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High-performance thin-layer chromatographic determination of lamotrigine in serum

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Abstract

A simple and rapid high-performance thin-layer chromatographic (HPTLC) determination of lamotrigine (LTG) in serum is reported. The method involves extraction of the drug by ethyl acetate followed by separation on TLC silica plates using a mixture of toluene-acetone-ammonia (7:3:0.5), as eluting solvent. Densitometric analysis was carried out at 312 nm with lamotrigine being detected at R_f of 0.54. The analytical method has excellent linearity ($r = 0.998$) in the range of 20–300 ng/spot. This assay range is adequate for analyzing human serum, as it corresponds to lamotrigine concentrations measured in human serum from epileptic patients. The method was validated for sensitivity, selectivity, extraction efficiency, accuracy and intra and inter-day reproducibility. The limit of detection and limit of quantification were found to be 6.4 and 10.2 ng, respectively. Good accuracy is reported in the range of 92.06–97.12% and high precision with %CV in range of 0.53–2.59. The method was applied for determination of serum lamotrigine levels in epileptic patients and in pharmacokinetic study of lamotrigine administered orally to rabbits.

Keywords: Lamotrigine; Serum; High-performance thin-layer chromatography

1. Introduction

Lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) (Fig. 1) is a new antiepileptic drug from triazine class of chemical compounds. It has recently been approved for the treatment of partial and generalized epileptic seizures as an add-on agent or as monotherapy and it is generally well tolerated [1]. It acts by blocking voltage dependent sodium channels in the neuronal membrane, thus reducing release of excitatory neurotransmitters, especially glutamate [2]. There are large interindividual variations in dose versus serum concentrations in patients on monotherapy, and pharmacokinetic variability plays a major role in the lamotrigine dosage requirements to achieve optimum serum concentrations depending on interacting antiepileptic drugs (AEDs) comedication [3].

Various analytical methods to estimate lamotrigine from biological fluids have been reported in the literature. These include HPLC [4–9], GC [10–12], GC–MS [13,14], radioimmunoassay [15], electrospray ionization-mass spectrometry (ESI-MS) [16], capillary electrophoresis [17–19], etc. Unlike HPTLC, all reported methods are cumbersome, time consuming and costlier techniques for estimation of drug in biological samples. HPTLC serves as an important tool in the assay of drugs. HPTLC enables qualitative, quantitative and preparative analysis with the same system, high speed quantitative analysis, co-chromatography, less analysis time and minimal sample clean up. In recent years, HPTLC has gained importance since it allows reliable quantitation of analytes at micro and even nanogram levels [20,21].

However, there is no reported HPTLC method for the estimation of lamotrigine in serum. Thus, taking into consideration, the simplicity and cost effectiveness of HPTLC in the analysis of drugs, HPTLC method was developed for estimation of lamotrigine in serum. This paper describes a reliable, sensitive and selective HPTLC method which enables

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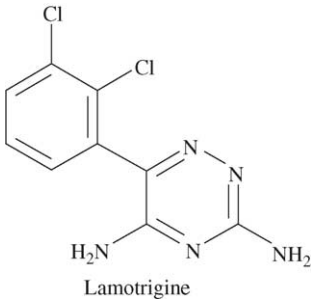

Lamotrigine

Fig. 1. Structure of lamotrigine.

the determination of lamotrigine serum levels for therapeutic drug monitoring in epileptic patients or pharmacokinetic studies conducted in humans or laboratory animals.

2. Materials and methods

2.1. Drug and chemicals

Lamotrigine was obtained from RPG Life Sciences Ltd., Mumbai, India. All reagents and chemicals used were of AR grade and procured from local sources.

2.2. Preparation of standards

A stock solution of lamotrigine (100 μg/ml) was prepared in methanol. The stock solution was diluted with methanol to obtain a standard solution of lamotrigine (10 μg/ml). The stock solution was also diluted with drug-free serum to obtain a working standard solution of lamotrigine (1 μg/ml) in serum.

