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Short communication

LC method for the therapeutic drug monitoring of lamotrigine: Evaluation of the assay performance and validation of its application in the routine area

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ABSTRACT

An accurate and precise high-performance liquid chromatographic method using diode array detection for the determination of lamotrigine in human plasma has been developed and validated for use in pharmacokinetic studies. A validation strategy based on the accuracy profiles was used to select the most appropriate regression model and to determine the limits of quantitation as well as the concentration range. On the other hand, the present paper also shows this validation approach as a suitable tool to guaranty the quality of the results obtained by the use of the analytical validated methodology for plasma lamotrigine determination in a routine setting and to ensure the risk of obtaining the future measurements outside the previously fixed acceptance limits.

1. Introduction

Lamotrigine, 3,5-diamino-6-(2,3 diclorophenyl)-1,2,4-triazine, is a broad-spectrum antiepileptic drug, unrelated chemically to other anticonvulsants in current use [1–3]. It is common practice that pharmacotherapy with anticonvulsants, including carbamazepine, ethosuximide, phenytoin, valproic acid, phenobarbital and lamotrigine, is accompanied by therapeutic drug monitoring (TDM) [4–7]. The goal of TDM is to optimize patients clinical outcome by managing their medication regimen with the assistance of measured drug concentrations. To this end TDM seeks to optimize the desirable properties of antiepileptics drugs (i.e. their anticonvulsant effect) increasing clinical efficacy while minimising their undesirable effects or adverse effects [8,9]. The concept rests on the assumption that drug concentration correlates better with clinical effects than the dose. Lamotrigine is well absorbed after oral administration and approximately 55% is bound to plasma proteins. It undergoes biotransformation by hepatic N-glucuronidation and elimination via the renal route, displaying first-order linear kinetics with a slight autoinduction period. Wide inter-individual differences exist in serum/plasma lamotrigine levels achieved at any given dose, largely because of their pharmacokinetic interactions with concurrently prescribed anticonvulsants. A large variation has been reported in the elimination half-life of lamotrigine: about 25 h in patients on monotherapy, decreased to an average of 14 h with coadministration of enzyme inducing drugs, including carbamazepine, phenytoin and phenobarbital and increased to an average of 60 h during concomitant valproic acid therapy. Thus, when lamotrigine is used as an add-on therapy, dosage depends on the type of concomitantly administered drugs (hepatic enzyme inducers or inhibitors) [10].

Various tentative target ranges of lamotrigine in plasma have been proposed and are being envisaged [7,10–16]. Further studies are required to assess the relationship between plasma/serum concentration and clinical effect and to determine the changes of lamotrigine kinetics in relation to the type of comedication [17].

Lamotrigine is typically monitored by high-performance liquid chromatography [4,7,10–16,18–24], but although a capillary zone electrophoresis assay [25], a radioimmunoassay [26], and an immunofluorimetric assay [27] for lamotrigine have been described, no convenient commercial immunoassay has been developed.

In the present work, a simple, rapid and sensitive HPLC method for determination of lamotrigine in plasma/serum has been developed and validated in our laboratory according to a novel validation strategy based on the use of accuracy profiles [28–33]. The notion of including the use of accuracy profiles as a decision tool to select the most appropriate response function, to estimate the limit of quantitation or to evaluate the concentration range, is in accordance with the objective of an analytical method that can be summa-

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rized as its ability to quantify as accurately as possible each of the unknown quantities in human samples that the laboratory will have to determine. Moreover, to the best of our knowledge, this is the first account of a lamotrigine HPLC assay for which a specific evaluation of the risk of the procedure related to the future use of the method during its daily use for the therapeutic drug monitoring of lamotrigine in the clinical setting is reported.

2. Experimental

2.1. Chemical and reagents

Lamotrigine (98% minimum purity) reference standard was from Sigma–Aldrich Chemical S.A. (Madrid, Spain). 3,5-Diamino-(6-methoxy phenyl)-1,2,4-triazine standard (98% minimum purity) used as internal standard was kindly supplied by GlaxoSmithKline (Tres Cantos, Spain).

