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Original article

VALIDATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF LAMOTRIGINE IN HUMAN PLASMA AND SALIVA

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Abstract

A simple, sensitive and precise high performance liquid chromatographic assay for lamotrigine (LTG) is described. Good chromatographic separation was achieved using a 5 µm ACE-5 C_{18} reverse-phase column and a mobile phase consisting of methanol:acetonitrile:0.01 M potassium phosphate monobasic (30:15:55, v/v/v) adjusted to pH 6.3 and delivered at a flow rate of 1 mL/min. Quantification was performed by measurement of the UV absorbance at a wavelenght of 304 nm. The method was linear in the range of 0.1-6.2 µg/mL with a coeffient of determination, (r^2 = 0.999). The retention time of LTG was 6.8 and 6.2 min in plasma and saliva, respectively. The limit of quantification was 0.10 µg/mL. Recovery from plasma and saliva ranged from 97 to 98 and 96 to 105 %, respectively. Between-day and within day precision expressed as CV % in plasma and saliva were in the range of 0.26 to 6.8. The method was showed to be appropriate for pharmacokinetic (PK) studies and monitoring LTG in saliva and plasma samples.

Key words: *Lamotrigine, High performance liquid chromatography (HPLC), Plasma, Saliva.*

İnsan Plazmasında ve Tükrüğünde Lamotrijinin Saptanması için Valide Edilmiş Bir Yüksek Performanslı Sıvı Kromatografi (HPLC) Yöntemi

Lamotrijin (LTG) için basit, duyarlı ve kesin bir yüksek basınç sıvı kromatografisi yöntemi tanımlanmıştır. 5 µm ACE-5 C_{18} revers-faz kolon ve pH'sı 6.3'e ayarlanmış, 1 mL/dak. akış debisinde metanol:asetonitril:0.01 M potasyum fosfat monobazik (30:15:55, h/h/h) mobil fazı kullanılarak iyi bir kromatografik ayırım başarılmıştır. Kantitatif saptama 304 nm dalgaboyunda UV absorbansının ölçülmesiyle gerçekleştirilmiştir. Yöntem 0.1-6.2 µg/mL derişim aralığında doğru1saldır (determinasyon katsayısı, r^2 = 0.999). LTG'nin plazma ve tükrükte alıkonma zamanları sırasıyla 6.8 ve 6.2 dakikadır. Kantitatif saptama sınırı 0.10 µg/mL'dir. Plazma ve tükrükten geri elde edilme yüzdeleri sırasıyla %97'den 98'e ve %96'dan 105'e kadar değişmiştir. Plazma ve tükrükte % varyasyon katsayısı olarak ifade edilen günlerarası ve güniçi kesinlik 0.26 ile 6.8 arasında değişmiştir. Yöntemin, farmakokinetik çalışmalar için ve LTG'nin tükrük ve plazma örneklerinde izlenmesi için uygun olduğu gösterilmiştir.

Anahtar kelimeler: *Lamotrijin, Yüksek performanslı sıvı kromatografisi (HPLC), Plazma, Tükrük.*

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INTRODUCTION

Lamotrigine, 3,5-diamino-6-(2,3-dichloropheny)-1,2,4-triazine (LTG) is a broad-spectrum antiepileptic drug (AED), that is chemically unrelated to other anticonvulsants in current use (1). It is indicated for use in the treatment of epilepsy and bipolar disorder (2,3). It exerts its efficacy by blocking voltage-sensitive sodium channels and inhibiting the release of excitatory neurotransmitter, predominantly glutamate (4,5). The efficacy of LTG against some common seizure types, such as partial with or without secondary generalization, primary generalized tonic-clonic seizures, absence seizures, drop attacks and seizures associated with the Lennox-Gastaut Syndrome has been demonstrated in several studies (6-8). Interindividual pharmacokinetic variability is prominent with this drug, and further amplified by the influence of age, pregnancy, disease states and comedication (9,10). This variability plays a major role in the dosage requirements for LTG that makes the drug a good candidate for therapeutic drug monitoring (TDM) (11).

