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Development and validation of an automated method for the liquid chromatographic determination of sotalol in plasma using dialysis and trace enrichment on a cation-exchange pre-column as on-line sample preparation

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Abstract

A fully automated method for the determination of sotalol in human plasma was developed, involving dialysis through a cellulose acetate membrane, clean-up and enrichment of the dialysate on a strong cation-exchange pre-column and subsequent liquid chromatographic (LC) analysis with UV detection. All sample handling operations were carried out by means of an ASTED system. Before starting dialysis, the trace enrichment column (TEC) was conditioned. The plasma sample, to which the internal standard (atenolol) was automatically added, was then loaded in the donor channel and was kept static while the dialysis liquid, consisting of 0.017 M acetic acid, was passed through the acceptor channel in successive pulses. After each pulse, the dialysate was dispensed onto the TEC. When dialysis was discontinued, the analytes were eluted from the TEC by the LC mobile phase by rotation of a switching valve and transferred to the analytical column packed with octyl silica. The LC mobile phase was a mixture of methanol and pH 7.0 phosphate buffer containing 1-octanesulfonate at a concentration of 7.5×10^{-4} M (19:81; v/v). The UV detection was performed at 230 nm. The influence of several parameters of the dialysis and trace enrichment processes on analyte recovery and method selectivity was investigated. The method was then validated. The mean absolute recovery for sotalol was about 60%. The limit of quantitation was 25 ng/ml and R.S.D. for repeatability and intermediate precision obtained at a concentration level of 50 ng/ml were 4.3 and 5.8%, respectively.

Keywords: Sotalol; Atenolol; Plasma; Liquid chromatography; Sample preparation; Dialysis; Validation

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1. Introduction

Sotalol, *N*-[4-[1-hydroxy-2-[(1-methylethyl)amino]ethyl]phenyl]methanesulphonamide (Fig. 1), therapeutically used as the monohydrochloride salt, is a non-selective β-adrenoreceptor antagonist without intrinsic sympathomimetic activity. It is indicated as an antiarrhythmic agent for the prevention and treatment of both supraventricular and ventricular tachyarrhythmias [1].

From an analytical point of view, sotalol presents a hydrophilic character (log $P = -0.79$ [2]). Furthermore, the acidic nitrogen of the methanesulphonamide group ($pK_a = 8.3$) and the basic nitrogen of the amino group in the side-chain ($pK_a = 9.8$) yield a zwitterionic character to the molecule [3].

Several LC procedures have been reported for the measurement of sotalol concentrations in biological fluids and especially in human plasma [2–15]. Detection was achieved by UV [3–10] or by fluorescence owing to the native fluorescence properties of sotalol [11–15].

Prior to the chromatographic analysis, the sample preparation techniques consisted of deproteinisation of the plasma samples [11], liquid–liquid extraction after alkalinisation [3–6,12–14], followed by a back extraction in an acidic medium

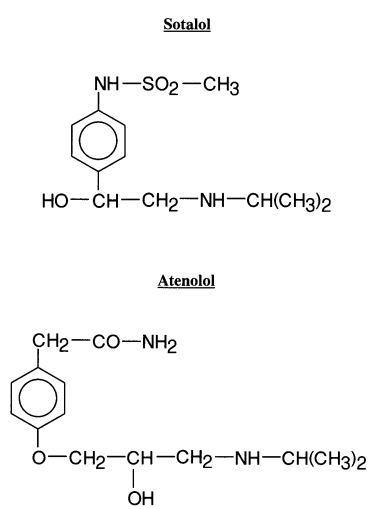

Fig. 1. Structures of sotalol and atenolol.

[3–6,12,13] as well as solid phase extraction on disposable cartridges after deproteinisation [2] or alkalinisation [7–9,15]. These off-line procedures were often performed manually and were, therefore, laborious and time-consuming. When the number of samples to be analysed is particularly large, the automation of sample preparation often becomes a necessity. An on-line automated LC procedure based on a column-switching technique has already been developed for the biodetermination of sotalol [10].

An interesting alternative to this on-line precolumn technique is dialysis, which offers the possibility of removing easily the plasma proteins as well as other macromolecular sample constituents owing to the use of a semi-permeable membrane. If, in addition, a trace enrichment system is incorporated to overcome the dilution of the sample caused by dialysis and to improve method selectivity, an efficient sample clean-up and analyte enrichment can be combined in a fully automated way [16–20].

In the last few years, this on-line dialysis has been successfully applied to the LC determination of several drugs in biological fluids and especially in plasma [16,18,20–30]. The sample preparation was carried out using the ASTED (Automated Sequential Trace Enrichment of Dialysates) system connected on-line with an LC system.

The purpose of this paper is to describe such an automated procedure for the chromatographic determination of sotalol in human plasma. The method applied on-line dialysis, enrichment of the dialysate on a precolumn prepacked with a strong cation-exchange material and subsequent LC analysis using UV detection. For this procedure, atenolol, another hydrophilic β-blocker, was selected as internal standard (Fig. 1). Until now, only a few publications have reported the combination of dialysis with the enrichment of the dialysate on a cation-exchange sorbent [25,30,31]. Therefore, the effect of different parameters of dialysis and trace enrichment processes on the recoveries of sotalol and atenolol was studied. The composition of the sample, the composition and the volume of the dialysis liquid, the volume

of dialysed plasma sample and the dialysis mode were the main parameters investigated. Moreover, the influence on analyte recovery and method selectivity of the addition of an organic solvent to the dialysis liquid as well as the introduction of a washing step after loading the TEC with the dialysate were analysed. The breakthrough volumes of sotalol and atenolol obtained with different dialysis liquids were also determined. These parameters were then optimised with respect to analyte recovery and method selectivity. Finally, the procedure was validated and analytical data are presented.

2. Experimental

2.1. Chemical and reagents

Sotalol hydrochloride was purchased from Sigma (St Louis, MO, USA) and atenolol was kindly supplied by Zeneca (Delstelbergen, Belgium). They were used without further purification. Potassium dihydrogenphosphate, sodium dihydrogenphosphate, sodium hydroxide, potassium hydroxide, phosphoric acid (85%), hydrochloric acid (32%), glacial acetic acid (100%), Triton® X-100 and sodium azide were of analytical grade from Merck (Darmstadt, Germany). 1-Octanesulphonic acid sodium salt was obtained from Sigma. Methanol and acetonitrile, both of LC grade, were purchased from Acros Chimica (Geel, Belgium). The water used in all experiments was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

The analytical column and the guard column were prepacked with Alltima C_8 bonded silica (particle size: 5 μm) from Alltech (Deerfield, IL, USA). The TEC contained 27 mg of *S*-hydroxyethylmethacrylate-BIO 1000 sulphobutyl (HEMA) and was obtained from Gilson Medical Electronics (Villiers-le-Bel, France).

