



ORIGINAL ARTICLE

Optimisation of a high-efficiency liquid chromatography technique for measuring lamotrigine in human plasma

N. Rivas,^{a,*} A. Zarzuelo,^b and F.G. López^c

^aFarmacocinética, PKPD, Salamanca, Spain

^bUSALA, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain

^cUniversidad de Salamanca, Salamanca, Spain

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Abstract

Objective: The purpose of this study was to optimise the HPLC-UV bio-analytical method currently used by the Salamanca University Clinical Hospital for determining lamotrigine plasma levels.

Material and methods: The developed HPLC-UV analytic technique currently in use was shown to be linear, exact and precise, and suitable for use in routine monitoring of lamotrigine levels. The drawback of this method has always been the time required for analysing samples, so our aim was to improve on that elapsed time.

That improvement involved using a different chromatographic column from the one used up until now. We replaced the column that was normally used (Kromasil-100C18-5 µm-15*0.4 cm with a LiChroCART-RP18e-3 µm-5.5*0.4 cm); in both cases, a liquid-liquid extraction was performed and the same sample extraction protocol was followed.

Results: Both validation methods showed that the two column types are valid for routine lamotrigine monitoring.

Conclusion: The decrease in retention time, in addition to a lower quantification limit and better precision and accuracy parameters obtained with the LiChorCART column, suggest that this unit is ideal for use in clinical practice because it enables a large number of determinations to be performed in less time and the greater precision of LTG measurements.

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*Corresponding author.

E-mail address: nuria.m.rivas@gmail.com (N. Rivas).

PALABRAS CLAVE

Lamotrigina;
Cromatografía líquida
de alta eficacia con
detector ultravioleta;
Estudios de validación

Optimización de una técnica de cromatografía líquida de alta eficacia para la determinación de lamotrigina en plasma humano

Resumen

Objetivo: Optimizar el método bioanalítico HPLC-UV empleado hasta el momento en el Hospital Clínico Universitario de Salamanca, para la determinación de los niveles plasmáticos de la lamotrigina (LTG).

Material y métodos: La técnica analítica de HPLC-UV desarrollada y utilizada hasta el momento demostró ser lineal, exacta y precisa, siendo apta para su empleo en la monitorización rutinaria de la LTG. Sin embargo, presentaba un prolongado tiempo para el análisis de las muestras, por lo que se optó por una mejora en la misma. Dicha mejora consistió en el empleo de una columna cromatográfica alternativa a la usada hasta el momento. Para ello se sustituyó la habitualmente empleada (Kromasil-100C18 – 5 μm – 15 x 0,4 cm por la LiChroCART-RP18e – 3 μm – 5,5 x 0,4 cm) realizando en ambos casos una extracción líquido-líquido y siguiendo el mismo protocolo de extracción de muestra.

Resultados: Ambas validaciones demostraron que los dos tipos de columnas son válidos para la monitorización rutinaria de la LTG.

Conclusión: La disminución en el tiempo de retención, junto con el menor límite de cuantificación y los mejores parámetros de precisión y exactitud obtenidos con la columna LiChroCART, sugieren a ésta como una candidata ideal para la práctica clínica debido al gran número de determinaciones que pueden realizarse en un menor tiempo y la mayor precisión en la cuantificación de la LTG.

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Introduction

Lamotrigine (LTG) (3,5-diamine-(6[2,3-dichlorophenyl]-1,2,4-triazine) is an antiepileptic agent whose chemical structure is not related to other habitually used antiepileptics.

Lamotrigine presents a pharmacological activity similar to other antiepileptic drugs such as phenytoin and carbamazepine.^{1,2} It is an effective medication as a coadjutant for the treatment of simple partial and tonic-clonic seizures, with secondary resistances to other drug treatments.³

Routine monitoring of antiepileptic drugs is a common practice in hospital pharmacy departments due to the high level of help these give to patients, since an optimal control of plasma concentrations is one of the key tools for proper seizure control, helping to widen the knowledge base on the different interactions that are produced when taken with other antiepileptic drugs, especially enzymatic inductors and inhibitors.⁴⁻⁷

From the pharmacokinetic point of view, these are of great usefulness due to the fact that, although the therapeutic range is not well defined,⁸⁻¹⁰ being so wide, a deeper understanding of the drug would allow a better adjustment to its use. Furthermore, this type of research can be applied to population studies.¹¹ Along with an optimisation and reduction in time required for LTG plasma level readings, these are some reasons for which the current study has focused on the effect of column length and particle size in time required for analysis, the limit for quantification, and the accuracy and precision of the two techniques using high efficiency liquid chromatography with ultraviolet detection

(HPLC-UV) using the concentration limits of 0.5-20.0 $\mu\text{g}/\text{ml}$ (range of normal plasma concentrations in humans).

