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Knowledge-based system for the automated solid-phase extraction of basic drugs from plasma coupled with their liquid chromatographic determination Application to the biodetermination of β-receptor blocking agents

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Abstract

Techniques for the preparation of biological samples are often based nowadays on solid-phase extraction (SPE). The different SPE steps can be performed automatically on disposable extraction cartridges (DECs) by means of a sample processor. A knowledge-based system was developed to facilitate the development of fully automated methods for the solid-phase extraction of relatively hydrophobic basic drugs from plasma, coupled with their determination by high-performance liquid chromatography (HPLC). The DEC filled with 50 mg of cyanopropyl-bonded silica phase is first conditioned with methanol and buffer solution (pH 7.4). After sample application, the DEC sorbent is washed with the same buffer. The analytes are then desorbed with an appropriate eluent and the eluate is finally diluted with the same buffer as used in the HPLC mobile phase before injection. Under these conditions, only three variables are still to be optimized: the composition and volume of the elution solvent and the volume of buffer to be added to the eluate. On the basis of this general strategy, a decision tree providing information about suggested starting conditions and guidelines for the optimization of the three variables was developed and implemented by use of a hypermedia software. This didactic expert system was evaluated using several β-receptor blocking agents as model compounds and the operating conditions obtained for the automated SPE of these compounds are presented. A method for the determination of propranolol in plasma using the SPE conditions deduced from the knowledge-based system was validated. The absolute recovery of propranolol is *ca.* 93% and the limit of detection is 1.3 ng ml^{-1}. The mean within-day and between-day reproducibilities are 2.3 and 3.6%, respectively.

1. Introduction

When traces of drugs must be determined in complex matrices such as biological fluids, a sample handling procedure is usually needed prior to the HPLC analysis. The aims of the sample pretreatment are the release of the analyte from a conjugate or from proteins in the biological matrix, the elimination of proteins, which can clog the chromatographic column, and

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of endogenous compounds that can interfere in the assay (sample clean-up) and an increase in the concentration of the analyte to reach the detection range of the detector (trace enrichment) [1–3].

In current bioanalytical methods, solid-phase extraction (SPE) is increasingly used for sample preparation instead of traditional methods such as deproteinization or liquid–liquid extraction [3]. With the use of sample processors [4–9] such as the ASPEC (automatic sample preparation with extraction cartridges) system, the determination of drugs in plasma can be fully automated, the SPE on disposable extraction cartridges (DECs) being directly coupled with HPLC [6–8]. This is of particular interest when the number of samples is large. Most often, the use of an automated sample handling procedure leads to better results with respect to accuracy and precision than manual techniques. This holds especially true when the analyses must be performed in the low concentration range (ng ml^{-1}). However, the development of a new application is not straightforward and can be relatively time consuming [9]. For such automated systems, the lack of information about method development probably represents the main limitation.

On the basis of our expertise [8,10–16], a knowledge-based computer system for the development of automated SPE methods was elaborated. The expert system was until now restricted to the isolation of relatively hydrophobic basic drugs from plasma. The first choice for the DEC sorbent is cyanopropyl-bonded silica [10–12,14,16–19] and for the conditioning and washing steps a buffer solution of pH 7.4. After elution of the analyte with an appropriate solvent, the eluate is diluted with the same buffer as used in the HPLC mobile phase. Following this simple scheme, only three parameters are still to be optimized: the composition and volume of the elution solvent and the volume of buffer added to the eluate.

To build such an expert system, a decision tree providing information about suggested starting conditions and guidelines for the optimization of the variables mentioned above was constructed and implemented by use of hypermedia software in which the user is guided to the appropriate information through a series of questions.

For the determination in plasma of the β-adrenoreceptor antagonists chosen as model compounds in this study (Fig. 1), several methods have been proposed using either gas chromatography after derivatization [20–23] or high-performance liquid chromatography (HPLC) [18,19,24–38]. In the HPLC methods, fluorescence detection has often been preferred owing to the native fluorescence properties of most of these compounds [18,24–29,31–34,36]. UV

