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Automated liquid chromatographic determination of atenolol in plasma using dialysis and trace enrichment on a cation-exchange precolumn for sample handling

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Abstract

A fully automated method involving dialysis combined with trace enrichment was developed for the liquid chromatographic (LC) determination of atenolol, a hydrophilic β-blocking agent, in human plasma. The plasma samples were dialysed on a cellulose acetate membrane and the dialysate was reconcentrated on a short trace enrichment column (TEC) packed with a strong cation-exchange material. All sample handling operations can be executed automatically by a sample processor (ASTED system). After TEC conditioning, the plasma sample, to which the internal standard (sotalol, another hydrophilic β-blocker) was automatically added, was introduced in the donor channel and dialysed in the static/pulsed mode. The dialysis liquid consisted of 4.3 m*M* phosphoric acid. When the dialysis process was discontinued, the analytes were eluted from the TEC in the back-flush mode by the LC mobile phase and transferred to the analytical column, packed with octyl silica. The LC mobile phase consisted of phosphate buffer, pH 7.0–methanol (81:19; v/v) with 1-octanesulfonate. Atenolol and the internal standard were monitored photometrically at 225 nm. The different parameters influencing the dialysis and trace enrichment processes were optimised with respect to analyte recovery. The influence of two different kinds of cation-exchange material on analyte recovery and peak efficiency was also studied. The method was then validated in the concentration range 25–1000 ng/ml. The mean recovery for atenolol was 65% and the limit of quantitation was 25 ng/ml.

Keywords: Dialysis; Trace enrichment; Atenolol

1. Introduction

Atenolol, [4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetamide] (Fig. 1), is a cardioselective β_1-adrenergic receptor blocking agent prescribed for the treatment of hypertension, angina pectoris and cardiac arrhythmias [1].

A few gas chromatographic methods have been described for the determination of atenolol in plasma [2,3]. Owing to the rather high hydrophilic character of atenolol (log $P=0.23$ [4,5]), liquid chromatography (LC) is a simpler and faster technique, since the derivatization step can be avoided. Several LC procedures for the quantitative analysis of atenolol in human plasma have been reported [6–18]. Due to the native fluorescence properties of atenolol, detection was often achieved by fluorescence [6–14]. However, in some LC methods, UV detection was also used [15–17].

In these methods, various sample preparation procedures were employed: liquid–liquid extraction [12] after alkalisation [7,9–11], possibly followed by

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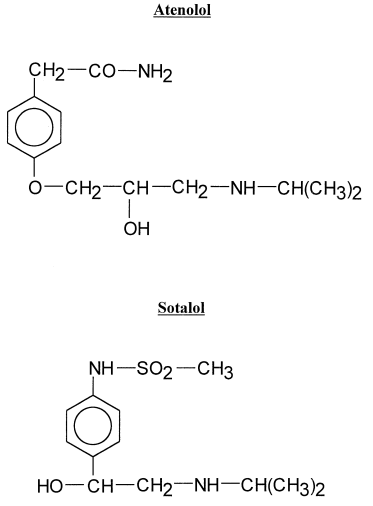

Fig. 1. Structures of atenolol and sotalol.

a back-extraction in an acidic medium [8,13]; solid-phase extraction [6,15,16] or ultrafiltration [17]. However, these off-line procedures are unsuitable when a large number of samples have to be handled. Such problems can be avoided by the automation of the sample preparation. An automated LC procedure has recently been developed for the determination of atenolol in biofluids [14]. This method is based on a column-switching technique using a precolumn packed with a biocompatible material.

Dialysis is another alternative for automated sample pretreatment, which leads to the removal of proteins and other macromolecular components from biological samples. A sample processor, such as the ASTED (automated sequential trace enrichment of dialysates) system, equipped with a dialysis membrane and with a trace enrichment precolumn (TEC), has been shown to be useful for the determination of drugs in biological fluids, especially plasma [18–31].

The on-line coupling of dialysis to LC has already been applied to the determination of atenolol in plasma, by using a TEC packed with octadecyl silica [18]. However, the selectivity of this method was not really satisfactory.

The purpose of this work was to develop an automated procedure for the LC determination of atenolol in human plasma using dialysis and the enrichment of the dialysate on a precolumn packed with a strong cation-exchange material as sample pretreatment. We have already developed a method for the LC determination of sotalol (see Fig. 1), another hydrophilic β-blocker, in plasma, based on the same handling technique [31]. In this method, atenolol was selected as internal standard (I.S.). However, the recovery of the latter compound (about 40%) was not sufficient to provide a method sufficiently sensitive for its quantification. Therefore, it was necessary to adapt the LC method for the determination of atenolol in plasma in order to obtain a satisfactory recovery for this compound.

In the present study, the effect of different parameters of dialysis and trace enrichment processes on the recoveries of atenolol and sotalol has been studied. The influence of two kinds of cation-exchange material on analyte recovery and peak efficiency has also been considered. The volume and composition of dialysis liquid as well as the dialysis time have been particularly investigated. These parameters have been optimised with respect to analyte recovery, peak efficiency and method selectivity. The whole procedure has been validated and the results of the validation are presented.

2. Experimental

2.1. Chemical and reagents

Atenolol (base) was kindly supplied by Zeneca (Delstelbergen, Belgium) and sotalol hydrochloride was obtained from Sigma (St. Louis, MO, USA). They were used without further purification. Potassium dihydrogenphosphate, potassium hydroxide, phosphoric acid (85%), glacial acetic acid (100%), Triton X-100 and sodium azide were purchased from Merck (Darmstadt, Germany) and were of analytical grade. 1-Octanesulphonic acid sodium salt was obtained from Sigma. Methanol and far UV acetonitrile, both of LC grade, were purchased from Fisher Scientific (Leicestershire, UK). The water used in all experiments was purified by means of a Milli-Q system (Millipore, Bedford, MA, USA).

