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Fully automated LC method for the determination of sotalol in human plasma using restricted access material with cation exchange properties for sample clean-up

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Abstract

A simple and rapid fully automated bio-analytical method for the liquid chromatographic (LC) determination of sotalol in human plasma has been described. The method is based on the use of a new kind of porous silica restricted access material (RAM) with cation exchange properties for sample clean-up. 100 μl of plasma samples were directly injected into the precolumn coupled on-line to a reversed-phase column (RP-Select B) by means of column switching system. The plasma matrix was washed out for 10 min using a washing liquid composed of 2 mM lithium perchlorate and methanol (97:3; v/v). By rotation of the switching valve, the analytes were then eluted in back-flush mode for 2 min and transferred to the analytical column by the LC mobile phase constituted of a mixture of methanol and 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM 1-octanesulphonic acid sodium salt (20:80; v/v). The flow-rate was 1.0 ml/min and sotalol was detected using fluorescence detection at 235 and 300 nm as excitation and emission wavelengths, respectively. The method was then validated using a new approach based on accuracy profile over a concentration range from 5 to 500 ng/ml. The limit of quantitation (LOQ) was 5 ng/ml and the total analysis time was 19 min.

Keywords: Sotalol; Restricted access material; Accuracy profile; Fluorescence detection; Plasma; Sample preparation

1. Introduction

Sotalol, *N*-[4-1-hydroxy-2-[(1-methylethyl)-amino]ethyl]phenyl]methane-sulphonamide (Fig. 1), is a non-selective β-adrenoreceptor antagonist used for the treatment of hypertension, cardiac arrhythmias and angina pectoris [1].

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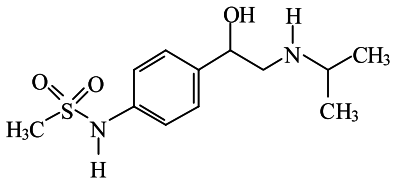

Fig. 1. Chemical structure of sotalol.

Several liquid chromatographic (LC) methods have been described for measuring sotalol concentration in plasma using UV detection [2,3] as well as fluorescence detection [4,5]. Sotalol enantiomers were also determined in plasma and urine by LC methods [6,7] using UV and fluorometric detection with sample derivatisation [8]. Prior to all the LC methods, different sample preparation procedures for sample clean-up such as liquid–liquid extraction (LLE), [3,5,9] solid phase extraction (SPE), [2] or precipitation of plasma proteins [4]. These off-line procedures were carried out manually and were, therefore, labour intensive and time consuming. Moreover, low recoveries of sotalol were obtained by LLE after alkalinisation followed by back-extraction in acidic medium [9]. Due to its hydrophilic character ($\log P = -0.79$), it is also difficult to extract sotalol from plasma using SPE cartridges [10]. A recent on-line LC method for the determination of sotalol enantiomers using cellobiohydralse chiral (CBH) column and ADS C18 precolumn for sample clean-up was described. Although the method was used for pharmacokinetic studies, due to the lower stability of protein based chiral columns, however, its applicability for routine analysis would not be satisfactory [11]. Another on-line LC method for determination of sotalol in human plasma using dialysis and trace enrichment on cation exchange precolumn for sample clean-up was also described. Beside its reliability, however, the mean recovery was only 60% [12].

The objective of this work was to develop an automated method based on the coupling of a precolumn to the analytical column by means of the column switching technique. Among the on-line extraction procedures, the use of a precolumn packed with restricted access material (RAM) has proved to be a useful approach for the analysis of drugs in plasma [13–17].

In the present work, a novel RAM material with a strong cation exchange properties, namely XDSc, was tested.

The access restriction is obtained by the use of silica particles (particle size of 25 μm) with an appropriate pore diameter (6 nm), the molecular weight cut-off is about 15 kDa. Due to this physical diffusion barrier, macromolecules such as proteins have no access to the internal surface and are directly eliminated. Moreover, some hydrophilic diol groups are bound to the external surface of the particles, which prevents the adsorption and the denaturation of proteins. On the other hand, low molecular weight compounds have free access to the internal surface on which sulphonate groups are bound and can be retained. This precolumn has been recently evaluated by our group for the on-line SPE of basic compounds including hydrophilic ß-blockers and quaternary ammonium compounds from directly injected plasma samples prior to their determination by reversed-phase liquid chromatography using the column switching technique [18].