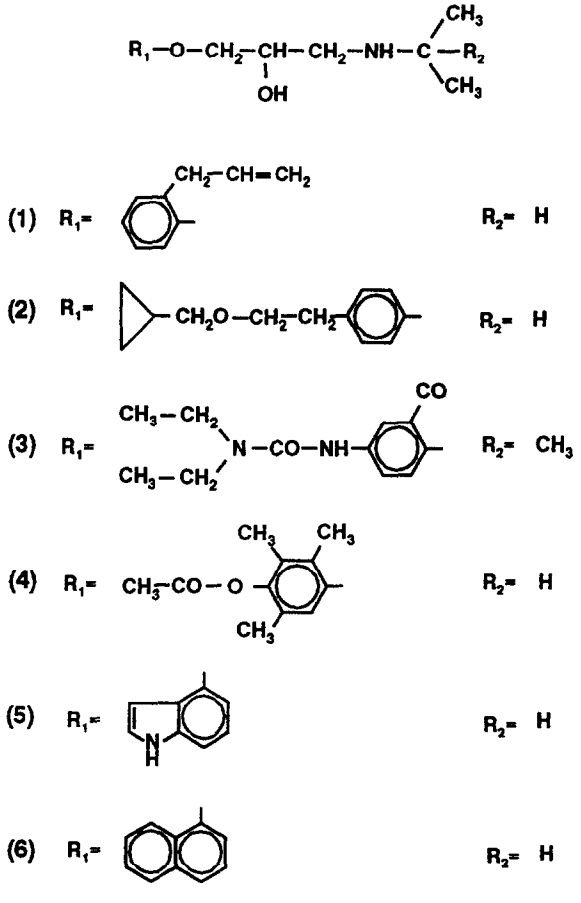

Fig. 1. Structures of the β-blocking receptor drugs investigated. 1 = Alprenolol; 2 = betaxolol; 3 = celiprolol; 4 = metipranolol; 5 = pindolol; 6 = propranolol.

[18,27,30,35,37] and amperometric [38] detection have been used in some instances.

In most bioanalytical methods mentioned above, the sample pretreatment consists of liquid–liquid extraction after alkalinization [29,31,36], followed by back-extraction into an acidic aqueous solution [24,26–28,30,32,33, 35,36]. In some instances, an additional extraction with an organic solvent after increasing the pH of the aqueous solution is performed [20–22]. Sample preparation by deproteinization has also been proposed [25]. The isolation of some β-blocking drugs by SPE, using either an Extrelut-1 column [34] or DECs packed with octadecyl- [23] or cyanopropyl-bonded silica [18], has also been considered. Finally, an HPLC method using SPE in a column-switching system has been described [38]. Except in the last instance, all sample preparation procedures were performed manually.

The principal aim of this work was to test the usefulness of the proposed knowledge-based system for the development of automated methods for the SPE of several β-receptor blocking drugs from plasma, coupled with their HPLC determination. The operating conditions obtained for automated SPE of these compounds are presented. In addition, a fully automated method for the determination of propranolol in plasma using the SPE conditions deduced from the expert system was validated with respect to recovery, linearity, precision and detectability.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Gilson (Villiers-le-Bel, France) Model 305 pump coupled to a Model F-1050 fluorescence detector from Merck–Hitachi (Darmstadt, Germany) and a Spectra 200 UV–Vis programmable wavelength detector from Spectra-Physics (San Jose, CA, USA).

A Manu-CART system, which contained a LiChroCART analytical column (250 × 4 mm I.D.) and a short LiChroCART guard column (4 × 4 mm I.D.) from Merck, was thermostated at 37 ± 0.1°C in a Julabo (Seelbach, Germany) VC 12 B water-bath.

The preparation of plasma samples was performed by use of an ASPEC system [6–8] from Gilson. This system consists of three components: a sample processor equipped with an *XYZ*-motion robotic arm, a Model 401 diluter/pipetter connected to a needle attached to the robotic arm by a transfer tube and a set of racks and accessories for disposable extraction cartridges and solvents. The SPE operations are performed on a specific rack which consists of a DEC holder, a drain cuvette and collection tubes. The DEC holder can be moved in such a way that each extraction cartridge is automatically located above the drain cuvette during the conditioning, loading and washing steps and above a collection tube during the elution step. The collected fraction is then introduced into a 0.25-ml loop of an electrically actuated injection valve for on-line HPLC analysis. The dispensing flow-rates of the different liquids used in the sampling handling procedure can be varied from 0.18 to 96.0 ml min^{-1}. The ASPEC system uses positive air pressure to push the different liquids through the DECs. The latter are covered with a special cap ensuring an air-tight fit while solvents, sample or air are dispensed through the needle.

The control of the HPLC and ASPEC systems and also the data collection were performed by use of an IBM compatible computer (CPU type 80486) equipped with two sets of Gilson software: GME-715 version 1.1. (HPLC System Controller) and GME-718 version 1.1. (Sample Manager). The hypermedia software used for the implementation of the knowledge on automated SPE was Toolbook 1.0 (Asymetrix, Bellevue, WA, USA).

2.2. Chemicals and reagents

Alprenolol hydrochloride was purchased from Sigma (Brussels, Belgium). Propranolol hydrochloride, betaxolol, pindolol, metipranolol and celiprolol hydrochloride were kindly supplied by

different pharmaceutical companies and were used as received.

Potassium dihydrogenphosphate, phosphoric acid (minimum 85%) and sodium hydroxide were of analytical-reagent grade from Merck. 2-Aminoheptane was obtained from Aldrich (Gillingham, Dorset, UK) and was doubly distilled before use [14]. Methanol and acetonitrile were of HPLC grade from Janssen (Geel, Belgium). Water was of Milli-Q quality (Millipore, Bedford, MA, USA).