99.9% purity HPLC grade solvents methanol and acetonitrile were obtained from Merkc (Barcelona, Spain). Potassium dihydrogen phosphate and triethylamine, analytical grade, were purchased from Panreac (Barcelona, Spain).

Stock standard solutions of lamotrigine and the internal standard were prepared by dissolving appropriate amounts of compounds in a known volume of methanol and stored at −30 °C.

Working standard solutions for the preparation of calibration, validation and quality controls standards were prepared by appropriate dilutions of the stock standard solutions in blank human plasma obtained from the University hospital blood bank.

ClinCheck lamotrigine plasma lyophilised controls purchased from Recipe (Munich, Germany) were reconstituted with HPLC water, aliquoted and frozen at −20 °C until analysis. All frozen samples were slowly defrosted and vortex-mixed prior to analysis.

2.2. Sample preparation by solid-phase extraction procedure (SPE)

The procedure of extraction of the analyte from plasma/serum was a solid-phase extraction pretreatment based on the one developed by Bugamelli et al. [34] with some important modifications.

A volume of 200 μL of a unknown serum/plasma sample, calibration, validation or quality control sample was placed in a 1.5 mL centrifuge tube and 200 μL of a plasma solution of 5-diamino-(6-methoxy phenyl)-1,2,4-triazine (8 mg/L) as internal standard, 200 μL of a solution of NaOH 1N and 200 μL of water were added and homogenized by vortex-mixed.

Waters OASIS HLB cartridges (30 mg, 1 mL) were used for lamotrigine sample pretreatment. The solid-phase extraction procedure was carried out in a Tecknokroma Vac Elut apparatus according to the following steps: (a) conditioning with 1 mL methanol; (b) equilibration with 1 mL water; (c) loading of 800 μL of the mixtured plasma/serum sample; (d) washing with 1 mL water; and (e) elution with 1 mL acetonitrile/methanol (90/10, v/v) mixture. The eluate was then dried under vacuum at 50 °C in a vortex evaporator. The residues were reconstituted in 100 μL of mobile phase and after vortex-mixing for 20 s, these samples were centrifuged at 10,000 × *g* for 3 min at room temperature. The clear supernatant was transferred to microvials and the autosampler programmed to inject 50 μL into the HPLC system.

2.3. High-performance liquid chromatography

The chromatographic system used in the study was a Hewlett-Packard 1100 series with a Model G1311A quaternary pump, Model G1313A autosampler, Model G1315A diode array detector, Model G1316A column compartment and Model G1322A degasser. Data were acquired and processed with HP Chem Station chromatography manager software from Agilent Technologies (Santa Clara, CA, USA).

Separation of compounds was achieved using a Teknokroma Tracer Excel 120 ODS-B (3 μm, 4.6 mm i.d. × 100 mm) analytical column protected by a Teknokroma ODS C_{18} precolumn.

The chromatographic separation was carried out using a mobile phase consisting of a mixture of 0.01 M potassium dihydrogen phosphate with 0.6% TEA and acetonitrile in a proportion 80:20 pumped at a constant flow rate of 1.2 mL/min.

The column was maintained at 40 °C and the eluent was monitored at a wavelength of 308 nm.

2.4. Preparation of standards

In order to validate the analytical method, we prepared two kinds of samples for calibration and validation in an independent way.

The calibration standards consist of plasma/serum samples, containing known concentrations of the analyte of interest. The samples are only used for calibration and they are prepared according to the protocol that will be applied routinely. Two calibration standards series of five concentrations levels replicated on three different days were performed. Spiked plasma samples used as calibration standards (0.5, 1.5, 9, 12.5 and 20 mg/L) were prepared by addition of different volumes of the corresponding standard solution of lamotrigine in blank human plasma obtained from the University hospital blood bank. The most appropriate response function was selected according to the accuracy profile approach in order to guaranty a reliable quantification.

The validation standards are also matrix samples containing known concentrations of the analyte of interest. They were independently prepared in the matrix simulating as much as possible the future routine analysis of lamotrigine samples. In the validation phase, the validation standards represent the future samples that the analytical procedure will have to quantify. The concentration levels selected for the validation standards were the same as the levels of the calibration standards. Six replicates were prepared at each concentration level for three days.