2.2. Apparatus

A Gilson ASTED XL combined on-line with an LC system was used. A schematic representation of the ASTED XL unit has been published previously [28]. It consisted of an auto-sampling injector, two model 401C dilutors equipped with 1-ml syringes, two Rheodyne model 7010 six-port switching valves (Berkeley, CA, USA) and one flat-bed dialyser with donor and acceptor channel volumes of 370 and 650 μl, respectively. The dialysis cell contained a cellulose acetate membrane (Cuprophan) with a molecular mass cut-off of 15 kDa. The TEC prepacked with the HEMA material consisted of a titanium tube (2.5×4.6 mm, i.d.) contained in a stainless steel holder (Gilson) and was connected with the acceptor channel of the dialysis cell or to the analytical column when the valve was switched. The TEC could be replaced by an injection loop of 100 μl.

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

The Alltech stainless-steel analytical column (150×4.6 mm, i.d.) was preceded by an All-guard holder, that contained a short guard column (7.5×4.6 mm, i.d.), 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' and the '722 keypad' software loaded in an IBM compatible computer (PC-AT; CPU 80486) were used to control the LC and the ASTED systems, respectively.

2.3. Chromatographic conditions

All chromatographic experiments were carried out in the isocratic mode, using a mobile phase consisting of a mixture of methanol and 0.05 M potassium phosphate buffer of pH 7.0 containing 1-octanesulphonic acid sodium salt at a concentration of 7.5×10^{-4} M (19:81; v/v). 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 and the analytes were monitored photometrically at 230 nm.

The potassium phosphate buffer pH 7.0 was prepared in a 1000-ml 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 a 0.1 M potassium hydroxide. The buffer solution was then transferred quantitatively to a 1000-ml volumetric flask and water was added to the mark. Before use, the phosphate buffer (pH 7.0) was passed through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel, Germany).

2.4. Standard solutions

Stock solutions of sotalol hydrochloride and atenolol 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 was prepared once a month [15].

2.4.1. Solutions used for the determination of breakthrough volumes

For the determination of breakthrough volumes, 0.2 ml of each stock solution was diluted to a final volume of 200 ml with different solutions (0.017 M or 0.0085 M hydrochloric acid, 0.017 or 0.0085 M phosphoric acid and 0.017 M acetic acid) or with different buffers prepared by dissolving in water phosphoric acid and potassium or sodium hydroxide (0.085 M potassium phosphate buffer of pH 3.0 and 0.017 or 0.0085 M sodium phosphate buffers of pH 3.0).

2.4.2. Solutions used for method development

During method development, a mixed solution of sotalol and atenolol 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 remained stable for at least 1 week.

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

2.4.3. Solutions used for method validation

The methanolic solution of sotalol was diluted with water to obtain concentrations of 10, 1 and 0.5 μg/ml, respectively. These three solutions were prepared each day and were used to spike plasma samples (0.65 ml) for calibration curves (concentration range from 15 or 25 ng/ml to 1000 ng/ml). The stock solution of atenolol was also diluted in water to obtain a final concentration of 10 μg/ml. This latter solution was used as internal standard solution and was made each day.

2.5. Automated sample preparation

After centrifugation of the plasma sample at 4500 rev./min for 10 min, a 0.6-ml volume of plasma was introduced into a polypropylene vial (0.85 ml) placed on the sample rack of the autosampler. The automatic procedure was then started.

Between each step, the needle was rinsed with 1.0 ml of 0.017 M acetic acid (flow-rate: 30 ml/min) and an air-gap volume of 5 μl was generated inside the transfer tubing before pipetting the next liquid in order to avoid cross-contamination.

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

TEC conditioning (flow-rate: 2.0 ml/min): the TEC was successively conditioned with 1.0 ml of 0.017 M acetic acid containing 0.005% (w/v) of sodium azide and 0.01% (v/v) of Triton® X-100 and 1.0 ml of 0.017 M acetic acid devoid of additives.

Addition of the internal standard to the plasma sample: a 40 μl-volume of a solution of atenolol 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 a collector vial. Afterwards, 460 μl of plasma was introduced into the same vial at a flow-rate of 1.0 ml/min. The sample was homogenised by bubbling (air volume: 0.3 ml; flow-rate: 0.5 ml/min).

Dialysis (aspirating and dispensing flow-rates: 2.0 and 1.0 ml/min, respectively): 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 the second dilutor was pumping 5.2 ml of the dialysis liquid (0.017 M acetic acid) through the acceptor channel, divided in eight successive 0.65-ml pulses. After dialysis, each pulse was dispensed onto the TEC.

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 a mixture 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.

Dialyser washing (flow-rate: 3.0 ml/min): the donor channel was successively rinsed with 1.0 ml of the 0.017 M acetic acid solution containing sodium azide and Triton® X-100 and 1.0 ml of the same acetic acid solution devoid of additives, while the acceptor channel was washed twice with 1.0 ml of 0.017 M acetic acid.

After the elution of the analytes, the handling of the next sample was started during the chromatographic analysis of the previous one (concurrent mode).

3. Results and discussion

3.1. Chromatographic conditions and selection of the detection mode

Sotalol and atenolol present a secondary amino group (cf. Fig. 1) and are charged positively during the chromatographic separation. As could be expected with this kind of compound, broad and asymmetrical peaks would be obtained on alkyl-bonded silica, due to interactions with residual silanol groups at the silica surface. Therefore, the chromatographic analysis was performed on a fully endcapped stationary phase (Alltima C_8 bonded silica), which reduces this kind of interaction and excellent results with respect to peak symmetry and efficiency were obtained. Moreover, since the analytes were present in the mobile phase in an ionised form, a low concentration of an ion-pair reagent, such as the anion octanesulphonate, was added to the LC mobile phase in order to increase the retention of the analytes on the stationary phase.