Bond Elut DECs (1-ml capacity) packed with 50 mg of cyanopropylsilica (CN) with a particle size of 40 μm were used as supplied by Analytichem (Harbor City, CA, USA). When loaded with plasma the DECs were used only once, whereas they could be used several times by application of aqueous standard solutions of the analytes.

The LiChroCART analytical column was packed with Superspher 100 RP-18 (particle size 4 μm) and the LiChroCART guard column was filled with LiChrospher 100 RP-18 (particle size 5 μm) from Merck.

2.3. Chromatographic technique

The HPLC mobile phases were mixtures of 0.05 *M* phosphate buffer (pH 3.0) and acetonitrile or methanol, containing 0.5% (v/v) of 2-aminoheptane. The flow-rate was 1.2 ml min^{-1}. The nature and percentage of organic modifier in the mobile phase and the wavelengths selected for the fluorescence or UV absorbance detection of the different β-blocking drugs are given in Table 1.

The pH 3.0 phosphate buffer was prepared in a 1.0-l volumetric flask by dissolving 4.0 g of sodium hydroxide in 700 ml of water. The pH was adjusted to 3.0 with phosphoric acid (minimum 85%) and water was then added to the mark. The buffer solution was filtered through a nylon filter (0.45 μm) from Schleicher & Schüll (Dassel, Germany).

2.4. Standard solutions

Stock standard solutions of each analyte were prepared once a month in methanol at a concentration of 1.0 mg ml^{-1}. They were then stored in a refrigerator at 4°C. The methanolic solutions were first diluted with water to 10 μg ml^{-1}. The latter solutions were further diluted with water for spiking the plasma samples and with the HPLC mobile phase to measure the recoveries. New dilute solutions were prepared each day. Working standard solutions and spiked plasma samples were prepared in the concentration range 2.5–500 ng ml^{-1}.

2.5. Automatic solid-phase extraction procedure

The same operations as in a classical SPE procedure are performed automatically by use of an ASPEC system. After thawing of the plasma sample, the only manual operations are centrifu-

Table 1
Chromatographic conditions for β-blocking drugs

Analyte	Organic modifier[a] (%)	Fluorescence		UV absorbance: λ (nm)
		λ_{ex} (nm)	λ_{em} (nm)	
Alprenolol	ACN 30	230	300	–
Betaxolol	ACN 30	230	300	–
Celiprolol	ACN 25	350	480	–
Metipranolol	ACN 25	–	–	230
Pindolol	MeOH 30	255	315	–
Propranolol	ACN 30	255	340	–

[a] ACN = Acetonitrile; MeOH = methanol.

gation of the sample at 6000 rpm for 20 min and the introduction of an aliquot (*e.g.*, 2.0 ml) of the latter into a vial located in the appropriate rack of the sample processor. The automatic procedure starts by the washing of the needle and the external tubing of the injection valve with 2.0 ml of water. Between each step, the needle is rinsed with the same volume of water (flow-rate 24 ml min^{-1}) and a 10-mm air gap is generated inside the transfer tubing before the aspiration of other liquids in order to avoid cross-contamination.

The automatic sequence (see Table 2) is performed in the following way:

(i) DEC conditioning (flow-rate 6.0 ml min^{-1}; air volume 0.3 ml). At the beginning of the SPE procedure, the DEC holder is located above the drain cuvette (front position). The sorbent (cyanopropylsilica, 50 mg) is first treated with 1.0 ml of methanol; the excess of methanol is then removed with 1.0 ml of phosphate buffer (pH 7.4) in order to prepare the extraction cartridge for the application of the plasma sample.

(ii) Loading with plasma sample (flow-rate 0.18 ml min^{-1}; air volume 0.3 ml). A 1.0-ml volume of plasma sample is aspirated by the needle from the sample vial and applied on the corresponding extraction cartridge at the minimum dispensing flow-rate [8].

(iii) Washing (flow-rate 1.5 ml min^{-1}; air volume 0.6 ml). The sorbent bed is washed with 1.0 ml of phosphate buffer (pH 7.4).

(iv) Elution (flow-rate 1.5 ml min^{-1}; air volume 0.6 ml). The DEC holder is pushed by the needle above the collection tubes. The analyte is eluted from the sorbent bed with a suitable solvent. Even if another organic modifier is used in the HPLC mobile phase, methanol is selected as a starting elution solvent. The nature and volume of the latter are given in Table 3 for each compound tested. The eluate is collected in the corresponding collection tube positioned under the DEC.

(v) Dilution (flow-rate 1.5 ml min^{-1}; air volume 0.6 ml). A volume of the same buffer as used in the HPLC mobile phase (see Table III) is passed through the cartridge [14]. The DEC holder is then replaced in its front position for the following steps.