2.3. Serum sample preparation

To 1 ml of serum in a 15 ml centrifuge tube, 10 ml of ethyl acetate was added. After mixing for 10 min, the sample was centrifuged for 10 min at 4000 × *g*. The aqueous phase was discarded and the organic phase was evaporated to dryness under a flow of nitrogen. The residue was reconstituted in 100 μl of methanol and 2–3 μl was spotted on precoated Silica Gel F_{254} plates (E-Merk) with the chromatographic conditions as mentioned in Section 2.4.

2.4. Instrumentation and chromatographic conditions

The chromatographic estimation was performed by spotting the standards or concentrated reconstituted samples of lamotrigine on silica gel 60 F_{254} TLC plates (10 cm × 10 cm) using Camag Linomat IV sample applicator (Camag, Muttenz, Switzerland) and a 100 μl Hamilton syringe. The samples were streaked in the form of narrow bands of lengths 5 and 10 mm from the bottom, 10 mm from margin and 4 mm apart at a constant flow rate of 10 s μl^{-1} by using a nitrogen aspirator. Camag Twin Trough Chamber was saturated for 10 min with the mobile phase containing mixture of acetone: toluene: ammonia in the ratio of 7:3:0.5 (v/v/v). After chamber saturation the plates were developed to a distance of 80 mm and then dried in hot air. Densitometric analysis was carried out using Camag TLC Scanner III (Camag, Muttenz, Switzerland) in the absorbance mode of 312 nm with a slit dimension of 4.0 mm × 0.45 mm and scanning speed of 10 mm/s. Lamotrigine was detected at an R_f of 0.51. The chromatograms were integrated using CATS evaluation software (Version 4.06).

2.5. Method validation

The HPTLC method developed was validated for following parameters.

2.5.1. Sensitivity

The sensitivity of the method was determined in terms of LOD, LOQ, linearity range and correlation coefficient. Lamotrigine was extracted from working stock solution (1 μg/ml) and spotted on TLC plate in the range of 5–300 ng. The LOD was calculated as three times the noise level and LOQ was calculated as ten times the noise level. A graph was obtained by plotting the area under the peak of lamotrigine against the amount of lamotrigine (ng) to determine the linearity range and correlation coefficient.

2.5.2. Selectivity

The selectivity of the assay was determined in relation to interferences from endogenous substances and other commonly used antiepileptic drugs in human serum.

2.5.3. Recovery study

Recovery of lamotrigine was determined by spiking lamotrigine in drug free human serum to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The recovery was calculated by comparing the resultant peak areas with those obtained from pure standards in methanol at the same concentrations.

2.5.4. Precision and accuracy

Different amount of lamotrigine covering the low, medium and higher ranges of the calibration curve were spotted on the TLC plate. These spots were analyzed ($n = 5$) by using above described HPTLC method. Precision was expressed as the %coefficient of variation (%CV) and accuracy was expressed as a percentage (observed concentration × 100/theoretical concentration).

2.5.5. Reproducibility

The intra day reproducibility was evaluated by analyzing the amount of lamotrigine spotted on TLC plate covering low, medium and higher ranges of calibration curve in replicates ($n = 5$). The inter day reproducibility was evaluated by

analyzing the same amount of analyte over period of seven days ($n = 7$).

2.6. Application

2.6.1. Therapeutic drug monitoring

Twenty five epileptic patients participated in the study after giving informed consent. Venous blood samples were withdrawn from epileptic patients, collected in centrifuge tubes and processed within 30 min to separate serum. The serum levels of lamotrigine in the samples were analyzed using the above described HPTLC method.

2.6.2. Pharmacokinetic Study

Six healthy New Zealand rabbits of either sex, weighing between 2.0 and 2.5 kg were used in the study. After overnight fasting a single oral dose (20 mg kg^{-1}) of lamotrigine suspended in normal saline was administered orally. Blood samples (1 ml) were collected into centrifuge tubes through marginal ear vein just before dosing and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12 and 24 h. The blood samples were further processed to obtain serum, which was stored at −20 °C till the time of estimation. Pharmacokinetic parameters for lamotrigine were obtained from time versus concentration profile using the computer program KINETICA (Inna Phase Corporation, 1999).

