# Rapid Quantification of Lamotrigine in Human Plasma by Two LC Systems Connected with Tandem MS

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## Abstract

**A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method has been developed and validated for the determination of lamotrigine in human plasma using multiplexing technique (two HPLC units connected to one MS–MS). Lamotrigine was extracted from human plasma by solid-phase extraction technique using Oasis Hydrophilic Lipophilic Balance (HLB) or *N*-vinylpyrrolidone and divinylbenzene cartridge. A structural analog, 3,5-diamino-6-phenyl-1,2,4-triazine, was used as an internal standard (IS). A BetaBasic $C_8$ column was used for the chromatographic separation of analytes. The mass transition $[M+H]^+$ ions used for detection were *m/z* 256.0 → 211.0 for lamotrigine and *m/z* 188.0 → 143.0 for IS. The method involved a simple multiplexing, rapid solid-phase extraction without evaporation and reconstitution. The proposed method has been validated for a linear range of 0.025 to 10.000 µg/mL with a correlation coefficient ≥ 0.9991. The limit of quantification for lamotrigine was 0.025 µg/mL, and limit of detection was 50.000 pg/mL. The intra-run and inter-run precision and accuracy were within 10.0% for intra-HPLC runs and inter-HPLC runs. The overall recoveries for lamotrigine and IS were 97.9% and 92.5%, respectively. Total MS run time was 1.4 min per sample. The validated method has been successfully used to analyze human plasma samples for applications in pharmacokinetic, bioavailability, bioequivalence, or in vitro in vivo correlation studies.**

## Introduction

Lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) is a phenyltriazine derivative. In clinical trials, lamotrigine has been found to be an effective antiepileptic agent with a broad spectrum of activity (1).

Lamotrigine is an anticonvulsant drug found to be effective as adjunctive therapy and as monotherapy for partial seizures (2–6). It protects against lesions produced by kainate (7), and at high doses it can reduce cortical infarct volume and protect against global cerebral ischemia (8–11). Lamotrigine is also labeled for prevention of depressive episodes in patients with bipolar disorders. It is rapidly and completely absorbed after oral administration with negligible first-pass metabolism (absolute bioavailability = 98%). Peak plasma concentrations were found to be between 1.4 and 4.8 h following drug administration. It is approximately 55% bound to human plasma proteins at plasma lamotrigine concentrations from 1 to 10 µg/mL (12). Extensive metabolism of lamotrigine molecule takes place in the liver, predominantly via *N*-glucuronidation by uridine-glucuronyl-transferase isoenzymes. After an oral dose of lamotrigine, 70–94% is excreted in the urine; 80–90% of the dose is found as 2-*N*-glucuronide metabolite while the rest is eliminated as the 5-*N*-gluronide metabolite, 2-*N*-methyl metabolite, unidentified metabolite and unchanged drug (12). The metabolites are not thought to be pharmacologically active. Hence, metabolites do not need to quantified for the pharmacokinetics of lamotrigine.

The actual plasma concentration of a drug is of major interest in pharmacokinetic studies. Several high-performance liquid chromatographic (HPLC) analytical methods have been reported in literature for monitoring plasma and serum levels of lamotrigine and with other antiepileptic drugs (16–23). The reported HPLC methods are not sensitive enough to quantify 25.000 ng/mL levels of lamotrigine in human plasma. Hence, this single method used for 100 mg and 200 mg of immediate release dosage forms as well for (in vitro in vivo) IVIVC for modified release dosage form. Also, the reported methods follow long run time (> 8.0 min) and involve a time-consuming drying and reconstitution step during extraction. A previously reported LC–MS–MS method (24) monitored a metabolite that is not commonly detected. This LC–MS–MS method further had a limit of detection (LOD) (0.05 µM/L) that is higher than the present method. Reported method used protein precipitation method, which contaminates the electrospray ionization (ESI) source rapidly and run time reported (20 min/sample) with gradient mobile phase. We report a new validated method for quantification of lamotrigine in human plasma that includes a simple solid-phase extraction (SPE) technique without a drying and reconstitution step to assay rapid analysis without compromising sensitivity (limit of quantitation, LOQ 25.000 ng/mL and LOD 50.000 pg/mL). The reported methods do not have any multiplex LC–MS–MS (two HPLC units connected to one MS–MS or parallel two column liquid chromatography in conjunction with a conventional single-source electrospray mass spectrometer) method for quantification of lamotrigine in human plasma. Also, the use of LC–MS–MS for analysis of drug candidates in biological matrices has grown exponentially in the last decade due to its

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unmatched sensitivity, extraordinary selectivity, and rapid rate of analysis. Therefore, it was necessary to develop a sensitive, selective and rapid multiplexing analytical technique for the quantification of lamotrigine in human plasma by using lowest volume of plasma with isocratic mobile phase and 1.4 min/sample run time.

