Acetazolamide working stock solution was prepared bulk in acetonitrile at a concentration of 1,000 nM, and was used as the protein crash solvent in sample preparation.

Table 1. Agilent 1290 Infinity II LC method parameters.

Parameter	Value	
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl 2.1 × 50 mm 1.8 µm at 50 °C	
Mobile phase	A) 10 mM Ammonium acetate and 0.1 % formic acid in water B) 10 mM Ammonium acetate and 0.1 % formic acid in methanol	
Gradient	Time (min)	%B
	0	3
	0.5	15
	3	45
	5	70
	5.25	95
	6.5	95
	6.6	3
Post run	3 minutes	
Flow rate	0.5 mL/min	
Injection volume	1 µL (multiwash: needle wash using acetonitrile (S2), methanol (S1), and mobile phase initial composition (S3) for 10 seconds each)	

Table 2. Agilent 6495 Triple Quadrupole LC/MS parameters.

Parameter	Value
Ion source	AJS ESI
Polarity	Positive/Negative
Peak Filter	0.02 minutes
Drying gas temperature	270 °C
Drying gas flow	12 L/min
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Nebulizer pressure	40 psig
Capillary voltage positive	4,500 V
Nozzle voltage	0 V
High pressure RF	200 V
Low pressure RF	100 V
Fragmentor voltage	380 V
Cell accelerator voltage	7 V
MS1/MS2 Resolution	Wide/unit

Sample preparation by protein precipitation

To an aliquot of 50 µL of blank/calibration standards/study sample, 200 µL of acetonitrile containing internal standard was added. Samples were vortexed for 1 minute, followed by centrifugation at 13,000 rpm for 5 minutes at 4 °C. The 1 µL supernatant obtained was injected onto the LC/MS/MS system for analysis.

Partial validation

Bioanalytical methods for GBP, TGB, and LTG were partially validated for selectivity, precision, accuracy, linearity, and reproducibility. The selectivity of the analytes towards the *in vivo* matrix components was determined by comparing the response of the least concentrated sample (LLOQ) with that of the blank matrix. The intra-day precision and accuracy of the methods were assessed by analyzing area and retention time precision of six repeat preparations of the linearity levels. A linear regression model was used to plot the linearity relation between analyte concentration and instrument response ratio for the individual analyte to that of the internal standard. *In vivo* samples were analyzed, and AEDs were quantitated using the corresponding standard curve.

Table 3. MRM Details of all 26 antiepileptic drugs.

Compound group	Sl no.	Compound	Precursor ion (m/z)	RT (min)	Quant	Qual	Collision energy (V)
1	1	10-OH-Carbamazepine	255	3.02	237	194	20
	2	Carbamazepine	237	3.94	194	179	25
	3	Carbamazepine-10,11-epoxide	253	3.29	180	210	15
	4	Carbamazepine-diol	271	2.81	253	180	5
	5	Oxcarbazepine	253	3.29	236	208	10
2	6	Felbamate	239	2.43	117	178	5
	7	Lacosamide	251	2.38	108	91	5
	8	Lamotrigine	256	2.34	211	159	30
	9	Levetiracetam	171	1.23	126	154	5
	10	Rufinamide	239	2.47	127	222	10
	11	Theophylline	181	1.31	124	69	20
3	12	Gabapentin	172	1.23	154	137	10
	13	Pregabalin	160	1.16	142	125	10
	14	Sultiam	308	2.03	291	274	5
	15	Tiagabine	376	4.62	149	247	20
	16	Topiramate	357	3.07	264	282	10
	17	Vigabatrin	130	0.42	84	113	1
4	18	N-desmethylmesuximide	190	2.9	120	145	15
	19	PEMA	207	1.63	162	119	10
	20	Phenytoin	253	3.29	182	225	10
	21	Primidone	219	2.24	162	119	10
	22	Stiripentol	217	5.12	159	187	10
5	23	Ethosuximide	140	1.59	140		1
	24	Phenobarbital	231	2.82	188	85	5
	25	Valproic acid	143	4.2	143		1
	26	Zonisamide	211	1.95	119	147	5