3. Results and discussion

Now a day, HPTLC has become an important analytical tool for estimation of drugs in biological samples. The HPTLC method offers several advantages over the reported methods [4–19]. It facilitates automatic application and scanning in situ. Several samples can be run simultaneously using a small quantity of mobile phase. This lowers analysis time and cost per analysis. Furthermore, the developed TLC plates can be scanned for several times with same or different parameters. As this method concentrates (×10) the serum samples in sample preparation step and since more than 10 samples are analyzed in one run, this method proves to be very sensitive, relatively fast, inexpensive and suitable for therapeutic drug monitoring and pharmacokinetic studies.

3.1. Sensitivity

Under the experimental conditions used, the lowest amount of drug which could be detected was found to be 6.5 ng/spot and the lowest amount of drug which could be quantified was found to be 10 ng/spot, with coefficient of variation <6%. The calibration curve in serum was found to be linear in the range of 20–300 ng as shown in Fig. 2.

3.2. Selectivity

Fig. 3 shows representative chromatograms of A) drug free human serum and B) Serum sample of patient on lamotrigine (100 mg/day) therapy, spiked with carbamazepine. The sample was further processed as described in procedure and 3 μl was spotted on to TLC plate and quantified using developed method. Estimated concentrations for LTG and CBZ were 3.3 μg/ml and 4 μg/ml, respectively. Table 1 shows the R_f values of commonly used antiepileptic drug and carbamazepine metabolite which were tested for potential interference after spiking in drug-free human serum. The method can also effectively estimate lamotrigine when spiked in drug-free serum of rabbit or rat. Thus at the R_f of lamotrigine, no interfering peaks are observed thereby confirming the selectivity of the method.

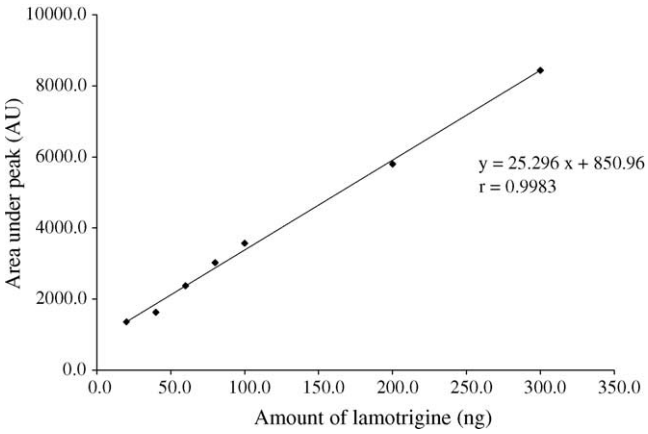

Fig. 2. Calibration curve of lamotrigine in serum.

3.3. Recovery study

Results showed high extraction efficiency due to selective extraction of drug in serum. The recovery of lamotrigine ranged from 87 to 91%, average of 89%.

3.4. Precision and accuracy

The result shown in Table 2 depicts good accuracy and high precision. The accuracy was found in the range of 92.06–97.12% and %CV in range of 0.53–2.59.

Table 1
R_f values of the antiepileptic drugs tested by HPTLC

Compound	R_f
Lamotrigine	0.54
Carbamazepine	0.65
Carbamazepine-10, 11-epoxide	ND
Phenobarbitone	ND
Phenytoin	ND
Primidone	ND
Ethosuximide	ND
Valproic acid	ND
Oxcarbamazepine	ND
Gabapentine	ND

R_f: retention factor; ND: not detected.