The purpose of this paper is to present a useful application of this kind of sorbent for sample clean-up of drugs in order to obtain a fully integrated LC method for the determination of sotalol in plasma with improved selectivity and sensitivity.

2. Experimental

2.1. Reagents and materials

Sotalol hydrochloride was kindly donated by Profarmaco (Milan, Italy), potassium dihydrogenphosphate, potassium hydroxide, sodium hydroxide, sodium perchlorate, lithium perchlorate, potassium perchlorate, magnesium perchlorate, sodium octanesulphonate and orthophosphoric acid (85%) were all analytical grade and purchased from Merck (Darmstadt, Germany). Perchloric acid was obtained from Acros (New Jersey, USA). Methanol was Lichrosolv gradient LC grade and was purchased from Merck. Deionised water was purified on a Milli-Q system (Millipore, Bedford, USA).

2.2. Chromatographic system

The components of the chromatographic system Merck Hitachi comprised: a model L-6200A pump (pump 2), a model AS-2000 autosampler equipped with a 100 μl injection loop, a model L-5025 column oven, a model L-4250 UV–vis detector and a model F-1050 fluorescence 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 manually controlled external LC pump 420 from Kontron Instruments (Schlieren, Switzerland) (pump 1) was also used.

The LiChrocart precolumn (25 × 4 mm, i.d) prepacked with RAM Lichrospher 60 XDSc (particle size 25 μm) supplied as research sample by Merck and the LiChrocart analytical column (125 × 4 mm, i.d.) pre-packed with LiChrospher 60 RP-Select B (particle size: 5 μm) were coupled by using of a Valco model VICI AG six port switching valve (Valco, Shenkon, Switzerland).

The chromatographic separations were performed using the isocratic mode with a flow-rate of 1.0 ml/min. The analytical column was thermostated at 37 °C.

The LC mobile phase consisted of a mixture of methanol and 50 mM potassium dihydrogen phosphate of pH 7.0 containing 1-octanesulphonic acid sodium salt at a concentration of 1 mM (20:80; 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 dihydrogen phosphate and 1.0 g of potassium hydroxide in 900 ml of water. The pH was adjusted to pH 7.0 with 0.1 M potassium hydroxide, the content was then transferred quantitatively into 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 Schuel (Dassel, Germany).

The washing liquid for sample clean-up was a mixture of 2 mM lithium perchlorate and methanol (97:3; v/v).

During method development, a UV detector was used and set at a wavelength of 230 nm, except for the determination of the elution profile of plasma that was performed at 280 nm. For method validation, the analyte was measured fluorometrically at an excitation wavelength of 235 nm and an emission wavelength of 300 nm.

2.3. Standard solutions

A stock solution of sotalol hydrochloride was prepared by dissolving 10 mg in methanol to obtain a concentration of 1 mg/ml. The solution was stored at 4 °C.

2.3.1. Solutions used for method development

Different diluted standard solutions were made up by diluting 1.0 ml of a stock solution with water to obtain a concentration of 50 μg/ml and were prepared daily.

2.3.2. Plasma solutions

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. The aliquots were spiked with the diluted standard solutions of sotalol and were prepared daily.

2.3.3. Solution used for method validation

2.3.3.1. Solutions used in the pre-validation step. From the stock solution of sotalol, different diluted solutions of 10, 1 and 0.1 μg/ml were prepared in water. Free plasma aliquots were spiked by these solutions to obtain three series (calibration curves), each was composed of six concentration levels as shown in Table 1. One non-biological calibration curve was also performed for the determination of absolute recovery. The first concentration level was close to the expected limit of quantitation (LOQ).

2.3.3.2. Solutions used in the validation step. For the validation step, two kinds of samples were prepared: calibration samples and validation sam-

Table 1
Experiments performed in the prevalidation and validation steps

Levels	Prevalidation step	Validation step	
	Calibration samples (ng/ml)	Calibration samples (ng/ml)	Validation samples (ng/ml)
1	5	5	5
2	10	10	10
3	20	20	50
4	100	100	250
5	250	250	500
6	500	500	–
Series	3	3	3
Replicates	3	2	4
Runs	$6 \times 3 \times 3$	$6 \times 2 \times 3$	$5 \times 4 \times 3$
Days	3	3	3

ples. As shown in Table 1, the calibration samples were the same as those used in prevalidation. For the validation samples, three series were prepared independently, five concentration levels were selected, the lowest been the LOQ. Each validation sample was analysed four times for three different days.