Carryover analysis using the Agilent 1290 Infinity II Multisampler for three selected AEDs

Sample carryover can be an issue for sensitive LC/MS analysis, and can impact method development. Typically, the autosampler is the source for carryover. The Agilent 1290 Infinity II multisampler, with advanced multiwash feature, offers the option to clean the inside and outside of the injection needle by active backflushing with three different washing solvents, and can achieve ultra low carryover. For quantitating carryover using various injector cleaning options, a blank sample was injected after the highest concentrated calibration standard using various injector cleaning options (Table 4). Methanol, acetonitrile, and a premixed solution of both mobile phases at initial gradient ratio were used as the three individual wash solvents. Carryover of the 1290 Infinity II autosampler for the AED analysis was calculated from the analyte peak area in a blank injection, which was acquired immediately following an injection of the highest concentration calibration standard (ULOQ). The percentage of the analyte peak area observed in blank trace was compared to the corresponding peak area from ULOQ as the analyte carryover.

Table 4. Injector wash options employed to assess carryover.

Experiment condition	Wash mode
1	Off – no needle wash
2	Standard wash vial repeat 3
3	Standard wash flush port for 10 seconds
4	Multiwash (3 solvents) 10 seconds needle wash
5	Multiwash (3 solvents) 10 seconds seat backflush
6	Multiwash (3 solvents) 10 seconds each both needle wash seat backflush