Quality control samples were prepared in human plasma at the concentrations of 1.5, 9 and 18 mg/L as described above for the calibration and validation standards. ClinChek purchased plasma controls from Recipe with mean lamotrigine concentrations of 4.8 and 13.9 also were employed for internal quality assurance. Calibration and validation standards and quality control samples were analyzed in the same way as patient plasma samples.

2.5. Application to clinical pharmacokinetic studies

To demonstrate that this HPLC method is applicable to pharmacokinetic studies, it has been used to determine lamotrigine concentrations in plasma/serum from patients undergoing therapy with lamotrigine under the specific protocol used and developed in the Neurology Department in the University Hospital of Navarra (Spain). The results showed that this method is sensitive, specific and accurate enough to follow plasma/serum levels of lamotrigine in patients for TDM purposes.

3. Results and discussion

3.1. HPLC assay development

Lamotrigine has been measured in biological fluids by gas chromatography [35] and by immunoassays [36]. Several HPLC methods have been reported for the determination of lamotrigine concentrations in human plasma/serum. These include normal

and reversed-phase procedures after liquid–liquid or solid-phase extraction under alkaline conditions [37–39].

During the phase of optimization of the assay, different stationary and mobile phase compositions were evaluated for their performance in separating lamotrigine, its internal standard and endogenous compounds. The mixture already described of a buffer 0.01 M potassium dihydrogen phosphate containing 0.6% triethylamine and acetonitrile as organic modifier 80:20, v/v; produced optimal separation with retention times of 2.1 and 6.0 min for the internal standard and lamotrigine, respectively. Under these conditions both compounds exhibit good retention and very sharp and symmetrical peak shapes. Typical chromatograms obtained with extracted drug-free human plasma, samples of plasma spiked with lamotrigine (0.5 and 12.5 mg/L) and internal standard (8 mg/L) and one patient's plasma treated with lamotrigine (300 mg/day) and spiked with internal standard (8 mg/L) are illustrated in Fig. 1.

We chose 3,5-diamino-(6-methoxy phenyl)-1,2,4-triazine as internal standard for lamotrigine because of its similar chemical structures and properties. So, it is suitable to test the extraction efficacy and the chromatography. The 3,5-diamino-(6-methoxy phenyl)-1,2,4-triazine peak appears in an area of the chromatogram which is almost free from interfering peaks and near to lamotrigine.

The absorption spectra of lamotrigine exhibited two absorbance maxima at approximately 205 and 308 nm and its internal standard presented maximum absorption at about 205, 237, and 308 nm. The wavelength of 308 nm (the second of the absorbance maxima for lamotrigine), was selected for the simultaneous determination of lamotrigine and the internal standard. This wavelength permits us to visualize a clean chromatogram without any interference from endogenous substances and other antiepileptic drugs; so, this detection was selected as the best compromise in terms of sensitivity and selectivity to accomplish lamotrigine quantification.

Lamotrigine is a lipophilic weak base with a pk_a value of 5.7. Different extraction procedures for lamotrigine and the internal standard were tested in our laboratory (data not shown). Because of its lipophilicity and weak basicity, lamotrigine can be efficiently extracted after alkalinizing the sample into various organic solvents, such us diethyl ether, dichloromethane, ethyl acetate and chloroform. Several solvents, solutions and volumes were assayed. Some solvents showed interferences of endogenous compounds with some of the compounds of interest, others; had an insufficient extraction yield to allow proper pharmacokinetic application or at least, worse than the one obtained for the solid-phase extraction procedure described before. Also the protein precipitation by addition of organic solvents or by the formation of insoluble salts was investigated as a suitable pretreatment procedure of plasma samples, however our results showed cleaner extracts with the solid-phase extraction procedure. This, combined with the disadvantage of diluting the sample and lowering the limit of quantification of the protein precipitation procedure let us to review a solid-phase extraction procedure as an alternative sample pretreatment for the analysis of lamotrigine in plasma/serum. The efficiency of several polymeric sorbents was evaluated. C18 cartridges showed satisfactory extraction yields but a low repeatability of the measurements. Contrary to what was described by Saracino et al. [40] good results were found using Oasis HLB (hydrophilic–lipophilic balanced copolymer) cartridges. The procedure was done according to the sample preparation treatment described in Section 2.2. Sample pH adjustment is necessary to ensure that weak bases such as our analytes exist in the un-ionised form thereby guaranteeing extraction with a neutral sorbent, so alkalinizing the sample with 200 μL of a solution of NaOH 1N is an essential prerequisite for achieving a successful recovery. Due to the lipophilicity of the analytes a washing step with 1 mL water was applied to the OASIS HLB cartridges and 1 mL of elution mixture acetonitrile/methanol 90/10, v/v; was finally selected for simultaneous extraction with the best recovery for both compounds. The extraction yield data obtained with the Oasis HLB cartridges are reported in Table 1. Very high and reproducible values of absolute recovery were obtained. The mean extraction yield of the analyte and the internal standard was 98.27 ± 5.94 and 95.24 ± 3.96%, respectively.