2.4. Automated sample preparation and LC chromatographic separation

2.4.1. Loading and washing step

100 μl of plasma samples were directly injected by the autosampler into the precolumn, with a washing liquid composed of a mixture of 2 mM lithium perchlorate and methanol (97:3; v/v) delivered by pump 1 for 10 min with a flow-rate of 1.0 ml/min.

2.4.2. Transfer step

The switching valve was turned to allow the precolumn to be coupled to the analytical column, the analyte was then desorbed and transferred in back-flush mode to the top of the analytical column for 2 min by the LC mobile phase delivered by pump 2 at a flow-rate of 1.0 ml/min.

2.4.3. Chromatographic separation step

After 2 min, the switching valve was turned to its original position allowing the equilibration of the precolumn with the washing liquid before the next injection. Simultaneously, the analyte transferred to the analytical column was separated and quantified using fluorescence detection.

3. Results and discussion

Since this kind of RAM is made of cation exchange material, the retention of sotalol can be expected to be mainly due to electrostatic interactions with the sulphonic acid groups bonded to the inner surface of the sorbent. This main retention mechanism should give rise to a more selective and efficient sample clean-up compared with that obtained by using more hydrophobic RAM, such as RP-18 ADS precolumns, for which stearoyl moieties have been bonded to the inner surface. Moreover due to the relatively high hydrophilicity of sotalol, it would be difficult to retain this compound sufficiently using a RP-18 ADS precolumn and an ion-pairing agent should be added to the washing liquid to extract it quantitatively from plasma.

3.1. Determination of the breakthrough volume (V_b)

The breakthrough volume corresponded to the beginning of the elution of the analyte from the precolumn. Low breakthrough volumes resulted in an early elution of the analyte and poor clearance of the sample matrix; on the other hand, large breakthrough volumes can be observed due to

strong interactions of the analyte to the inner surface of the precolumn.

The breakthrough volume has been determined elsewhere [19] by visual estimation of the beginning of the elution of the analyte peak using a UV detector set at 230 nm coupled directly to the precolumn. However, the measurement of the breakthrough volume is sometimes difficult.

Therefore, by using the retention parameters of the analyte on the precolumn, the breakthrough volume can be correctly determined according to the following equation [20]:

$$V_b = (1 + k)(1 - 2.3/\sqrt{N})V_M$$

where, V_M is the void volume of the precolumn and N is the number of theoretical plates.

As shown in Fig. 2, the breakthrough volume of sotalol was equal to 20 ml using a washing liquid consisted of a mixture of 2 mM of lithium perchlorate and methanol (97:3; v/v) at a flow-rate of 1.0 ml/min.

3.2. Determination of plasma elution profile

The determination of the time for a complete elution of plasma from the precolumn is very important in order to avoid the introduction of sample matrix into the analytical column after the rotation of the switching valve, which can result in the lack of selectivity and specially a rapid degradation of the analytical column. The elution profile of blank plasma was obtained using the same conditions mentioned above by setting the UV detector at 280 nm because the plasma proteins have maximum UV absorption at this wavelength. As shown in Fig. 3, the protein

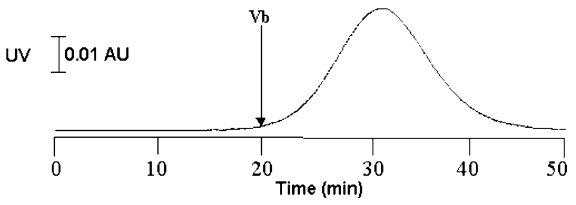

Fig. 2. Typical elution profile of sotalol; samples, aqueous solution of sotalol (50 μg/ml); injection volume, 100 μl; washing liquid, 2 mM lithium perchlorate/MeOH (97:3; v/v); flow-rate, 1.0 ml/min; detection, UV at 230 nm; temperature, 25 °C; V_b, breakthrough volume (ml). Other conditions: see Section 2