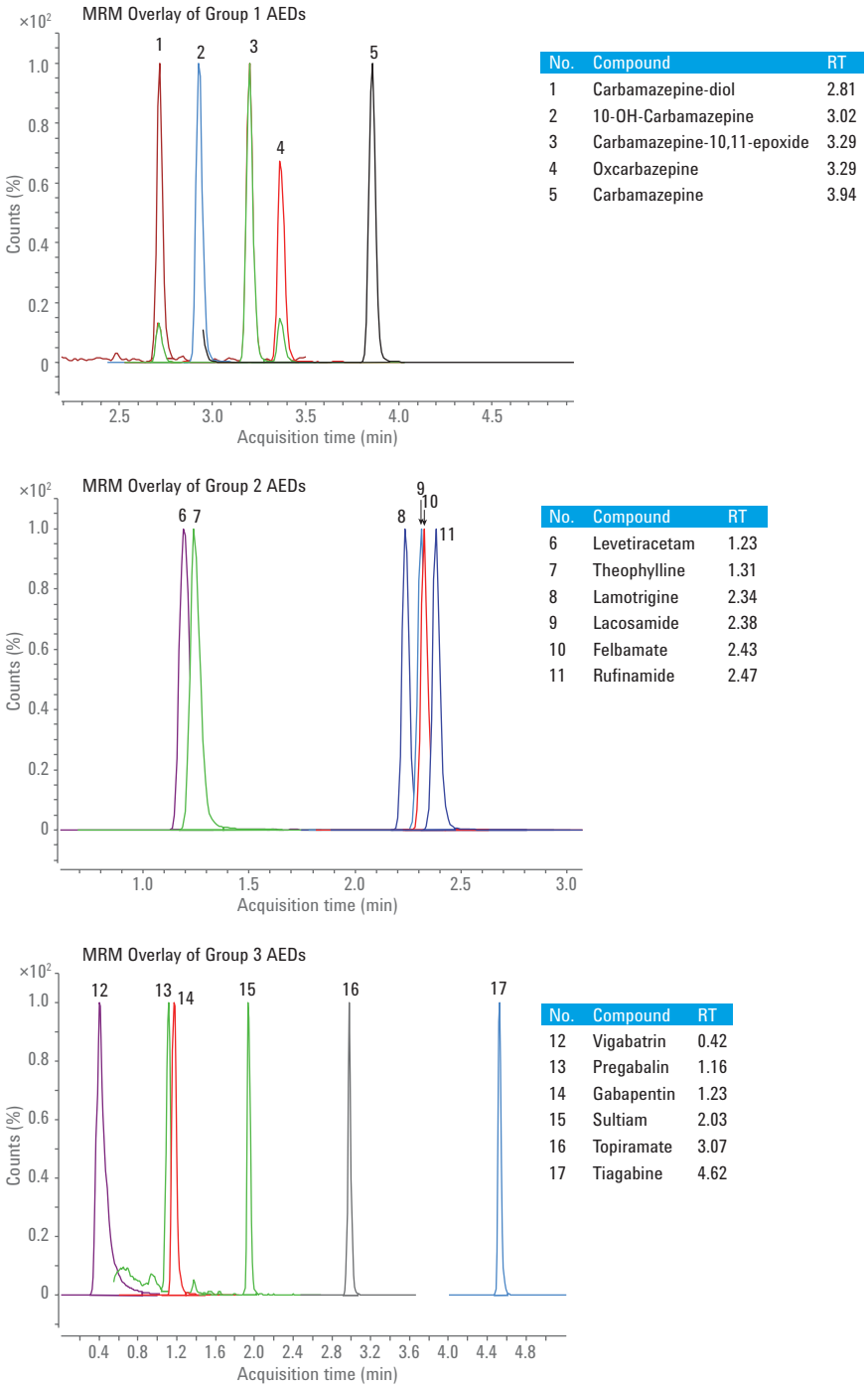
Rat PK study design

Jugular vein cannulated rats (n = 3, body weight range: 250–300 gm) were used to study PK parameters of three selected AEDs. A cassette approach wherein all three drugs were administered in a single IV dose was adopted to study their pharmacokinetics. Cassette formulation of GBP, TGB, and LTG was prepared by dissolving in 10 % water and 90 % PEG400. The formulation was administered through the catheter with silicone tubing inserted in a jugular vein (dose:1 mg/kg for each drug; dose volume: 2 mL/kg; administered as 10-minute infusion). Blood samples (200 µL) were collected from the same jugular vein catheter at 0.17, 0.25, 0.5, 1, 3, 5, 7, 10, 24, 32, and 48 hours post dose. After removal of each blood sample, approximately 0.3 mL of heparinized saline was injected into the animal using a fresh prefilled 1-mL syringe. Blood samples were collected into tubes containing K2EDTA (1.33 mM), and centrifuged at 10,000 g for 5 minutes at 4 °C to collect the plasma. Separated plasma samples were stored at –80 °C until analysis.

Results and Discussion

The Agilent 1290 Infinity II effectively separates all 26 AEDs using a single generic method

AEDs are typically grouped according to their major mechanism of action. All 26 AEDs are segregated into five different groups per Chromsystems kit recommendation. The Agilent 1290 Infinity II system could resolve all 26 AED analytes in a single chromatographic method. An Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column, along with DMRM analysis, offered well resolved chromatographic separation within 6 minutes. Excellent chromatographic peak shapes for all analytes were observed, with good method reproducibility (Figure 1). The sample preparation procedure used was quick, simple, and efficient.



No.	Compound	RT
1	Carbamazepine-diol	2.81
2	10-OH-Carbamazepine	3.02
3	Carbamazepine-10,11-epoxide	3.29
4	Oxcarbazepine	3.29
5	Carbamazepine	3.94

No.	Compound	RT
6	Levetiracetam	1.23
7	Theophylline	1.31
8	Lamotrigine	2.34
9	Lacosamide	2.38
10	Felbamate	2.43
11	Rufinamide	2.47

No.	Compound	RT
12	Vigabatrin	0.42
13	Pregabalin	1.16
14	Gabapentin	1.23
15	Sultiam	2.03
16	Topiramate	3.07
17	Tiagabine	4.62

Figure 1. LC/MS elution profile of all 26 AEDs. AED Groups 1 to 4 were measured in positive ionization, and Group 5 was analyzed in negative ionization mode (continued on next page).