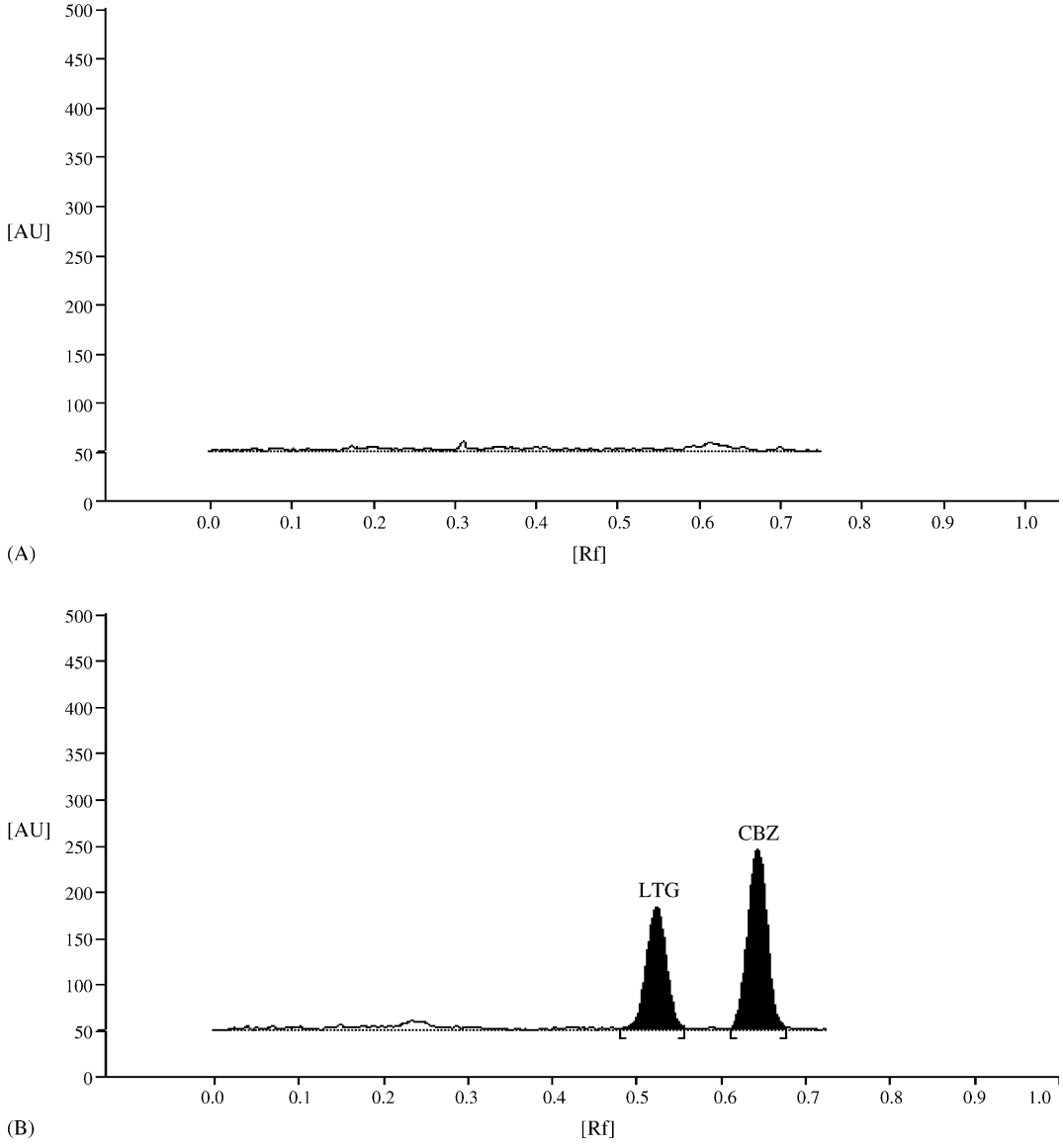

Fig. 3. Representative chromatogram of (A) blank serum (drug-free human serum) (B) serum sample of patient on lamotrigine therapy spiked with carbamazepine (160 ng/spot).

Table 2
Accuracy and precision of the assay

Amount of lamotrigine spotted (ng)	Amount detected (ng) (mean ± S.D., $n = 5$)	CV (%)	Accuracy (%)
30	27.6 ± 0.7	2.51	92.06
60	58.6 ± 0.4	0.78	97.73
100	97.1 ± 0.5	0.53	97.12

3.5. Reproducibility

Table 3 shows the inter- day and intra- day reproducibility studies of lamotrigine at different levels. The percentage CV was found to range from 1.41 to 3.70%, averaging to 2.5%.