Although the analytes present native fluorescence properties, UV detection was used to determine these compounds in plasma samples. The analytes were monitored photometrically at 230 nm [8,9], i.e. at the wavelength corresponding to the maximum absorption of sotalol under the analysis conditions. Since the oral administration of 80–320 mg sotalol is associated with mean C_{max} values of 0.6–2.8 μg/ml [1], the UV detection mode presents sufficient sensitivity to measure such concentrations in plasma and to determine a pharmacokinetic profile, as shown in previous studies [1,6,7,9].

3.2. Determination of breakthrough volumes

The breakthrough volume of a compound is defined here as the volume of liquid needed for its 50% elution from the TEC [16,28]. When the nature and the amount of sorbent contained in the TEC remains identical, the breakthrough volume of an analyte only depends on the composition of the liquid in which it is dissolved and on the dispensing flow-rate and can be determined using the method described previously [28]. Its determination allows the selection of the most suitable composition for the dialysis liquid and its maximal volume.

Since the HEMA material contained in the TEC is a strong cation exchange phase, the retention of the cationic analytes (sotalol and atenolol) can be expected to be mainly due to electrostatic interactions with the sulfonyl groups of this phase, although apolar van der Waals forces could also occur between the apolar groups of the sorbent and the analytes as well as dipole–dipole interactions [32].

In the adsorption step, both the cation exchange phase and the analytes must have opposite charges. Therefore, the pH of the solution needs to be two pH units below the pK_a value of the analytes. As can be seen in Table 1, the pH of the different liquids selected for the determination of the breakthrough volumes was lower than the pK_a values of sotalol (9.8) and atenolol (9.6). As for the sorbent, since the pK_a of the sulphonic acid group is very low, it was completely ionised under the working conditions.

Table 1 gives the breakthrough volumes obtained with different dialysis liquids. As expected, an increase of the ionic strength of the solutions gave rise to a decrease in the breakthrough vol-

Table 1
Breakthrough volumes of sotalol and atenolol[a]

Dialysis liquid	Breakthrough volume (ml)	
	Atenolol	Sotalol
0.085 M potassium phosphate buffer of pH 3.0	0.5	0.9
0.0085 M potassium phosphate buffer of pH 3.0	2.5	6.5
0.017 M sodium phosphate buffer of pH 3.0	1.6	4.0
0.0085 M sodium phosphate buffer of pH 3.0	3.1	7.6
0.017 M hydrochloric acid (pH 1.8)	2.5	2.7
0.0085 M hydrochloric acid (pH 2.1)	4.4	4.8
0.017 M phosphoric acid (pH 2.1)	3.8	4.4
0.0085 M phosphoric acid (pH 2.3)	6.0	7.0
0.017 M acetic acid (pH 3.5)	62	77

[a] Dispensing flow-rate, 1.0 ml/min; detection, 230 nm. Other conditions given in Section 2.

umes of both analytes, due to competition effects with the co-ions. The higher the concentration of co-ions was, the more important these competition effects were. Lower breakthrough volumes were obtained in the presence of K^+ ions, due to a stronger competition effect from this co-ion, compared to that of Na^+ or H^+ ions. The analytes were thus more rapidly eluted with a potassium phosphate buffer than a sodium phosphate buffer or an acidic concentration at the same concentration. The ordering of the elution power of these inorganic co-ions is in agreement with data quoted in a previous publication reporting the use of cation-exchange extraction cartridges [33]. The results obtained with acidic solutions also show that the breakthrough volumes of the analytes increased with decreasing acidity (higher pH) due to a reduction of the concentration of H^+ ions in the solution. Moreover, the breakthrough volumes of sotalol were in all cases higher than that obtained with atenolol, indicating a stronger affinity of sotalol for the HEMA sorbent.

A solution of acetic acid at a concentration of 0.017 M was finally selected as dialysis liquid in order to guarantee a sufficient retention of the analytes during the enrichment step of the dialysate.

3.3. Dialysis and trace enrichment

3.3.1. Influence of the volume of dialysis liquid and of the composition of the dialysed samples on analyte recovery

Fig. 2A,B shows the variation of analyte recoveries according to the volume of dialysis liquid and the composition of the dialysed samples for sotalol and atenolol, respectively. The analyte recoveries were expressed in terms of relative recoveries (%), calculated by comparing the peak areas

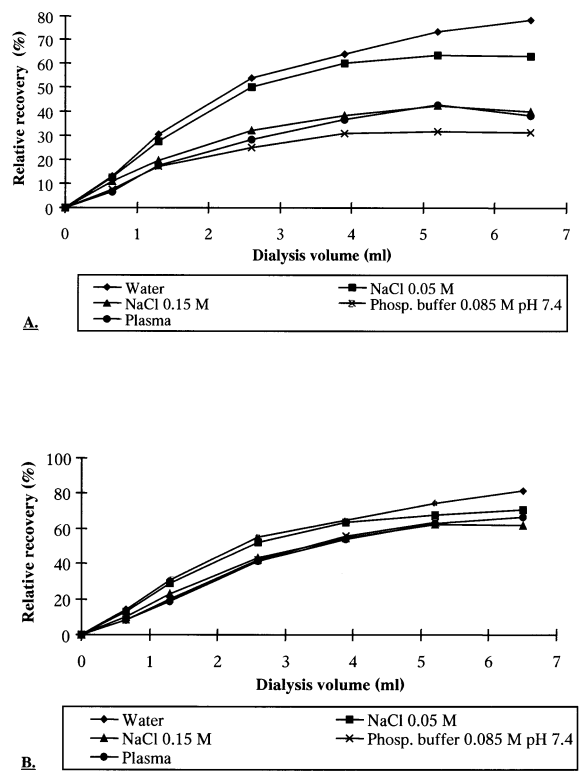

Fig. 2. Evolution of analyte recovery according to the volume of dialysis liquid and the composition of the dialyzed samples. (A) Atenolol; (B) Sotalol; aspirating flow-rate of the dialysis liquid: 1.0 ml/min; number of pulses: from 0 to 10; analytes dissolved in different media (conc., 500 ng/ml). Other conditions given in Section 2.

obtained after dialysis and trace enrichment with those found by direct injection of aqueous solutions of the analytes at the same concentration on the TEC.