Method reproducibility evaluation

Selectivity

To assess the selectivity of the developed method, the retention time of three selected AED analyte peaks were compared with the blank sample. Interfering peaks were not observed in the blank sample, thus confirming that the method is selective for the simultaneous separation and quantitation of all three AEDs in plasma matrix.

Accuracy and precision

All calibration standards were injected in replicate (n = 5), and accuracy values for each calibration level were back-calculated from the linear equations of each analyte. Accuracy values for all analytes at all concentration levels were within 90–100 % (Table 5). Precision was determined by calculating the relative standard deviations (RSDs) of retention time (RT) and peak area from repeat injections. Excellent RT and peak area precision were observed for all analytes throughout the calibration range, providing supporting evidence of method reproducibility. RT RSDs were less than 0.5 % for all analytes at all concentrations. Peak area RSDs for all analytes at all concentration levels, except for LOQ, were less than 5.0 %; for LOQ, the peak area RSD values were less than 10 %. Therefore, the measurements made using the LC/MS/MS instrument were determined to be accurate and precise for repeated injections, and suitable for analysis of *in vivo* PK samples.

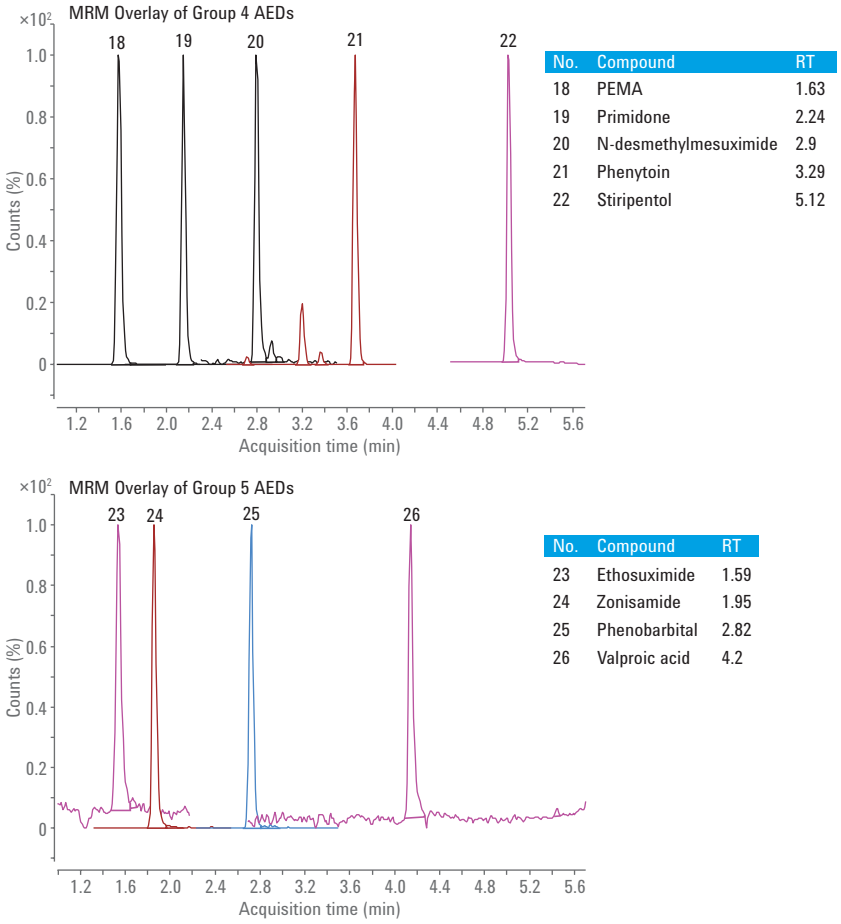


Figure 1 (continued). LC/MS elution profile of all 26 AEDs. AED Groups 1 to 4 were measured in positive ionization, and Group 5 was analyzed in negative ionization mode.