Table 3
Precision data of HPTLC assay for lamotrigine

Amount of lamotrigine spotted (ng)	Amount detected (ng) (mean ± S.D.)	CV (%)
Inter-day ($n = 5$)		
30	27.4 ± 0.8	3.13
60	58.2 ± 0.8	1.41
100	97.2 ± 1.1	1.16
Intra-day ($n = 7$)		
30	27.9 ± 1.0	3.70
60	58.2 ± 1.3	2.27
100	97.7 ± 2.1	2.21

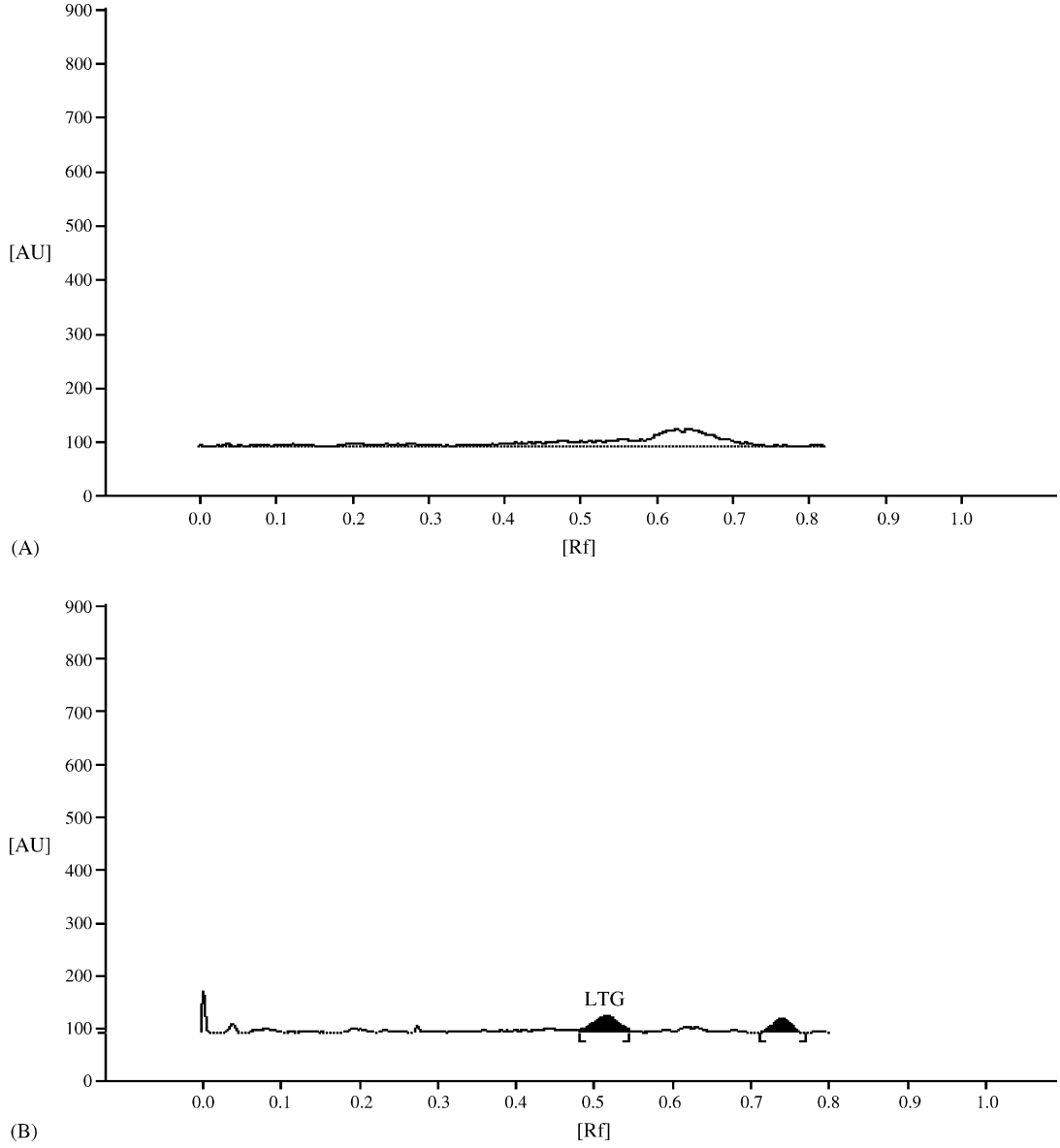

Fig. 4. Chromatogram of (A) drug free rabbit serum and (B) extracted serum sample taken 12 h after oral administration of lamotrigine (20 mg/kg) to rabbit.