The analytical column was pre-packed with LiChrospher 60 RP Select B (particle size: 5 μm) (Merck). Another column filled with Alltima C_8 bonded silica (particle size: 5 μm) from Alltech (Deerfield, IL, USA) was also tested.

The studies on the enrichment of the dialysate

were performed with two kinds of strong cation-exchange material. A TEC was laboratory-packed with propylsulfonic acid silica (PRS) (particle size: 50 μm) obtained from International Sorbent Technology (IST) (Mid Glamorgan, UK), while the column containing HEMA (*S*-hydroxyethylmethacrylate-BIO 1000 sulphobutyl) material (particle size: 10 μm) was supplied by Gilson Medical Electronics (Villiers-le-Bel, France).

2.2. Apparatus

The addition of the I.S. and the sample clean-up were carried out automatically by using a Gilson ASTED XL module with on-line combination to the LC system. A schematic representation of the ASTED system has been already published [20,22,25,28,30]. It consists of a XYZ autosampler, two model 401C dilutors equipped with 1-ml syringes, one flat-bed dialyser with donor and acceptor channel volumes of 370 and 650 μl, respectively and two Rheodyne model 7010 six-port switching valves (Berkeley, CA, USA), one of which was fitted with a 100-μl injection loop or with a TEC. The dialyser contained a cellulose acetate membrane (Cuprophan) with a molecular mass (M_r) cut-off of 15 000. The TEC prepacked with the HEMA material (2.5×4.6 mm I.D.) consisted of a titanium tube contained in a stainless steel holder (Gilson), while the other TEC (11×4 mm I.D.) was a stainless steel tube contained in a stainless steel holder from Macherey-Nagel (Düren, Germany).

The chromatographic system was composed of a model 305 pump coupled with a model 805 manometric module (Gilson) and of a model Dynamax UV-1 variable-wavelength UV–visible absorbance detector (Rainin, Woburn, MA, USA).

The analytical column consisted of a LiChroCART column (125×4 mm, I.D.) preceded by a guard column (4×4 mm I.D.) (Merck), both packed with the same stationary phase. The other analytical column tested (150×4.6 mm I.D.) was also preceded by a guard column (7.5×4.6 mm I.D.) contained in an All-guard holder, both from Alltech. These columns were thermostated at 35±0.1°C in a model 20 B/VC Julabo waterbath (Seelbach, Germany).

The 715 HPLC SYSTEM CONTROLLER software loaded on an IBM compatible computer (PC-AT; CPU 80486) and the 722 KEYPAD software were used to control the LC and the ASTED systems, respectively.

2.3. Chromatographic conditions

Unless otherwise stated, the mobile phase consisted of methanol–0.05 *M* phosphate buffer, pH 7.0 (19:81; v/v) and contained 1-octanesulphonic acid sodium salt at a concentration of 1.5 m*M*. Prior to use, the mobile phase was degassed for 15 min in an ultrasonic bath. The chromatographic separation was performed at 35°C using a constant flow-rate of 1.0 ml/min. Atenolol and its I.S. (sotalol) were monitored photometrically at 225 nm.

The 0.05 *M* phosphate buffer (pH 7.0) was prepared in a 1-l beaker by dissolving 6.8 g of potassium dihydrogenphosphate and 3.5 g of potassium hydroxide in 900 ml of water. The pH was adjusted to 7.0 with 0.1 *M* potassium hydroxide. The buffer solution was then transferred quantitatively to a 1-l volumetric flask and water was added to the mark. Before use, the phosphate buffer was passed through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel, Germany).

2.4. Standard solutions

Stock solutions of atenolol and sotalol hydrochloride were prepared in methanol at a concentration of 1.0 mg/ml. Each standard solution was stored in a refrigerator at 4°C when not in use and were prepared once a month.

2.4.1. Solutions used for method development

During method development, a mixed solution of atenolol and sotalol was prepared by diluting 1.0 ml of each stock solution with water (10 μg/ml for each compound). This intermediate solution was stored in a refrigerator at 4°C and was found to remain stable for at least 1 week.

This solution was then diluted with water or plasma to reach a final concentration of about 500 ng/ml for each analyte. The latter solutions were prepared daily.

2.4.2. Solutions used for method validation

The stock solution of sotalol was first diluted with water to obtain a solution at a concentration of 10 μg/ml. The latter was used as I.S. solution. The stock solution of atenolol was then diluted with water in order to reach concentrations of 10, 1 and 0.5 μg/ml, respectively. These diluted solutions were stored in a refrigerator at 4°C and were prepared daily. They were used to spike plasma samples (0.65 ml) in order to obtain calibration samples at concentrations of 20, 25, 30, 50, 100, 225, 450 and 1000 ng/ml as well as validation samples corresponding to the quality control samples used in routine analysis. The validation samples were prepared in pools with final concentrations of 25, 75, 450 and 850 ng/ml. Individual aliquots of 1 ml were then stored in polypropylene tubes at −20°C.

2.5. Automated sample preparation

After centrifugation of the plasma sample at 4500 rpm for 10 min, a 0.6-ml volume of plasma was manually transferred into a polypropylene vial (0.85 ml) placed on the sample rack of the ASTED system. All other sample handling operations were then executed automatically by the sample processor.