A critical review is reported for published work in optimization of a method development strategy, sample preparation techniques, chromatography, and parallel analysis to achieve high throughput using LC–MS–MS methods (25). Parallel analysis approach for achieving high throughput, either using multiple inlets in the mass spectrometer source or multiplexing LC units into one MS, is well-established. Although the multiple inlets in the mass spectrometer allow four-fold increase in throughput, sensitivity loss and potential cross-inlets contamination are major drawbacks (26). Multiplexing LC unit set-up enables the ability to overcome these two shortcomings and demonstrates that when two LC units feed one MS, the run time is reduced by 50%. This approach has been successfully used for analysis of drug candidates in plasma (27–31).Therefore, in the present work, two HPLC units connected with one MS–MS set-up are utilized to achieve high throughput.

## Experimental

### Chemicals and reagents

Lamotrigine (99.95%) and 3,5-diamino-6-phenyl-1,2,4-triazine (99.67%) were obtained from Torrent Pharmaceutical Ltd. (Ahmedabad, India). The chemical structures are presented in Figure 1. High-purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Billerica, MA). Gradient-grade methanol (Ranbaxy, Delhi, India), suprapure formic acid (Merck, Darmstadt, Germany), and sodium hydroxide (Merck) were purchased and used. Drug-free heparinized human plasma (blank) was obtained from clinical research department of Torrent Research Centre (Gandhinagar, India) and was stored at –70°C until use.

### Calibration curves

The stock solutions of lamotrigine and internal standard (IS) were prepared in water–methanol (50:50, v/v). Secondary standard solutions and working standard solutions for lamotrigine and IS were prepared from stock solutions using water–methanol (50:50, v/v) by subsequent dilutions. These diluted working standard solutions were used to prepare the calibration curve and quality control samples.

A nine-point standard calibration curve for lamotrigine was prepared by spiking the blank plasma with appropriate amount of lamotrigine. The calibration curve ranged from 0.025 to10.000 µg/mL. This range is applicable for 200 mg lamotrigine dispersible to modified release formulations. Low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples were prepared at three concentration levels of 0.075 µg/mL, 3.000 µg/mL and 7.000 µg/mL, respectively, for lamotrigine in plasma the standard from the stock solution.

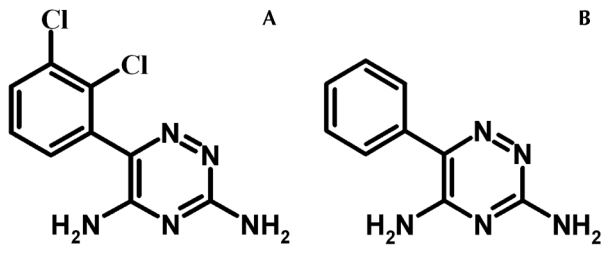


**Figure 1.** Chemical structure for (A) lamotrigine and (B) IS (3,5-diamino-6-phenyl-1,2,4-triazine).

### Sample preparation

A 100 µL aliquot of human plasma sample was mixed with 5 µL of IS working solution (1.0 µg/mL of 3,5-diamino-6-phenyl-1,2,4-triazine) followed by addition of 0.2 mL of 0.05 M sodium hydroxide. For SPE, an Oasis HLB (Hydrophilic Lipophilic Balance) cartridge has been used. In this cartridge, *N*-vinylpyrrolidone (hydrophilic) and divinylbenzene (lipophilic) chemicals stationary phase (patented technology) have been used by Waters (Milford, MA). The sample mixture was loaded onto an Oasis HLB (1 $cm^3$, 30 mg) extraction cartridge that was pre-conditioned with 1.0 mL methanol followed by 2.0 mL water. The extraction cartridge was washed with 1.0 mL water followed by 1.0 mL methanol–water (20:80; v/v). Lamotrigine and IS were eluted with 300 µL methanol. 2.0 µL of the extract was injected into the LC–MS–MS system without the drying and reconstitution step.