(vi) Mixing (flow-rate 1.5 ml min^{-1}). The homogenization of the final extract is performed by aspirating and dispensing it successively in the collection tube. These operations are repeated three times [15].

(vii) Injection. The total volume of the final extract or an aliquot thereof is aspirated from

Table 2
Starting scheme for the SPE of basic drugs from plasma on DEC

SPE step	Liquid	Volume (ml)	Dispensing flow-rate (ml/min)
Conditioning	Methanol	1.00	6.00
	Buffer (pH 7.4)	1.00	6.00
Sample loading	Plasma	1.00	0.18
Washing	Buffer (pH 7.4)	1.00	1.50
Elution	[a]	[a]	1.50
Buffer addition	HPLC buffer	[a]	1.50
Mixing	Plasma extract	[b]	3.00
Filling of the injection loop	Plasma extract	[b]	0.75

DEC: Bond Elut CN (50 mg; 1-ml capacity).
[a] To be optimized.
[b] Depends on the volume of the final extract. For the determination of propranolol in plasma, a 0.65-ml volume was introduced in the loop filler port of the injection valve.

Table 3
Nature and volume of the solvent used in the elution step and volume of buffer added to the organic eluate

Analyte	Elution solvent		HPLC buffer volume (μl)
	Nature[a]	Volume (μl)	
Alprenolol	MeOH + 0.3% AH	300	700
Betaxolol	MeOH + 0.3% AH	300	700
Celiprolol	MeOH + 0.2% AH	250	750
Metipranolol	MeOH	250	750
Pindolol	MeOH + 0.1% AH	300	700
Propranolol[b]	MeOH + 0.3% AH	240	410

HPLC buffer: phosphate buffer (pH 3.0).
[a] MeOH = Methanol; AH = 2-aminoheptane.
[b] Fully optimized experimental conditions.

the collection tube by the needle and introduced in the loop filler port of the injection valve. The excess is directed to waste. By automatic switching of the injection valve, 0.25 ml of the final extract is finally injected on to the analytical column of the HPLC system.

The chromatographic separation of a prepared sample is performed during the preparation of the next sample (concurrent mode).

Phosphate buffer (pH 7.4) was prepared in a 1.0-l volumetric flask by mixing 250 ml of 0.1 *M* potassium dihydrogenphosphate with 195.5 ml of 0.1 *M* sodium hydroxide and diluting to the mark with water. The pH of the buffer solution was controlled before filtration through a nylon filter (0.45 μm) from Schleicher & Schüll.

3. Results and discussion

3.1. General strategy for the development of fully automated bioanalytical methods

The general strategy summarized in Table 4 has been used successfully for developing methods in which automated SPE is coupled to HPLC for the determination of drugs in plasma [8,10–16,39].

The first step is the selection of an appropriate detection mode according to the properties of the analytes and the sensitivity and selectivity required. Fluorescence or electrochemical detection is preferred to UV detection, owing to their higher sensitivity and selectivity, when the analytes have native fluorescence or electroactive properties [40,41]. However, relatively few compounds have such properties and a derivatization step must then be introduced, making the bioanalytical procedure more complicated. For such compounds, UV detection is often a useful alternative, especially when the concentrations to be determined in the biological samples are not lower than 1 ng ml^{-1}.

Table 4
General strategy for the development of bioanalytical methods

(1) Selection of an appropriate detection mode
(2) Selection of appropriate HPLC conditions
(3) Elimination of possible memory effects
(4) Development of the SPE procedure with aqueous samples
(5) Control of method selectivity and analyte recovery with spiked plasma samples
(6) Method validation

The HPLC system is optimized by use of aqueous standard solutions of the analytes. Well documented in the literature [42–44], the selection of suitable HPLC conditions is often relatively straightforward. With basic drugs, the detrimental effects due to interactions of these compounds with the residual silanol groups on silica-based stationary phases can be avoided by use of highly deactivated modified silica [10,11,16,44] and/or by addition of a competing amine to the mobile phase [12,14,16,44]. In a bioanalytical procedure using UV detection, the retention of the analytes should be sufficiently high (capacity factors higher than 3) in order to avoid interferences with the front peak in the chromatogram, which is often relatively large in the high-sensitivity range. The use of a guard column and its frequent replacement are essential to maximize the lifetime of the analytical column [8].

At this stage of development, the presence of possible memory effects must be investigated by performing successive (at least six) direct injections of a standard solution of the analytes with the autosampler [14]. The aqueous buffer used as the dissolution medium is injected immediately afterwards. In the presence of memory effects, poor reproducibilities are often obtained on successive injections of the analytes (R.S.D.s >10%) and residual analyte peaks are generally observed on the blank chromatogram [14,45]. Such effects are due in most instances to the limited solubility of the analytes in the dissolution medium and in the rinsing and/or the washing liquids. These effects can usually be eliminated by adapting the pH of the aqueous buffer used as the dissolution medium or adding a certain percentage of organic modifier (*e.g.*, methanol) to the buffer. The use of phosphate buffer (pH 7.4) as washing liquid was found to be adequate in most instances [10,11,16,39].