Material and methods**Material**

The BW430C78 LTG (3,5-diamine-(6[2,3-dichlorophenyl]-1,2,4-triazine) and its internal standard BW725C78 ([3,5-diamine-6-[2-metoxipheryl]-1,2,4-triazine (SI) were supplied by Wellcome Research Laboratories (Cardiff, United Kingdom).

The reagents used (dihydrogen potassium phosphate, sodium hydroxide, and triethylamine) were analytical grade, and the methanol was HPLC grade, both of which were supplied by Merck (MerckKGaA, Darmstadt, Germany). The purified water was obtained in the laboratory with a Milli-Q purification system. The human plasma was obtained from the blood bank at the Salamanca University Clinical Hospital (HUSAL).

Standard preparation

Firstly, a parent solution of LTG in methanol at a concentration of 500 $\mu\text{g}/\text{ml}$ is prepared. This solution is then diluted in plasma in order to prepare the working solution, at a concentration of 20.0 $\mu\text{g}/\text{ml}$. From this working solution, standard concentrations are prepared at 15.0; 10.0; 8.0; 6.0; 4.0; 2.0; 1.0; 0.5; 0.25; 0.15 and 0.1 $\mu\text{g}/\text{ml}$ of LTG.

In the case of SI, a parent solution was prepared at 500 $\mu\text{g}/\text{ml}$ in methanol from which a working solution was prepared at a concentration of 20.0 $\mu\text{g}/\text{ml}$.

Chromatography

We used the HPLC analysis technique. The chromatographic system used was the HP1050 system with automatic injector and UV detector (Waters 486), and Clarity® software.

The chromatographic separation was performed with two column types: Kromasil 100 C18 5 μm 15 \times 0.4 and LiChroCART RP18e 5.5 \times 0.4 μm (TeknoKroma).

The chromatographic conditions were: the mobile phase consisted of a mix of 0.1M KH₂PO₄, triethylamine, and methanol (62%:3%:35% v/v) with a pH=6.2. The mobile phase was prepared daily, including degasifying and filtering the solution with a 0.45 μm filter membrane.

The chromatography process took place at ambient temperature with a workflow of 1 ml/min and with UV radiation at a wavelength of 206 nm.

Extraction procedure for LTG/SI

The extraction process was liquid-liquid. Five hundred μl of the sample were added to a solution of 50 μl of SI+50 μL of 2M NaOH; the mixture was then agitated for 30 s and 2ml of ethyl acetate was added as an organic solvent. Following 30 seconds of vortex agitation, the organic mixture was

centrifuged for 10 min. at 3,500 rpm. As a final step, the solution was evaporated in an N₂ atmosphere and reconstituted with 100 μl of mobile phase being injected into the chromatograph at a volume of 50 μl .¹²

Validation of bioanalytical techniques

The selectivity, accuracy, and precision have been studied according to the premises laid forth by the FDA.¹³

In order to study the selectivity of the technique, we studied 6 target plasmas from distinct sources. The linearity study was done by preparing 5 calibration curves with concentration ranges between 0.5 and 20.0 $\mu\text{g/ml}$ (0.5; 1.0; 2.0; 4.0; 6.0; 8.0; 10.0; 15.0 and 20.0 $\mu\text{g/ml}$). With the data obtained we calculated the value of r , the coefficient of variation for the factor relative to the response, and the deviation from the mean of the response factor for each concentration.

From the study data on linearity, we determined the accuracy of the technique as a percentage of recuperation from the quantity of LTG added to each sample.