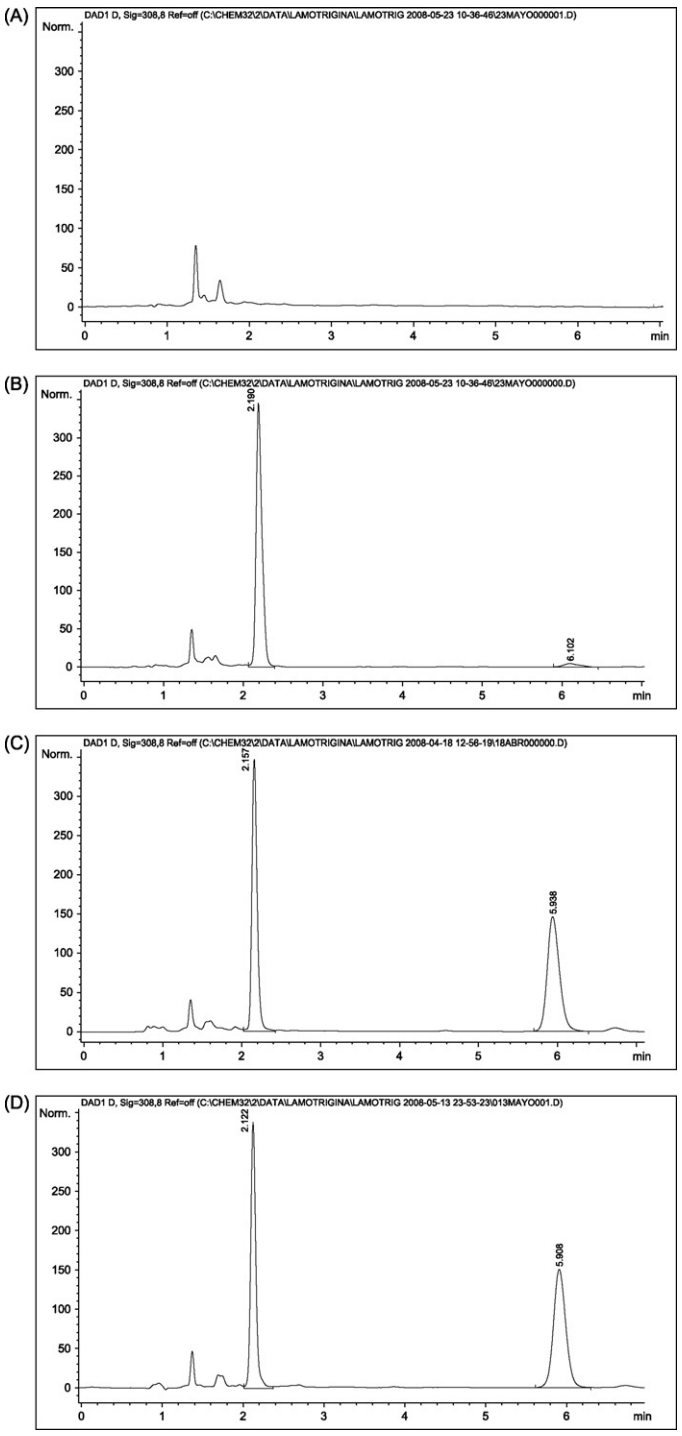

Fig. 1. Chromatographic separation of a blank plasma extract (A) without lamotrigine, from a blank plasma extract (B) at the LLOQ (0.5 μg/mL) and (C) after the addition of a concentration of 12.5 μg/mL of lamotrigine and 8 μg/mL of its internal standard and (D) from a patient plasma extract in treatment with 300 mg/day of lamotrigine. The experimental conditions are described in Section 2.3.

