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Integrated on-line sample clean-up using cation exchange restricted access sorbent for the LC determination of atropine in human plasma coupled to UV detection

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Abstract

A new, simple and fully automated liquid chromatographic (LC) method with UV detection has been developed for the direct determination of atropine in plasma. Sample clean-up was based on the use of cation exchange restricted access material (RAM) in a pre-column, coupled to LC by means of a column switching system. After direct injection of a 200 μl-volume of plasma sample, the biological matrix was washed out for 10 min using a washing liquid composed of 2 mM lithium perchlorate adjusted to pH 3.0 and methanol (97:3; v/v). By rotation of the switching valve, atropine was then eluted in the back-flush mode for 2 min and transferred to the analytical column packed with octadecyl silica by the LC mobile phase constituted of a mixture of acetonitrile and potassium phosphate buffer (pH 3.0; 50 mM) containing 2 mM sodium heptanesulfonate (16:84; v/v). The UV detection was performed at 220 nm.

The method was validated according to a new approach based on accuracy profile over a concentration range from 25 ng/ml, corresponding to the limit of quantitation, to 1000 ng/ml. The method was then applied for the determination of atropine in plasma after intravenous administration to hospitalised patients.

Keywords: Atropine; Plasma; Sample preparation; Cation exchange restricted access material; Reversed-phase liquid chromatography; Column-switching

1. Introduction

Atropine (Fig. 1) is an alkaloid, naturally occurred in plants, such as *Atropa belladonna* and *Datura stramonium*, as DL-hyoscyamine [1].

This compound is mainly used as antidote in case of intoxication with organophosphorous insecticides and chemical warfare nerve gases [2]. Due to the use of herbal medicines containing atropine, intoxication symptoms were reported in many cases [3]. Atropine is also used in small doses in pre-anaesthetic medication, for the treatment of cardiopathy, parkinsonism and in ophthalmic diagnosis [4].

Several analytical methods in combination with different detection systems have been developed for the analysis of atropine in plant extracts, pharmaceutical dosage forms and in biological fluids [5–11]. Although the chromatographic methods could determine atropine in small concentrations, some complex and time consuming off-line procedures, such as liquid–liquid extraction (LLE) or solid phase extraction (SPE) using conventional sorbents, were used to extract atropine from various sample matrices.

The coupling of pre-columns packed with restricted access material (RAM) to LC by means of the column-switching technique represents an interesting alternative [12–14]. A family of restricted access sorbents, namely alkyl diol silica (ADS), belonging to the group of internal surface reversed-phase (ISRP) supports, has been applied successively for

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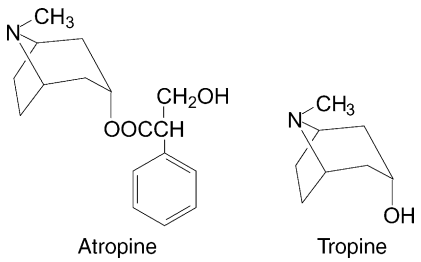

Fig. 1. Chemical structures of atropine and tropine.

on-line sample clean-up prior to the determination of compounds in different biological matrices [15–19]. After direct injection of biological samples, macromolecules, such as proteins, are flushed to the waste due to an appropriate pore diameter (about 6 nm) of the silica particles, while low molecular-mass compounds can penetrate into the pores and be retained by interaction with butyryl (C_4), capryoyl (C_8) or stearoyl (C_{18}) moieties bonded to the inner surface. The molecular weight cut-off is approximately 15 kDa. Moreover, the outer surface of these RAM particles contains hydrophilic and electroneutral diol groups, which prevents the adsorption and denaturation of proteins.

A new type of restricted access material, for which sulfonate groups are bonded to the inner surface of the porous sorbent, has been recently introduced. The mean pore diameter of the silica particles is also about 6 nm and the external surface is biocompatible. This support, namely XDS (SO_3/diol) sorbent, presents the properties of a strong cation exchanger towards basic compounds and has been evaluated in our previous works [20,21] for on-line sample handling prior to the LC determination of some basic compounds in plasma.

