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Validation of a liquid chromatographic-tandem mass spectrometric method for the determination of loperamide in human plasma

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Abstract

A sensitive and selective method based on liquid chromatography-tandem mass spectrometry (LC–MS/MS) has been developed for the quantitative determination of loperamide in human plasma. Automated solid-phase extraction (SPE) on disposable extraction cartridges (DEC) is used to isolate the compounds from the biological matrix and to prepare a cleaner sample before injection and analysis in the LC–MS/MS system. After conditioning, the plasma sample is loaded on the DEC filled with endcapped ethyl silica ($C2^{EC}$) and washed twice with water. The analytes are therefore eluted by dispensing methanol. The eluate is then collected and added with ammonium acetate solution in order to inject an aliquot of this final extract in the LC–MS/MS system. On-line LC–MS/MS system using atmospheric pressure chemical ionization (APCI) has been developed for the determination of loperamide. The separation is obtained on a octadecylsilica based stationary phase using a mobile phase consisting in a mixture of methanol and 5 mM ammonium acetate solution (25:75, v/v). Clonazepam is used as internal standard (IS). The MS/MS ion transitions monitored are *m/z* 477 → 266 and 316 → 270 for loperamide and clonazepam, respectively. The most appropriate regression model of the response function as well as the limit of quantitation were first selected during the pre-validation step. These latter criteria were then assessed during the formal validation step. The limit of quantitation (LOQ) was around 50 pg/ml for loperamide. The method was also validated with respect to recovery, precision, trueness, accuracy and linearity.

Keywords: Loperamide; Pharmacokinetics; Validation

1. Introduction

Loperamide (4-(p-chlorophenyl)-4-hydroxy-*N*,*N*,-dimethyl-α,α-diphenyl-1-piperidine butyramide hydrochloride, is an opiate agonist widely used as an effective drug for the control and symptomatic relief of acute non-specific diarrhea [1]. More recently, it has also been reported that loperamide could have some interest as an antihyperalgesic agent reducing pain without any central nervous system side effects [2].

Loperamide is orally administered and is moderately absorbed (about 40%) from the gastrointestinal tract to undergo first-pass metabolism in the liver and excretion in the faeces via the bile as inactive conjugates (sulfo- and glucuronocombination). Only a little intact drug reaches the systemic circulation. Loperamide is highly bound to plasma proteins (around 95%). Due to the low dosage of loperamide administered to humans and the metabolic pathway leading to glucuronides metabolites, the plasma concentrations of loperamide is situated in the ng/ml range. This very low concentration level represents a challenging task for analysts.

The analysis of loperamide in bulk drugs and pharmaceutical products is well described in the litterature [3–7] and

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it is mentioned either in the European [8] and the United States Pharmacopoeias [9]. The determination of loperamide in biological fluids for pharmacokinetics studies has also been reported. Radioimmunoassays (RIAs) were used for the quantitation of loperamide in serum and urine [1,10,11] but liquid chromatographic methods involving electrochemical [12], UV [13] or MS detection [14] seem to be preferred due to their higher selectivity. Since the introduction of atmospheric pressure ionization interfaces, the combination of liquid chromatography and mass spectrometry allows to dispose very sensitive and selective methods. This technique is therefore particularly useful for bioanalytical determinations which are generally characterised by very small drug concentrations and numerous samples to analyze. However, even if LC and MS are separative techniques, the sample handling step remains preferable prior a chromatographic analysis in order to remove proteins and to increase the selectivity and sensitivity of the method.

The extraction of loperamide from plasma samples has generally been carried out by liquid–liquid extraction [14] using organic solvents after alkalinisation of the sample or by protein precipitation [13]. An interesting alternative to this tedious and time-consuming sample preparation approach consists in the isolation of drugs by solid phase extraction (SPE). The SPE procedure can be easily automated by using column-switching systems or by using sample processors such as the ASPEC system (Gilson) allowing therefore the treatment of a great number of samples to be analyzed. These automated sample processors can be coupled at-line [15–17] or off-line [18–20] to liquid chromatographic determinations.

This study describes a new validated method combining automated SPE and liquid chromatography coupled to tandem mass spectrometry to determine loperamide in human plasma. The SPE procedure has been optimized in order to obtain sufficiently high recoveries for loperamide, regarding particularly the selection of the extraction sorbent. The LC and MS/MS conditions were also investigated in order to achieve very low concentrations for this substance. The method has been fully validated according to the new strategy proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedure [21]. The validation strategy consists in two steps. The first step, the so called prevalidation step, is focussed on the selection of the most appropriate regression model using the accuracy profile as decision tool [21–25]. The second step, representing the validation itself, consists of testing the method selectivity towards endogenous components and the assessment of method precision, trueness and accuracy [21,26] at different concentration levels over the range investigated as well as the confirmation of the limit of quantitation (LOQ) and the method linearity [21–23]. The relative standard deviation values for repeatability and intermediate precision were between 3.6 and 8.6%. Moreover, the method was found to be accurate over the whole calibration range. Indeed, the 95% one-sided confidence limits of the mean bias did not exceed the acceptance limits of 85 and 115% [27,28]. The LOQ was found to be 50 pg/ml for loperamide. Finally the method reported was successfully used to perform the quantitative determination of loperamide in real human plasma samples and was found to be applicable for the quantification of the compound in pharmacokinetics studies, which requires high sensitivity and selectivity.