Between each step, the needle was rinsed with 1.0 ml of 4.3 m*M* phosphoric acid (flow-rate: 30 ml/min) and an air-gap volume of 5 μl was generated inside the transfer tubing before pipetting the next solution in order to avoid cross-contamination.

Unless stated otherwise, the automatic sequence of operations was performed in the following way

- TEC conditioning (flow-rate: 2.0 ml/min): the TEC was successively conditioned with 1.0 ml of 4.3 m*M* phosphoric acid containing 0.005% (w/v) of sodium azide and 0.01% (v/v) of Triton X-100 followed by 1.0 ml of the same phosphoric acid solution devoid of additives.
- Addition of the I.S. to the plasma sample: 460 μl of plasma were introduced into a vial placed on the collector rack at a flow-rate of 1.0 ml/min. A 40-μl volume of a solution of sotalol (10 μg/ml) was aspirated by the needle of the first dilutor at a flow-rate of 0.36 ml/min and then dispensed at the same flow-rate in the collector vial. Afterwards, the sample was homogenised by air bubbling (air volume: 0.3 ml; flow-rate: 0.5 ml/min).
- Dialysis: the donor channel of the dialyser was filled with 0.37 ml of the sample at a flow-rate of 1.0 ml/min. During the dialysis process, the sample was kept static while 5.2 ml of the dialysis liquid (4.3 m*M* phosphoric acid) divided into eight successive 0.65-ml pulses were passed through the acceptor channel at a flow-rate of 1.0 ml/min. After dialysis, each pulse was dispensed onto the TEC at the same flow-rate.
- TEC washing (flow-rate: 1.0 ml/min): when dialysis in the static-pulsed mode was discontinued, the TEC was washed with 1.0 ml of water–methanol (95:5, v/v).
- Elution (flow-rate: 1.0 ml/min): by rotation of a switching valve, the analytes were eluted from the TEC to the analytical column in the back-flush mode with the LC mobile phase.
- Washing of the dialyser (flow-rate: 3.0 ml/min): the donor channel was rinsed with 1.0 ml of the 4.3 m*M* phosphoric acid solution containing sodium azide and Triton X-100 followed by 1.0 ml of the same phosphoric acid solution devoid of additives, while the acceptor channel was washed twice with 1.0 ml of 4.3 m*M* phosphoric acid.

The LC analysis of the prepared sample was then performed during the handling of the next sample (concurrent mode).

3. Results and discussion

3.1. Selection of the LC conditions and of the detection mode

First, the chromatographic conditions applied for the determination of sotalol in plasma were tested [31], since atenolol had been selected as I.S. for this method. The analytes were separated on an Alltima C_8 stationary phase by means of a mobile phase consisting of methanol–phosphate buffer, pH 7.0, and containing 1-octanesulfonate at a concentration of 0.6 m*M*. However, under the conditions selected for dialysis, a rapid loss of column efficiency was observed in spite of the frequent replacement of the

guard column, resulting in an insufficient resolution of the two analytes.

Therefore, another stationary phase (60 RP-Select B) designed for the analysis of basic compounds was used. In order to obtain an adequate separation of the two compounds, several mobile phases were tested, as can be seen in Table 1. In order to increase the retention of the analytes on the stationary phase, a low concentration of an ion-pairing agent, such as the anion octanesulphonate, was added to the LC mobile phase. The capacity factor, the symmetry factor as well as the resolution were calculated according to the standard expressions presented in the European Pharmacopoeia [32]. In order to avoid interferences with the front peak in the chromatogram, which is often relatively large, the retention of the analytes should be sufficiently high (capacity factors >4). Taking into account the results presented in Table 1, the best compromise between resolution, retention and peak symmetry was obtained by using a mobile phase consisting of methanol–phosphate buffer, pH 7.0 (19:81, v/v) and containing 1-octanesulfonate at a concentration of 1.5 m*M*.

Although they present native fluorescence properties, the analytes were monitored photometrically at 225 nm [33], i.e. at the wavelength corresponding to the maximum absorption of atenolol under the experimental conditions. Since the peak plasma concentration was about 400 ng/ml after oral administration of 50 mg of atenolol [34], the UV detection mode was found to provide a sufficient sensitivity to determine the main pharmacokinetic parameters.

3.2. Dialysis and trace enrichment

In order to compensate for the dilution of the sample caused by dialysis, a TEC which contained a strong cation-exchange material was introduced into the system as a precolumn. Two kinds of TECs were tested: one packed with a propylsulfonic acid silica (PRS) and the other with a *S*-hydroxyethylmethacrylate-BIO 1000 sulphobutyl (HEMA) phase.

3.2.1. Influence of the volume of dialysis liquid and the kind of TEC on analyte recovery

Fig. 2 shows the changes in analyte recoveries in aqueous and plasma samples caused by altering the total volume of dialysis liquid for atenolol (A) and sotalol (B). By using a dialysis liquid made of 17 m*M* acetic acid (HAc), atenolol and sotalol were positively charged and their retention on the TECs was mainly due to electrostatic interactions with the sulfonyl groups of the phases [31].

The experiments were performed with the two types of cation-exchange material. The analyte recoveries were expressed in terms of relative recoveries (%), calculated by comparing the peak areas obtained after dialysis and trace enrichment with those found by direct injection of aqueous solutions of the analytes on the TEC at the same concentration.