The aim of this paper is to develop and validate an automated method for the determination of atropine in human plasma using this kind of sorbent for on-line sample clean-up coupled to a simple LC-UV system. As far as we know, such procedure has not been reported for the direct determination of atropine in plasma.

2. Experimental

2.1. Chemical and reagents

Atropine sulfate was obtained from Acros (New Jersey, USA), potassium dihydrogenphosphate, sodium perchlorate, lithium perchlorate, potassium perchlorate, magnesium perchlorate, 1-heptanesulfonic acid sodium salt were all analytical grade and were purchased from Merck (Darmstadt, Germany). Perchloric acid and phosphoric acid (85%; m/m) were obtained from Acros. Methanol, acetonitrile were Lichrosolv gradient LC grade and were purchased from Merck. Deionised water was purified on a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2. Apparatus

The components of the chromatographic system from Merck-Hitachi comprised: a model L-6200A pump (pump 2), a model AS-2000 auto-sampler equipped with a 200 μl injection loop, a model L-5025 column oven and a model L-4250 UV–vis detector. The different parts were connected through an interface (D-6000, Merck) with an IBM compatible computer (PC-AT; CPU type Pentium) in which the D-7000 HPLC manager software was loaded for the control of the analytical system and data collection. A model 420 LC pump from Kontron Instruments (Schlieren, Switzerland) (pump 1) was also used and was controlled manually.

The integrated LC system was composed of a LiChroCART pre-column (25 mm × 4 mm, i.d.) pre-packed with restricted access material LiChrospher 60 XDS (SO_3/diol) (particle size: 25 μm) supplied as research sample by Merck and an analytical column Alltima C_{18} (150 mm × 4.6 mm, i.d.; particle size: 5 μm) purchased from Alltech (Deerfield, IL, USA). The pre-column and the LC column were coupled by using a Valco model VICI AG six port switching valve (Schenkon, Switzerland). The e.novel® software (Arlenda, Belgium) was used to obtain the accuracy profiles as well as all validation results.

2.3. Chromatographic conditions

The chromatographic separation was performed in the isocratic mode at a flow-rate of 1.3 ml/min. The analytical column was thermostated at 30 °C.

The LC mobile phase consisted of a mixture of acetonitrile and potassium phosphate buffer (pH 3.0; 50 mM) containing 1-heptanesulfonic acid sodium salt at a concentration of 2.0 mM (16:84; v/v). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath and 5 min with helium gas.

The phosphate buffer was prepared by dissolving 6.8 g of potassium dihydrogenphosphate in 900 ml of water. The pH was adjusted to 3.0 with 1 M phosphoric acid, the content was then transferred quantitatively into a 1000 ml volumetric flask and water was added to the mark. The buffer was filtered through a membrane filter (0.45 μm) from Schleicher and Schuell (Dassel, Germany).

The analyte was monitored photometrically at 220 nm.

2.4. Standard solutions

A stock solution of atropine sulfate was prepared by dissolving 10 mg in methanol to obtain a concentration of 1 mg/ml. The solution was stored at 4 °C and was used for a week.

2.4.1. Solutions used for method development

A diluted standard solution was made up by diluting 1.0 ml of the stock solution with water to obtain a concentration of

50 μg/ml and was prepared daily. Free drug human plasma samples were obtained from the Blood Transfusion Centre of Liège (Liège, Belgium) and were stored under −20 °C. Before use, the plasma samples were thawed at room temperature and were centrifuged at 4500 × *g* for 10 min. Aliquots were spiked with the diluted standard solution of atropine and were prepared daily.