An increase of the acceptor volume from 0.65 to 6.5 ml (1–10 pulses) resulted in higher relative recoveries for both analytes irrespective of the sample composition. By increasing the dialysis time and maintaining a steep concentration gradient across the membrane owing to the use of the static/pulsed mode, a relatively high dialysis efficiency was obtained. Maximum recoveries were observed for both analytes when they were dissolved in water. When the sample contained K^+ or Na^+ ions, the relative recoveries were lower irrespective of the dialysis volume. This decrease was more pronounced for atenolol than for sotalol. These results confirm the role of these co-ions in the competition effects with the analytes for the sulfonyl groups on the TEC.

The same experiments were performed with plasma samples spiked with the analytes. The relative recoveries obtained were quite lower than those found with analyte solutions in water. This substantial decrease in recovery is a problem commonly encountered using dialysis for the treatment of plasma samples and is often caused by the binding of the analytes to plasma proteins. However, atenolol is characterised by a very low degree of plasma protein binding (less than 5%) and sotalol is not significantly bound to proteins in plasma [34]. Consequently, they could diffuse easily through the membrane. Moreover, the difference in viscosity between the aqueous and plasma samples did not explain this loss of recovery, because the effect of the higher viscosity of plasma samples on the rate of dialysis has been found to be negligible [18,22]. On the other hand, the decrease of recovery is certainly related to the presence of relatively high concentrations of inorganic cations in plasma. It is considered that the ionic strength of plasma corresponds to a sodium chloride solution at a concentration of about 0.15 M [18]. These ions diffuse through the membrane during the dialysis process and could compete with the analytes for the sulphonic groups of the sorbent. Moreover, the relative recoveries obtained with plasma samples were similar to those observed when the analytes were dissolved in a sodium chloride solution at a concentration of 0.15 M.

Table 2
Influence of the volume of dialysed plasma sample on analyte recovery[a]

Volume of the dialysed plasma sample (μl)	Analyte recovery (%) ($n = 2$)	
	Atenolol	Sotalol
370	42.4	66.8
270	60.4	82.8
185	71.5	89.2

[a] Aspirating flow-rate of the dialysis liquid: 1.0 ml/min; plasma samples spiked with sotalol and atenolol (conc., 500 ng/ml). Other conditions given in Section 2.

3.3.2. Influence of the volume of plasma sample to be dialysed on the analyte recovery

Since the presence of competitive co-ions in plasma gave rise to a decrease of the retention of the analytes on the TEC, it was interesting to investigate if the analyte recovery would increase by reducing the volume of plasma introduced into the dialyser. Therefore, three different plasma volumes (0.37, 0.270 and 0.185 ml) were dialysed. As can be seen in Table 2, the relative recoveries for both analytes increased significantly when the sample volume treated decreased. However, the increase of 30 and 20% observed for atenolol and sotalol, respectively, when a 0.185-ml plasma volume was used, did not compensate the effective decrease of the analyte amount dialysed. If recovery is an important factor to be considered, the amount of analytes of interest introduced into the analytical system is essential in order to obtain an acceptable limit of quantitation.

3.3.3. Effect of the addition of an organic solvent to the dialysis liquid and of the introduction of a washing step on analyte recovery and method selectivity

When the volume of the dialysis liquid was lower than 3.9 ml (six pulses), no interfering endogenous components were observed at the retention times of the analytes. On the other hand,

Table 3
Influence of the addition of an organic solvent to the dialysis liquid on analyte recovery and method selectivity[a]

Solvent	Concentration (%)	Analyte recovery (%) ($n = 2$)	
		Atenolol	Sotalol
Number of pulses: 6 (3.9 ml)			
–	–	37.0	Interference
Methanol	5	37.6	67.0
Methanol	7	35.0	66.5
Methanol	10	29.6	52.5
Acetonitrile	5	28.6	56.6
Number of pulses: 8 (5.2 ml)			
–	–	42.7	Interference
Methanol	5	42.1	Interference
Methanol	7	40.1	Interference
Methanol	10	27.3	53.1
Acetonitrile	5	31.7	66.5

[a] Dialysis liquid, 0.017 M acetic acid containing or not an organic solvent; aspirating flow-rate of the dialysis liquid, 1.0 ml/min; number of pulses, 6 or 8; no washing step of the TEC after loading with the dialysate; plasma samples spiked with sotalol and atenolol (conc., 500 ng/ml). Other conditions given in Section 2.

when the volume of the acceptor solution was changed from 3.9 to 5.2 ml (eight pulses), an interfering peak was observed at the retention time of the sotalol peak (Table 3). In order to eliminate this plasma interference, an organic solvent (methanol or acetonitrile) was added to the acceptor solution at a concentration varying from 5 to 10% (v/v). With a 3.9-ml volume of dialysis liquid, only 5% of methanol was sufficient to eliminate this interference. Higher concentrations of methanol or the addition of acetonitrile gave rise to a loss of the affinity of the analytes for the HEMA sorbent and led to a significant decrease in analyte recovery. When the dialysis was carried out with 5.2 ml of the dialysis liquid, at least 10% of methanol or 5% of acetonitrile were needed to eliminate the interference. Under these conditions, the relative recoveries obtained were however inferior to those observed with a lower concentration of methanol.

Moreover, instead of adding an organic solvent to the dialysis liquid, a washing step was introduced directly after loading the TEC with the dialysate. The washing liquid was mixtures of water or 0.017 M acetic acid with methanol. The concentration of organic solvent was varied from 5 to 10% (v/v). As can be seen from Table 4, with a dialysis volume of 5.2 ml, the interfering endogenous component at the retention time of the sotalol peak was eliminated by the washing of the TEC sorbent at the end of the dialysis. No analyte losses were observed with the three washing liquids tested, the analyte recover-

Table 4
Effect of the TEC washing after loading with the dialysate on analyte recovery and method selectivity[a]

Washing liquid of the TEC	Analyte recovery (%) ($n = 2$)	
	Atenolol	Sotalol
–	42.7	Interference
0.017 M HAc/MeOH (90:10; v/v)	42.8	67.5
0.017 M HAc/MeOH (95:5; v/v)	42.6	67.6
Water/MeOH (95:5; v/v)	42.4	66.8

[a] HAc, acetic acid; MeOH, methanol; aspirating flow-rate of the dialysis liquid, 1.0 ml/min; volume of the washing liquid of the TEC, 1.0 ml; plasma samples spiked with sotalol and atenolol (conc., 500 ng/ml). Other conditions given in Section 2.

ies being similar in all cases. Consequently, in order to improve method selectivity, a washing step with a mixture of water and methanol (95:5; v/v) was incorporated in the procedure directly after loading the TEC with the dialysate.