2. Experimental

2.1. Chemicals

Loperamide hydrochloride was obtained from Welding GMBH & Co. (Hamburg, Germany) and the internal standard (clonazepam-5-2(-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one) was supplied by Sigma (Saint-Louis, MO, USA). Ammonium acetate and glacial acetic acid were of analytical grade from Merck (Darmstadt, Germany). Methanol and water were of HPLC grade from Merck. Nitrogen was produced by an on-site nitrogen generator from Air Liquide (Milmort, Belgium).

Isolute DECs (1 ml capacity) filled with 50 mg ethylsilica encapped ($C2^{EC}$) were obtained from IST (International Sorbent Technology, Mid-Glamorgan, UK). Other Isolute DECS filled with 50 mg of other sorbents such as ethyl (C2), octyl (C8), endcapped octyl ($C8^{EC}$), octadecyl (C18) and endcapped octadecyl ($C18^{EC}$) were also tested. Bond Elut Cyano 50 mg DECs (CN) filled with 50 mg of cyanopropyl sorbent (Varian Inc., Palo Alto, CA, USA) were also used in the present study.

The analytical column Zorbax SB-C18 Stable Bond (150 mm × 4.6 mm i.d.) was prepacked with octadecylsilica (particle size 3.5 μm) from Agilent Technologies (Palo Alto, CA, USA). The analytical column was preceded by a LiChroCart guard column (4 mm × 4 mm i.d.) prepacked with Purospher RP-18e column (5 μm) from Merck.

2.2. Apparatus

The automated sample preparation with extraction cartridges (ASPEC) system from Gilson (Villiers-le-Bel, France) consisted of an automatic sampling injector module equipped with four needles, four model 401 dilutor pipettors and a set of racks and accessories for handling DECs, plasma samples and solvents.

The LC system consisted in a Model 1100 Series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted column compartment and a thermostatted autosampler, all from Agilent Technologies.

Mass spectrometric detection was carried out using an Applied Biosystems API 3000 Triple Quadrupole instrument (Thornhill, Toronto, Canada) equipped with an APCI interface. A PC Dell Optiplex GX1 (Round Rock, TX, USA) equipped with a Analyst 1.1 version software from Applied Biosystems was used to control the LC–MS/MS system and

to collect and treat the data. The e-Noval® software (Arlenda, Belgium) was used to determine the accuracy profiles as well as all the validation results.

2.3. Chromatographic technique

All chromatographic experiments were carried out in the isocratic mode. The Zorbax SB-C18 Stable Bond analytical column (150 mm × 4.6 mm i.d.) and the precolumn were thermostatted at 35 °C. The mobile phase consisted of a mixture of methanol and 5 mM ammonium acetate adjusted to pH 3.0 (25:75, v/v). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 1.0 ml/min and the volume injected was 50 μl. The thermostatted autosampler was set to 15 °C.

2.4. Mass spectrometric detection

Mass spectrometric detection was carried out using an Applied Biosystems API 3000 apparatus equipped with an APCI interface operating in the positive ion mode. The heated nebuliser temperature was 450 °C, the auxiliary gas (N_2) flow-rate was 2 l/min, the curtain gas flow-rate was 1.2 l/min at 60 psi and the corona discharge was 3 μA. The mass spectrometer was set to generate and select the pseudomolecular ion [MH^+] at *m/z* 477 for loperamide and 316 for clonazepam (IS) via the first quadrupole mass filter (Q1). The MS-MS fragmentation was then achieved by introducing the pseudomolecular ions into the collision cell (Q2) with a collision energy of 29 eV (collision gas: N_2). Signals were monitored from the third quadrupole (Q3) at *m/z* 266 and 270 for loperamide and IS, respectively.

2.5. Standard solutions

2.5.1. Solutions used for method development

A stock solution of loperamide was prepared by dissolving the appropriate amount in methanol in order to obtain a final concentration of 1.0 μg/ml. This solution was then diluted with methanol in order to achieve a final concentration of 200 ng/ml.

2.5.2. Solutions used for method validation and routine analysis

Six solutions of loperamide were prepared by diluting the stock solution with the mobile phase to reach concentrations ranging from 1.0 to 50.0 ng/ml. These solutions were then used to spike plasma samples either for calibration curves ranging from 50 to 2500 pg/ml ($m = 6$) or for quality control during the pharmacokinetic study. A stock solution of clonazepam (IS) was prepared in methanol. This solution was then diluted with the mobile phase to obtain a final concentration of 5.0 ng/ml.

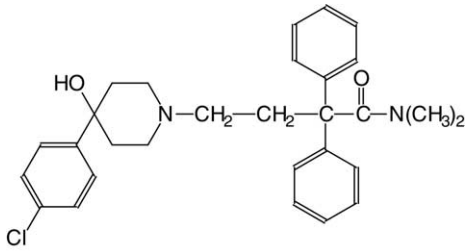
During the prevalidation and validation phases, three calibration curves ($k = 3$) were performed, every one by using new diluted solutions. Each calibration standards were injected in duplicate ($n = 2$). The independent validation standards were prepared at final concentrations of 50, 100, 500, and 2500 pg/ml ($n = 6$). The same calibration scheme was used in routine analyses.