Table 1
Absolute recoveries for lamotrigine extraction with OASIS HLB cartridges.

Concentration (mg/L)	Recovery (%)
0.5000	105.2
20.00	93.96
1.500	94.5
9.000	104.3
12.50	93.39

3.2. Assay validation

In the present study, the recent approach using accuracy profiles is applied [41]. It is based on β-expectation tolerance intervals for the total error measurement that includes trueness (bias) and intermediate precision (standard deviation). The first advantage of this strategy is the possibility to guaranty that the results of the measurement that will be obtained during the future use of the validated method will be included within the acceptance limits previously fixed. So, this strategy helps us to select the most appropriate method to be used in routine for therapeutic dug monitoring. In our work, β was 95%, indeed, it guarantees that at least, the 95% of future results will be included in the ±15% settled according to the FDA or Washington conference regulatory requirements [42–44]. So, this strategy helps us to determine the acceptability of a method to be used in routine for therapeutic drug monitoring.

According to this strategy, the analytical methodology was validated in order to ensure the reliability of the developed method in terms of selectivity, response function, absolute recovery, linearity, limits of quantification and detection, trueness, precision and accuracy. The validation data were processed by e-noval® software Version 2.0 (Arlenda, Liège, Belgium).

3.2.1. Selectivity

A useful analytical method should permit resolution and detection of the analytes of interest and the internal standard from other interfering metabolites and co-eluting endogenous compounds or co-administered therapeutic drugs. So, standard solutions of several antiepileptic agents which could be co-administered in polytherapy during the lamotrigine treatment of patients, such us: carbamazepine, oxcarbamazepine, phenobarbital, primidone, phenytoin, ethosuximide, valproic acid and levetiracetam were injected confirming the good method selectivity because no interference was found.

Possible interferences from endogenous constituents of human plasma were evaluated by analyzing a minimum of six blank plasma samples obtained from different donors. No interfering peaks were observed and no significant peaks were found at the retention times of the analyte or the internal standard. Fig. 1 shows, as we have mentioned in Section 3.1, a representative chromatogram from a human plasma extract without lamotrigine, from a plasma extract spiked with internal standard and with lamotrigine at the quantification limit (0.5 mg/L) and at a concentration of 12.5 mg/L and from a human plasma extract of a patient in treatment with 300 mg/day of lamotrigine. The approximate retention times for lamotrigine and the internal standard were 6.0, and 2.1, respectively.

3.2.2. Response function

This step is decisive since the reliability of the future validation results will depend on the selected regression model. The response function was evaluated from three calibration curves corresponding to three days, constructed from two calibration standards for each concentration level, five, ranging from 0.5 to 20 mg/L. Several regression models were fitted in order to analyze the relationship between concentration and analytical response. Independent validation standards, six, at similar concentration levels were also processed. The concentration of the validation standards were back-calculated for each response function in order to determine, by concentration level, the mean relative bias and the upper and the lower β-expectation tolerance limits by considering the estimation of the standard deviation for intermediate precision. From these data, different accuracy profiles were plotted to select the most suitable regression model (Fig. 2). As we have mentioned, the acceptance limits were settled to ±15%, since we are with a bioanalytical method and considering a risk of 5%. Linear regression simple or weighted models or linear regression after logarithm or square root transformation models were rejected because the lower β-expectation tolerance limit was out of the acceptance at the lowest concentration level. Other regression models such as weighted $1/x^2$ quadratic regression or weighted $1/x^2$ linear regression were also tested and as we can see, the β-expectation tolerance intervals were comprised within the acceptance limits over all concentration range. So, both of them could be selected because of the objective of the method, the ability to quantify lamotrigine in the 0.5–20 mg/L range remained fulfilled. Consequently, the $1/x^2$ linear regression model was chosen as the simplest model that met expectations and was then used to evaluate the different validation criteria.