From the results obtained, it can be concluded that the presence of a low concentration of an organic solvent in the dialysis liquid or the washing of the TEC with a liquid containing a low proportion of methanol were sufficient to remove some interfering impurities that were probably retained on the HEMA material by secondary interactions.

3.3.4. Comparison between the static/pulsed and static/continuous dialysis modes

In the present study, dialysis in the static/pulsed mode was selected. However, other dialysis modes can be used, especially the static/continuous mode, which is also frequently employed for a dialysis procedure.

In order to compare both modes, the effect of the dialysis volume on the recoveries of sotalol and atenolol obtained with aqueous and plasma samples was investigated.

As shown in Fig. 3A,B, the recoveries of the analytes in aqueous solutions increased when the volume of the dialysis liquid was varied from 4.0 to 8.5 ml, irrespective of the dialysis mode. However, the recoveries obtained with the static/continuous mode were lower than those observed with the static/pulsed mode. For an equivalent volume of the dialysis liquid, the dialysis time is greater with the static/pulsed mode than with the static/continuous mode, which facilitates the diffusion of the analytes through the membrane.

The same investigation was then carried out with plasma samples. Fig. 4A,B presents the results of this experiment. As can be seen, the recovery of both analytes was also higher with the static/pulsed mode. However, with this dialysis mode, the recovery of atenolol decreased and that of sotalol seemed to reach a level when the volume of the acceptor solution was varied from 5.2 to 8.5 ml, while the analyte recoveries increased under the same conditions with the static/continuous mode.

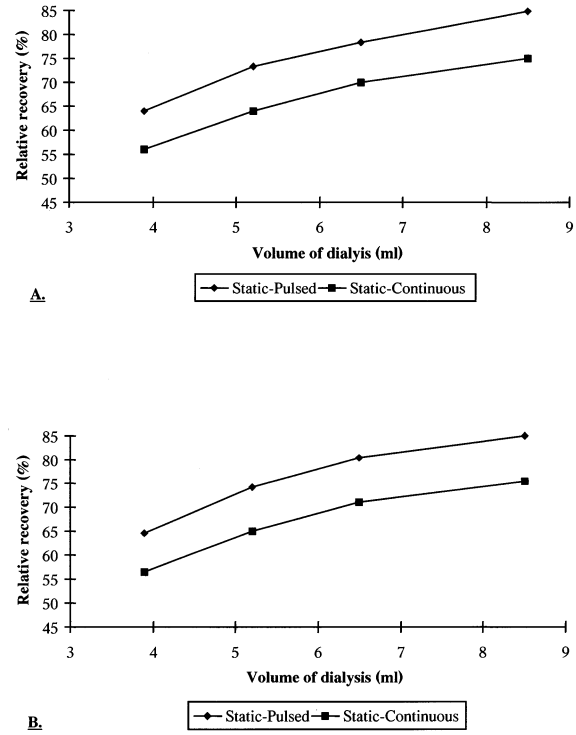

Fig. 3. Influence of the dialysis mode on the recovery of the analytes in aqueous samples; (A) Atenolol; (B) Sotalol; dialysis mode: static-pulsed or static-continuous; number of pulses (static-pulsed): from 6 to 13; volume of pulses (static-pulsed): 650 μl; aqueous samples of both analytes (conc., 500 ng/ml). Other conditions given in Section 2.

3.4. Method validation

The strategy applied for the validation of the determination procedure of sotalol in plasma consisted of a new approach proposed by a commission of the 'Société Française des Sciences et Techniques Pharmaceutiques' (SFSTP) [35,36] in order to obtain good estimates of the validation parameters in accordance with the acceptance criteria defined in a consensus report, known as 'The Guidelines of the Washington Conference' [37]. The validation results were presented in more detail elsewhere as an example of application of this strategy [38]. Taking into account the statistical requirements, the protocol validation developed by the SFSTP commission for bioanalytical methods involves two steps. The experiments achieved during the first step, the so-called preval-

idation step, mainly allow the selection of the most appropriate calibration curve model by means of a decision tree, the estimation of the limits of quantitation and of detection and determination of the extraction efficiency. The second step is the validation itself, involving the evaluation of method selectivity, the confirmation of the calibration model and of the limit(s) of quantitation and the assessment of method precision (repeatability, intermediate precision) and accuracy with respect to the concentration levels of quality control samples over the range investigated.

3.4.1. Method selectivity

Selectivity towards interferences from endogenous components present in biological fluids is usually established by processing a minimum of six independent sources of the same matrix [37]. In the present study, the absence of interfering endogenous components at the retention times of sotalol and atenolol was demonstrated in Fig. 5A–G which shows typical chromatograms obtained after the analysis of a plasma sample spiked with sotalol at a concentration of 50 ng/ml and six independent blank plasma samples.

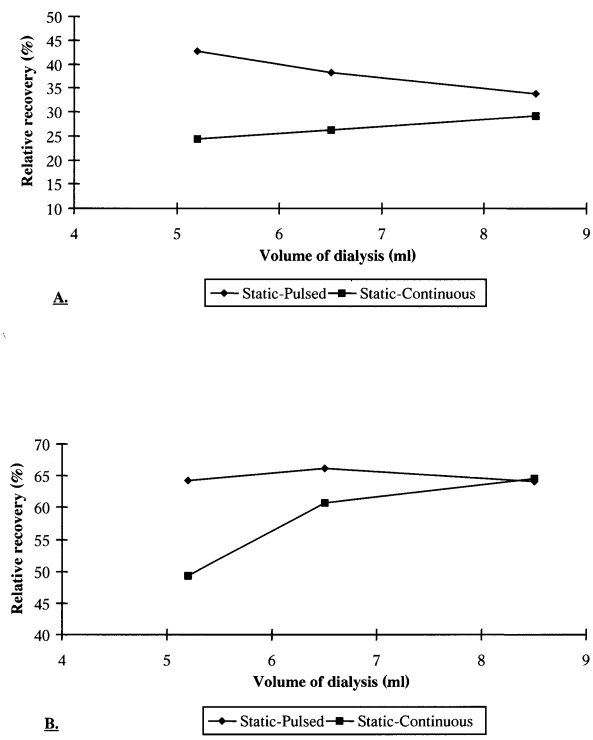

Fig. 4. Influence of the dialysis mode on the recovery of the analytes in plasma samples; (A) Atenolol; (B) Sotalol; dialysis mode: static-pulsed or static-continuous; number of pulses (static-pulsed): from 8 to 13; volume of pulses (static-pulsed): 650 μl; plasma samples containing both analytes (conc., 500 ng/ml). Other conditions given in Section 2.