2.6. Sample preparation

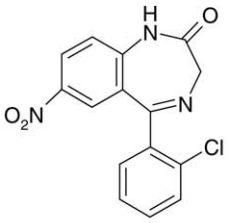
After thawing, plasma samples were first centrifuged at 3000 × *g* for 10 min and a 1.0 ml volume was transferred manually to a sample vial on the appropriate rack of the ASPEC system. A 1.0 ml volume of internal standard solution (5 ng/ml) was then automatically added and mixed. The conditioning of the sorbent was achieved by passing first 1.0 ml of methanol and then 1.0 ml of water through the DEC. The mixed sample (1.8 ml) was then aspirated by the autosampler needle from the corresponding vial and applied onto the DEC. The washing step was then performed by dispensing twice 1.0 ml of water. A 0.75 ml volume of methanol was then dispensed on the DEC and the eluate was collected in the tube positioned under the DEC. 0.25 ml of a 5×10^{-3} M ammonium acetate solution was then added directly to the collected eluate and the final extract was then successively aspirated and dispensed twice in the collection tube. All these operations were performed automatically by the ASPEC system in the batch mode and the final extract was transferred manually to the LC autosampler rack for analysis.

2.7. Pharmacokinetic study

The developed LC–MS/MS procedure was used to investigate the plasma profiles of loperamide after a single oral dose of an immediate release formulation of this compound. A clinical study on healthy caucasian volunteers of both



Loperamide (pKa = 8.7)



Clonazepam (pKa1 = 1.5 – pKa2 = 10.5)

Fig. 1. Structures of loperamide and clonazepam.

sexes, aged from 18 to 50 years, non smokers or smoking less than 10 cigarettes per day was conducted. The study was approved by a local ethics committee. The subjects received a single dose of 2 mg of loperamide (Imodium 2 mg capsules). Fifteen blood samples were withdrawn at different times until 48 h after the administration of the medication (Fig. 1).

3. Results and discussion

3.1. Optimization of MS conditions

The LC–MS/MS method for the determination of loperamide was first investigated. For the optimization of MS conditions, each compound was directly introduced in the MS detector using APCI ionisation and parameters such as corona discharge, orifice voltage, ring voltage, flow of nebulizer and auxiliary gas (N_2) and temperature of auxiliary gas (N_2) were investigated in order to obtain the protonated pseudomolecular ions of loperamide and clonazepam (IS).

The pseudomolecular ions [MH^+] observed on the full scan mass spectra of loperamide was *m*/*z* 477. Moreover, the collision energy in Q2 produced six significant fragment ions with *m*/*z* ranging from 115 to 266 (Fig. 2). The MS/MS transition 477/266 was selected since the ion scan product with *m*/*z* 266 presented a higher abundance and stability.

3.2. LC optimisation

Even if the MS detection coupled to the LC separation can be considered as a very selective method which allows quantitation with a high level of precision even of some coeluting peaks, it is generally agreed that it is better to achieve a complete chromatographic separation of the compounds. The complete separation of loperamide and clonazepam was obtained on an octadecyl silica stationary phase using a mobile phase consisting of a pH 3.0 buffer–methanol (35:65, v/v) mixture. This separation was obtained by modifying the pH of the mobile phase since the loperamide and clonazepam present basic and neutral characteristics, respectively.

3.3. Selection of SPE sorbent

Different kinds of disposable extraction cartridges (DECs) containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of loperamide were determined (Table 1). The recoveries were calculated by comparing the peak areas obtained from freshly prepared samples extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–MS/MS system, using the same autosampler. As can be seen in Table 1, very low recoveries for clonazepam were observed with the cyano phase. This can be explained by analytes losses during the loading and washing steps due to the more polar character of the cyano phase in comparison with the octadecyl phase.