Table 1
Optimisation of the chromatographic conditions for the analysis of atenolol and sotalol[a]

Organic modifier (O.M.)	Buffer pH	O.M. (%)	$O.S.^-$ conc. (m*M*)	Atenolol		Sotalol		R_S
				k'	A_S	k'	A_S	
ACN	5.0	10	1.5	5.5	2.1	6.3	2.0	1.8
	5.0	12	1.5	3.3	2.3	4.0	2.0	2.5
	5.0	12	2.0	3.9	2.5	4.8	2.5	2.2
	6.5	10	2.0	6.3	1.7	7.0	1.5	1.4
	6.5	11	2.0	4.6	1.6	5.4	1.4	1.9
	7.0	11	1.5	5.2	1.6	5.8	1.6	1.5
MeOH	6.5	20	1.5	5.2	1.8	4.1	1.7	2.7
	7.0	19	1.5	6.0	1.7	4.5	1.6	3.4

[a] Mobile phase: phosphate buffer–O.M.+octanesulfonate ($O.S.^-$). Other conditions as given in Experimental. ACN, acetonitrile; MeOH, methanol; k', capacity factor; A_S, symmetry factor; R_S, resolution.

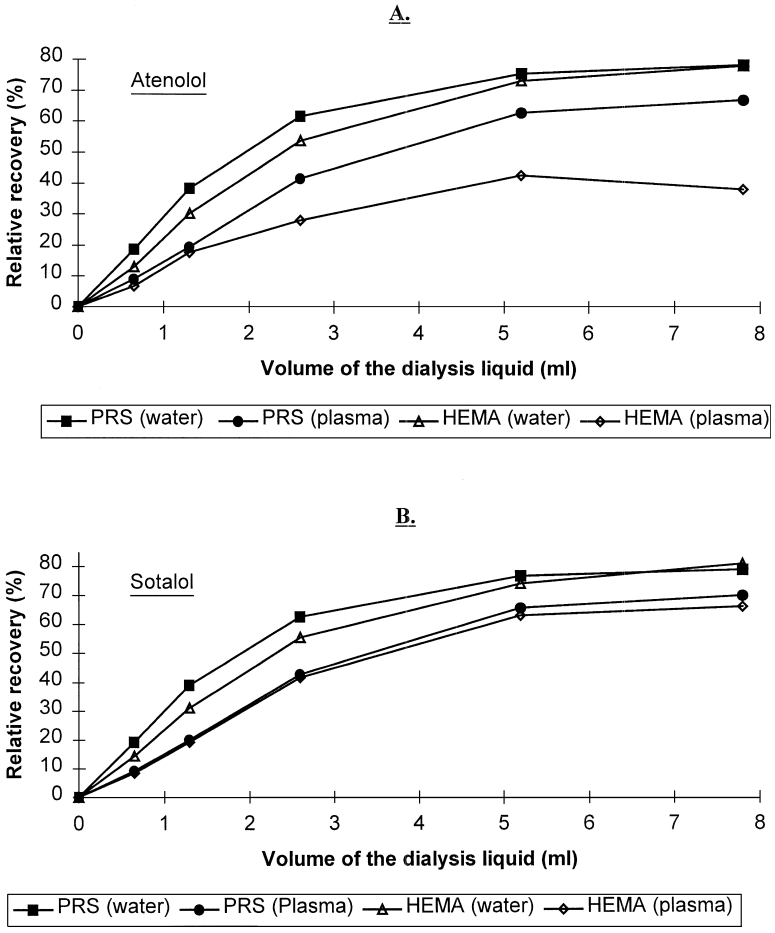

Fig. 2. Influence of the volume of dialysis liquid and the kind of TEC on analyte recovery. (A) Atenolol (B) sotalol. Dialysis liquid: HAc 17 m*M*. TEC conditioning: HAc 17 m*M* (1.0 ml). Number of pulses: 0–12. Aqueous solutions of analytes or spiked plasma samples. Other conditions as given in Experimental.

An increase of the volume of dialysis liquid from 1.3 to 5.2 ml (2–8 pulses) resulted in higher relative recoveries for both compounds. Indeed, by increasing the dialysis time and maintaining a steep concentration gradient across the membrane due to the use of the static/pulsed mode, a relatively high dialysis efficiency could be obtained [21,23,24,28]. The highest recoveries were observed with the PRS phase for the two compounds. However, when using volumes of dialysis liquid $>$5.2 ml (10–12 pulses), no further increase in the analyte recoveries were observed.

Irrespective of the volume of dialysis liquid and the kind of TEC, the relative recoveries obtained in plasma were always lower than those found with aqueous solutions. Since the compounds are very weakly bound to plasma proteins [35,36], this decrease in recovery is certainly related to the presence of relatively high concentrations of inorganic cations in plasma, which can compete with the cationic analytes for the sulfonyl groups on the exchangers [23,31]. Nevertheless, when using volumes of dialysis liquid $>$1.3 ml (4–12 pulses), the recoveries of atenolol were clearly higher with the PRS phase than those obtained with the other cation-exchange material and were comparable to recoveries obtained for sotalol. The higher amount of PRS phase present in the corresponding TEC could probably explain the

higher retention of atenolol on this kind of support. As for sotalol, the use of the PRS phase only led to a slight increase of its recovery.

3.2.2. Effect of the volume of dialysis liquid and the kind of TEC on peak efficiency

Since the particle size was different for the two cation-exchange materials (10 and 50 μm for the HEMA and PRS phases, respectively), Fig. 3 shows, as expected, a decrease in peak efficiency by using the PRS material due to its larger particle size. The peak broadening (%) was calculated by comparing the peak widths at half height obtained after dialysis and trace enrichment with those observed after direct injection of aqueous solutions of the analytes on the TEC at the same concentration.