3.4.2. Analysis of the response function

In the prevalidation step, three calibration curves ($p = 3$) were constructed in the range 15–1000 ng/ml by selecting seven concentration levels ($m = 7$). Each calibration point was analysed in triplicate ($n = 3$). The peak area ratios of sotalol versus atenolol were first calculated. The most appropriate calibration curve model was selected by means of a decision tree [35,36]. Since the results obtained for Cochran's ($C_{\text{calc.}} = 0.7023$, $> C_{(0.05;\ m,p(n-1))} = 0.3726$) and Levene's tests ($F_{\text{calc.}} = 22.6$, $> F_{(0.05;\ m-1,\ m(pn-1))} = 2.27$) demonstrated that the variances were not homogenous at the P-level of 95%, the regression model using the least squares method could not be applied. So, in order to describe the relationship between the responses (y) and the concentrations (x), a transformation of data ($\sqrt{x} - \sqrt{y}$) was carried out and a weighted regression model was selected. As weight, $1/x^{\lambda}$ was chosen, with λ being the slope of the regression line adjusted between the Napierian logarithms of the square root of concentrations and of the square root of the responses variances. Under these conditions, λ was equal to 1.278 and the analysis of the response function for each series gave the following equations (concentration range: 15–1000 ng/ml; $N = 21$; $s_{y/x}$: Residual standard deviation):

Series 1: $\sqrt{y} = 0.04816\ \sqrt{x} - 0.0344$;

$s_{y/x} = 0.0118$;

Series 2: $\sqrt{y} = 0.04659\ \sqrt{x} - 0.0274$;

$s_{y/x} = 0.0183$;

Series 3: $\sqrt{y} = 0.05080\ \sqrt{x} - 0.0641$;

$s_{y/x} = 0.0497$.

3.4.3. Limit of quantitation

As shown in Fig. 6, the limit of quantitation (LOQ) was easily estimated by plotting as a func-

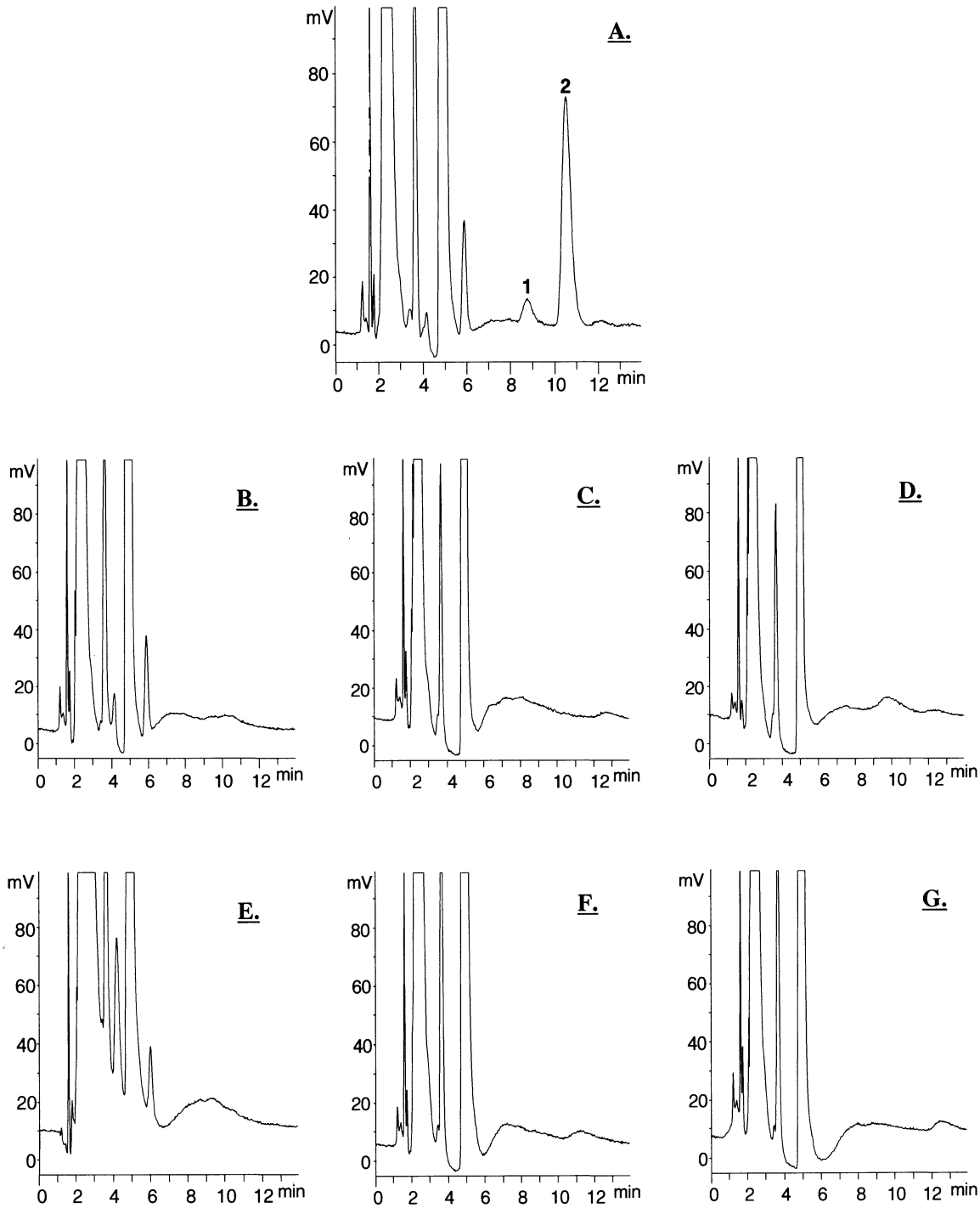

Fig. 5. Typical chromatograms obtained by using dialysis and trace enrichment coupled on-line to LC. (A) chromatogram of a plasma sample spiked with sotalol (50 ng/ml). (B)–(G), chromatograms obtained from six different blank plasma samples. Chromatographic and dialysis conditions given in Section 2. Peaks: 1, Sotalol (conc., 50 ng/ml); 2. Atenolol (IS; conc., 800 ng/ml).