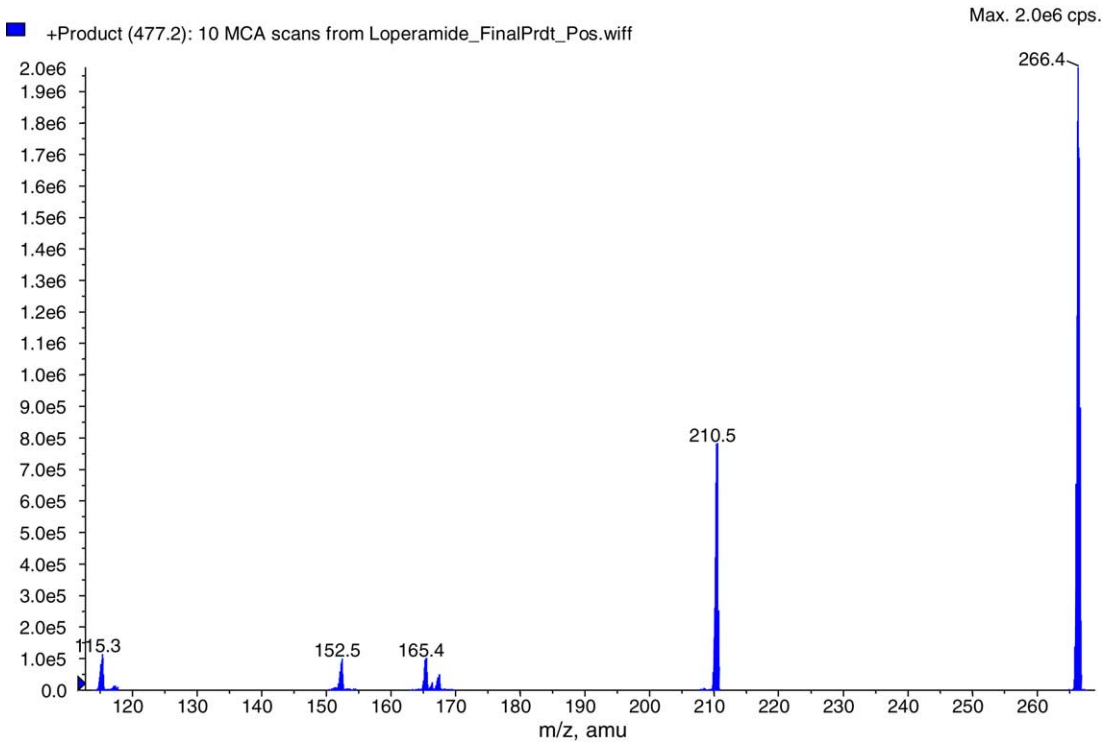


Fig. 2. Product ion mass spectrum of loperamide illustrating the ion *m*/*z* 266.

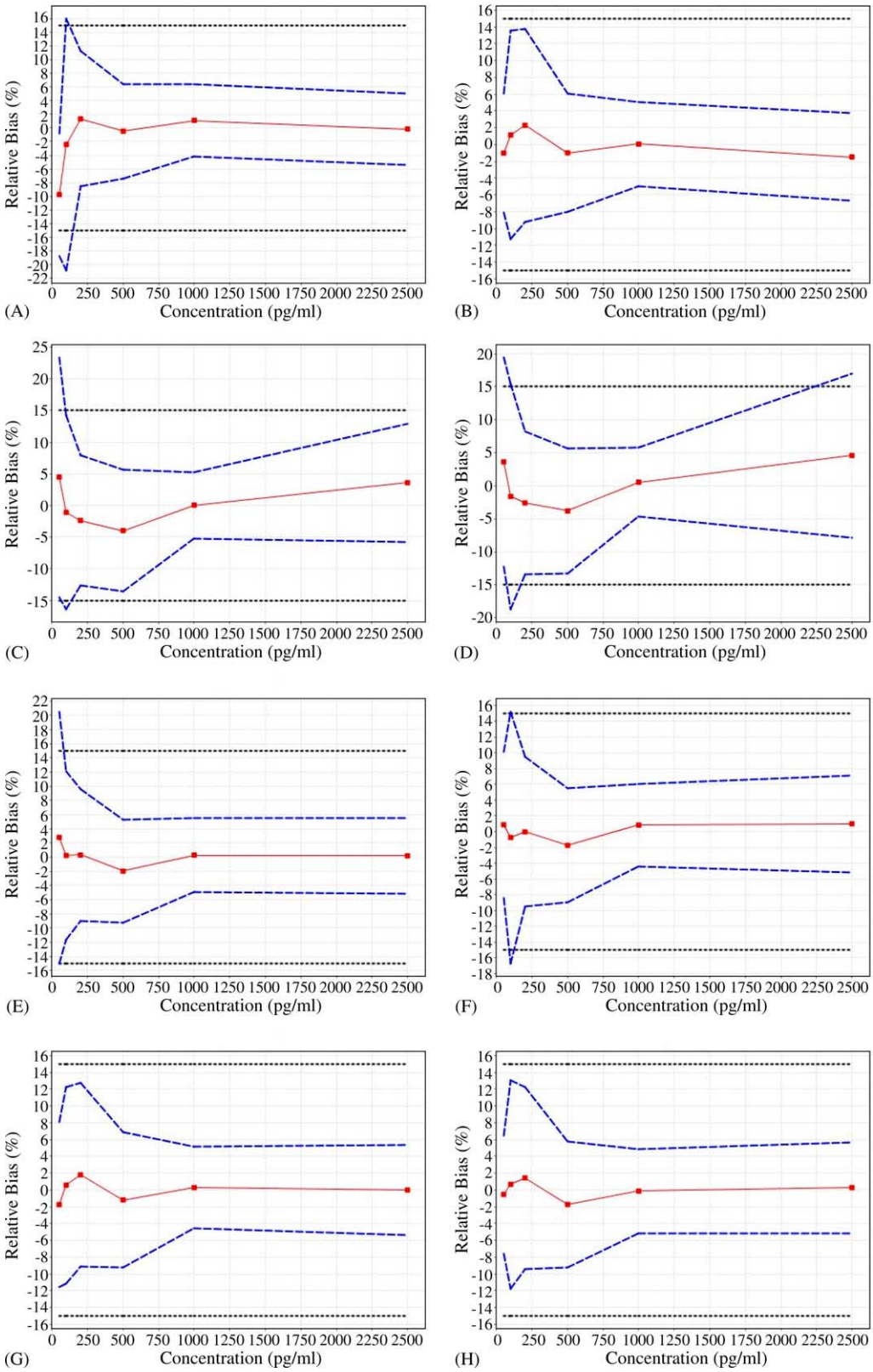


Fig. 3. Accuracy profiles of loperamide (concentration pg/ml) using (A) linear regression model, (B) weighted linear regression model with a weight equal to $1/X^2$, (C) linear regression model after logarithm transformation, (D) weighted linear regression model after logarithm transformation, (E) linear regression model after square root transformation, (F) linear regression model after square root transformation, (G) quadratic regression, (H) weighted quadratic regression.