Furthermore, LTG crosses saliva. Several studies have shown a strong and highly significant correlation between salivary and plasma/serum LTG concentrations over a wide range. Therefore, it was suggested that saliva, instead of blood may be used for TDM of LTG in adults and pediatric patients (12-16). This is an attractive alternative, because its collection is noninvasive, painless, simpler and cheaper than blood drawing. Hence, it is necessary to develop a specific and rapid method for the quantification of LTG in biological fluids. Several normal-phase (17,18) and reverse phase (RP) high performance liquid chromatographic (HPLC) methods (19-47) for the quantification of LTG in biological fluids have been described. Among these methods, most of them make use of liquid-liquid extraction for the isolation of LTG from endogenous compounds in the samples prior to HPLC analysis (17-26), except for the ones (27-34) that use solid-phase extraction. A RP-ion-pairing assay after solid-phase extraction (35) and RP chromatographic assay after solvent demixing sample preparation have also been reported (36). An HPLC method based on on-line sample preparation by column-switching procedure to monitor LTG and oxcarbazepine has been published (37). However, the method has the disadvantage of poor column life. In these procedures, extensive sample preparations with tedious extraction and separation steps are time consuming (17,19-27,36), while the solid-phase extraction may be expensive (27-30,38). The use of ion-pairing reagents often involves the need for elevated column temperatures, long analysis times and problems with rapid column deterioration (35). One requires the use of a special system, automated sequential trace enrichment of dialysates, prior to HPLC to prepare plasma samples for the estimation of LTG (39). Procedures based on direct HPLC analysis after sample deproteinization with acetonitrile or methanol have also been reported (40-46). However, some of these are lack of detailed validation data (41), have high limit of quantification (LOQ) values (40,42,44) or time consuming (46). A RP-HPLC method based on direct injection into a column with an internal surface reversed phase has been published (47). The main problem of this method is the short column life, which makes the analysis impossible to be performed after 300 to 400 injection of plasma samples. Moreover, very few methods exist for the determination of LTG simultaneously in various biological fluids, such as blood and brain (23) or blood and urine (17,27,35). No validated methods have been published for the determination of LTG in saliva.

Therefore, in the present paper, a rapid, specific, sensitive and simple HPLC method for the measurement of plasma and saliva levels of LTG was developed and validated, making it suitable, not only for pharmacokinetic (PK) studies, but also for TDM. The method requires a small speciman volume, minimal handling for sample preparation, and short times for chromatographic separation.

EXPERIMENTAL

Chemicals

Pharmaceutical grade LTG was kindly supplied by GlaxoSmithKline, Durham, UK (Figure 1). Acetonitrile and methanol HPLC grade; and potassium phosphate monobasic analytical grade, were purchased from Riedel-de Haën (Seelze, Germany). Sodium hydroxide was purchased from Merck. Human plasma was kindly provided by Gazi University Hospital blood bank and pooled human-saliva was obtained from healthy volunteers (16).

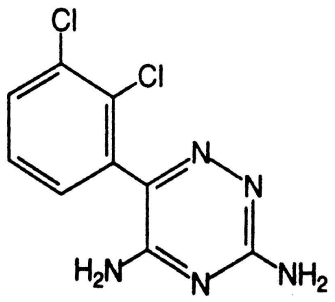

Figure 1. Chemical structure of LTG.