3.2.3. Absolute recovery

Absolute recoveries (%) were determined according to the ratio of the areas of extracted samples treated according to the described procedure to those found after the direct injection of corresponding phosphate standard solutions at the same concentrations. The overall mean recoveries of lamotrigine and its internal standard (8 mg/L) were (mean ± SD) 98.27 ± 5.94 and 95.24 ± 3.96%, respectively. As shown in Table 1, recoveries for lamotrigine were not only high, but also similar for every concentration studied all over the range.

3.2.4. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [29,45]. As can be seen in Table 2, trueness is expressed in terms of absolute or relative bias and recoveries (%) and was assessed by means of six validation standards at five concentration levels ranging from 0.5 to 20 mg/L during three days. According to regulatory requirements [41], trueness was good since the bias did not exceed the value of ±15%, irrespective of the concentration level.

Table 2
Validation results referred to lamotrigine by considering weighted $1/x^2$ linear regression model.

Trueness (n = 6, p = 3)	Absolute bias (mg/L)	Relative bias (%)	Recovery (%)
0.5000	0.002128	0.4257	100.4
20.00	−0.02152	−0.1076	99.89
1.500	0.02488	1.659	101.7
9.000	−0.08743	−0.9715	99.03
12.50	−0.01426	−0.1141	99.89

Precision (n = 6, p = 3)	Repeatability (RSD%)	Intermediate precision (RSD%)
0.5000	3.084	3.543
20.00	4.262	4.678
1.500	4.090	4.150
9.000	3.167	3.167
12.50	4.923	4.923

Accuracy (n = 6, p = 3)	Beta-expectation tolerance limit (mg/L)	Relative beta-expectation tolerance limit (%)
0.5000	[0.4606, 0.5436]	[−7.877, 8.728]
20.00	[17.85, 22.11]	[−10.75, 10.54]
1.500	[1.389, 1.661]	[−7.407, 10.72]
9.000	[8.294, 9.532]	[−7.849, 5.906]
12.50	[11.15, 13.82]	[−10.80, 10.58]

n = replicates; p = days.