Blood samples were also taken from 4 hospitalised persons before intravenous administration of 1 mg atropine sulfate and after 10, 30 and 60 min and were transferred to test tubes containing heparin as anti-coagulant. After centrifugation at 4500 × *g* for 10 min, the supernatant was stored at −20 °C. Before use, the samples were thawed at room temperature and centrifuged at 4500 × *g* for 10 min.

2.4.2. Solutions used for validation

2.4.2.1. Solutions used in the pre-validation step. A stock solution of atropine sulfate was prepared in methanol at a concentration of 1 mg/ml. This solution was then diluted with water in order to obtain two solutions at 10 and 1 μg/ml. These intermediate solutions were used to spike free drug plasma samples at six concentration levels covering a range from 25 to 1000 ng/ml. The first concentration level was close to the expected limit of quantitation. Two other series were then prepared from independent stock solutions according to the same protocol. One non-biological calibration curve was also performed for the determination of absolute recovery.

2.4.2.2. Solutions used in the validation step. Two types of plasma samples were prepared: calibration standards covering the same concentration range as used in the pre-validation step and validation standards. The latter represented the quality control samples to be used in routine analysis. Four concentration levels were selected, the lowest one being the limit of quantitation. Each validation sample was analysed four times for three different days.

2.5. On-line sample clean-up and chromatographic separation

The time events of the column-switching valve for on-line sample clean-up and chromatographic separation are indicated in Table 1.

2.5.1.1. Loading and washing of sample matrix

200 μl of plasma samples were directly injected by the autosampler into the pre-column with the washing liquid delivered by pump 1 at a flow-rate of 1.0 ml/min for 10 min. The washing liquid was a mixture of 2 mM lithium perchlorate adjusted to pH 3.0 with 1 M perchloric acid and methanol (97:3; v/v).

2.5.1.2. Elution and transfer of atropine to the analytical column

The switching valve was turned to allow the pre-column to be coupled to the analytical column, the analyte was then desorbed and transferred in the back-flush mode to the top of the analytical column by the LC mobile phase delivered by pump 2.

2.5.1.3. Chromatographic separation and re-equilibration of the pre-column

Two minutes later, the switching valve was turned to its initial position allowing the re-equilibration of the pre-column with the washing liquid before the next injection. Simultaneously, the analyte transferred to the analytical column was separated and quantified.

3. Results and discussion

The determination of polar compounds characterised by an absorption maximum at low wavelengths and/or by low molar absorptivities in plasma is generally difficult. However, when an efficient and selective sample handling procedure is used, a fraction free from interference can be obtained and a satisfactory detectability can be achieved. Due to the use of restricted access material with cation exchange properties, method selectivity should be improved, since only the positively ionized compounds can be retained through electrostatic interactions with sulfonate groups bonded to the inner surface of the sorbent.

3.1. Optimisation of the LC mobile phase composition

As shown in Fig. 1, the nitrogen of atropine is protonated at acidic pH and can be associated with a negatively charged counter ion. Consequently, an ion-pair reversed-phase LC method is expected to be a suitable method.

1-Heptanesulfonic acid sodium salt was selected as ion-pairing reagent at a concentration of 2 mM. The chromatographic separation was finally performed using a mobile phase composed of a mixture of acetonitrile and phosphate buffer (pH 3.0) containing heptanesulfonate (16:84; v/v). Under these conditions, the retention factor of atropine was 4.3 and the chromatographic peak was separated sufficiently from the solvent front.

Table 1
Time events of the column-switching valve for on-line sample clean-up and chromatographic separation

Step	Process	Switching valve connection		Time (min)
1	Sample clean-up	Pre-column – waste	LC column – UV detector	0–10
2	Elution and transfer	Pre-column – LC column – UV detector		10–12
3	Equilibration of the pre-column and LC separation	Pre-column – waste	LC column – UV detector	12–15

Other conditions: see Section 2.

3.2. Method development for sample clean-up

The procedures for the determination of the breakthrough volume (Vb), the washing time (Tw) and the time needed for the analyte transfer from the pre-column to the analytical column (Tt) were briefly explained in our previous work [20] about the use of this kind of RAM prior to the determination of some basic drugs in plasma.