After selection of suitable detection and HPLC conditions, the automated SPE procedure using DECs can also be developed by use of aqueous solutions of the analytes instead of spiked plasma samples in order to limit the consumption of DECs [11,14,39,45]. It was found that the DECs could be used several times with aqueous solutions whereas they could be employed only once after loading with plasma. By using the starting scheme presented in Table 2, very few parameters need to be optimized and the whole sample handling procedure can be developed in a relatively short time.

Subsequently, the fully automated SPE procedure coupled with HPLC determination is applied to blank and spiked plasma samples in order to confirm that method selectivity and the analyte recoveries are satisfactory. In most applications developed so far [8,10–16] according to this strategy, good results with respect to selectivity and analyte recovery have been obtained when spiked plasma samples were tested, so that no changes in the operating conditions selected by use of aqueous solutions of the analytes were necessary. However, if interfering peaks and/or a significant decrease in analyte recovery is observed with plasma samples, the SPE or HPLC parameters must be modified accordingly.

Finally, a complete validation of the bioanalytical procedure is performed.

3.2. Automated SPE procedure

Table 2 shows the different steps of a fully automated SPE procedure with DECs: the conditioning of the DEC with methanol and buffer, the application of the plasma sample, the washing step, the elution of the analytes from the cartridge, the addition to the eluate of a small volume of the same buffer as in the HPLC mobile phase, the homogenization of the extract and finally the filling of the injection loop with this extract.

The starting conditions given in Table 2 were found to be suitable in most applications [8,10–16]. The use of 50-mg DECs is particularly interesting as these cartridges can be loaded with 1 ml of plasma like the 100-mg DECs and they give rise to recoveries similar to those of the latter. However, roughly half the volumes are needed for the elution of the analytes from 50-mg DECs. The total volume of plasma extract is then reduced and the fraction of this volume that is injected into the HPLC system is proportionally larger [11,14,16,39].

The conditioning of the DECs is made in two steps. In the first step, the solvation or wetting of the sorbent is performed by passing several bed volumes of a solvent such as methanol through the DEC. Methanol is an effective solvating agent because it can interact with both the silanol groups at the silica surface and the carbon atoms of the bonded alkyl chains. In the second step, the excess of methanol is removed with a solvent similar to the sample solution to be extracted. In this respect, phosphate buffer (pH 7.4) is particularly suitable for preparing the solid phase before the application of the plasma sample.

In the sample loading step, a 1.0-ml plasma sample is applied on the solid phase. The analytes are then adsorbed on the solid phase while the proteins and other hydrophilic endogenous compounds pass through the sorbent bed. In the loading step, an important factor with respect to the analyte recovery is the dispensing flow-rate of the plasma sample on the DEC. As previously reported, while the dispensing flow-rates used in all the other steps of the SPE procedure are not critical, the use of a very low dispensing flow-rate during the application of a plasma sample is essential to obtain high analyte recoveries, especially when the analytes are strongly bound to plasma proteins [8,15,45]. Indeed, at higher dispensing flow-rates, the recoveries of the analytes decrease drastically because the residence time of the plasma sample in the DEC is reduced to such an extent that only part of the analytes is displaced from its binding to proteins and can be distributed to the solid phase. Consequently, the minimum dispensing flow-rate available (0.18 ml min^{-1}) has been systematically selected for the application of plasma in order to obtain sufficiently high recoveries (>90%). Under these conditions, the air volume introduced into the DEC after the sample application has no signifi-

cant influence, because the plasma delivery is so slow that there is virtually no residual volume of plasma at the top of the sorbent when air is dispensed [8,45].

Next, matrix components that might interfere with the analytes are washed from the DEC with a suitable solvent. In this washing step, phosphate buffer (pH 7.4) was selected because of its good clean-up efficiency: very clean chromatograms, devoid of interferences from plasma components, have been obtained in most instances [8,10–16,39]. With 50-mg cartridges, the volume of buffer used in the washing step should be limited to 1.0 ml [11,39]. On the other hand, the addition of methanol to the washing liquid gives rise to a significant decrease in the analyte recovery, especially when DECs packed with cyanopropylsilica are used in the SPE procedure [11,16].

After the elution of the analytes from the DEC with a limited volume of solvent, a volume of the same buffer as used in the HPLC mobile phase is added to the eluate in order to obtain a final extract with an eluting strength equivalent to that of the HPLC mobile phase, or lower if a concentration effect at the top of the HPLC column is wanted. It should be emphasized that when small volumes of solvent are used for the elution of the analytes, a constant volume of eluate, equivalent to the initial volume of solvent dispensed, is only obtained if the buffer to be added is also passed through the DEC [11,14,44].