Table 5. Accuracy results summary.

Cal. level	Conc. (nM)	Accuracy (%) GBP	TGB	LTG
1	0.15			
2	0.31		109	
3	0.61		107	98
4	1.22		97	98
5	2.44		95	94
6	4.88		90	93
7	9.77	109	95	91
8	19.53	106	98	96
9	39.06	105	104	105

Cal. level	Conc. (nM)	Accuracy (%) GBP	TGB	LTG
10	78.13	97	98	98
11	156.25	93	101	96
12	312.50	98	98	95
13	625.00	98	108	108
14	1,250.00	107	109	108
15	2,500.00	102	102	107
16	5,000.00	98	100	110
17	10,000.00	98	107	91
18	20,000.00	104		91

Limit of detection (LOD), limit of quantitation (LOQ) and linearity range

The lowest concentration of each AED peak with a signal-to-noise ratio (S/N) of at least 10:1 was determined to be the LOQ, and the LOD at an S/N of 3:1. The linearity curve for GBP, TGB, and LTG was plotted from the least concentrated calibration level to the upper calibration level. To determine the best linearity response function, various regression models were evaluated, and the best calibration model was with Type: Linear, Origin: Ignore, Weight: 1/y (R^2 >0.99). Figure 2 gives the linearity curves of all three AEDs. Table 6 summarizes the LOD, LOQ, and linearity results using the 6495 Triple Quadrupole system.

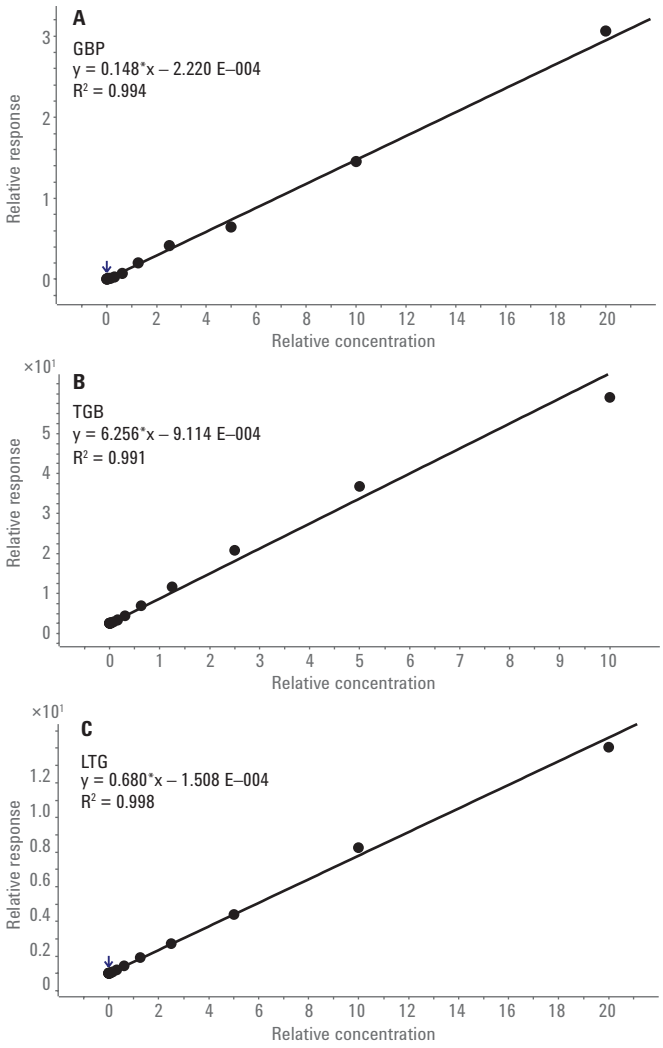


Figure 2. Linearity curve of (A) GBP (9.7–20,000 nM), (B) TGB (0.31–10,000 nM), and (C) LTG (0.61–20,000 nM). Type: Linear, Origin: Ignore, Weight:1/y.