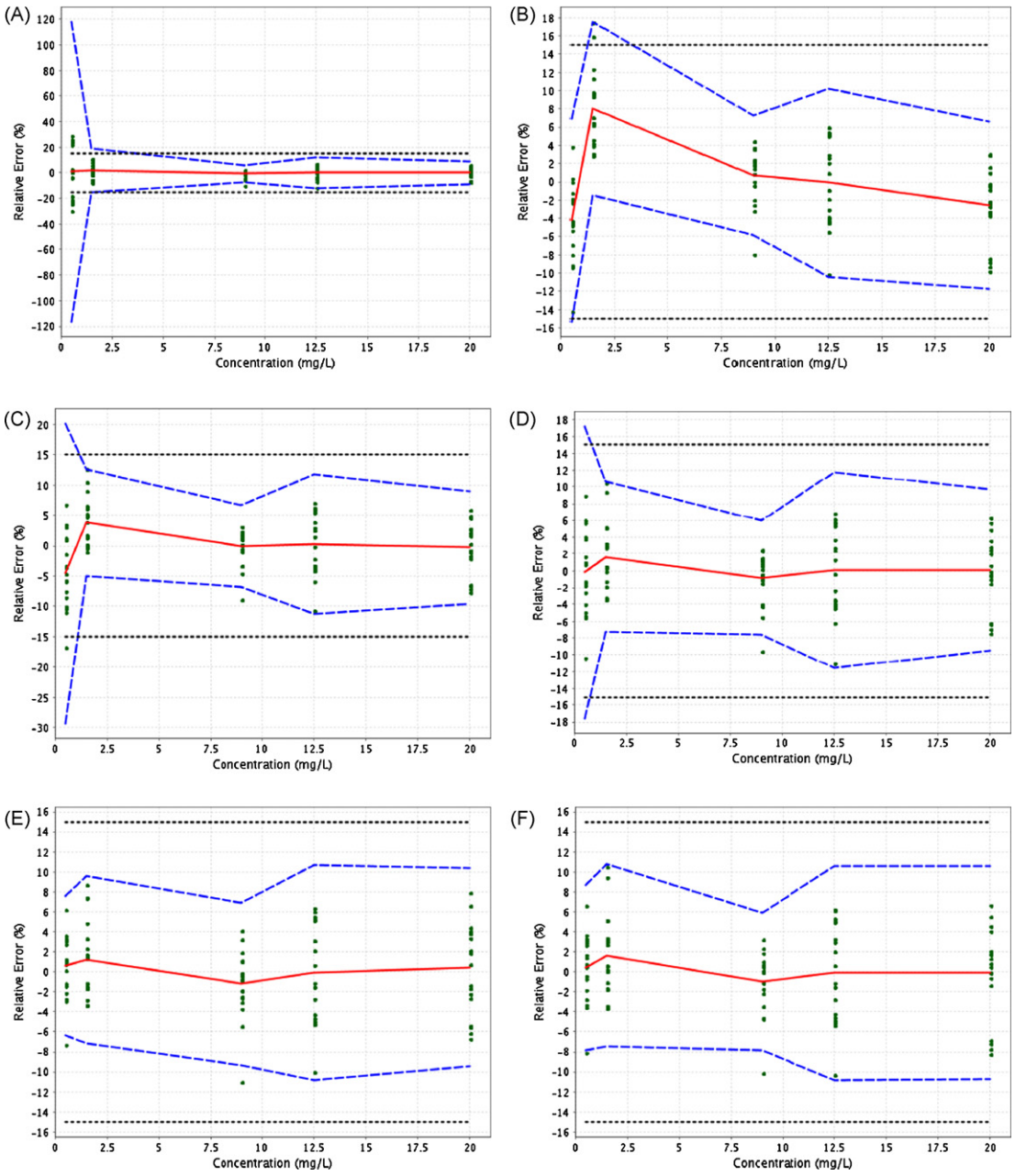

Fig. 2. Accuracy profiles for lamotrigine using (A) a simple linear regression model, (B) a linear regression after logarithm transformation model, (C) a linear regression after square root transformation model, (D) a weighted $1/x$ linear regression model, (E) a weighted $1/x^2$ quadratic regression model or (F) a weighted $1/x^2$ linear regression model. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits and the dotted curves represent the acceptance limits (15%). The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

3.2.5. Precision

The precision of the method is evaluated by calculating the relative standard deviation for repeatability (RSD%) and intermediate precision (RSD%) at each concentration level of the validation standards. The RSD values presented in Table 2 were low for both the repeatability and the intermediate precision, demonstrating the good precision of the developed method.

3.2.6. Accuracy and the lower limit of quantification and detection

Accuracy refers to the closeness of agreement between the test result and the accepted reference value or the conventionally true value. Takes into count the total error, i.e. systematic and random errors, related to the test result [29,45,46]. It is represented from the accuracy profile illustrated in Fig. 2F. As can be seen from the results in Table 2, the different limits of tolerance of bias (the upper and lower β-expectation tolerance limits) did not exceed the acceptance limits settled at ±15% for each concentration level. Consequently, the proposed method was accurate over the concentration range investigated.

Usually, only the lower limit of quantitation (LLOQ) is defined as the smallest quantity in the sample that can be assayed under experimental conditions with a well defined accuracy [28–33]. The concept of total error also introduces the upper limit of quantification (ULOQ) given by the intersection between the accuracy profile and the upper acceptance limit. The intersection with the lower limit defines the LLOQ. In our developed method, according to these results, as the accuracy profile was included inside the acceptance

limits (Fig. 2C), the lowest concentration investigated (0.5 mg/L) was considered as the LLOQ and the highest concentration investigated (20 mg/L) was the ULOQ.

The limit of detection (LOD) is the smallest quantity that can be detected, but not accurately quantified in the sample [28–33]. The LOD (0.1515 mg/L) was estimated using the mean intercept of the calibration model and residual variance of the regression.

3.2.7. Linearity

The linearity of an analytical method is its ability within a given range to obtain results directly proportional to the concentrations of the analyte in the sample [28–33]. For all three series, a regression line was fitted on the calculated concentrations versus the introduced concentrations by applying the linear regression model for which the determination coefficient (r^2), the slope and the intercept were 0.9953, 0.9980 and −0.001921, respectively. The absolute β-expectation tolerance limits were within the absolute acceptance limits demonstrating the linearity of the present method.