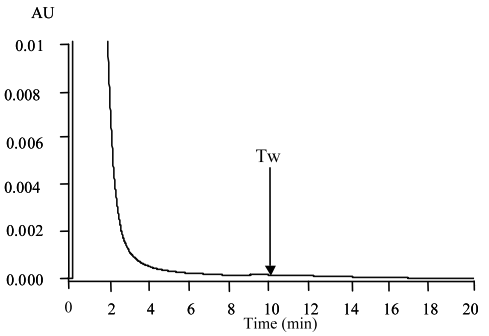

Fig. 3. Typical elution profile of blank plasma; washing liquid, 2 mM lithium perchlorate/MeOH (97:3, v/v); flow-rate, 1.0 ml/min; injection volume, 100 μl; detection, 280 nm; temperature, 25 °C; T_w, washing time (min). Other conditions: see Section 2.

fraction produced a large signal. However, the sample matrix was completely eluted from the precolumn over a period of 10 min as indicated by the return of the signal to the baseline value. Accordingly, the washing time (T_w) was setup at 10 min, which was enough to produce an efficient clean-up of the sample matrix without the elution of the analyte.

3.3. Effect of the washing liquid composition on the breakthrough volume of sotalol

The composition of the washing liquid plays a major role on the analyte recovery and the sample clean-up efficiency. Therefore, an optimal composition for the washing liquid should be specified.

3.3.1. Effect of the nature of the competing ions on the breakthrough volume and the retention factor of sotalol

As expected in ion-exchange chromatography, the competition for the sulphonate groups between the analytes and the co-ions depend on the charge and the size of the individual ions. Different washing liquids with the same concentration of ions were tested. As shown in Table 2, a small breakthrough volume of sotalol was obtained with a solution of magnesium perchlorate due to the greater affinity of magnesium ions for sulphonate groups. On the other hand, when a solution of lithium perchlorate was used as washing liquid a larger breakthrough volume was obtained due to

Table 2
Influence of the nature of the co-ion present in the washing liquid on the breakthrough volume (V_b) and the retention factor (k) of sotalol

Co-ions	Breakthrough volume (V_b)	Retention factor (k)
Lithium	20	106
Sodium	17	81.0
Potassium	15	76.0
Magnesium	0.75	5.0

Washing liquid: 2 mM lithium, sodium, potassium or magnesium perchlorate containing 3% v/v MeOH. Sample, aqueous solution of sotalol; concentration, 50 μg/ml. Flow-rate, 1.0 ml/min; injection volume, 100 μl; detection, UV at 230 nm; temperature, 25 °C. Other conditions: see Section 2.

much weaker competition effects from the co-ion. Since sotalol is sufficiently retained (breakthrough volume of 20 ml) under these conditions, a solution of lithium perchlorate (2 mM) was selected as washing liquid.

3.3.2. The effect of the addition of organic modifier in the washing liquid

In the bioanalytical methods based on the direct injection of biofluids into RAM precolumns, it is essential to incorporate a determined amount of organic modifier (methanol, acetonitrile and 2-propanol) in the washing liquid to enhance the selectivity. However, the proportion of the organic modifier should be limited [21] in order to avoid the precipitation and denaturation of plasma proteins that could lead to the blocking of the pores and a rapid degeneration of the precolumn and the analytical column. In Fig. 4, the retention of sotalol was decreased with increasing content of methanol in the washing liquid. A proportion of 3% (v/v) of methanol can be considered as adequate to enhance method selectivity without giving rise to decrease in analyte recovery.

3.3.3. Effect of the washing liquid pH on the retention of sotalol

Neutral and acidic solutions of sodium perchlorate (5–25 mM) were used as washing liquids. Fig. 5 illustrates the relationship between the reciprocal of the retention factor (1/k) and the concentration of sodium ions. As can be seen, two lines with different slopes were obtained. By using neutral

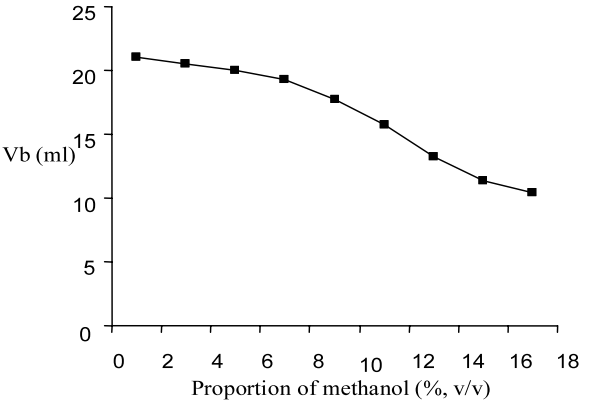

Fig. 4. Effect of the addition of methanol to the washing liquid; washing liquid, 2 mM lithium perchlorate/MeOH; flow-rate, 1.0 ml/min; injection volume, 100 μl; detection, 230 nm; temperature, 25 °C. Other conditions: see Section 2.