3.2.1. Determination of the optimum washing liquid composition

The washing liquid composition was optimized by studying separately the influence of the concentration of competing co-ions, the pH of the washing liquid and the addition of organic modifier in order to obtain an efficient washing of the sample matrix and a sufficient retention of atropine in the pre-column during the washing step.

3.2.1.1. Effect of the type and the concentration of competing co-ions on the breakthrough volume of atropine. Since the extraction sorbent contains sulfonate groups, atropine is likely to be retained through electrostatic interactions with these groups. Accordingly, the type and the concentration of the competing co-ions in the washing liquid can influence the retention of atropine and should be closely tested in order to allow a sufficient washing time of sample matrix. Different washing liquids containing the same concentration of competing co-ions, such as lithium, sodium, potassium and magnesium, were tested. A small breakthrough volume (8 ml), corresponding to the beginning of elution of atropine, was observed by using a solution of 2 mM magnesium perchlorate due to the greater affinity of magnesium ions for sulfonate groups. On the other hand, by using a solution of lithium perchlorate, a larger breakthrough volume (30 ml) was obtained due to much weaker competition effects for the sulfonate groups. Consequently, a solution of 2 mM lithium perchlorate was selected as the main component of the washing liquid. The effect of endogenous co-ions present in the sample matrix (plasma) was then tested by comparing the breakthrough volume of atropine in aqueous solution with that obtained from the elution profile of spiked plasma samples. The breakthrough volume of the analyte in plasma was 1.5 ml smaller than that observed in aqueous solution, which would indicate a weak competition effect of endogenous co-ions.

3.2.1.2. Effect of the washing liquid pH on the retention of atropine. The effect of the washing liquid pH on the retention of atropine was tested by using 2 mM lithium perchlorate in a pH range from 2.5 to 6.5.

As shown in Fig. 2, the relationship between the inverse of the retention factor (1/*k*) and the pH was constructed. A weak increase in the retention of atropine was observed from pH 2.5 to 5 followed by a more pronounced increase at higher pH values. This increase in retention can be attributed to interactions of atropine with the sulfonate groups and the residual silanol groups. In a low pH range, the silanol groups are not ionized and their effects are much less important, which results in a lower retention.

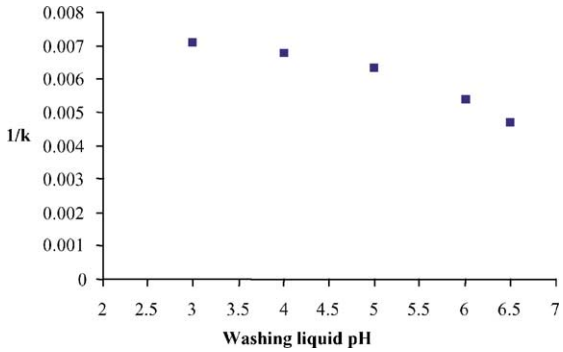

Fig. 2. Effect of the washing liquid pH on the retention of atropine, Sample: aqueous solution of atropine (conc.: 100 μg/ml) – washing liquid: 2 mM lithium perchlorate containing 3% (v/v) of methanol – flow-rate: 1.0 ml/min – detection: UV at 220 nm – temperature: 25 °C – other conditions: see Section 2.

During the washing step, it is important that the analyte of interest is extracted and enriched on the sorbent. However, a too strong retention is not recommended. Indeed, the desorption process would be slower or a small amount of analyte might be not eluted, giving rise to carry-over effects. Therefore, a washing liquid adjusted to pH 3.0 was finally selected.

3.2.1.3. Effect of the addition of organic modifier to the washing liquid. The addition of a low percentage of an organic modifier, such as methanol, is important to achieve the release of the drug from the binding sites of the plasma proteins and to improve method selectivity [22]. A content of 3% (v/v) of methanol was added to the washing liquid without affecting the breakthrough volume of atropine.