### Instrumentation

The multiplexing set-up consisted of two Shimadzu class VP series HPLCs (Figure 2) and one flow change over valve FCV-AH12. Chromatographic separation was carried out with BetaBasic $C_8$ (5.0 µm, 100 × 4.6 mm) column purchased from Thermo Fisher (Auchtenmuchty, UK). A mobile phase consisting of methanol–0.03% formic acid in water (90:10; v/v) was delivered with a flow rate of 0.45 mL/min without a splitter. The

**Table I. Ion Source and Analyte-Dependent Parameters of MS**

| **Ion source** | | |
|---|---|---|
| Spray voltage | 3000 V | |
| Capillary temperature | 300°C | |
| Sheath gas | 40 (arbitrary) | |
| Auxillary gas | 20 (arbitrary) | |
| Polarity mode | Positive | |
| **Analyte dependent** | **Lamotrigine** | **IS** |
| Precursor ion (*m/z*) | 256.00 | 188.00 |
| Product ion (*m/z*) | 211.00 | 143.00 |
| Tube lens off set (V) | 95 | 143 |
| Q1 Pw* (amu) | 0.35 | 0.5 |
| Q3 Pw† (amu) | 0.35 | 0.5 |
| Collision energy | 32 | 29 |

* Quadrupole 1 peak width.
† Quadrupole 3 peak width.

column oven temperature was kept at 50°C. The total run time for each sample analysis was 3.1 min for both HPLCs and 1.4 min for MS. The sample injection volume was 2.0 µL. Mass spectra were obtained using a TSQ Discovery mass spectrometer, a triple quadrupole mass analyzer with electron multiplier detector, equipped with electrospray ionization (ESI) source (Thermo Fisher). The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. The MS and HPLC parameters for analytes are listed in Table I and Table II, respectively. HPLC-2 time program is the same as HPLC-1 except the first line was not applicable for HPLC-2. Class-VP software (version 6.14 SP2) was used for HPLC function whereas MS data acquisition was ascertained by Xcaliber LCquan 2.5 SUR1 software. The HPLC and MS data were synchronized by contact closure using a RS232 cable for communication and triggering. Within a single chromatographic run time in the multiplex LC–MS–MS, sample injections were made alternately onto each of the two analytical columns parallelly at specified intervals with a mass spectrometer data file opened at every injection. Thus, the mass spectrometer collected data from two sample injections into separate data files within a single chromatographic run time. Therefore, without sacrificing the chromatographic separation or the SRM, the sample throughput was increased by a factor of two. Comparing the method validation results obtained using the two-column system with those obtained using the corresponding conventional single-column approach, the methods on the two systems were found to be equivalent in terms of accuracy and precision. The parallel-column system is simple and can be implemented using existing laboratory equipment with no additional capital outlays. For quantification, the peak-area ratios of the target ions of the drug were compared to those of the internal standard with weighted (1/*x*) least squares calibration curves in which the peak-area ratios of the calibration standards were plotted versus their concentrations.

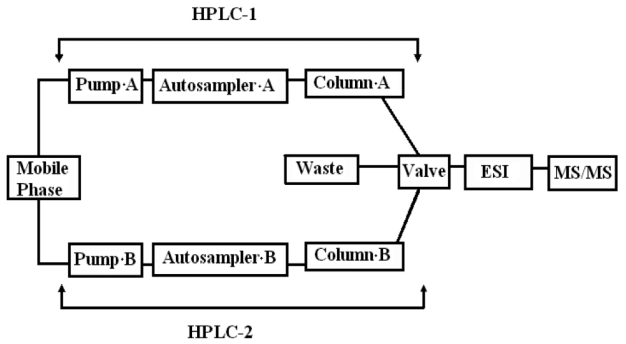


**Figure 2.** Simple multiplexing set up.

**Table II. Shimadzu LC Method Parameters (Time Program) for Multiplexing System**

| Time (min) | Module | Event | Value |
|---|---|---|---|
| *Time program for HPLC-1* | | | |
| 0.89 | Subcontroller vp | RV.B (2.Pos.) | 1* |
| 0.90 | SCL-10Avp | Event | 1 |
| 1.00 | SCL-10Avp | Event | 0† |
| 2.30 | SCL-10Avp | Event | 3‡ |
| 2.40 | SCL-10Avp | Event | 0 |
| 3.10 | SCL-10Avp | Stop | 0 |

* Event value 1 = Giving signal to the MS to start acquisition.
† Event value 0 = All events off.
‡ Event value 3 = Giving signal to the next HPLC to start run.