HPLC apparatus and conditions

Chromatography was performed using a chromatographic system consisting of a Hewlett Packard, series 1050 Chromatographic System equipped with a HP 3396 series manual injector, 50 µL loop, a UV variable-wavelength detector and Hewlett Packard 3396 Series II Integrator (Waldbronn, Germany). The column effluent was monitered using ultraviolet detector at 304 nm. Solutions and mobile phases were prepared at the time of use. The mobile phase was methanol:acetonitrile:0.01 M potassium phosphate monobasic (30:15:55, v/v/v), adjusted to pH 6.3 with sodium hydroxide. It was filtered through a 0.22 µm membrane and degassed by sonication prior to use and delivered at a flow rate of 1 mL/min at room temperature. Separations were carried out using ACE-5 C_{18} reverse-phase column; 25 cm x 4.6 mm, 5 µm particle size [Advanced Chromatography Technologies (ACT), Aberdeen, Scotland].

Preparation of stock and standard solutions

A stock solution of LTG was prepared by dissolving the appropriate amount of pure drug substance in bidistilled water to yield a final drug concentration of 104 µg/mL and stored at 4°C until analysis and was stable for at least two months. Further solutions were obtained by serial dilutions of stock solutions with bidistilled water. Calibration standards of LTG in drug free human plasma/saliva were made by spiking with an appropriate volume of the working solutions, giving final concentrations of 0.10, 0.21, 0.62, 1.0, 2.1, 3.1, 4.1, 5.2, 6.2 µg/mL for plasma or 0.10, 0.21, 0.41, 0.62, 1.0, 3.1, 4.1, 5.2, 6.2 µg/mL for saliva. Calibration curves were constructed by plotting peak-height as a function of the drug concentration in the calibrators in the range of 0.10-6.2 µg/mL. In the same manner, quality control samples (QC samples) in plasma and saliva were prepared to measure the precision and the accuracy of the

method. The equation of the calibration line was calculated by least-square linear regression analysis and was used to calculate the drug concentration in the unknowns.

Preparation of plasma and saliva samples

Preparation of samples was based on the deproteinization procedure (40-42). The plasma/saliva samples were stored in a deep freezer at –70°C and allowed to thaw at room temperature before processing. 500 µL acetonitrile was added to 500 µL plasma/saliva. The mixture was vortexed for 5 sec. and allowed to stand 5 min to complete precipitation of the plasma proteins and the oral mucus, mucopolysaccharides and muco-proteins which contribute to saliva's highly mucous, string and sticky consistency. The mixture was centrifuged at 3000 rpm (740 g) for 10 min and 50 µL of supernatant was introduced onto the column for HPLC analysis. This procedure was also described in the previous paper by the authors (16).

Analytical method validation

The method was validated according to the reliability parameter, linearity, sensitivity, precision, accuracy, recovery, specificity and stability. The linearity was tested between 0.10-6.2 µg/mL for plasma and saliva. Calibration data were plotted as a graph of LTG peak height versus LTG concentration.

In order to study the specificity of the present method, blank human plasma and saliva samples were subjected to the same sample processing and were analysed. Stability samples were freshly prepared by adding a known amount of LTG to a drug free matrix and stored in a refrigerator (4°C) and in a deep freezer (-70°C) for five days and six months in three replicates, respectively. The method was validated by the samples prepared at different concentrations spanning the concentration range.

QC samples in plasma and saliva at three different concentrations (0.31, 1.6, 4.6 µg/mL) covering the low, medium, and high ranges of the calibration curve, used to assess between-day precision were freshly prepared and assayed with each calibration curve. Separate three different concentrations (0.62, 4.1, 6.2 µg/mL) were prepared to assess within-day precision. Between and within-day precision tests were performed in six replicates for three consecutive days and five replicates, respectively. Precision was expressed as the coeffient of variation (CV) for the concentrations back-calculated from the regression analysis within a single run (within-day) and between different assays (between-day). Accuracy was expressed as the percentage (%) of (found concentration/spiked concentration x 100). Recoveries of LTG were determined by spiking drug-free human plasma/saliva with known amounts of the LTG (1.0, 2.1, 4.1 µg/mL and 0.41, 3.1, 5.2 µg/mL for plasma and saliva, respectively). The samples were extracted and analyzed in five replicates with the developed procedure. The limit of quantification (LOQ) was defined as the lowest LTG concentration that could be determined with a precision below 15 % and with an accuracy between 85 and 115 %. The limit of detection (LOD), defined as the lowest concentration of the analyte that can be detected above the baseline signal, is estimated as three-times noise level.