Table 1
Selection of the sorbent of the disposable extraction cartridge

Type of sorbent	Analyte recovery (Mean ± S.D.%, $n = 3$)	
	Loperamide	Clonazepam
CN	63 ± 4	16 ± 1
C2	75 ± 5	71 ± 4
C2 endcapped	78 ± 4	101 ± 5
C8	75 ± 6	103 ± 3
C8 endcapped	66 ± 6	99 ± 6
C18	69 ± 8	99 ± 16
C18 endcapped	67 ± 4	105 ± 11

DECS: isolute (50 mg), conditioning: methanol and water (1.0 ml of each), washing: 1.0 ml of water twice, eluting: 0.75 ml of methanol, buffer addition: 0.25 ml of ammonium acetate pH 3.0, sample: spiked plasma solution of loperamide and IS.

The best recovery for loperamide was observed when DECs filled with ethyl endcapped (C2) sorbent was used. Consequently, the latter extraction sorbent was finally selected for the present study.

3.4. Pre-validation step

Before the formal validation phase, an important step consists in the assessment of the relationship between the response and concentration in order to avoid serious difficulties in the estimation of other validation criteria. In order to select the most appropriate response function, the SFSTP approach based on two-sided 95% confidence intervals for total measurement error—including both bias and precision—of validation samples has been used [21–23]. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e. 15% according to the Washington conference [27] or FDA document [28]. As illustrated in Fig. 3, once the pre-validation experiments have been performed, the response function can be determined by applying different regression models and, from both analytical responses and regression line obtained, selecting the most suitable accuracy profile for the intended use of the analytical method [21,24,25]. Regarding these latter accuracy profiles (Fig. 3), regression analysis could be performed in the present study using three different regression models: weighted linear regression (Fig. 3B), the quadratic regression (Fig. 3G) and the weighted quadratic regression (Fig. 3H). Among these three possibilities, the well-known weighted least-square model with weight equal to $1/X^2$ (where X is the theoretical concentration) was selected since it represents the simplest model adequately describing the concentration-response relationship and gives better results at the lower concentration levels.

The following equation was obtained (concentration range: 50–2500 pg/ml):

$$Y = 7.93 \times 10^{-4}(\pm 5.54 \times 10^{-5})\, X + 1.09 \times 10^{-2}(\pm 9.19 \times 10^{-3}) \qquad r^2 = 0.9991$$

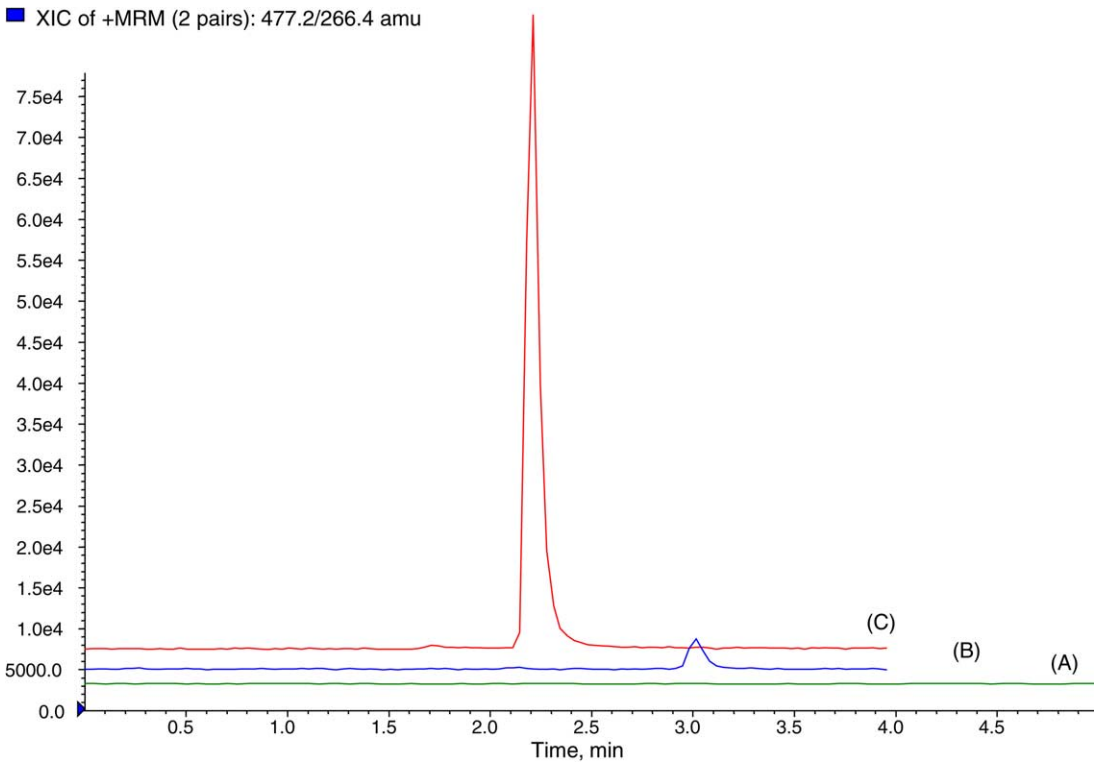


Fig. 4. SRM ion chromatograms of blank plasma (A) and spiked plasma samples with 50 pg/ml for loperamide (B) and IS (C). See text for operating conditions.