**Validation**

The method has been validated for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability, matrix effect, and carryover check. Selectivity and specificity was performed by analyzing and comparing the blank plasma samples and spiked LOQ samples from different sources (or donors) to ensure absence of any possible interference at the retention time of lamotrigine and IS. Sensitivity was determined by analyzing six replicates of spiked plasma with the analyte at the lowest level of the calibration curve. The intra-run and inter-run accuracy was determined by replicate ($n$ = 6) analysis of quality control samples and at LOQ that were extracted from the sample batch. Inter-run precision and accuracy of the calibration standards were assessed using the six calibration curves used for assay validation.

Accuracy is defined as the percent relative error (% RE) and was calculated using the formula % RE = (E – T) × (100/T), where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula % RSD = (SD/M) × 100, where M is the mean of the experimentally determined concentrations and SD is the standard deviation.

The extraction efficiencies of lamotrigine and IS were determined by comparing the peak area of extracted analytes to the peak area of equivalent non-extracted standards (drug spiked in extracted blank plasma) and equivalent aqueous standard.

The processed sample stability was evaluated by comparing the extracted plasma samples injected immediately (time 0) with the samples re-injected after keeping it in the autosampler at 5°C for 72 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 12 h and compared with freshly prepared samples. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times with freshly spiked quality control samples. The stability of spiked human plasma stored at –70°C (long-term stability) was evaluated by analyzing LQC, MQC, and HQC samples that were stored at –70°C for 98 days together with freshly spiked calibration standard and quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable if the deviation of the mean test results were within 15% of freshly prepared or comparison sample.

The importance of including evaluation of matrix effect in an LC–MS–MS method is outlined in an excellent paper by Matuszewski and co-workers (32). Their data strongly emphasizes the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this assay were performed with matrices obtained from different individuals. Matrix effect was checked with total six different lots of plasma. Three samples each of LQC

and HQC were prepared from different lots of plasma (total 36 QC samples) and checked for the accuracy in all QC samples. This was performed to see the matrix effect of these different lots of plasma on the back-calculated value of QCs versus their nominal concentrations. It is considered that there is no significant matrix effect if the deviation of the mean test results were within 15% of nominal concentration.

Carryover check of autosampler and system was also performed by sequential injections of extracted LOQ, extracted blank plasma, extracted upper limit of quantification (ULOQ, 10.000 µg/mL), extracted blank plasma, extracted ULOQ, and extracted blank plasma. These sequential runs were performed in parallel on both the HPLC systems to ensure absence of any carryover from individual system as well as multiplexed system.

## Results

### Selectivity

Chromatographic selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times for lamotrigine and IS in six different lots of extracted blank plasma. The area observed at the retention time (RT) of lamotrigine was much less than 20% at the LOQ (25.000 ng/mL) area, whereas the area observed at the RT of IS was less then 5% the area of IS concentration used in sample preparation. The aim of performing a selectivity check with these different types of plasma samples was to ensure the integrity of the results of actual study sample analysis. Representative chromatograms of extracted blank plasma, zero standard (drug free spiked with IS in plasma), extracted plasma samples containing 25 ng/mL lamotrigine (LOQ), and plasma samples from subjects (2.523 µg/mL) are presented in Figure 3A–3D, respectively.

### Linearity

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Six linearity curves containing nine non-zero concentrations (0.025, 0.100, 0.200, 1.000, 2.000, 4.000, 6.000, 8.000, 10.000 µg/mL) were analyzed. The calibration curves appeared linear and were well-described by regression line equation $y = mx + c$, where $y$ = area ratio of lamotrigine to IS, m = slop of calibration curve, $x$ = concentration of lamotrigine and c = intercept of calibration curve. A weighting factor of 1/concentration (i.e., $1/x$) was chosen to achieve homogeneity of variance. The correlation coefficients were ≥ 0.9991 ($n$ = 6) for lamotrigine. The observed mean back-calculated concentrations with accuracy (% RE) and precision (RSD) of six linearities are within limit (± 20% for LOQ and ± 15% for others).