Table 6. Linearity summary of all three AEDs.

Validation parameter	GBP	TGB	LTG
LOD (nM)	4.88	0.15	0.31
LOQ (nM)	9.77	0.31	0.61
Linear range (nM)	9.7–20,000	0.31–10,000	0.61–20,000
Correlation coefficient (R^2)	0.99	0.99	0.99

The multiwash feature of Agilent 1290 Infinity II LC offers the least carryover

The bioanalytical method described has a wide linearity range, which may pose carryover challenges. The Agilent multisampler has a flow-through injection needle design where the needle is part of the fluid path during operation. Additionally, the outside of the needle can be cleaned and rinsed in a flush port with up to three different solvents for a predefined duration. Fresh wash solvents are delivered to the flush port using a peristaltic pump. Seat backflush is enabled by a second pump: an integrated high-pressure flush pump. Carryover was defined as the percentage area of each analyte observed in the matrix blank, which was injected immediately after injection of the highest calibration standard. Carryover was evaluated for all three analytes under all experimental conditions. Investigation of percentage carryover using the various available options of the 1290 Infinity II LC multisampler resulted in interesting carryover results; percentage carryover results for injections with the 1290 Infinity II multiwash feature enabled are significantly lower than conventional injector wash modes (Figure 3).

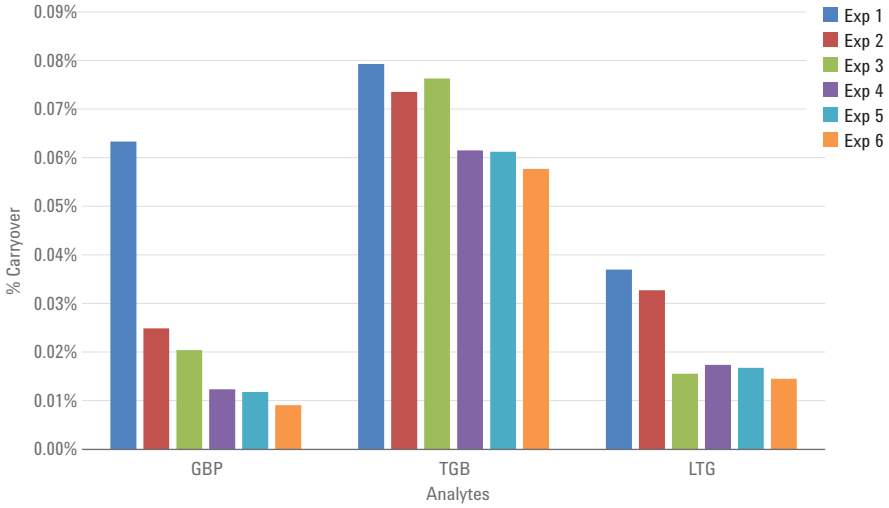
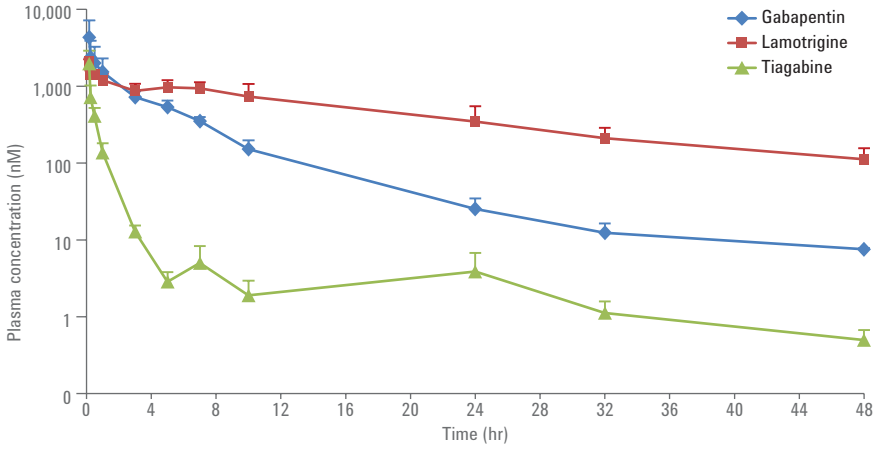


Figure 3. Carryover evaluation for GBP, TGB, and LTG using various needle wash options, as listed in Table 4.