This figure also shows that an increase of the volume of dialysis liquid gave rise to a lower peak efficiency with the two kinds of TEC. Moreover, the peak broadening was similar for both compounds on the PRS phase, while by using the HEMA material the decrease in peak efficiency was more important for sotalol than for atenolol.

Although the peak broadening of atenolol was more pronounced with the PRS phase, this support was selected, since the recovery of this compound was clearly higher.

3.2.3. Influence of the composition of the dialysis liquid on analyte recovery and peak efficiency

Table 2 gives the relative recoveries obtained for both compounds in water and in plasma when using

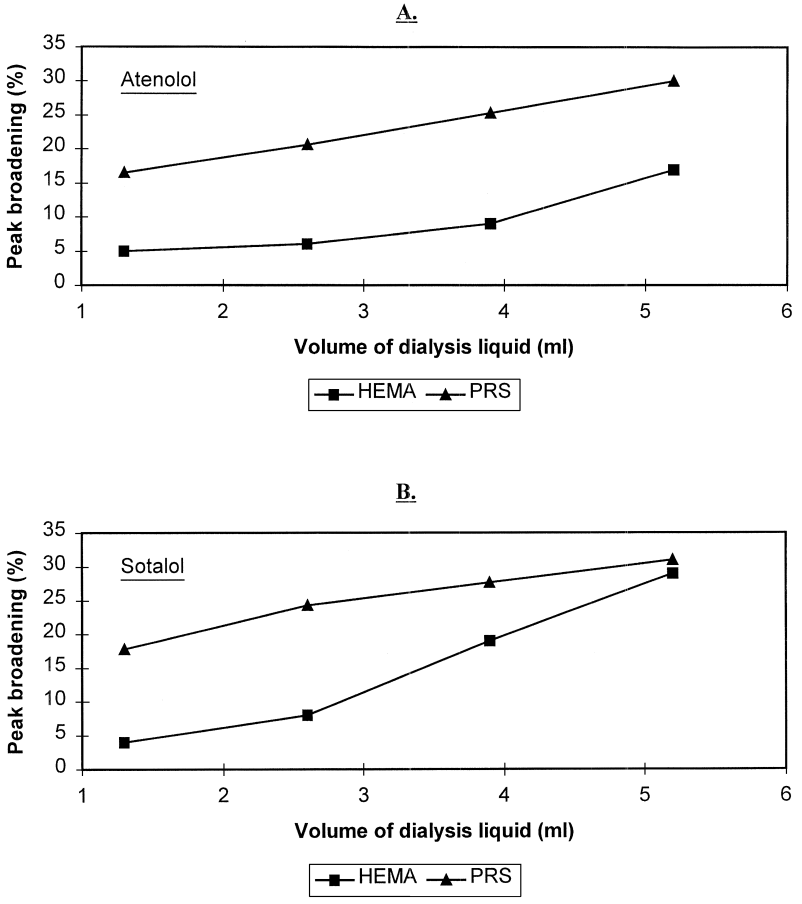

Fig. 3. Influence of the volume of dialysis liquid and the kind of TEC on peak efficiency (A) Atenolol (B) sotalol. Dialysis liquid: HAc 17 m*M*. TEC conditioning: HAc 17 m*M* (1.0 ml). Number of pulses: 2–8. Spiked plasma samples. Other conditions as given in Experimental.

Table 2
Influence of the composition of the dialysis liquid on analyte recovery[a]

Dialysis liquid	Analyte recovery (%, $n=2$)			
	Atenolol		Sotalol	
	Aqueous sample	Plasma sample	Aqueous sample	Plasma Sample
17 m*M* HAc	77.5	62.0	78.6	67.8
8.5 m*M* HAc	78.5	63.6	79.5	66.8
3.4 m*M* HAc	74.5	57.6	76.6	59.4
1.7 m*M* HAc	72.9	52.9	75.2	55.0
8.6 m*M* H_3PO_4	74.2	72.0	74.6	73.1
4.3 m*M* H_3PO_4	78.8	69.7	80.3	72.8

[a] TEC conditioning: with the same liquid as for dialysis; other conditions as given in Experimental.

acetic acid or phosphoric acid solutions at different concentrations as dialysis liquids. In all cases, the recoveries for sotalol were higher than those for atenolol, especially in plasma. With acetic acid solutions as dialysis liquids, the recoveries for both compounds were found to decrease with decreasing concentration of the dialysis liquid. This decrease was more pronounced with plasma samples than with aqueous solutions. On the other hand, with phosphoric acid solutions, a decrease of the concentration resulted in higher recoveries with aqueous solutions, while the analyte recoveries were found to decrease slightly in plasma.

If an increase of the concentration of the dialysis liquid generally led to an improvement in analyte recoveries, peak efficiency was also influenced, as shown in Table 3. Indeed, broader peaks were obtained for both analytes with increasing concentration of the dialysis liquid. This phenomena is probably related to competition effects of H^+ ions with the analytes for the sulfonyl groups of the exchanger.

Table 3
Effect of the composition of the dialysis liquid on peak efficiency[a]

Dialysis liquid	Peak broadening (%, $n=2$)	
	Atenolol	Sotalol
17 m*M* HAc	30	31
8.5 m*M* HAc	19	21
3.4 m*M* HAc	1	1
1.7 m*M* HAc	1	1
8.5 m*M* H_3PO_4	23	21
4.25 m*M* H_3PO_4	20	20

[a] TEC conditioning: with the same liquid as for dialysis. Samples: spiked plasma. Other conditions as given in Experimental.