**Table III. Intra-run (within-batch) and Inter-run (between-batch) ($n$ = 6) Precision and Accuracy of Lamotrigine in Human Plasma in HPLC-1 and HPLC-2**

| HPLC No. | $n$ | Spiked conc. (µg/mL) | Mean calculated conc. (µg/mL) | %RSD ($n$ = 6) | % RE |
|---|---|---|---|---|---|
| *Intra-run (within-batch)* | | | | | |
| (HPLC-1) | 6 | 0.025 | 0.026 | 4.32 | 5.6 |
| | 6 | 0.075 | 0.073 | 5.30 | –3.2 |
| | 6 | 3.000 | 3.174 | 3.02 | 5.81 |
| | 6 | 7.000 | 7.260 | 3.67 | 3.72 |
| (HPLC-2) | 6 | 0.025 | 0.027 | 3.36 | 6.4 |
| | 6 | 0.075 | 0.073 | 5.73 | –2.67 |
| | 6 | 3.000 | 3.127 | 2.47 | 4.22 |
| | 6 | 7.000 | 7.101 | 0.88 | 1.44 |
| *Inter-run (between-batch)* | | | | | |
| (HPLC 1) | 36 | 0.025 | 0.026 | 8.26 | 2.56 |
| and | 36 | 0.075 | 0.073 | 5.07 | –2.88 |
| (HPLC-2) | 36 | 3.000 | 3.124 | 4.89 | 4.14 |
| | 36 | 7.000 | 7.136 | 4.02 | 1.94 |

### Sensitivity

The LOQ is defined as the lowest concentration of the calibration standard yielding accuracy ± 20% RE and precision of ≤ 20%

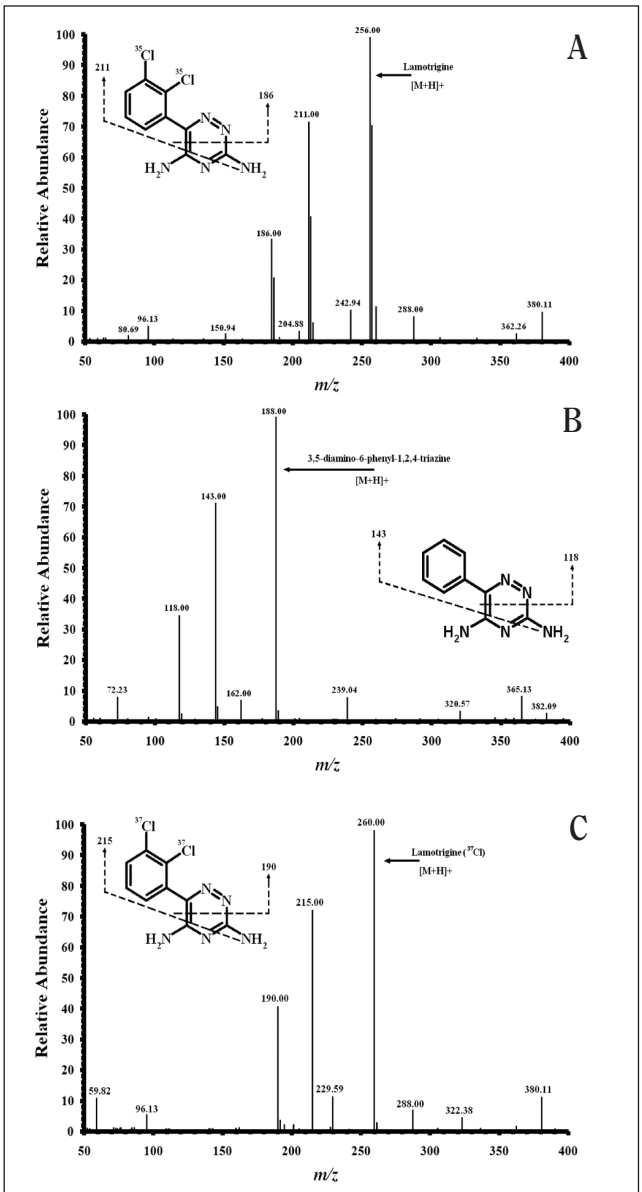


**Figure 3.** Full-scan positive product ion mass spectra and the proposed patterns of fragmentation of lamotrigine with $^{35}Cl$ isotope (A), IS (3,5-diamino-6-phenyl-1,2,4-traizine) (B), and lamotrigine with $^{37}Cl$ isotope (C).

RSD. The LOQ for lamotrigine was 0.025 µg/mL, and LOD was 50.000 pg/mL. The intra-run precision of the LOQ plasma samples containing lamotrigine were 4.32% and 3.36% for HPLC-1 and HPLC-2, respectively. The mean intra-run accuracy of the LOQ plasma samples containing lamotrigine were 5.60% and 6.40% for HPLC-1 and HPLC-2, respectively (Table III).