3.2.2. Sample matrix clean-up

The efficiency of the selected washing liquid to wash out the sample matrix was tested several times by injecting 200 μl of plasma directly into the pre-column connected to a UV detector monitored at 280 nm. From the elution profile, the elimination of the biological matrix was considered as complete when the detector signal reached the baseline. Under these conditions, a washing time of 10 min was sufficient for sample clean-up without any loss of analyte, since its breakthrough time was much higher (30 min).

3.2.3. Determination of the transfer time

The determination of the transfer time was performed by connecting directly the UV detector with the switching valve. Due to the high ionic strength of the LC mobile phase, a time period of 2 min was sufficient to perform the complete transfer of atropine from the pre-column to the analytical column. Twelve minutes after sample application, the switching valve returned to its initial position, allowing the sorbent to be re-equilibrated with the washing liquid.

3.3. Validation

The method validation was performed in two steps: the prevalidation phase and the validation phase. In the first step, the objective was to demonstrate method selectivity, to determine the absolute recovery, to analyse the response function, to evaluate the range, to estimate the limits of quantitation (LOQ) and of detection (LOD). As for validation, the experiments allowed the assessment of method trueness, precision and accuracy over the concentration range considered, the confirmation of the LOQ and the verification of linearity.

3.3.1. Prevalidation phase

3.3.1.1. Method selectivity. The method selectivity towards endogenous components of plasma was evaluated by injecting blank plasma samples from six different sources of the sample matrix into the LC-integrated sample clean-up system. As shown in Fig. 3(A), no interference was observed at the retention time of the peak corresponding to atropine.

Moreover, method selectivity was also demonstrated towards two metabolites of atropine, namely tropic acid and tropine. Plasma samples spiked with atropine, tropine and tropic acid were analysed under the described operating conditions. Due to the retention capability of the sorbent for basic compounds, only atropine and tropine were extracted. These two compounds were separated completely, as shown in Fig. 3 (B). The structure of tropine is illustrated in Fig. 1.

3.3.1.2. Absolute recovery. The absolute recovery of atropine at four concentration levels was determined by comparing the peak areas measured after analysis of spiked plasma samples according to the whole procedure with those found after direct injection into the chromatographic system of non-biological samples at the same concentration levels. As shown in Table 2, the analyte recoveries were close to 100% and the extraction efficiency was relatively constant over the considered range according to the relative standard deviation values obtained.

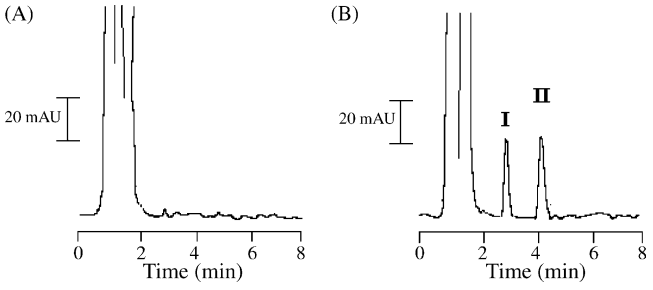

Fig. 3. Typical chromatograms obtained after on-line coupling of the XDS (SO_3/diol) pre-column to LC for the analysis of a blank plasma sample and a plasma sample spiked with atropine and tropine, A: chromatogram of a blank plasma sample, B: chromatogram of a plasma sample spiked with atropine and tropine (concentration: 200 ng/ml). Operating conditions given in Section 2. Peaks: (I) tropine – (II) atropine.

Table 2
Determination of absolute recovery

Concentration (ng/ml)	Absolute recovery	
	Mean (%; n = 9)	R.S.D. (%; n = 9)
50	89.8	2.8
100	96.3	2.5
500	93.5	3.8
1000	94.4	2.1

3.3.1.3. Analysis of the response function, estimation of the range and the LOQ. Three independent calibration curves were constructed from 25 to 1000 ng/ml by selecting six concentration levels. Different regression models, such as the linear regression, the linear regression through 0, the weighted linear regression and the linear regression after square root transformation, were tested for calibration. The most appropriate regression model was selected on the basis of the accuracy profile [23]. This concept was also used to estimate the range and the LOQ.