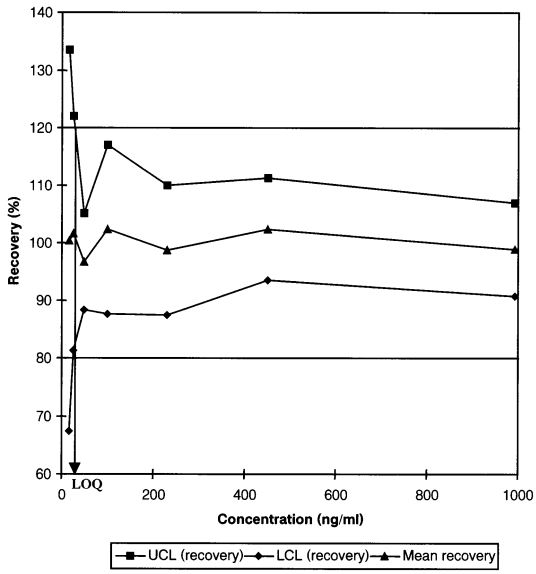

Fig. 6. Accuracy profile for the estimation of the limit of quantitation and of the calibration range.

tion of concentration levels the mean recoveries as well as their one-sided confidence limits at 95% by including the estimation of the S.D. for intermediate precision. Indeed, the LOQ corresponds to the concentration for which the confidence limits of the mean recovery were equal to 120 or 80%. In the present study, the LOQ was 28 ng/ml. For reasons related to the preparation of plasma samples, the LOQ was settled to 25 ng/ml. Moreover, the lowest concentration level equivalent to 15 ng/ml had to be eliminated, the limits of confidence of the mean recovery exceeding widely the limits of 80 and 120%. Consequently, the new calibration range was comprised between 25 and 1000 ng/ml and an analysis of the response function was achieved again since one concentration level was eliminated. Under these conditions, for the three series of determinations, the analysis of the response function gave the following equation by applying a weighted regression model (weight: $1/x^{1.783}$) after transformation of data ($\sqrt{x}-\sqrt{y}$) (concentration range: 25–1000 ng/ml; $N=54$):

$$\sqrt{y} = 0.04855\ \sqrt{x} - 0.04234;\ s_{y/x} = 0.01159$$

In order to test the quality of the fitting (F_2), an analysis of variance (ANOVA) of the regression was carried out. The assessment of the linear model was confirmed at the P-level of 99% ($F_2 = 1.97,\ < F_{(0.01;\ m-2,\ N-m)} = 2.72$).

3.4.4. Limit of detection

The limit of detection (LOD) was estimated on the basis of the intercept and the residual standard deviation of the regression line ($s_{y/x}$) [39]. Under these conditions, the LOD was equal to 2.5 ng/ml.

3.4.5. Absolute and relative recoveries

The relative recoveries of the analyte were determined at six different concentrations ranging from 25 to 1000 ng/ml. The mean relative recovery was 60.4% (R.S.D.: 7.4% ($n=6$)). These relative recoveries were calculated by comparing peak areas for sotalol obtained from freshly prepared 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 concentration. Moreover, by comparison of the peak areas obtained after direct introduction on the TEC of 370 μl of aqueous standard solutions with those found by direct injection of the same solutions using the same autosampler equipped with a sample loop of 100 μl instead of the TEC, it was demonstrated that all the analyte was eluted from the TEC. Indeed, recovery was 98.4% (R.S.D.: 2.6% ($n=6$)). Consequently, the absolute recoveries were equivalent to the relative recoveries.

3.4.6. Precision and accuracy

These two parameters were estimated in the validation step. During this phase, six concentration levels varying from 25 to 1000 ng/ml were selected and each calibration sample was treated in duplicate. Three independent series of analyses were performed for 3 days. The same regression model as that used in the prevalidation step described the relationship between the responses and the concentrations.

Moreover, quality control (QC) samples were prepared; four concentration levels representing the entire range of the calibration were chosen (Table 5). Each QC sample was treated four times and these analyses were performed for 3 days.

Table 5
R.S.D. for repeatability and intermediate precision obtained at each concentration level of quality control samples

Actual concentration (ng/ml)	R.S.D. for repeatability (%)	R.S.D. for intermediate precision (%)
25.4	5.4	5.4
48.2	4.3	5.7
437.8	3.0	3.9
838.6	2.4	3.8

Table 5 shows the R.S.D. for repeatability and for time-different intermediate precision obtained from the interpolated concentrations. The observed values varied from 2.5 to 5.4% for repeatability and from 3.8 to 5.7% for intermediate precision, respectively. At the LOQ, the R.S.D.s for repeatability and intermediate precision were 5.4%.

Then, at each concentration level of QC samples, the mean recoveries as well as their one-sided confidence limits at 95% were calculated by including the estimation of the S.D. for intermediate precision. A method is considered acceptable with respect to precision and accuracy if the confidence limits of the mean recoveries are between 80 and 120% [35,36]. As shown in Fig. 7, the proposed method is precise and exact, since the different limits of confidence of the recovery do not exceed the values of 80 and 120% irrespective of the concentration level. In addition, the LOQ estimated in the prevalidation step was confirmed. Indeed, precision and accuracy were also assessed at this concentration level.

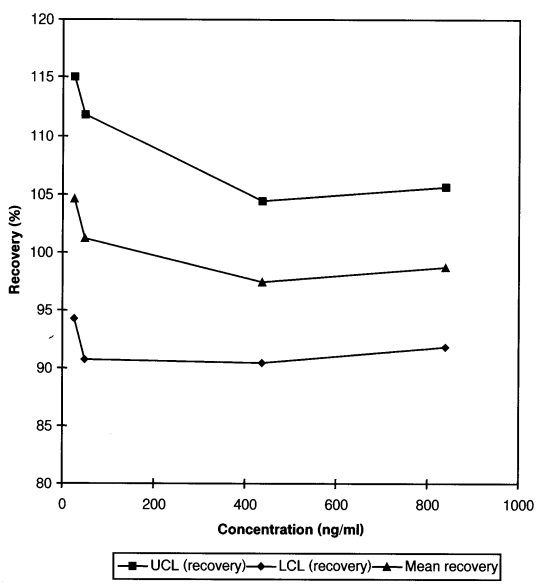

Fig. 7. Accuracy profile for the determination of method accuracy.