Considering these results, 4.3 m*M* phosphoric acid was selected as dialysis liquid. Indeed, with this dialysis liquid, the recoveries of both compounds (about 70% in plasma and 80% in water) were relatively high for a sample preparation method based on dialysis. Moreover, peak broadening was limited.

In the present study, the recovery for atenolol was higher than that observed in the method previously published [18]. In addition, method selectivity was improved.

3.2.4. Dependency of analyte recovery on the dialysis time

By using the static-pulsed dialysis mode, an increase of the dialysis volume led to an increase in analyte recovery, if the flow-rates for aspirating the dialysis liquid and for dispensing the dialysate were kept constant (see section 3.2.1.). However, during these experiments, the dialysis time was also increased, as shown in the following equation

$$\text{Dialysis time} = \text{Dialysis volume} \cdot \left(\frac{1}{\text{Aspirating flow-rate}} + \frac{1}{\text{Dispensing flow-rate}} \right)$$

with dialysis time (min); dialysis volume (ml); aspirating and dispensing flow-rates (ml/min).

Under these conditions, the recoveries of the two compounds increased about 30% by altering the dialysis volume from 2.6 to 7.8 ml (Table 4). Moreover, the recovery was slightly higher for sotalol than for atenolol. On the other hand, if the dialysis time was kept constant (10.4 min), the simultaneous increase of the volume and the aspirating flow-rate of the dialysis liquid led to a increase in analyte recoveries of only 15%. Still, the volume of dialysis liquid was varied in the same way as during previous experiments. Moreover, the recoveries of both compounds were nearly identical.

Consequently, these experiments clearly indicate that the dialysis time has a more important influence on analyte recovery than the volume of the dialysis liquid. However, this influence is more pronounced

Table 4
Dependency of analyte recovery on the dialysis time[a]

Dialysis time (min)	Aspirating flow-rate (ml/min)	Volume of dialysis liquid (ml)/ no. of pulses	Analyte recovery (%; $n=2$)	
			Atenolol	Sotalol
5.2	1	2.6/4	41.2	43.5
7.8	1	3.9/6	53.6	56.2
13.0	1	6.5/10	70.6	72.8
15.6	1	7.8/12	70.0	73.6
10.4	0.33	2.6/4	53.2	53.2
10.4	0.6	3.9/6	62.7	63.1
10.4	1.67	6.5/10	67.7	68.0
10.4	3.0	7.8/12	67.6	68.5

[a] Flow-rate for dispensing the dialysate: 1.0 ml/min; samples: spiked plasma; other conditions as given in Experimental.

when the volume is lower. Indeed, the comparison of recoveries obtained for both compounds with volumes of dialysis liquid of 2.6 or 3.9 ml shows that an increase of the dialysis time coupled to a decrease of the aspirating flow-rate leads to a relatively high increase in recoveries for both compounds (about 10%). On the other hand, when the volume of dialysis liquid is higher ($\geq$6.5 ml), the variation of the dialysis time does not seem to be so critical.

To summarise, it is preferable to decrease the aspirating flow-rate in order to increase analyte recovery when the volume of dialysis liquid is relatively low. On the other hand, with a high volume of dialysis liquid, since the influence on analyte recovery is limited, it would be interesting to increase the aspirating flow-rate in order to decrease the dialysis time and therefore the time required for sample preparation.

Experiments of the same kind were carried out by altering the flow-rate for dispensing the dialysate on the TEC in order to change the dialysis time. Similar results were obtained.

3.3. Method validation

The widely recognised criteria commonly used for the validation of bioanalytical methods were analysed by applying a strategy proposed by a Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [37,38]. Validation results for the method developed in this paper were presented in more detail elsewhere in order to demonstrate the applicability of the new strategy [39].

3.3.1. Evaluation of method selectivity towards endogenous components

Selectivity towards interferences from endogenous components present in biological fluids is usually established by analysing a minimum of six independent sources of the same matrix [40]. In the present study, the absence of interfering endogenous components at the retention times of atenolol and the I.S., sotalol, was demonstrated in Fig. 4A–G.

3.3.2. Analysis of the response function

In order to determine the response function, three calibration curves ($p=3$) were constructed in the concentration range 20–1000 ng/ml by selecting seven concentration levels ($m=7$). Each calibration point was analysed in triplicate ($n=3$). The ratio of the peak area for atenolol to the peak area for sotalol (I.S.) was taken as the analytical response. Since the C and F values obtained from the statistical Cochran's and Levene's tests [41] ($C=0.67$; $F=9.51$) were higher than the critical values of the tables at the 5% significance level ($C_{(0.05;\ m,p(n-1))}=0.37$; $F_{(0.05;\ m-1,\ m(pn-1))}=2.27$), the variances were not homogeneous at this significance level. Therefore, the regression model using the least squares method could not be applied. In order to describe the relationship between concentration (x) and response (y), the most appropriate regression model was obtained by performing a 'square root' transformation of the data ($\sqrt{x}-\sqrt{y}$) before the application of the least squares method. With this mathematical transformation, the variances were found to be homogeneous at the 5% level ($C_{\text{calc.}}=0.31$, $<C_{\text{table}}$ and $F_{\text{calc.}}=1.80$, $<F_{\text{table}}$). Moreover, the adequacy