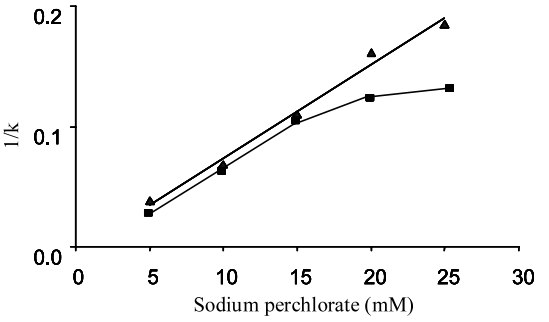

Fig. 5. Effect of the washing liquid pH on the retention of sotalol; Washing liquid:■ solution of sodium perchlorate containing 3% (v/v) of methanol; ▲ solution of sodium perchlorate containing 3% (v/v) of methanol adjusted to pH 3.0 with 1 M perchloric acid; flow-rate, 1.0 ml/min; sample, aqueous solution of sotalol (50 μg/ml); detection, UV at 230 nm; temperature, 25 °C. Other conditions: see Section 2.

solutions, both sulphonic acid groups and residual silanol groups can contribute to the retention of the analyte giving higher retention factors. On the other hand, when the washing liquid was adjusted to pH 3.0 with perchloric acid, smaller retention factors were obtained, this could be due to the absence of the silanol groups effects since they were unionised at this pH range. Therefore, the relationship appears to be linear, which demonstrates that the interaction was mainly due to one kind of anionic groups. Since the objective was to obtain a high retention and a large breakthrough

volume for the analyte, the washing liquid was not adjusted to pH 3.0. Under these conditions, a stronger affinity of sotalol for the cationic sorbent was observed due to the combination of the electrostatic interactions with both sulphonic acid groups and residual silanol groups.

3.4. Optimisation of the composition of the LC mobile phase

Since sotalol was present in the LC mobile phase in ionised form, a low concentration of an ion-pairing agent, such as the anion octanesulphonate, was added to the mobile phase in order to increase the retention of this analyte on the stationary phase. The concentrations of octanesulphonic acid sodium salt were varied from 0.05 to 2 mM. At a concentration of 1 mM, a suitable retention factor ($k = 6.5$) was obtained and the peak asymmetry was equal to 1.1. The use of shorter alkylsulphonates did not improve the separation [12].

3.5. Detection mode

In bioanalytical procedures using UV detection, a low selectivity can be observed, especially at low wavelengths. Unless an efficient sample clean-up procedure using a suitable packing material is developed, a high background is usually obtained which leads to a major decrease in method sensitivity. Consequently, in order to improve method specificity and to increase sensitivity and due to the native fluorescence of sotalol, the method was validated using fluorescence detector monitored at 235 and 300 nm as excitation and emission wavelengths, respectively.

3.6. On-line determination of sotalol by coupling of the XDSc precolumn to the analytical column

The optimised conditions for the washing liquid as well as the chromatographic separation were tested by on-line coupling of XDSc precolumn to the LC column by means of column switching system. At a concentration level of 50 ng/ml, the mean recovery was 96% (± 1.1, $n = 6$).

Since the extraction was almost complete, the use of an internal standard in this method was not considered. Fig. 6 illustrates typical chromatograms obtained after the analysis of blank plasma and spiked plasma samples (concentration of sotalol 50 ng/ml) using the optimal operating conditions.

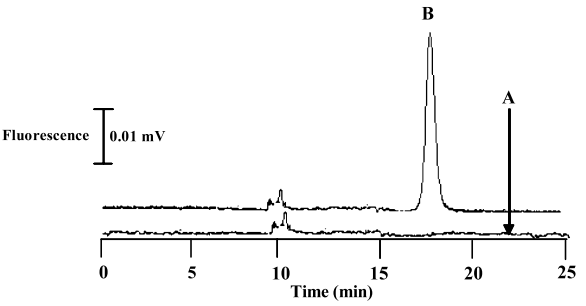

Fig. 6. Typical chromatogram obtained after on-line coupling of the cation exchange precolumn to LC column for the on-line determination of sotalol in plasma. (A) Chromatogram of a blank plasma sample; (B) chromatogram of a plasma sample spiked with sotalol; concentration, 50 ng/ml. Operating conditions given in Section 2.