Regarding precision, we studied the repeatability and reproducibility of both methods. In order to evaluate repeatability, or intraday precision, the same person

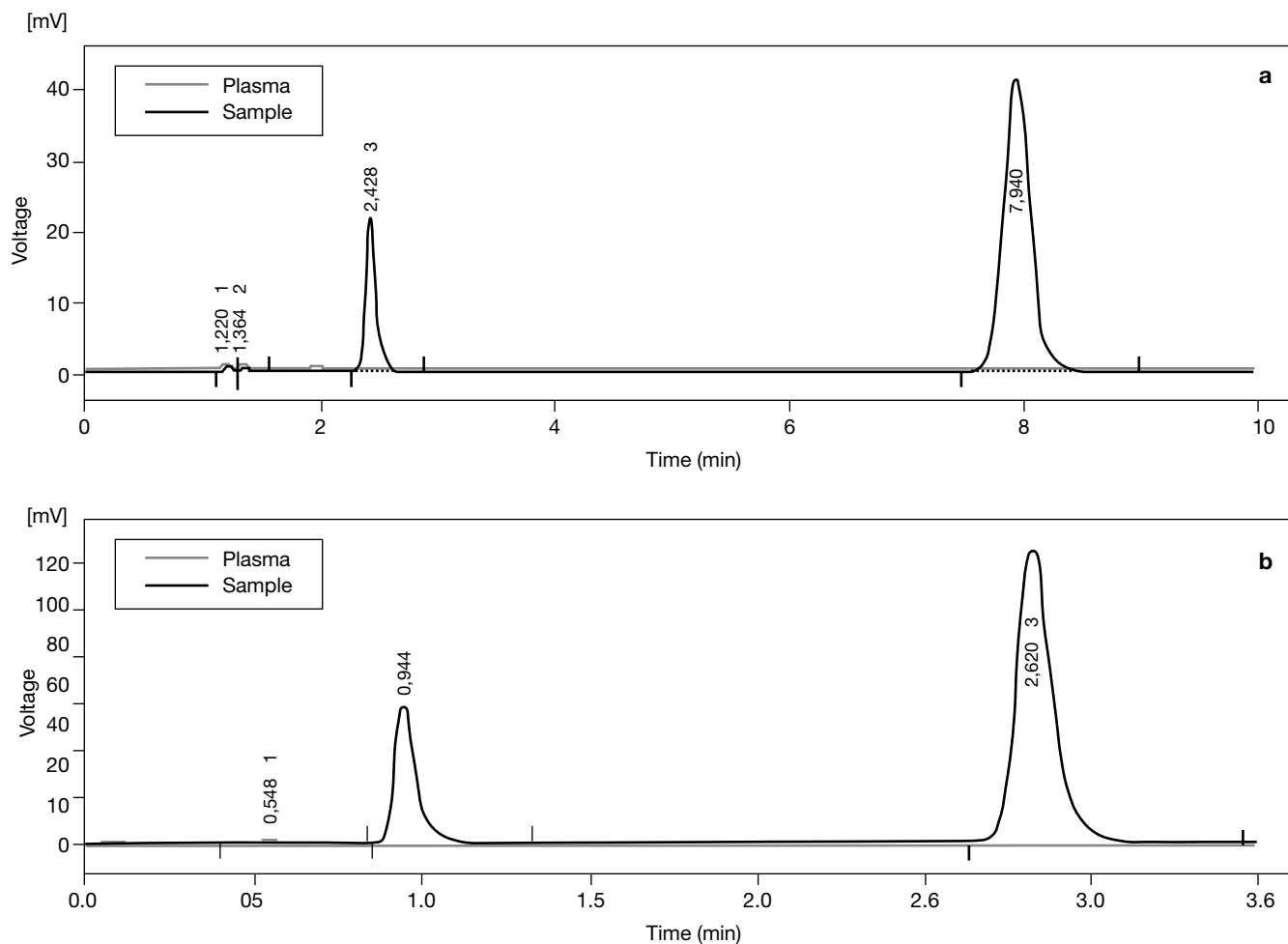


Figure Chromatogram for a blank and a sample identifying both LTG and SI. a) Kromasil. b) LiChroCART.