3.6. Application

3.6.1. Therapeutic drug monitoring study

Patients included in study comprised of 20 males and five females (age of 20.46 ± 15.38 years and weight 41.04 ± 21.82 kg). All patients had well documented diagnosis of epilepsy and epilepsy syndrome comprised of generalized tonic clonic seizures (9), complex partial seizures (10), simple partial seizures (2) and myoclonic seizures (4). The serum concentration of lamotrigine ranged from 0.5 to 5.1 μg/ml and included six patients on monotherapy and nineteen patients on concomitant therapy of AEDs like phenytoin, carbamazepine and valproate. We found lamotrigine serum level significantly correlated with the respective dosage in milligram per day ($r = 0.54$, $P < 0.001$). This shows the usefulness of present method for monitoring the serum levels in patients receiving therapeutic doses of lamotrigine.

3.6.2. Pharmacokinetic studies

Fig. 4 depicts the chromatogram of (A) drug free rabbit serum and (B) extracted serum sample taken 12 h after oral administration of lamotrigine (20 mg/kg) to rabbit. The mean plasma concentration–time profile after a single lamotrigine oral dose (20 mg/kg) to six healthy New Zealand rabbits is shown in Fig. 5. The absorption of lamotrigine in rabbits is rapid, reaching peak plasma concentration (3.88 ± 0.29) in about 1.0 h. The computed pharmacokinetic parameters are shown in Table 4. The pharmacokinetic trend is very much in agreement with the HPLC method reported earlier by Matar et al. [5] for determination of lamotrigine in plasma.

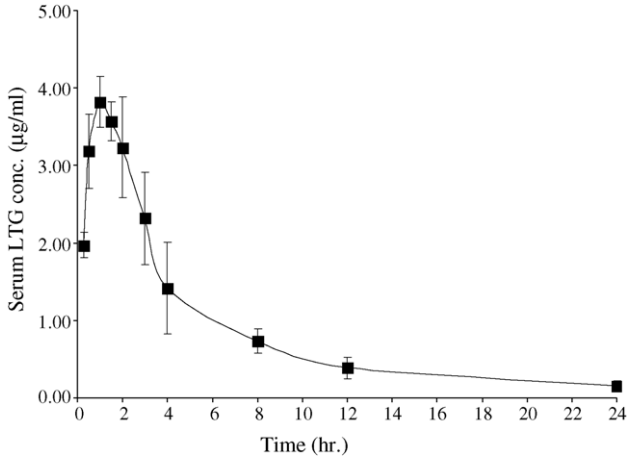

Fig. 5. Mean plasma concentration-time profile of lamotrigine (20 mg/kg) following oral administration to six rabbits.

Table 4
Pharmacokinetic parameters (mean ± S.D.) of lamotrigine after an oral administration of lamotrigine (20 mg kg^{-1}) to six rabbits

Parameter	Mean ± S.D.
C_{max} (µg ml^{-1})	3.88 ± 0.29
T_{max} (h)	1.00 ± 0.2
$T_{1/2}$ (h)	7.02 ± 2.35
AUC_{0-t} (µg h ml^{-1})	20.61 ± 3.78
$AUC_{0-\infty}$ (µg h ml^{-1})	22.54 ± 4.92
Vd/f (l kg^{-1})	8.94 ± 2.43
Cl/f (l h^{-1} kg^{-1})	0.92 ± 0.17

The one way ANOVA test showed no statistical difference ($P > 0.05$) between pharmacokinetic parameters estimated by HPLC and HPTLC methods.

4. Conclusion

A simple, sensitive, specific, accurate, precise and rapid HPTLC method for determination of lamotrigine in serum is reported. The method proving its utility in the analysis of AEDs is suitable for therapeutic drug monitoring studies and pharmacokinetic studies conducted in humans and animals.

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