The final extract is then homogenized by three successive pumping steps [15] and the total volume of this extract is generally introduced into the injection loop, the excess being directed to the waste.

Most SPE applications can be developed according this simple starting scheme. For the isolation of basic drugs from plasma, cyanopropylsilica was found to be the most suitable sorbent with respect to selectivity and analyte recovery [10–12,14,16,17–19]. Under these conditions, only three SPE parameters are still to be optimized: the composition and volume of the eluting solvent and the volume of buffer to be added to the eluate before injection [39,46].

3.3. Optimization scheme for automated solid-phase extraction

According to the starting scheme given in Table 2, 1.0 ml of plasma (or aqueous standard solution) is used in the SPE procedure and, in a first approach, the aim is to obtain the same volume for the final extract. Methanol was selected as starting elution solvent as it was found to give high recoveries in most instances [14,39]. The volumes of methanol and of HPLC buffer to be added to the eluate are then calculated in order to obtain a 1.0-ml volume of final extract with the same eluting strength as that of the HPLC mobile phase.

As can be seen in Fig. 2, once these standard SPE conditions have been settled, the first experiments are carried out and the absolute recovery of the analyte is determined. If the recovery is ≥90%, the selectivity of the auto-

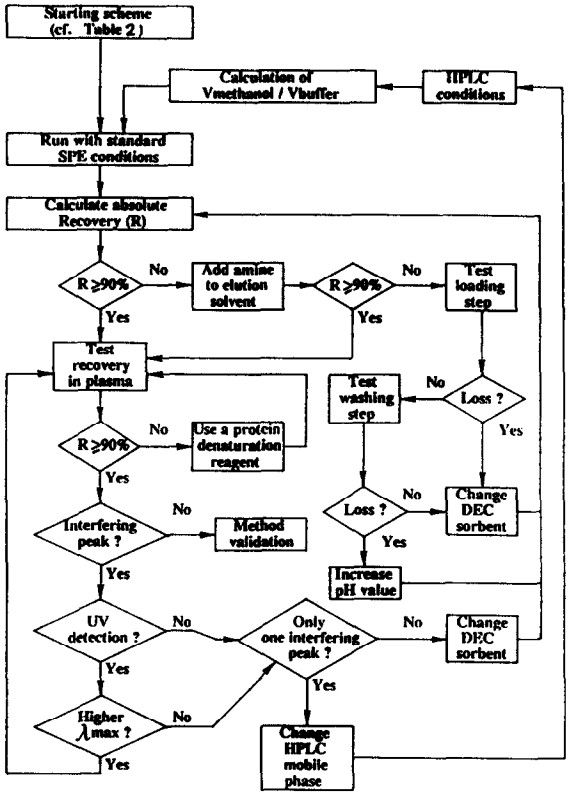

Fig. 2. Optimization scheme for automated SPE.

mated SPE procedure and the analyte recovery can be immediately evaluated with a spiked plasma sample. When the recovery obtained with the standard operating conditions and aqueous solutions of the analyte is not satisfactory (<90%), other options should be chosen.

A low recovery can be caused by several factors: the analyte may be too weakly retained on the solid phase, the analyte may be partially eluted during the washing step or the choice of methanol as elution solvent may be inappropriate. The latter possibility is the first to be investigated (*cf.*, Fig. 2). The primary interaction forces responsible for the retention of basic hydrophobic analytes on the cyanopropylsilica phase are in principle apolar Van der Waals forces, but secondary electrostatic interactions with the residual silanol groups are also very important, giving rise to very high retention and therefore low analyte recoveries in the SPE procedure. In order to minimize interactions with silanol groups, the addition to methanol of a competing amine, *e.g.*, 2-aminoheptane, as in the HPLC mobile phase, at a starting concentration of 0.1% is suggested. Table 5 shows the influence of the addition of this silanol masking agent at different concentrations on the recoveries of the different β-adrenoreceptor antagonists tested. As can be seen, a high extraction efficiency (>90%) was obtained in all instances by selecting a suitable percentage of competing amine to be added to the methanol.

If the addition of a competing amine to the eluent does not improve the recovery, possible losses in the loading and the washing steps are investigated (*cf.*, Fig. 2). Losses in the loading step may occur when the analyte is too weakly bound to the solid phase and it is then suggested that the cyanopropyl-bonded phase be replaced with the less polar phenyl or C_8 phases. When the analyte elutes partially with matrix components during the washing step, it is advisable to increase gradually the pH of the buffer used as the washing liquid. If the low recovery does not seem to be due to one of these three factors, it is concluded that the analyte is too strongly bound to the cyanopropyl-bonded phase and it is suggested that bare silica be used instead.