For each regression model, the concentrations of the calibration standards were back-calculated and an accuracy profile was obtained from the mean relative bias as well as the upper and lower β-expectation tolerance limits at 95% level by introducing the estimation of the standard deviation for intermediate precision [23]. Since it is a bioanalytical method, the acceptance limits were set at 15%.

As can be seen in Fig. 4, the upper tolerance limit exceeded the acceptance limit at one or two concentration levels by considering the simple linear regression or the linear regression through 0, respectively. These two models should be rejected. On the other hand, the tolerance intervals were totally included inside the acceptance limits with the two other models tested. The weighted regression model with a weight equal to $1/x$ was finally selected, since the mean bias was a little lower at the first concentration level. Consequently, the concentration range was defined and the LOQ, corresponding to the first concentration level, was estimated.

3.3.1.4. Determination of the LOD. The LOD was determined as the analyte concentration giving rise to a signal-to-noise ratio of 5. The LOD was evaluated at 10 ng/ml.

3.3.2. Validation phase

In order to describe the relationship between peak area and concentration, the same regression model as determined in prevalidation was selected, namely a weighted linear regression. The slope, the intercept and the coefficient of determination (r^2) for each series are indicated in Table 3. This table also presents the other results obtained.

3.3.2.1. Trueness. Trueness expressed in terms of relative bias (%) was assessed from the validation standards at four concentration levels. According to the regulatory requirements [24], trueness was quite acceptable since the bias did not exceed the value of 15%, irrespective of the concentration level.