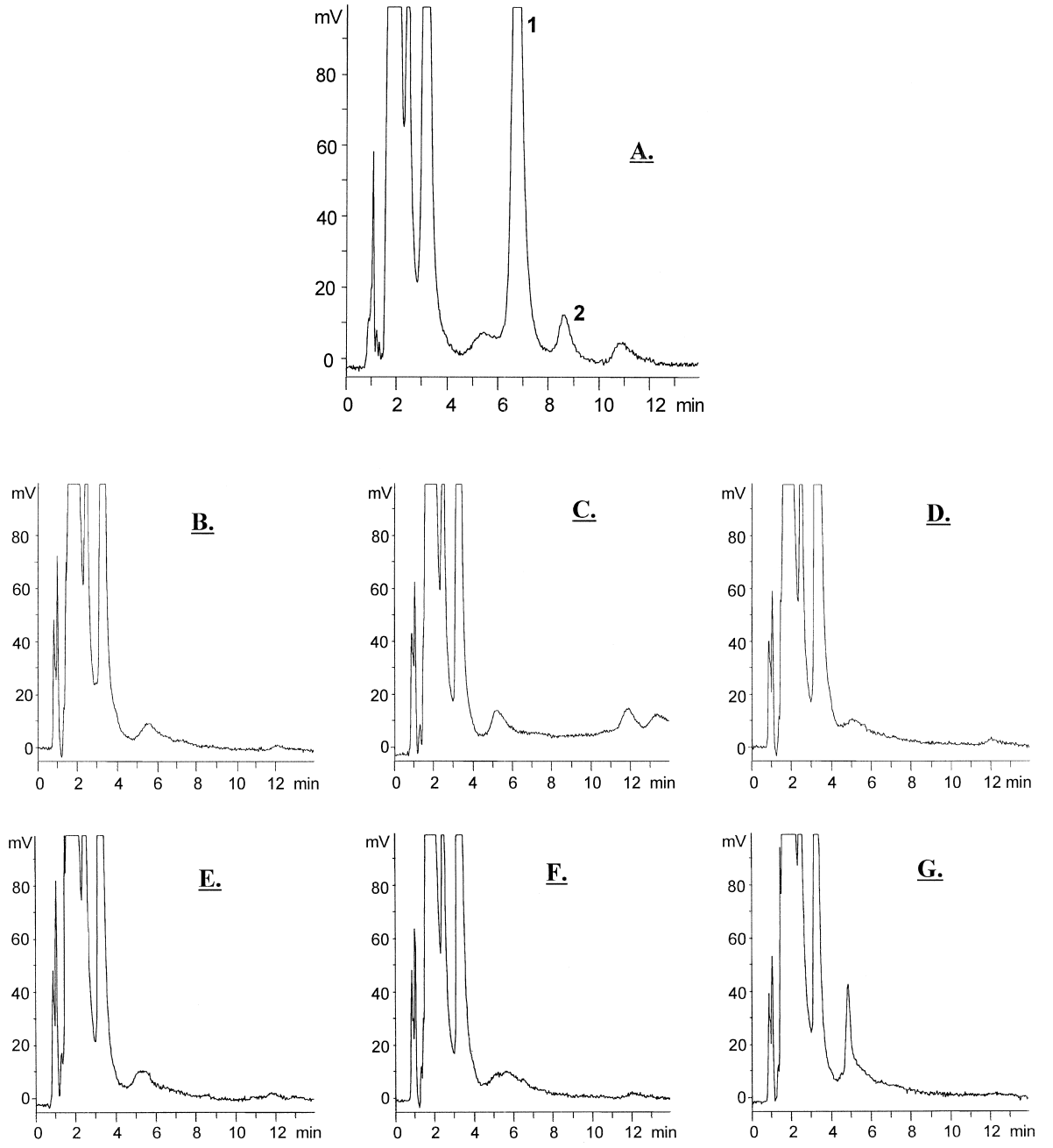

Fig. 4. Typical chromatograms obtained by on-line coupling of dialysis and trace enrichment to LC. (A) chromatogram of a plasma sample spiked with atenolol (75 ng/ml). (B–G) Chromatograms obtained from six different blank plasma samples. Chromatographic and dialysis conditions as given in Experimental. Peaks: 1 = Sotalol (I.S.; concentration 800 ng/ml); 2 = atenolol (concentration 75 ng/ml).

of the linear model was confirmed by a lack of fit test, since the F value obtained for this test ($F=2.4$) was lower than the critical F value of the table ($F_{0.99;\ 15,42}=2.5$) with the corresponding degrees of freedom at the 1% level.

3.3.3. Limit of quantification and determination of the concentration range

As shown in the accuracy profile presented in Fig. 5, the limit of quantitation (LOQ) can easily be estimated by plotting as a function of the analyte concentration the mean recoveries (%) obtained for the responses at each calibration level as well as their one-sided confidence limits at the 95% level by introducing the estimation of the standard deviation for intermediate precision. Indeed, the LOQ corresponds to the concentration for which the confidence limits of the recovery are equal to 120 or 80%. In the present study, the LOQ for atenolol was estimated to be equal to 25 ng/ml. Moreover, the lowest concentration level (20 ng/ml) had to be eliminated from the range since the upper confidence limit exceeded the limit of 120%. Consequently, the calibration range was now between 25 and 1000 ng/ml. As the calibration range had changed, an analysis of the response function was again performed. Under these conditions, the selected regression model was again a linear model with data transformation (square roots). For the three series of analyses ($N=54$), the following equation was obtained for the regression line ($s_{y/x}$ = residual standard deviation)

$$\sqrt{y} = 0.0320\ \sqrt{x} - 0.0697;\ s_{y/x} = 9.98 \cdot 10^{-5}$$

Moreover, the adequacy of the model was confirmed, the F value obtained from the lack of fit test ($F=1.96$) being lower than the critical value of the table at the 1% level ($F_{(0.99;\ 12.36)}=2.72$).