3.3. Application of the analytical method to biological samples in clinical pharmacokinetic studies

We developed our method to determine lamotrigine concentrations in plasma of patients receiving this drug orally under the protocol used in the Neurology Department in the University Hospital of Navarra (Spain). This assay was useful to quantify plasma/serum levels of lamotrigine in patients undergoing lamotrigine therapy. None of the determined samples posed any matrix interference and no problem for the analyte quantitation was found. The chromatogram obtained by injecting a plasma sample from a patient under lamotrigine therapy is shown in Fig. 1D. We have confirmed the applicability of our method which provides fast quantitative results with specificity, accuracy, and precision. Internal quality control has been assessed over 2.5 year time period. A total of 96 sets of samples with 96 quality control samples were analyzed monitoring a total of 339 lamotrigine samples of 183 patients. All controls were essentially found to provide lamotrigine concentrations within the target range defined as ±15% settled according to the FDA or Washington conference regulatory requirements [42–44] (Table 3). RSD values calculated for each quality control were found to be ≤10% (Table 3). An accurate and precise quantification of drugs and a fully validated analytical method to quantify the drugs is critical in therapeutic drug monitoring. Detailed pharmacokinetic data will be reported in a separate article.

3.4. Profile of risk

The risk profile expresses the expected probability to have measurements falling outside the acceptance limits during routine use and is computed according to Mee [47].

The risk was investigated at each concentration level of the validation standards by taking into account the most appropriate regression model previously determined by use of the accuracy profile as decision tool. The maximum risk tolerated was set at 5%. Fig. 3 shows the risk profile.

Table 3
Imprecision data and statistical evaluation of internal quality control data.

Quality control level (mg/L)	n^a	Mean calculated level (mg/L)	RSD (%)
1.500	22	1.54	9.8
9.00	22	9.37	6.3
18.00	8	18.94	6.7
4.80	22	4.48	6.9
13.90	22	13.54	6.1

[a] Number of repeats or number of data sets.

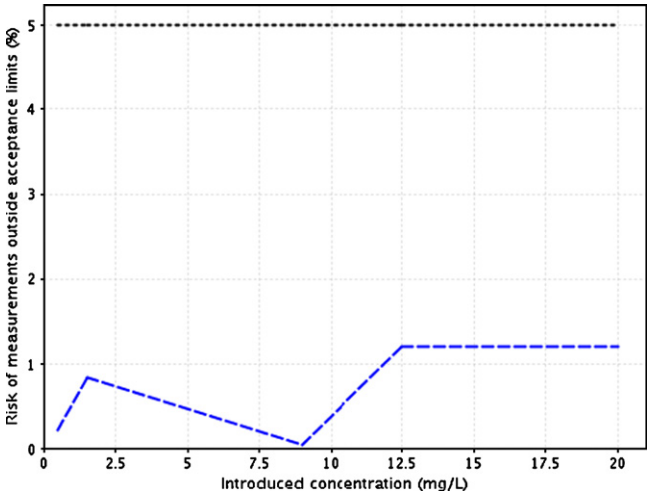

Fig. 3. Risk profile obtained by considering weighted ($1/x^2$) linear regression. The dotted line represents the maximum level chosen set at 5%.

4. Conclusions

We report a sensitive assay for the rapid and accurate determination of lamotrigine. The assay was successfully validated using the approach based on the accuracy profile, strategy proposed by the SFSTP commission which has been applied to demonstrate the ability of our developed method to quantify lamotrigine.

The validated method has a sufficiently rapid turnaround time and its results are good enough to enable the laboratory to routinely provide useful and accurate pharmacokinetic data in time to adjust patient regimens. Moreover, the profiles of risk were also investigated in order to evaluate the probability that a future measurement obtained during routine use of the method will fall outside the defined acceptance limits. This was a very interesting tool to assist the method development and the quality assurance because it allows us to asses the reliability of our analytical method according to its intended use. Apart from therapeutic drug monitoring, the assay should facilitate pharmacokinetic research in clinical laboratory settings. Clinical pharmacokinetics of lamotrigine will be studied in detail in further investigations.

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