Once an acceptable SPE method has been developed for aqueous solutions of the analyte, the analyte recovery and the selectivity of the automated SPE method are evaluated with spiked plasma samples. If a decrease in the analyte recovery below 90% is observed with the plasma samples, this can probably be attributed to very strong binding of the analyte to plasma proteins. As shown in Fig. 2, it is then advisable to add a small volume of a concentrated acidic buffer which can displace the protein binding, so that the analyte can be more easily adsorbed on the solid phase.

Subsequently, the chromatogram of the final extract is evaluated with respect to the presence of interfering peaks. If the determination of the

Table 5
Influence of the addition of 2-aminoheptane on the recoveries of β-blocking drugs

Analyte	Recovery (%)[a]			
	MeOH	MeOH +0.1% AH	MeOH +0.2% AH	MeOH +0.3% AH
Alprenolol	79.0	82.0	91.3	95.5
Betaxolol	76.6	79.7	80.9	100.0
Celiprolol	87.0	88.5	97.0	–
Metipranolol	96.0	–	–	–
Pindolol	85.5	99.5	–	–
Propranolol	62.9	68.5	88.6	91.5

Concentration: 100 ng ml^{-1}.
[a] MeOH = Methanol; AH = 2-aminoheptane.

analyte is disturbed by interfering peaks, some options are given, such as the use of a more selective detection system or, with UV detection, the use of a higher measuring wavelength (λ_{max}). The analyst can also change the nature of the DEC sorbent or adapt the HPLC mobile phase composition in order to separate the interfering peaks from that of the analyte. In this instance, however, the whole SPE procedure must be optimized again by taking these changes into account.

Typical chromatograms obtained with plasma samples containing different β-blocking drugs are presented in Fig. 3.

3.4. Minimum volume of eluent

If a further improvement of the detectability for the analytes is wanted, an additional parameter in the development of such an automated sample preparation method is the selection of the minimum volume of solvent that still gives a satisfactory elution of the compounds [11,14,16].

In this work, this parameter was only optimized for propranolol. Fig. 4 shows that a significant decrease in the recovery of the analyte was only obtained when the volume of eluent was smaller than 0.20 ml. A volume of 0.24 ml of methanol containing 0.3% of 2-aminoheptane was finally selected for the elution of propranolol. In order to obtain a final extract with an eluting strength comparable to that of the HPLC mobile phase while minimizing dilution, 0.41 ml of pH 3.0 buffer should then be passed through the DEC [15], giving the extract a total volume of 0.65 ml (concentration factor = 1.54).

3.5. Validation of the automated method for the determination of propranolol in plasma

Absolute recovery

Table 6 gives the absolute recoveries of the analyte at six different concentrations ranging from 10 to 250 ng ml^{-1}. The mean absolute recovery for propranolol was *ca.* 93 ± 2%. This absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection

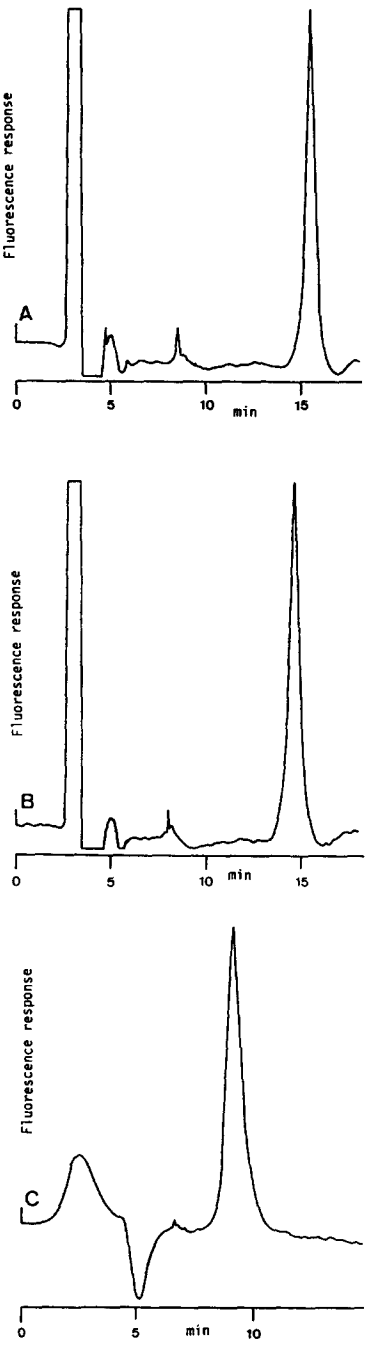

Fig. 3. Typical HPLC traces for plasma samples containing 100 ng ml^{-1} of (A) alprenolol, (B) betaxolol and (C) pindolol. Fluorescence response (arbitrary units) as a function of time (min). Extraction cartridge, Bond Elut CN (50 mg; 1-ml capacity). For SPE and chromatographic conditions, see Experimental (*cf.*, Tables 1–3).