Table 1 Results from the validation study with the Kromasil column for concentrations of the study range (0.5-20 µg/ml)

	Mean±SD	CV %
r	0.998	
Rf (response factor)	0.55±0.03	6.07
Accuracy, % recuperation	101.83±7.39	7.25
Repeatability (area LTG/area SI)		
0.5 µg/ml	0.27±0.01	1.99
6.0 µg/ml	3.35±0.21	6.40
20.0 µg/ml	11.31±0.15	1.29
Reproducibility (area LTG/area SI)		
0.5 µg/ml	0.29±0.01	4.55
6.0 µg/ml	3.55±0.27	7.68
20.0 µg/ml	11.49±0.27	2.31
Quantification limit	0.25 µg/ml	

LTG indicates lamotrigine; SD, standard deviation.

Table 2 Results of the validation study performed with the LiChroCART column for range of concentrations (0.5-20 µg/ml)

	Mean±SD	CV %
r	0.999	
Rf (response factor)	0.55±0.03	6.07
Accuracy, % recuperation	100.13±3.42	3.41
Repeatability (area LTG/area SI)		
0.5 µg/ml	0.33±0.01	3.90
6.0 µg/ml	3.60±0.05	1.31
20.0 µg/ml	11.82±0.36	3.05
Reproducibility (area LTG/area SI)		
0.5 µg/ml	0.32±0.02	6.64
6.0 µg/ml	3.49±0.14	4.12
20.0 µg/ml	11.56±0.37	3.20
Quantification limit	0.1 µg/ml	

LTG indicates lamotrigine; SD, standard deviation.

prepared and analyzed 5 high concentration replicates, 5 medium, and 5 low, on the same day using the same reagents. For the reproducibility study, or interday precision, the same analyst prepared the same 5 replicates for the high, medium, and low LTG concentrations, but in this case on different days and with different reagents. In both cases, the CV in the area (LTG area/SI area) was calculated in the 5 analyses.

Furthermore, we determined the limits of quantification using the Lang and Bolton method.¹⁴ The concentration

margins between 0.5 and 0.1 µg/ml (0.5; 0.25; 0.15 and 0.1 µg/ml) were prepared in triplicate.

Results

In order to study the selectivity of the technique, we analyzed 6 plasma targets from distinct origins observing that, given the Figure representation, the LTG and SI retention times do not display ghost peaks in plasma components. In the same fashion, different studies were performed with plasma from patients with distinct polytherapy with different antiepileptic drugs observing the same effect, not interference in any of the cases. In the same way, we observed an excellent separation between the LTG and SI peaks, and between SI and the other components of the plasma samples. The retention times for the LTG were 7.94 and 2.62, and for SI: 2.43 and 0.95 min for the Kromasil and LiChroCART, respectively.

Quantification limits results were 0.25 and 0.1 µg/ml for kromasil and LichroCART columns respectively.

The results of both techniques provided results that can be observed in Tables 1 and 2.

Discussion

The optimisation study of the analysis technique for HPLC-UV for determining plasma concentrations of LTG has demonstrated that it is the correct method for pharmacokinetic monitoring, within the limits of the therapeutic ranges (3-14 µg/ml), which is the typically found range in clinical practice.

The selection of both columns must be made according to the similarities in physical/chemical characteristics, the principal difference between the two being smaller particle size and length. Performing a comparison of the two columns is based on the attempt to improve the bioanalytical technique, thus allowing an increase, as has been mentioned before, in both the number and precision of determinations.

The ability to count on an exact bioanalytical method that is precise, repeatable, reproducible, and quick allows the establishment of a routine monitoring system in clinical practice, as well as the possibility of performing pharmacokinetic studies in order to determine and study both the behaviour of the drug itself and its possible interactions with other medications.

From the results obtained in this study and taking into account the complexity of the treatment of the samples, once this method has been validated, a maximum variability of the SI area of 15% has been assumed as a criteria for proper treatment of the data, with respect to the mean value of the data obtained in validation. All samples that surpass this value must be repeated since it is possible that some error may have been made during the preparation of the samples. These errors are reflected in the SI area.

Thus this study does not allow us to conclude that the LiChroCART column will be the most adequate candidate for the routine monitoring of LTG in clinical practice, due to the reduction in analysis time and the adequacy of the technique.

Conflict of interest

The authors affirm that they have no conflicts of interest.

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