Application of the method

The assay was also applied to a single dose PK study in healthy volunteers to demonstrate the reliability of this HPLC method for the study of LTG pharmacokinetics in plasma and saliva. A single dose of 200 mg of LTG was administered orally. The detailed study design can be found in the previous paper by the authors (16).

RESULTS

Chromatography, specificity

Figure 2 and 3 show representive chromatograms of drug-free human plasma/saliva, spiked plasma/saliva samples and samples from a healthy volunteer treated with a 200 mg single oral dose of LTG. Under the described RP-HPLC procedure, LTG was sufficienly resolved from plasma and saliva compounds with a retention time of 6.8 and 6.2 min, respectively. Normal plasma and saliva constituents do not interfere in the assay. The tested blanks were free from endogenous saliva components at the retention time of the drug.

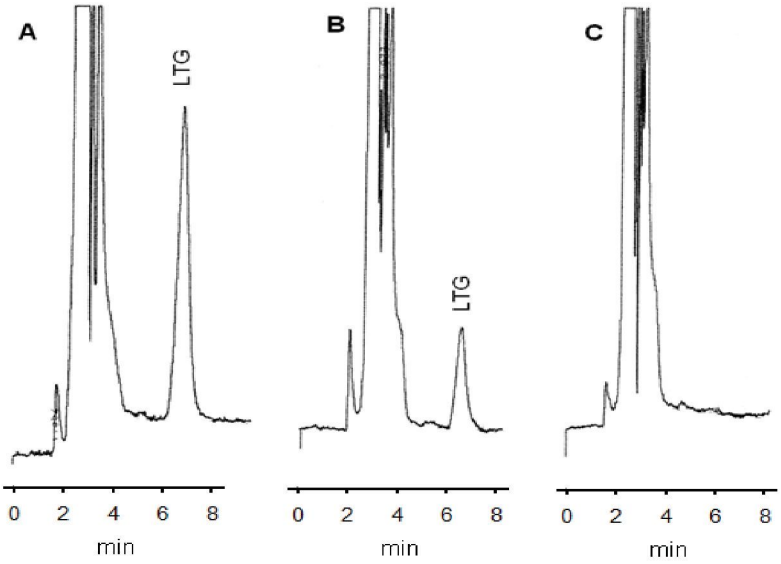

Figure 2. Chromatogram of (A) a sample of plasma spiked with LTG (2.1 μg/mL); (B) a plasma sample from a volunteer treated with a single dose of 200 mg LTG; (C) a drug free human plasma.

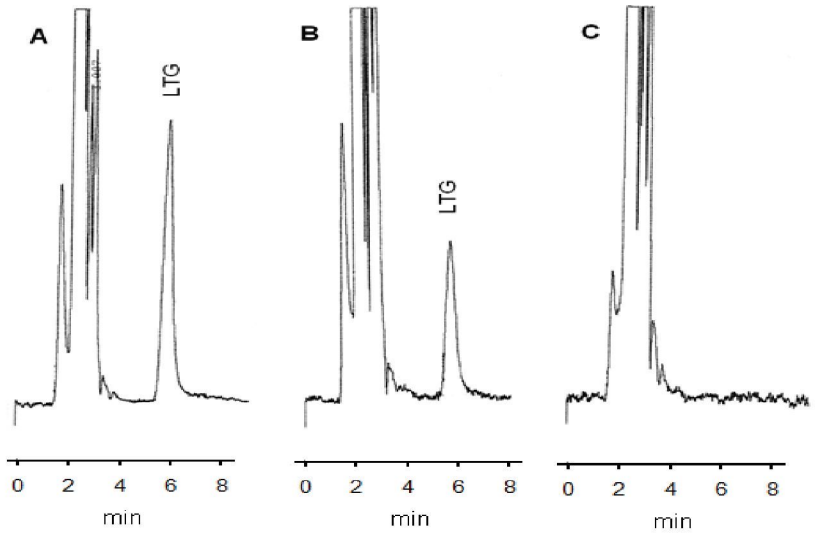

Figure 3. Chromatogram of (A) a sample of saliva spiked with LTG (3.1 µg/mL); (B) a saliva sample from a volunteer treated with a single dose of 200 mg LTG; (C) a drug free human saliva.