### Precision and accuracy

The intra-run precision ($n$ = 6) was ≤ 5.30% for HPLC-1 and ≤ 5.73% for HPLC-2. The intra-run accuracy was ≤ 5.81% for HPLC-1 and ≤ 6.40% for HPLC-2 (Table III). The inter-run precision and accuracy were determined by pooling all individual assay results of replicate ($n$ = 6) QC samples over the six separate batch runs. The inter-run precision was ≤ 8.26%. The inter-run accuracy was ≤ 4.14% for lamotrigine (Table III).

### Recovery

Five replicates at LQC, MQC, and HQC concentrations for the lamotrigine were prepared for recovery determination. The mean absolute recovery for lamotrigine was 97.89% with a precision of 2.56%. The mean recovery for IS was 92.50% with a precision of 4.62%. No significant difference (± 2.00%) was observed for absolute recovery and external spike sample in blank plasma eluent. Recoveries of the analytes and IS were good, and it was consistent, precise, and reproducible.

### Stability

The results of the stability studies are enumerated in Table IV. The benchtop stability, process stability and freeze-and-thaw stability of lamotrigine in plasma were investigated by analyzing quality control samples in replicates ($n$ = 6) at LQC and HQC level. Process stability results indicated that the difference in the back-calculated concentration from time 0 to 72 h is ≤ 5.92%, which concludes that processed samples are stable at least for 72 h at 5°C in autosampler. Benchtop stability results allowed us to conclude that lamotrigine is stable for at least 12 h at room temperature in plasma samples. Freeze-and thaw-stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability of lamotrigine. Long-term stability of lamotrigine in plasma at –70°C was performed at LQC, MQC, and HQC levels, and it was found to be stable for at least 98 days at –70°C.

**Table IV. Stability Results for Lamotrigine ($n$ = 6)**

| Stability | Spiked conc. (µg/mL) | Mean (± SD) obtained conc. (µg/mL) | %RE |
|---|---|---|---|
| Process* | 0.075 | 0.076 (± 0.003) | 1.33 |
| | 7.000 | 6.980 (± 0.342) | –0.29 |
| Bench top† | 0.075 | 0.072 (± 0.002) | –4.53 |
| | 7.000 | 7.069 (± 0.046) | 0.98 |
| Freeze/Thaw‡ | 0.075 | 0.079 (± 0.002) | 5.33 |
| | 7.000 | 7.143 (± 0.046) | 0.98 |
| Long-term§ | 0.075 | 0.073 (± 0.002) | –2.40 |
| | 3.000 | 2.955 (± 0.085) | –1.49 |
| | 7.000 | 6.963 (± 0.129) | –0.53 |

* After 72 h in autosampler at 5°C.
† After 12 h at room temperature.
‡ After three freeze/thaw cycles at –70°C/room temp.
§ After stored at –70ºC for 98 days.

### Matrix effect

Three quality control samples at each level along with the set of calibration standards were analyzed, and the % bias of the samples analyzed was found within ± 15% for each QC level for lamotrigine (Table V), proving that the elution of endogenous matrix peaks during the run has no effect on the quantification of lamotrigine. Therefore, the method of extraction of lamotrigine from plasma was rugged enough and gave accurate and consistent results when applied to real patient samples.