3.7. Method validation

The method has been validated according to the strategy for the validation of bioanalytical methods proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [22]. The validation protocol comprises two steps: the prevalidation and the validation steps.

3.7.1. Determination of method specificity

The aim is to demonstrate that there is no interference in the chromatographic region of the analyte, by injecting blank plasma samples from six different batches into the LC-integrated coupled sample clean-up system using the optimised operating conditions. As shown in Fig. 6, no interference was observed at the retention time of the peak corresponding to sotalol, which demonstrates the selectivity of this method.

3.7.2. The prevalidation step

After the method specificity is tested, and in order to obtain consistent results in terms of precision and accuracy, the prevalidation step is

required to obtain a formal validation protocol. Therefore, in the prevalidation step, the selection of the most appropriate calibration curve model that describes the relationship between the response (peak area) and the concentration of the analyte, the estimation of the LOQ and the determination of the absolute recovery were performed.

3.7.2.1. Selection of the regression model. Four different regression models were tested: linear regression passing through 0, weighted linear regression, linear regression after logarithmic transformation and quadratic regression. In order to select an adequate regression model the approach based on the accuracy profile was applied. The estimated concentrations from the calibration samples were first back-calculated by means of each regression model. The mean bias as well as its 90% upper and lower confidence limits (CL) by using the standard deviation for intermediate precision were then computed for each concentration level and the acceptance limits were settled at $\pm 20\%$ in order to obtain an accuracy profile.

As shown in Fig. 7, the weighted linear regression model (weighting factor = 2) was selected and considered adequate, where the confidence limits were included within the acceptance limits whatever the concentration level. The equation of a typical regression line was $y = 193.3x + 104.3$, with a coefficient of determination (r^2) equal to 0.9996 and the linear range was comprised between 5 and 500 ng/ml, the estimated LOQ being 5 ng/ml.

3.7.2.2. Determination of the absolute recovery. The absolute recovery of the analyte at different concentration levels was then calculated from the ratio of the response measured for spiked samples treated according to the whole analytical procedure to those of non-biological samples directly injected into the chromatographic system. Table 3 gives the analyte recoveries at four concentration levels covering the entire range. The mean recoveries were close to 100% and the extraction efficiency was constant over the considered range according to the relative standard deviation values obtained.

Table 3
Determination of absolute recovery of sotalol from plasma

Concentration (ng/ml)	Absolute recovery	
	Mean (%), $n = 9$	RSD (%) $n = 9$
10	94.2	1.5
50	96.6	1.2
250	94.7	1.3
500	98.2	0.9

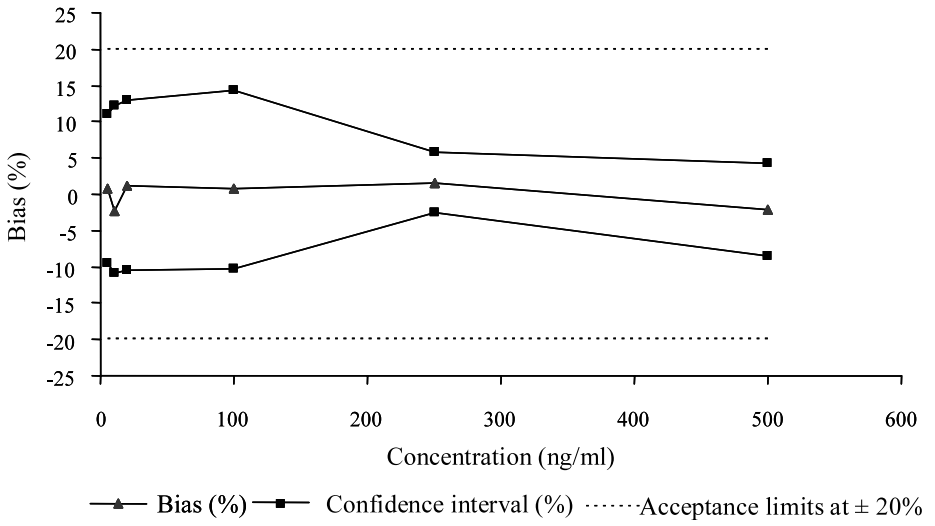

Fig. 7. Prevalidation step: accuracy profile obtained by applying the weighted linear regression model for calibration.