The determination coefficient (r^2) obtained for the regression line demonstrates the good relationship between peak area ratio and concentration.

In addition, by use of the weighted linear regression model, the procedure was able to quantify over the whole range under investigation. This was of particular interest in the present study since very low concentrations for loperamide should be measured.

3.5. Validation step

3.5.1. Stability

The stability of the whole procedure was studied by considering the different steps of the method. The stability of stock solutions (30 days at 4 °C), autosampler eluate (24 h at 20 °C), plasma sample (24 h at 20 °C), plasma storage (5 months at −80 °C) and after three freeze and thaw cycles was investigated. The determination of loperamide and IS were performed at the beginning and at the end of each storage period. The results obtained were all comprised between 95 and 105% of the initial value. No significant degradation of loperamide and internal standard was observed.

3.5.2. Selectivity

Potential interfering substances in a biological matrix include endogenous matrix components, related substances, metabolites and concomitant medication drugs such as OTC drugs (aspirin, acetaminophen, caffeine, ibuprofen). The selectivity was studied by injecting aqueous solutions of these compounds in the chromatographic system and by analyzing six different sources of plasma. No endogenous source of interference was observed at the retention times of the analytes. Typical chromatograms obtained with a blank plasma and a plasma containing 50 pg/ml of loperamide are presented in Fig. 4. A chromatogram obtained from the pharmacokinetics study representing a loperamide concentration around 170 pg/ml is illustrated in Fig. 5.

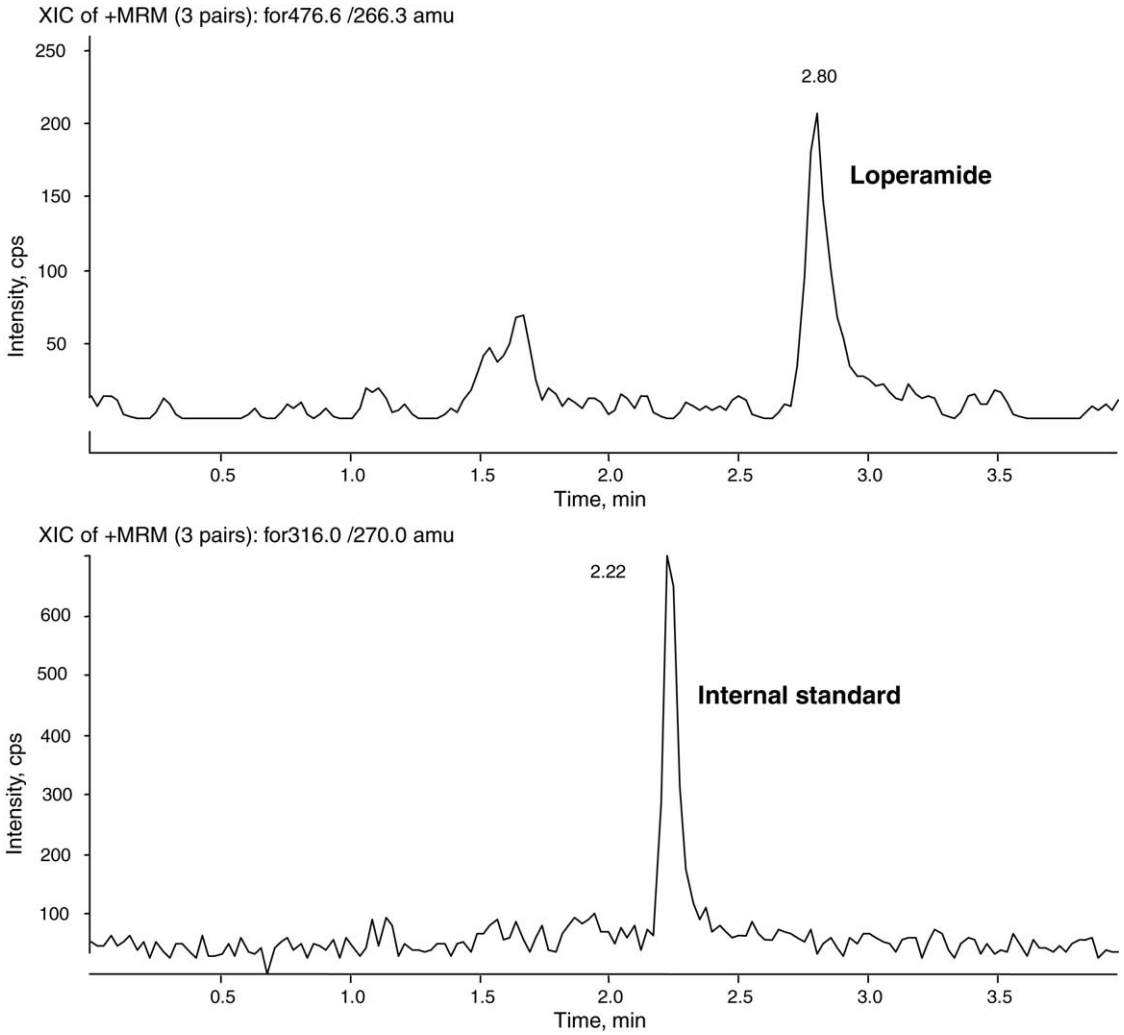


Fig. 5. SRM ion chromatograms of an actual plasma sample illustrating the separation of loperamide (173 pg/ml) and IS (5.0 ng/ml).