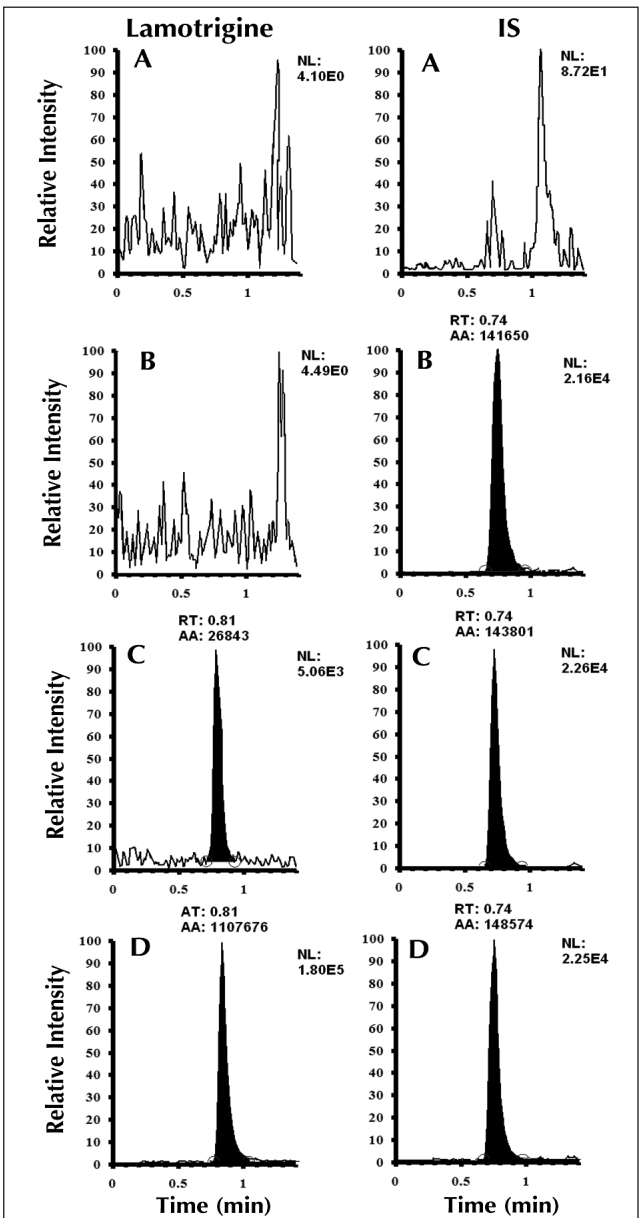


**Figure 4.** SRM chromatograms: (A) blank plasma (drug- and IS-free), (B) zero standard (drug-free spiked with IS in plasma), (C) 25 ng/mL (LOQ), and (D) resulting from the analysis of a subject's plasma sample after the administration of a single oral dose of 200 mg of lamotrigine. The sample concentration was determined to be 2.523 µg/mL.

### Carry over check

No carryover for analyte and internal standard was observed after sequential injections of blank plasma and extracted standards (10.000 µg/mL) as per validation section.

## Discussion

### Mass spectrometry

Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by MS–MS is becoming more common due to the improved sensitivity and selectivity of this technique. ESI and atmospheric pressure chemical ionization (APCI) were evaluated to get better response of analytes. It was found that the best signal was achieved with ESI positive ion mode. Lamotrigine accepts the proton in an acidic mobile phase and produces a protonated precursor ion ($[M+H]^+$) at *m/z* 256.0. The collisionally activated dissociation mass spectrum of lamotrigine and IS shows the formation of characteristic product ion at *m/z* 211.0, 186.0 and 143.0, 118.0, respectively. The major product ion was found at *m/z* 211.0 for lamotrigine and 143.0 for IS. The most sensitive and selective mass transition was from *m/z* 256.0 to 211.0 for lamotrigine and *m/z* 188.0 to 143.0 for IS. A proposed fragmentation pattern is also shown in Figure 4A–4B. It is known that Cl has two isotopes $^{35}Cl$ and $^{37}Cl$. Figure 4 shows fragmentation pattern by $^{35}Cl$ isotope. Lamotrigine fragmentation pattern was further confirmed by fragmentation of 260 molecular weight with consideration of $^{37}Cl$ isotope (Figure 4C).

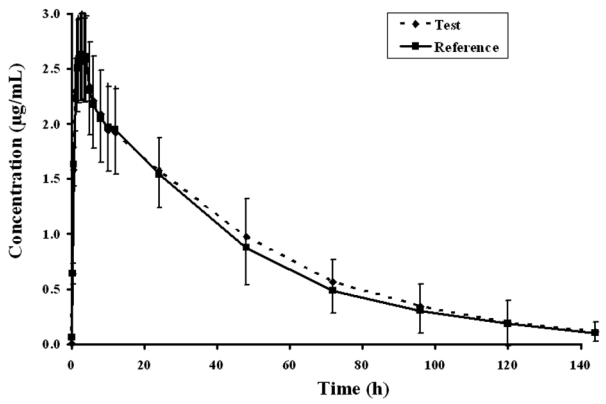


**Figure 5.** Representative data showing mean plasma concentration-time profiles of 30 male healthy volunteers after the administration of a single dose of 200 mg of lamotrigine under fasting conditions.