Moreover, the overall accuracy of the method was estimated over the range investigated by plotting the mean interpolated concentrations versus the actual concentrations for each series of analyses and by using a least-squares regression model to describe this relationship. Since the upper and the lower limits of confidence at 90% of the mean slope were 1.09 and 0.90, respectively and were comprised between 0.85 and 1.15, the overall accuracy was checked and the linearity was confirmed [40].

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References

[1] A. Fitton, E.M. Sorkin, Drugs 46 (1993) 678–719.
[2] G. Musch, Y. Buelens, D.L. Massart, J. Pharm. Biomed. Anal. 7 (1989) 483–497.
[3] B. Lemmer, T. Ohm, H. Winkler, J. Chromatogr. 309 (1984) 187–192.
[4] S. Käekkäinen, J. Chromatogr. 336 (1984) 313–319.
[5] J.-M. Poirier, P. Jaillon, G. Cheymol, Ther. Drug Monit. 8 (1986) 474–477.
[6] R. Urech, L. Chan, P. Duffy, J. Chromatogr. 534 (1990) 271–278.
[7] G.L. Hoyer, J. Chromatogr. 427 (1988) 181–187.
[8] R. Verbesselt, T.B. Tjandramaga, P.J. De Schepper, Ther. Drug Monit. 13 (1991) 157–165.
[9] S. Läer, J. Neumann, H. Scholz, P. Uebeler, N. Zimmermann, J. Chromatogr. B 681 (1996) 291–298.
[10] R. Herrmann, J. Pharm. Biomed. Anal. 13 (1995) 329–333.

[11] J. Bougaty, G.M. Shenfield, J. Chromatogr. 565 (1991) 523–528.
[12] W.P. Gluth, F. Sörgel, B. Gluth, J. Braun, M. Geldmacher-Mallinckrodt, Arzneim.-Forsch./Drug Res. 38 (1988) 408–411.
[13] R.G. Morris, Ther. Drug Monit. 11 (1989) 63–66.
[14] J.-M. Poirier, M. Lebot, G. Cheymol, J. Chromatogr. 493 (1989) 409–413.
[15] M.J. Bartek, M. Vekshteyn, M.P. Boarman, D.G. Gallo, J. Chromatogr. 421 (1987) 309–318.
[16] J.D.H. Cooper, D.C. Turnell, B. Green, F. Verillon, J. Chromatogr. 456 (1988) 53–69.
[17] F. Verillon, F. Qian, Analusis 19 (1991) 271–277.
[18] N.C. van de Merbel, J.M. Teule, H. Lingeman, U.A.T. Brinkman, J. Pharm. Biomed. Anal. 10 (1992) 225–233.
[19] N.C. van de Merbel, U.A.T. Brinkman, Trends Anal. Chem. 12 (1993) 249–256.
[20] N.C. van de Merbel, J.J. Hageman, U.A.T. Brinkman, J. Chromatogr. 634 (1993) 1–29.
[21] H. Irth, G.J. De Jong, H. Lingeman, U.A.T. Brinkman, Anal. Chim. Acta 236 (1990) 165–172.
[22] T. Agasøster, K.E. Rasmussen, J. Chromatogr. 564 (1991) 171–179.
[23] T. Agasøster, K.E. Rasmussen, J. Chromatogr. 570 (1991) 99–107.
[24] A.T. Andresen, P.B. Jacobsen, K.E. Rasmussen, J. Chromatogr. 575 (1992) 93–99.
[25] A.T. Andresen, M. Krogh, K.E. Rasmussen, J. Chromatogr. 582 (1992) 123–130.
[26] R. Herraes-Hernandez, N.C. van de Merbel, U.A.T. Brinkman, J. Chromatogr. B 666 (1995) 127–137.
[27] K. Johansen, M. Krogh, A.T. Andresen, A.S. Christophersen, G. Lehne, K.E. Rasmusen, J. Chromatogr. B 669 (1995) 281–288.
[28] A. Ceccato, P. Chiap, P. Hubert, B. Toussaint, J. Crommen, J. Chromatogr. A 750 (1996) 351–360.
[29] P. Chiap, B. Evrard, M.A. Bimazubute, P. de Tullio, P. Hubert, L. Delattre, J. Crommen, J. Chromatogr. A 870 (2000) 121–134.
[30] P. Chiap, B. Miralles Buraglia, A. Ceccato, P. Hubert, J. Crommen, J. Chromatogr. B 739 (2000) 205–217.
[31] B. Green, J.D.H. Cooper, D.C. Turnell, Ann. Clin. Biochem. 26 (1989) 361–367.
[32] M. Moors, B. Steenssens, I. Tielemans, D.L. Massart, J. Pharm. Biomed. Anal. 12 (1994) 463–481.
[33] K.C. Van Horne (Ed.), Sorbent Extraction Technology, Analytichem International, Harbor City, p. 105.
[34] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop (Eds.), Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Post-Mortem Material, 2nd edn., Pharmaceutical Press, London, 1986, pp. 362, 971.
[35] E. Chapuzet, N. Mercier, S. Bervoas-Martin, B. Boulanger, P. Chevalier, P. Chiap, D. Grandjean, P. Hubert, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Stp Pharma Prat. 7 (1997) 169–194.
[36] P. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135–148.
[37] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588–592.
[38] E. Chapuzet, N. Mercier, S. Bervoas-Martin, B. Boulanger, P. Chevalier, P. Chiap, D. Grandjean, P. Hubert, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Stp Pharma Prat. 8 (1998) 81–107.
[39] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 3rd edn, Ellis Horwood, New York, 1993.
[40] S.-C. Chow, J.-P. Liu, Statistical design and analysis in pharmaceutical sciences: validation, process controls and stability, in: Statistics Textbooks and Monographs, vol. 143, Marcel Dekker, New York, 1995.