Table 2
Validation of the method of determination of loperamide in human plasma

Recovery ($n = 3$)	Mean ± S.D. (%)
100 pg/ml	83 ± 3
500 pg/ml	77 ± 4
2500 pg/ml	80 ± 3

Response function ($k = 3$, $m = 6$, $n = 2$) (50–2500 pg/ml)	Day 1	Day 2	Day 3
Slope	7.39×10^{-4}	8.49×10^{-4}	7.91×10^{-4}
Intercept	2.04×10^{-2}	2.01×10^{-3}	1.04×10^{-2}
r^2	0.9990	0.9991	0.9992

Trueness ($k = 3$; $n = 6$) (pg/ml)	Absolute bias (pg/ml) (relative bias (%))
50	−1.86 (−3.7)
100	0.23 (0.2)
500	14.68 (2.9)
2500	73.73 (2.9)

Precision ($k = 3$; $n = 6$) (pg/ml)	Repeatability (R.S.D.%)	Intermediate precision (R.S.D.%)
50	5.2	5.5
100	5.2	8.6
500	3.6	4.1
2500	4.1	5.1

Accuracy ($k = 3$; $n = 6$) (pg/ml)	β-Expectation confidence limit (pg/ml) (%)
50	44–53 (−13.1–5.7)
100	80–120 (−19.8–20.2)
500	477–552 (−4.6–10.5)
2500	2316–2832 (−7.4–13.3)

Linearity ($k = 3$; $m = 6$; $n = 24$)	
Range (pg/ml)	50–2500
Slope	1.031
Intercept	−2.379
r^2	0.999
LOD (pg/ml)	10
LOQ (pg/ml)	50

3.5.3. Response function

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample system [20–23,27,28]. The validation results of the response function are presented in Table 2. As previously mentioned, weighted linear regression ($1/X^2$) with six concentration levels was used. During routine analysis, the calibration equation was computed and the concentration of each calibration sample was calculated. If the back-calculated concentration of a calibration sample did not fall within ±15% of the nominal value, the sample was discarded and the equation was recalculated. However, for the calibration and the run to be valid, no more than two calibration samples were discarded and at least five accepted calibration samples had to be kept.

3.5.4. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [21,26]. As can be seen from the results in Table 2, trueness was expressed in terms of absolute bias (in pg/ml) or relative bias (%) and was assessed by means of validation standards in the matrix at four concentration levels ranging from 50 to 2500 pg/ml ($k = 3$, $n = 6$). Compared to the regulatory requirements fixed [28,29], the proposed method was accurate enough since the bias did not exceed the values of 15% irrespective to the concentration level.

3.5.5. Precision

The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision at the same concentration levels as those mentioned above. The variance of repeatability and time dependent intermediate precision as well as the corresponding relative standard deviation (R.S.D.) were calculated from the estimated concentrations [22,23]. The R.S.D. values presented in Table 2 were relatively low, the relative standard deviation values for repeatability and intermediate precision were between 3.6 and 8.6%, illustrating the good precision of the proposed method.

3.5.6. Accuracy

The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result [21,26]. The upper and lower β-expectation confidence limits expressed in μg/ml are presented in Table 2 as a function of the introduced concentrations. As can be seen from the results, the proposed method was accurate, since the different limits of confidence of the bias did not exceed the acceptance limits (±15%) [28,29] for all the concentration levels tested including the lowest one (50 pg/ml).

3.5.7. Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample [21–23]. consequently, for all the series, a regression line was fitted on the estimated or back-calculated concentrations as a function of the introduced concentrations by applying the linear regression model based on the least squares method. the regression equation was presented in Table 2. Moreover, in order to demonstrate method linearity, the approach based on the absolute β-expectation confidence limits as illustrated in Fig. 6 can be applied [25]. The linearity of the present method was demonstrated since the absolute β-expectation confidence limits were within the absolute acceptance limits.

3.5.8. Detection and quantitation limits

In the present study, the limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression [29]. By applying this computation method, the LOD of the developed method was equal to 10 pg/ml. The limit of quantitation (LOQ) of an analytical procedure is the lowest amount of the targeted substance in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy [21], i.e. taking into account the systematic and random errors [27,28]. As the accuracy profile is comprised within the acceptance limits, the LOQ was fixed to 50 pg/ml, i.e. the smallest concentration level investigated. Indeed, precision and trueness were demonstrated at this concentration level (Table 2).

3.5.9. Method follow-up during routine analysis

In order to assess the method performances during routine analysis, quality control samples at different concentration levels have to be analysed. The procedure most widely used for the continuing evaluation of assay performance involves the construction of QC charts. In the present study, the acceptance limits have been fixed at ±15% of the observed bias according to the Washington conference [27] and the FDA document [28]. Three concentration levels for each method were monitored. The QC Charts presented in Fig. 7 demonstrates that the analytical procedures were under control during routine analysis. Indeed, at least 67% of the QC samples were within 15% of their nominal values and definitely less than 33% of the QC samples without replicates at the same concentration levels were outside the ±15% of the nominal value [27,28]. Moreover, the QC bias (%) and the QC R.S.D. values which are presented in Table 3 illustrates the very good reliability of the described method.