Linearity, sensitivity and stability

Under the optimized conditions, linear regression equations of plasma and saliva curves between 0.10-6.2 µg/mL was y (peak height) = 104 x (concentration, µg/mL)+16.9 and y (peak height) = 147 x (concentration, µg/mL)-15.6 with the value of 0.999 for the determination coeffients (r^2 value), respectively. LOD was 0.05 µg/mL. LOQ was 0.10 µg/mL with a coefficient of variance of 14 % and 5.2 % for five replicates in plasma and saliva, respectively. Sample stability was evaluated by calibrators at 4°C and -70°C. No significant loss of LTG (≤10%) was observed after storage of samples at 4°C for 5 days and -70°C for 6 months, which proves LTG stability under routine storage conditions. The method is appropriate for TDM and PK studies of LTG in human plasma and saliva and is competitive with some HPLC procedures reported in literature in terms of sensitivity.

Precision and accuracy

Table 1 and 2 show the within-day and between-day precision of LTG in human plasma and saliva expressed as CV(%). The within-day precision and between-day precision in plasma

Table 1. Within-day and between-day precision of LTG in human plasma.

Spiked concentration (µg/mL)	SD[a] (µg/mL)	CV[b] (%)
Within-day precision(n[c]=5)		
0.621	0.028	4.36
4.14	0.02	0.537
6.21	0.07	1.08
Between-day precion(n[c]=6)		
0.311	0.021	6.76
1.55	0.03	2.27
4.56	0.01	0.258

a: Standard deviation, b: Coefficient of variation(%),
c: Number of analyzed samples

Table 2. Within-day and between-day precision of LTG in human saliva.

Spiked concentration (µg/mL)	SD[a] (µg/mL)	CV[b] (%)
Within-day precision(n[c]=6)		
0.621	0.008	1.33
4.14	0.09	2.32
6.21	0.10	1.58
Between-day precision(n[c]=6)		
0.311	0.075	5.18
1.55	0.01	2.92
4.56	0.09	1.65

a: Standard deviation, b: Coefficient of variation(%),
c: Number of analyzed samples

samples ranged from 0.537 to 4.36 and 0.258 to 6.76 % CV, respectively. The within-day precision and between-day precision in saliva samples ranged from 1.33 to 2.32 and 1.65 to 5.18 % CV, respectively.

Table 3 and 4 show the mean recoveries of LTG ranged from 96.7 to 98.3 % and from 96.0 to 105 % in plasma and saliva, respectively.

Table 3. Recovery of LTG at different concentration levels in plasma(n^a=5).

Spiked concentration (µg/mL)	Found concentration (µg/mL)	CV^b (%)	% Recovery
1.04	1.00	5.02	96.7
2.07	2.03	0.948	97.9
4.14	4.07	1.93	98.3

a: Number of analyzed samples
b: Coefficient of variation(%)

Table 4. Recovery of LTG at different concentration levels in saliva(n^a=5).

Spiked concentration (µg/mL)	Found concentration (µg/mL)	CV^b (%)	% Recovery
0.414	0.436	4.21	105
3.11	2.98	1.16	96.0
5.18	5.25	3.39	101

a: Number of analyzed samples
b: Coefficient of variation(%)

Clinical case

The plasma and saliva concentration-time profiles of a healthy volunteer after the oral administration of a single dose of 200 mg LTG is illustrated in Figure 4.