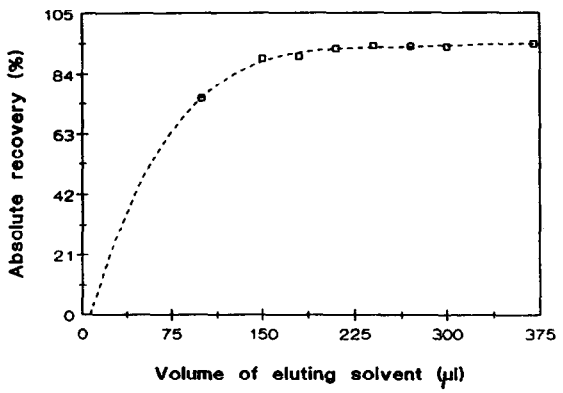

Fig. 4. Minimum volume of eluent for the elution step. Extraction cartridge, Bond Elut CN (50 mg; 1-ml capacity); eluent, methanol containing 0.3% of 2-aminoheptane; analyte concentration, 100 ng ml^{-1}. Other conditions as described under Experimental.

Table 6
Absolute recovery of propranolol using SPE on disposable extraction cartridges

Concentration (ng ml^{-1})	Recovery (%)
250	93.1
100	92.4
50	96.4
20	91.9
10	89.7
Mean	92.7
S.D.	2.5

of aqueous standard solutions at the same concentration [47], using the same autosampler.

Linearity

A calibration graph was constructed in the range 5–500 ng ml^{-1} ($n = 8$). Linear regression analysis made by plotting the analyte peak area (y) *versus* the concentration (x) in ng ml^{-1} gave the following equation:

$$y = 1465.1\ (\pm 7.8)x - 1740.4\ (\pm 1424.2)$$

$$r^2 = 0.99979$$

The linearity of the calibration graph is demonstrated by the good determination coefficient (r^2) obtained for the regression line.

Reproducibility

As shown in Table 7, the precision of the bioanalytical method was calculated by measuring the within-day and between-day reproducibilities of propranolol at four concentration levels ranging from 10 to 250 ng ml^{-1}. Mean values around 2.3% and 3.6% were obtained, respectively.

Accuracy

Accuracy was determined by analysing spiked plasma samples at four different concentrations and comparing the experimentally measured values with the nominal concentrations. The

Table 7
Precision and accuracy of the automated method for the HPLC determination of propranolol in plasma

Basis	Concentration (ng ml^{-1})	n	R.S.D.[a] (%)	R.E.M.[a] (%)
Within-day	250	5	1.6	1.2
	100	6	1.1	0.7
	50	5	2.4	2.2
	10	5	4.1	4.5
	Mean		2.3	2.2
Between-day	250	5	2.4	3.0
	100	5	2.1	2.3
	50	5	3.8	5.1
	10	5	5.9	4.2
	Mean		3.6	3.7

[a] R.S.D. = Relative standard deviation; R.E.M. = relative error of measurement.

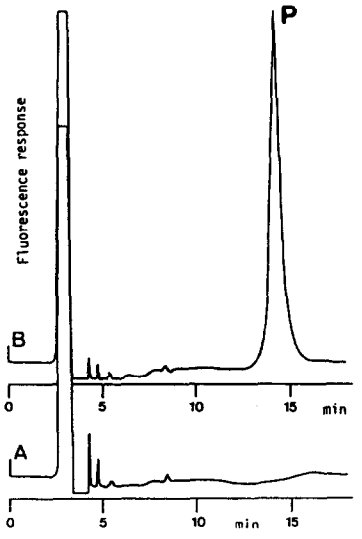

Fig. 5. Typical chromatograms of extracts from (A) blank plasma and (B) plasma spiked with 100 ng ml^{-1} of propranolol obtained by using SPE on DECs coupled with HPLC and fluorimetric detection. Fluorescence response (arbitrary units) as a function of time (min). For SPE and chromatographic conditions, see Experimental (*cf.*, Tables 1–3). Peak: P = propranolol.

accuracy of the automated method expressed as the relative error of measurement is given in Table 7. The mean values are very close to the nominal concentrations of propranolol, showing a method accuracy ranging from 0.7 to 5.1%.

Detectability

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the regression line [48] obtained with calibration graphs in the range 2.5–100 ng ml^{-1} (r^2 = 0.99986). The LOD and LOQ for propranolol were 1.3 and 4.5 ng ml^{-1}, respectively.

Selectivity

No endogenous sources of interference were observed at the retention time of the analyte. Typical chromatograms obtained with a blank plasma and with a spiked plasma containing 100 ng ml^{-1} of propranolol are presented in Fig. 5.

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