3.7.3. The validation step

The verification of the response function, the confirmation of the LOQ, as well as the determination of method accuracy and precision, were performed in the validation step. Fig. 8, illustrates the accuracy profile obtained from the validation samples by using the weighted linear regression model (weighting factor = 2) for calibration. According to the acceptance limits of ±20 (%) which are specified for bioanalytical procedures, method accuracy was demonstrated over the concentration range from 5 to 500 ng/ml. The LOQ estimated at 5 ng/ml in the prevalidation step was also confirmed and the LOD was less than 1 ng/ml.

3.7.3.1. Determination of method truness and precision. Moreover, as can be seen in Table 4, the mean bias was close to zero for each concentration level of the validation samples, which demonstrates the method trueness. No systematic error was observed. The relative standard deviation values were less than 4.0% for repeatability and 7.0% for intermediate precision and illustrate the very good precision of the proposal method.

3.8. Precolumn stability under long use

The stability of the cation exchange restricted access sorbent was found excellent. By coupling to the analytical column, 80 ml of untreated plasma samples (injection volume: 100 μl) were directly injected without decrease in the precolumn efficiency. The Peek filter was washed after 50 injections. Under these conditions, the precolumn back pressure never exceeded 6 bar during the whole analysis period.

4. Conclusion

A rapid, sensitive and selective fully automated bio-analytical method has been developed for the LC determination of sotalol in human plasma. The full automation of the method was performed by coupling a precolumn packed with cation exchange RAM to the analytical column by means of column switching technique.

The developed method was fully validated. The analyte recovery was almost total and an adequate regression model was selected for validation. The method trueness, precision and accuracy were determined over the working concentration range and a low LOQ was reached.

By using fluorescence detection, method selectivity and sensitivity were improved compared to the methods reported in the literature.

The method is applicable in pharmacokinetic studies and routine analysis due to the full automation and direct introduction of the sample into the LC-integrated system. The total analysis time was only 19 min.

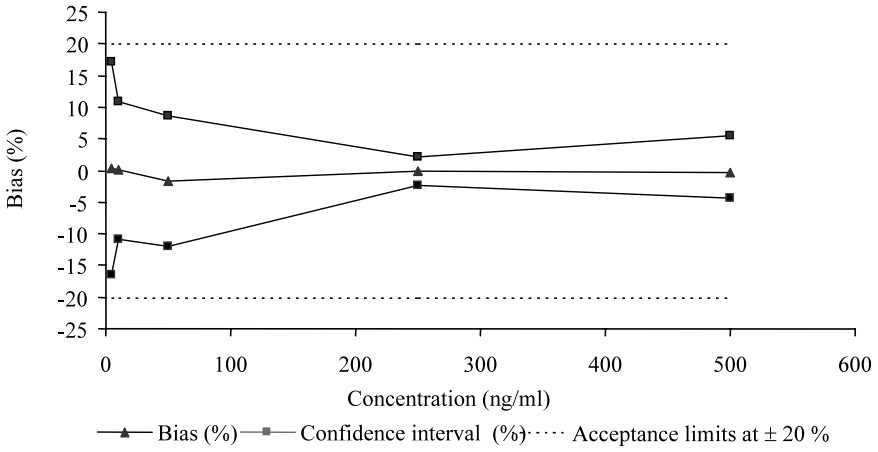

Fig. 8. Validation step: determination of method accuracy.

Table 4
Determination of method trueness and precision

Trueness		Precision	
Concentration (ng/ml)	Bias (%), $n = 12$	Repeatability RSD (%), $n = 12$	Intermediate precision RSD (%), $n = 4 \times 3$ days
5	0.36	4.0	6.9
10	0.07	1.4	3.7
50	−1.65	1.0	3.1
250	0.09	1.1	1.2
500	−0.04	0.5	1.4

The RAM with cation exchange properties has demonstrated its long use stability for the direct injection of large plasma volumes. More than 80 ml of untreated plasma have been introduced directly without loss of efficiency and increasing of the precolumn back pressure, which could be due to the sulphonate moieties attached to the inner surface of the packing material and the presence of diol groups on its outer surface.

This kind of RAM has proved its usefulness for the retention of hydrophilic and ionised analytes, which are usually, retained with difficulty by conventional extraction supports.

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