3.3.4. Limit of detection

The limit of detection (LOD) was estimated on the basis of the intercept of the regression line and the

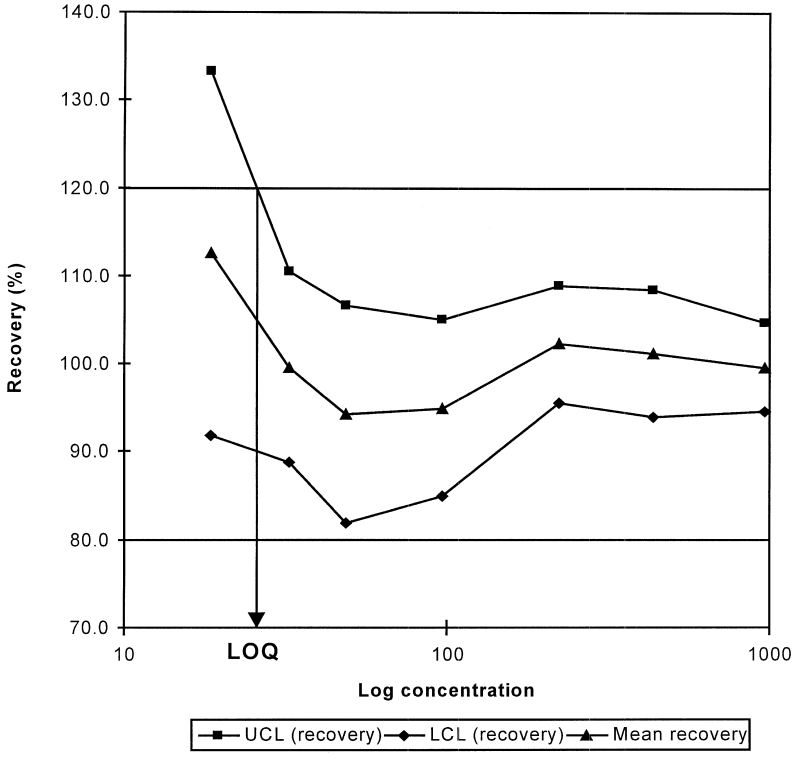

Fig. 5. Accuracy profile for the estimation of the limit of quantification and for the determination of the calibration range.

residual standard deviation ($s_{y/x}$) [42]. By applying this method, the LOD for atenolol was found to be 9 ng/ml.

3.3.5. Determination of the extraction efficiency

The analyte recoveries were determined at six different concentrations (30–1000 ng/ml). The mean recovery was about 65% (RSD: 4.1% ($n=6$)). These recoveries were calculated by comparing the peak areas obtained for atenolol from freshly prepared spiked plasma samples treated according to the described procedure with those found after the direct introduction on the TEC of aqueous standard solutions at the same concentrations. Moreover, a comparison of the peak areas obtained for atenolol after direct introduction on the TEC of aqueous standard solutions with those found by direct injection of the same solutions using an injection loop of 100 μl instead of the TEC demonstrated that the analyte was entirely eluted from the TEC.

3.3.6. Determination of precision and accuracy

For the determination of method precision and accuracy, new calibration curves were constructed in the range 25–1000 ng/ml. Moreover, validation samples corresponding to the quality control (QC) samples used in routine analysis were prepared. Four concentration levels representing the entire range investigated were selected, as can be seen in Table 5. Each validation sample was analysed four times for 3 consecutive days. Table 5 gives the relative standard deviations (RSDs) for repeatability and for time-different intermediate precision obtained at each concentration level. The RSD values are $<5\%$ for the medium concentration of the range and demonstrate the good precision of the described method.

Then, at each concentration level of the validation samples, the one-sided confidence limits of the mean recoveries (%) at the 95% level were computed by introducing the estimation of the standard deviation for intermediate precision. As can be seen in Table 5, the method is accurate, since the different confidence limits of the mean recoveries do not exceed 80 and 120% at the different concentration levels [37,38]. Moreover, the LOQ value estimated to be 25 ng/ml is confirmed, since precision and accuracy are also assessed at this concentration level.

4. Conclusions

An automated method involving dialysis combined with the enrichment of the dialysate on a precolumn packed with a strong cation-exchange material as sample pretreatment was developed for the LC determination of atenolol in human plasma. Sotalol was used as an I.S. Two different kinds of cation-exchange material (HEMA and PRS) were selected. In this study, the influence of the volume and the composition of the dialysis liquid as well as the effect of the type of TEC on analyte recoveries and on peak efficiency were demonstrated. Although the peak broadening of atenolol was more pronounced with the PRS phase, this support was selected, since the recovery of this compound was higher. The dependency of analyte recovery on the dialysis time was also investigated. The method was finally validated and the results of the validation demonstrate that the method is reliable and can be used for the

Table 5
Determination of precision and accuracy[a]

Actual concentration (ng/ml)	Precision (RSD)		Accuracy	
	Repeatability (%)	Intermediate precision (%)	LCL ($R_j^{\%}$)	UCL ($R_j^{\%}$)
25.4	9.0	9.0	83.4	116.5
77.5	7.1	7.8	82.0	109.3
438.4	4.3	4.3	94.0	110.1
839.7	2.7	3.0	97.8	109.4

[a] $R_j^{\%}$, mean recovery (%) at each concentration level j; LCL ($R_j^{\%}$), lower confidence limit at the 95% level of the recovery (%); UCL ($R_j^{\%}$), upper confidence limit at the 95% level of the recovery (%); RSD, relative standard deviation.

determination of atenolol in human plasma for routine analysis.

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