DISCUSSION

In the present paper, a RP liquid chromatographic assay was developed and validated for LTG quantification in plasma and saliva. The objective of this study was to develop of a rapid, specific and easy RP-HPLC assay with a total run time less than 10 min, while maintaining suitable sensitivity and specifity. The method involves an ordinary RP-HPLC system with very simple chromatographic conditions. Acetonitrile was used to precipitate plasma proteins and salivary mucus, mucopolysaccharides and muco-proteins before column separation in this study. Acetonitrile as a precipitation solvent gave clean chromatograms and good recovery of LTG. The mobile phase at pH 6.3 and flow rate used for the assay achieved optimum resolution of LTG with no interference from endogenous components in plasma/saliva. No internal standard was used.

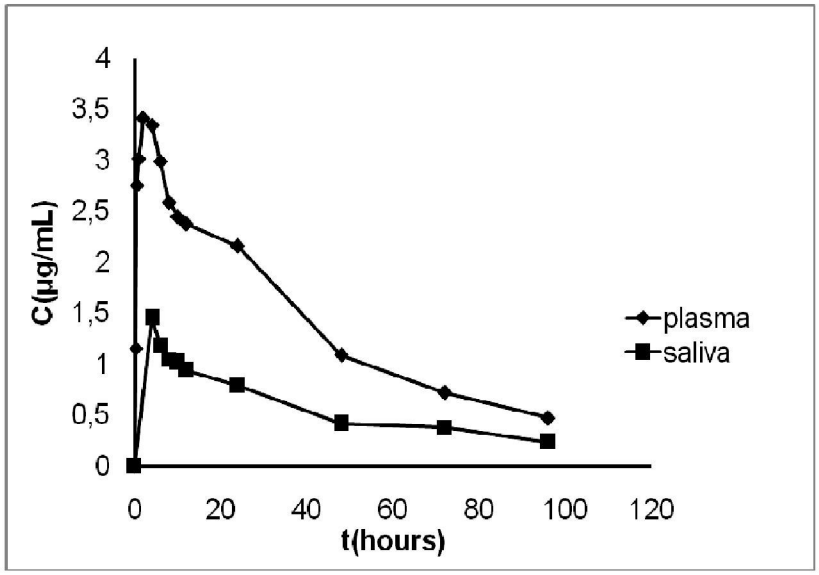

Figure 4. The plasma and saliva concentration-time profiles of LTG after oral single dose of 200 mg LTG tablets in a representative subject.

The present method offers a number of significant advantages. Sample processing is simple and rapid. Solvent evaporation and sample reconstitution are avoided, and the required sensitivity is retained without need for sample concentration steps. The short analysis time allow prolonged column life while maintaing good separation. Under the chromatographic conditions, the precision of the assay is fully satisfactory with a relative standard deviation ≤ 7 % at all tested concentrations, except at the limit of quantification.

Relative short time analysis was required. The UV detection at 304 nm provides excellent specificity, eliminating most of the interference from plasma and saliva and a better limit of detection. Under the experimental conditions described, the LOQ of plasma LTG concentration was much lower than those reported in other methods (19,25,26,34,42,44). The chosen working range of the assay is adequate for the determination of LTG concentrations in patients, as part of an eventual routine TDM protocol, because it covers the suggested tentative therapeutic range of the drug well which have been proposed to be 1-4 µg/mL (48). However, the potential for chromatographic interferences by the co-administered other AEDs in this method should be evaluated before applying the method for TDM of LTG in patients receiving comedication.

The clinical results showed that this simple and rapid method is sufficiently sensitive to follow blood and saliva levels of LTG in PK studies. The application of this method opens doors to monitoring salivary and plasma LTG levels clinically.

CONCLUSION

In conclusion, the HPLC method proved to be sensitive, simple, reproducible, rapid and precise, making it valuable in many applications, particularly in PK studies, TDM in a variety of seizure disorders and bioequivalency studies.

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