Application of the method to a rat PK study

A selective and highly sensitive bioanalytical method is a key requirement for measuring drug concentrations in low-dose PK studies. Time points in the elimination phase of the drug pose a challenge because of the very low concentrations involved. The LC/MS/MS method was applied to the estimation of GBP, TGB, and LTG in rat plasma samples in a cassette IV PK study (dose: 1 mg/kg). Figure 4 shows PK profiles of all three drugs; the accompanying table shows their respective PK parameters. As seen from the PK profiles, the developed method was suitable for measuring concentrations of all three drugs for up to 48 hours. In the case of TGB, the high analytical sensitivity of the method (LLOQ: 0.3 nM) was required to measure sub-nanomolar concentrations in the elimination phase of the drug.

Considering a rat hepatic blood flow of 70 mL/min/kg, GBP and LTG showed low plasma clearance, whereas TGB showed high plasma clearance in the rat. All compounds had a high volume of distribution, more than the total body water (0.7 L/kg). The half-lives of all three AEDs were long, and concentrations were observed in plasma up to 48 hours. The high volume of distribution resulted in the longer half-life of TGB despite high clearance.



PK Parameter	GBP Mean ±SD	TGB Mean ±SD	LTG Mean ±SD
Dose (mg/kg)	1	1	1
$AUC_{0-48\ hr}$ (μM*hr)	8.3 ±1.8	0.73 ±2.0	21.5 ±7.6
AUC_{tot} (μM*hr)	8.3 ±1.8	0.74 ±2.0	24.3 ±8.5
$\%AUC_{extra}$	1.0 ±0.5	1.4 ±1.4	11.3 ±4.2
t_{half} (hr)	7.3 ±2.1	11.5 ±6.9	17.0 ±4.8
MRT (hr)	6.1 ±1.4	4.2 ±1.6	2.3 ±3.3
Clearance (mL/min/kg)	12.0 ±2.5	62.6 ±14.4	3.0 ±1.2
V_{ss} (L/Kg)	4.55 ±1.9	16.4 ±8.7	3.7 ±1.1

Figure 4. PK profile and parameters of GBP, TGB, and LTG in SD rats.

Conclusions

The need for selective, sensitive, and high-throughput bioanalytical methods is critical for early drug discovery studies. Fast and generic LC methods coupled with sensitive mass spectrometry detection enables the performance of rapid PK screening of NCEs, thus aiding lead optimization. This study describes a generic LC/MS/MS method for quick screening and sensitive PK assessment of AEDs. Chromatographic separation of all 26 AEDs was achieved in less than 6 minutes using an Agilent 1290 Infinity II LC system with an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column. The utility and reliability of the Agilent 6495 Triple Quadrupole in a cassette PK study were assessed using a partially validated bioanalytical method for GBP, TGB, and LTG in DMRM mode. The selectivity was evaluated for all three analytes to ensure no interference from the blank sample matrix. Parameters such as accuracy, precision, and linearity range were evaluated for three selected drugs, and were found to be well within acceptable limits. With the multiwash feature included in the Agilent 1290 Infinity II autosampler, the current method demonstrated very minimal carryover for PK analytes.

The application of the newly developed LC/MS/MS method to the sample analysis of a low-dose rat cassette PK study was useful for determining accurate PK parameters. A simple protein precipitation sample preparation method gave adequate analytical sensitivity (LOQ), which enabled the measurement of all three analytes for up to 48 hours post dose. This approach allows collection of PK data for multiple drugs simultaneously from a single study, and facilitates rapid evaluation of many drug candidates with fewer animals.

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