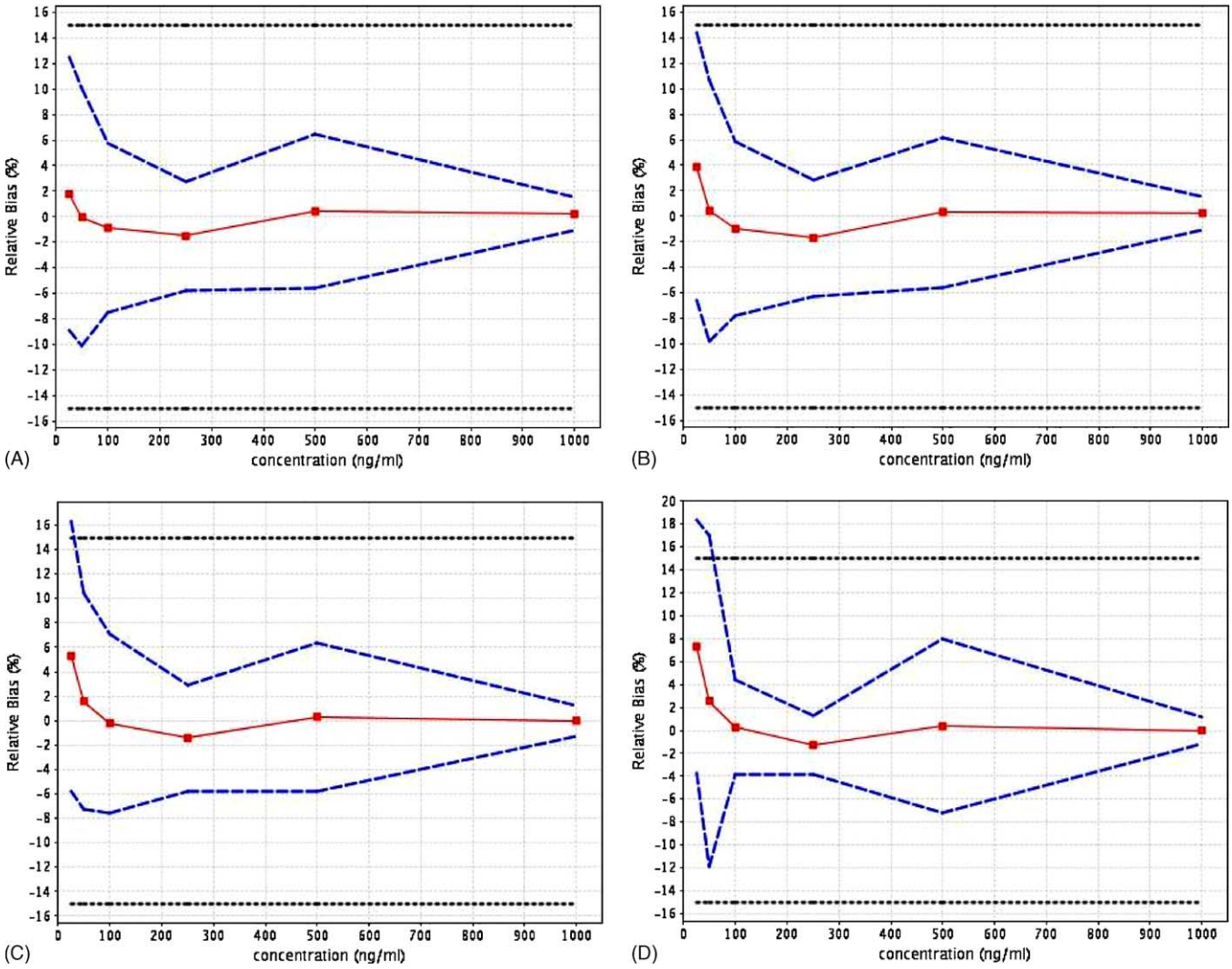

Fig. 4. Analysis of the response function from accuracy profiles obtained using different calibration models, A: weighted linear regression model with a weight equal to 1/*x*, B: linear regression model after square root transformation, C: linear regression model, D: linear regression through 0. The dotted lines represent the acceptance limits (15%), the dashed lines correspond to the accuracy profile, i.e. to the β-expectation tolerance limits, and the relative bias values (■) are located on the plain line.

3.3.2.2. Precision. Method precision was evaluated at two levels (repeatability and time-different intermediate precision) by computing the corresponding relative standard deviations (R.S.D.) for each concentration level of the validation standards. The R.S.D. values obtained (<4.5%) illustrate the very good precision of the developed method.

3.3.2.3. Accuracy. Accuracy takes into account the total error, i.e. the sum of systematic and random errors, related to the test result [23]. Instead of defining acceptance criteria only on estimates of observed bias and variance, the method capability was demonstrated using the approach based on the accuracy profile. The upper and lower β-expectation tolerance limits at 95% level were calculated at each concentration level of the validation standards from the mean relative bias and the standard deviation for intermediate precision. As can be seen in Table 3, the method was considered as accurate. Indeed, the tolerance limits did not exceed the acceptance limits settled at 15%, irrespective of the concentration level. Consequently, the concentration range was valid from 25 to 1000 ng/ml and the LOQ estimated at 25 ng/ml was confirmed, since accuracy was also assessed at this concentration level.

3.3.2.4. Linearity. In order to demonstrate method linearity, a regression line was fitted on the back-calculated concentrations of the validation standards as a function of the introduced concentrations by applying the linear regression model based on the least squares method [23,25]. The following equation was obtained: $y = 1.001x - 0.492$. The coefficient of determination (r^2) was 0.9999.