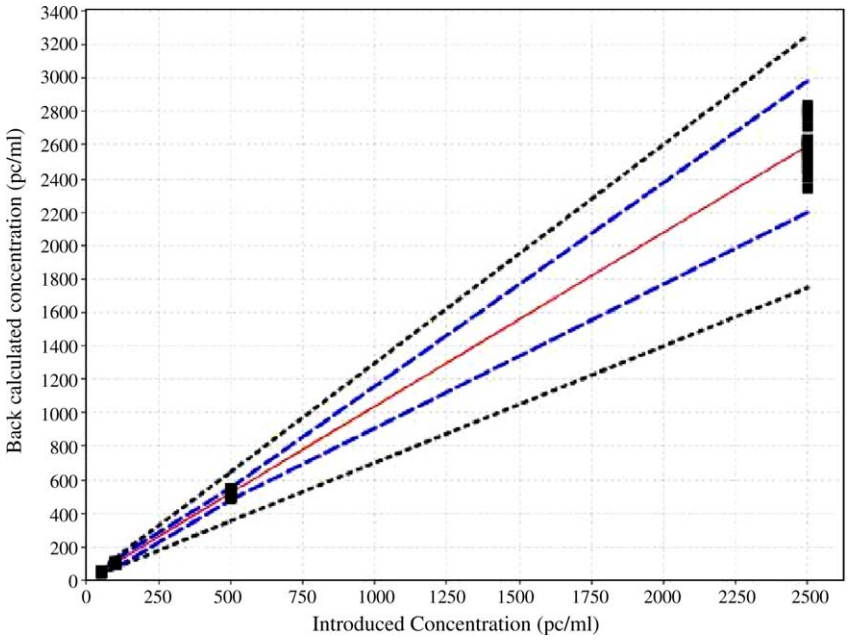


Fig. 6. Linear profile of loperamide. The dashed limits on this graph correspond to the accuracy profile, i.e. the β-expectation confidence limits expressed in absolute values. The dotted curves represent the acceptance limit at 15% expressed in the concentration unit.

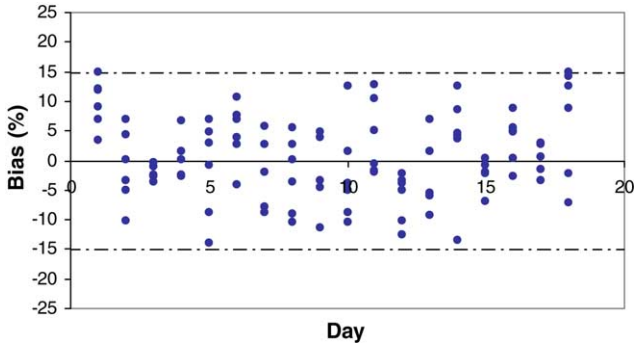


Fig. 7. Chart of frozen quality control during routine analysis ($N = 108$).

Table 3
QC interday precision, trueness and accuracy during routine analysis

Trueness ($k = 18$; $n = 2$) (pg/ml)	Absolute bias (pg/ml) (relative bias (%))
100	−1.01 (− 1.0)
500	6.92 (1.4)
1000	18.21 (1.8)

Precision ($k = 18$; $n = 2$) (pg/ml)	Repeatability (R.S.D.%)	Intermediate precision (R.S.D.%)
100	7.0	7.2
500	5.7	8.0
1000	5.4	6.0

Accuracy ($k = 18$; $n = 2$) (pg/ml)	β-Expectation confidence limit (pg/ml) (%)
100	87–111 (−13.4–11.4)
500	438–576 (−12.4–15.2)
1000	916–1121 (−8.4–12.1)

3.6. Pharmacokinetics

The LC–MS/MS procedure developed was used to investigate the plasma profile of loperamide after a single oral dose of an immediate release formulation containing 2 mg of loperamide. Plot of the plasma concentration of loperamide (pg/ml) versus post-dose sampling time (h) is presented in Fig. 8. Pharmacokinetics parameters calculated from these data are as follows: AUC_{0-48}: 4600 ± 2500 pg/ml h, C_{max}: 240 ± 120 pg/ml and T_{max}: 5.9 ± 1.7 h.

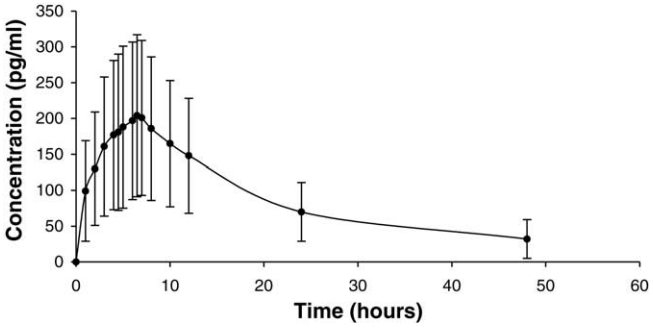


Fig. 8. Plasma concentration–time profile of loperamide following the administration of a single oral dose of 2 mg.

4. Conclusions

A sensitive and accurate procedure based on the solid-phase extraction coupled at-line to a LC–MS/MS determination has been developed for the assay of loperamide. The extraction procedure and the MS/MS conditions were optimized in order to have a sensitive method. The procedure was fully validated to meet the requirements of the pharmacokinetic investigation of this compound. The procedure developed was successfully applied to the determination of loperamide plasma levels for investigating a pharmacokinetic study.

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