**Table V. Matrix Effect (*n* = 3) for Lamotrigine**

| | | LQC (0.075 µg/mL) | | | HQC (7.000 µg/mL) | | |
|---|---|---|---|---|---|---|---|
| Sr. No. | Plasma Lot No.* | Mean calc. conc. | %RSD | %RE | Mean calc. conc. | %RSD | %RE |
| 1 | A1 | 0.077 | 3.19 | 3.11 | 7.180 | 1.13 | 2.58 |
| 2 | A2 | 0.078 | 3.30 | 3.56 | 7.214 | 0.89 | 3.05 |
| 3 | A3 | 0.080 | 4.08 | 6.22 | 7.239 | 2.36 | 3.41 |
| 4 | A4 | 0.077 | 3.37 | 2.67 | 7.285 | 2.02 | 4.07 |
| 5 | B | 0.081 | 6.57 | 7.56 | 7.332 | 2.90 | 4.73 |
| 6 | C | 0.079 | 3.61 | 4.89 | 7.223 | 1.90 | 3.18 |

* A = heparinized plasma; B = lipimix plasma; C = hemolyzed plasma.

### Method development

To develop a rapid, sensitive, and simple assay method for the extraction and quantification of lamotrigine, different options were evaluated to optimize chromatography parameters. The chromatographic condition, especially the composition of mobile phase, was evaluated through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS. It was found that a mixture of methanol–0.03% formic acid in water (90:10; v/v) could achieve this purpose and was finally adopted as the mobile phase used in this method. Use of BetaBasic $C_8$ (100 × 4.6 mm i.d., 5 µm) column resulted in reduced flow rate to 0.45 mL/min and reduced run time as low as 1.4 min with multiplex LC–MS–MS. Retention time of analyte and IS was at 0.81 and 0.74 min, respectively. The resulted signal with optimized chromatography and detection parameters enabled elimination of the laborious extraction steps of evaporation of eluent and reconstitution involved in generic SPE methods without compromising the sensitivity, which further resulted in reduced processing and analysis time.

SPE was used for sample preparation in this work. SPE can be helpful in producing a spectroscopically clean sample; it is essential for minimizing ion suppression and matrix effect in LC–MS–MS analysis. Oasis HLB (1 $cm^3$, 30 mg) extraction cartridge was used for sample extraction and washed with 1.0 mL methanol–water (20:80; v/v) in water for complete recovery. A good recovery was obtained by using 300 µL methanol as elution solvent.

Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC–MS–MS where matrix effects can lead to poor analytical results. Usually, an isotope-labeled compound is an ideal IS for the corresponding analyte. However, the isotope-labeled compound was not commercially available for lamotrigine. Therefore, we used 3,5-diamino-6-phenyl-1,2,4-triazine, structural analog of lamotrigine. In addition, its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained, and no interference in SRM channels at the relevant retention time was observed.

### Application of method

The present validated LC–MS–MS method (33) was successfully applied in the pharmacokinetic studies of 200 mg lamotrigine immediate release tablets in healthy male volunteers of 38 ± 9 years in age and 64 ± 6 kg in weight. The design of the study comprised of a randomized, open label, single dose, two treatments, two periods, two sequence crossover bioequivalence study of lamotrigine 200 mg dispersible formulation in 30 healthy male volunteers under fasting conditions. The study was conducted strictly in accordance with guidelines laid down by the U.S. FDA (34).The plasma samples were drawn at 0, 0.25, 0.50, 1, 1.5, 2, 2.5, 3,

3.5, 4, 5, 6, 8, 10, 12, 24, 48, 72, 96, 120, and 144 h. A total of 1700 human plasma samples from 30 male volunteers were analyzed along with calibration standards and QC samples in HPLC-1 and HPLC-2. One separate calibration curve was made for sample quantification on each HPLC-1 and HPLC-2. Total 600 samples were analyzed per day. No interference peak was found in predose samples for all volunteers. The mean (± SD) plasma maximum concentrations obtained for the lamotrigine test, and reference formulations (Lamictal) were 2.605 (± 0.282) µg/mL and 2.628(± 0.337) µg/mL, respectively. The mean lamotrigine plasma concentration-time profile following 200 mg oral dose of lamotrigine to human subjects is shown in Figure 5.

## Conclusions

A simple, specific, rapid, and sensitive LC–MS–MS method has been developed for the determination of lamotrigine in human plasma. The proposed method provided excellent specificity and reproducibility with a LOQ of 25.000 ng/mL for lamotrigine. Extraction method and results could also be used with a conventional LC–MS–MS setup.

It is concluded that this sensitive and specific method is applicable for the high throughput quantitative determination of lamotrigine in human plasma in pharmacokinetic, bioavailability, and IVIVC studies of lamotrigine.

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