Table 3
Validation results

Validation criterion	Atropine		
Response function (k = 3; m = 6; n = 2)	Weighted linear regression (weight = 1/x); Calibration range: 25–1000 ng/ml		
	Series 1	Series 2	Series 3
Slope	18.84	18.89	19.44
Intercept	29.08	29.41	48.24
r^2	0.9999	0.9999	0.9998
Trueness (k = 3; n = 4)			
Relative bias (%)			
25 ng/ml		1.0	
50 ng/ml		−2.8	
500 ng/ml		0.1	
1000 ng/ml		0.0	
Precision (k = 3; n = 4)			
Repeatability/Intermediate precision (R.S.D. %)			
25 ng/ml		4.3/4.3	
50 ng/ml		3.3/3.3	
500 ng/ml		0.4/0.9	
1000 ng/ml		0.3/1.0	
Accuracy (k = 3; n = 4)			
β-expectation lower and upper tolerance limits of the mean relative bias (%)			
25 ng/ml		−9.0/11.0	
50 ng/ml		−10.4/4.8	
500 ng/ml		−3.2/3.3	
1000 ng/ml		−4.7/4.7	
Linearity (k = 3; n = 4)			
Range (ng/ml)		25–1000	
Slope		1.001	
Intercept		−0.492	
r^2		0.9999	
LOD (ng/ml)		10	
LOQ (ng/ml)		25	

Note: k: number of series, *m*: number of concentration levels, *n*: number of replicates.

3.4. Application to real samples

The method was then tested for the determination of atropine after intravenous administration of 1 mg atropine sulfate to four hospitalised patients. Blood samples were collected just before and after 10, 30 and 60 min. Fig. 5 shows typical chromatograms obtained after analysis of a control sample (T_0) and a dosed sample ($T_{10\,min}$). The concentrations of atropine in the four samples collected after 10 min were 30, 27, 25 and 29 ng/ml, respectively.

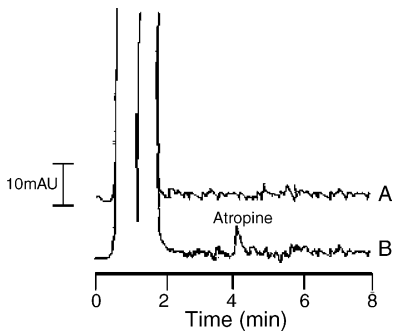

Fig. 5. Typical chromatograms obtained after on-line coupling of the XDS (SO_3/diol) pre-column to LC for the analysis of real plasma samples, A: chromatogram of a control sample (before the intravenous administration of 1 mg atropine sulfate), B: chromatogram of a dosed sample (10 min after the administration; atropine concentration: 30 ng/ml). Operating conditions given in Section 2.

Due to a very rapid decline in the level of atropine in blood [26], atropine was not quantified after 30 min by using UV detection. A more sensitive detection system should be employed in this case.

However, the developed method could be applied successively to the determination of atropine in plasma after administration of high doses [27] as antidote in the treatment of organophosphorous insecticide poisoning or after intoxication [28] due to the ingestion of herbal medicines.

4. Conclusions

A simple and selective bio-analytical method has been developed for the automated determination of atropine in human plasma, using a pre-column packed with cation exchange restricted access material as sample handling coupled to LC by means of the column switching technique.

Due to the use of this kind of extraction sorbent, a selective sample clean-up was obtained, which allowed achieving a relatively good detectability at a low wavelength in UV detection.

Then, the method was fully validated using a new approach based on the accuracy profiles taking into account the total error of measurement. The limit of quantitation (LOQ) was 25 ng/ml. As far as we know, such LOQ has not been achieved before by coupling LC to UV detection for the analysis of atropine in plasma.

The method was also applied for the determination of atropine in plasma after intravenous administration of a low dose of atropine sulfate. Ten minutes after administration, the concentration of atropine was about 30 ng/ml. Due to the rapid decline of atropine concentration in blood, UV detection is not the most appropriate detection mode. However, due to the simplicity of the developed method, it could be applied to the determination of atropine present in plasma at concentrations higher than 25 ng/ml, when this drug is used as antidote in the treatment of organophosphorous insecticide poisoning or in case of intoxication due to herbal medicines containing atropine.

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