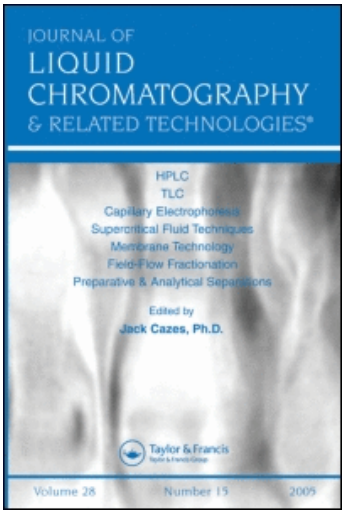

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HPLC Determination of Verapamil and Norverapamil in Plasma Using Automated Solid Phase Extraction for Sample Preparation and Fluorometric Detection

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HPLC DETERMINATION OF VERAPAMIL AND NORVERAPAMIL IN PLASMA USING AUTOMATED SOLID PHASE EXTRACTION FOR SAMPLE PREPARATION AND FLUOROMETRIC DETECTION

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ABSTRACT

A sensitive and fully automated method for the simultaneous determination of verapamil and its main metabolite norverapamil in plasma is described, which involves the solid phase extraction (SPE) of the analytes from plasma on disposable extraction cartridges (DECs) and reversed phase HPLC with fluorescence detection. The DEC filled with endcapped cyanopropyl silica (50 mg) was first conditioned with methanol and phosphate buffer of pH 7.4. A 1.0-mL volume of plasma sample containing the internal standard was then applied on the DEC. The washing step was performed with the same buffer. The analytes were eluted with 0.24 mL of methanol containing 0.2 % of 2-aminoheptane. A 0.41-mL volume of acetate buffer of pH 3.0 was then passed through the DEC and 0.25 mL of the resultant extract was directly introduced into the HPLC system. The absolute recoveries of the drugs were around 95 % and the limit of detection of verapamil was 1.0 ng/mL. The within-day and between-day reproducibilities at a plasma concentration of 100 ng/mL were 1.4 % and 1.9 %, respectively.

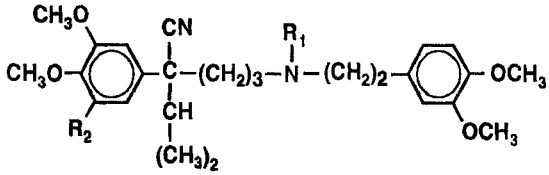
INTRODUCTION

Verapamil hydrochloride (cfr. Fig. 1), a synthetic papaverine derivative, is a well known calcium blocker compound which has anti-anginal, anti-hypertensive and anti-arrhythmic properties [1]. This drug is extensively metabolized. Norverapamil, its N-demethylated metabolite (cfr. Fig. 1), is pharmacologically active and can accumulate in plasma concentrations equal to or greater than those of verapamil itself [2].

Verapamil and its metabolites have been analyzed previously by conventional gas chromatography with flame ionization detection [3,4] or mass spectrometry [5], more recently by capillary gas chromatography with flame ionization detection or electron capture detection [6] and by high performance liquid chromatography using UV detection [6] or most often fluorescence detection [2,7-12] owing to the native fluorescence properties of these compounds.

A sample handling step is usually introduced prior to the HPLC analysis of drugs in biological fluids in order to remove proteins which may cause column clogging [13,14] and also to increase the selectivity and sensitivity of the method [15,16]. In most analytical procedures mentioned above, the sample pretreatment consists of liquid-liquid extraction of the analytes from plasma by organic solvents [10] after alkalization [2,6]. This is generally followed by a back-extraction into acidic solutions [3,4,7,8,9,11,12]. An alternative to this efficient but often tedious and time-consuming extraction technique is the isolation of drugs by liquid-solid or solid-phase extraction (SPE) using either column switching [13,14,16-20] or disposable extraction cartridges (DECs) [15,20-23]. The different SPE operations can be performed automatically on DECs by means of a sample processor, which can also inject the prepared sample on line into the HPLC system [23,24].

The purpose of this paper is to describe such a fully automated method developed for the determination of verapamil and its main metabolite norverapamil in plasma. The method involves sample handling by SPE on DECs, on-line injection of the extracts into a HPLC system and subsequent fluorometric detection of the analytes. The influence of the competing amine added to the mobile phase on the background fluorescence has been studied and the differents steps of the SPE procedure which may effect analyte recovery



	R_1	R_2
(1)	CH_3	H
(2)	H	H
(3)	CH_3	OCH_3

FIGURE 1

Structure :
(1) : Verapamil; (2) : Norverapamil; (3) : Gallopamil (internal standard)

have been investigated using aqueous standard solutions instead of spiked plasma samples in order to limit the consumption of DECs [25]. After elimination of memory effects and selection of the most appropriate dispensing mode for the liquids in the elution step, the composition of the eluent and of the washing liquid, the type of SPE sorbent and the volume of eluent have been optimized with respect to analyte recovery. Finally, the method developed has been validated.

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of a model 305 pump (Gilson, Villiers-le-Bel, France), an ASPEC system (Automatic Sample Preparation with Extraction Cartridges) from Gilson and a model F-1050 fluorescence detector from Merck-Hitachi (Darmstad, FRG / Tokyo, Japan) equipped with a mercury-xenon lamp (200 W). The fluorometer was set at an excitation wavelength of 275 nm, an emission wavelength of 310 nm [4,12] and a time constant of 1 sec.

The ASPEC system was composed of three components: an automatic sampling injector module, a model 401 dilutor/pipettor and a set of racks and accessories, necessary for handling DECs, plasma samples and solvents [23,24].

A Manu-CART system which was made up of a LiChroCART analytical column (250 x 4 mm, i.d.) preceded by a short LiChroCART guard column (4 x 4 mm, i.d.) from Merck (Darmstad, FRG), was thermostatted at 35.0 $\pm$ 0.1 ° C in a model 02PT923 water-bath from Heto (Birkeröd, Denmark).

An IBM compatible computer (PC-AT; CPU type 80386) equipped with GME-714 version 1.3 (HPLC system controller) and GME-718 version 1.1 (sample manager) softwares from Gilson, was used to control the HPLC and ASPEC systems as well as for data collection, storage and treatment. A model BD9 two-channel recorder from Kipp and Zonen (Delft, The Netherlands) was used simultaneously for data collection.

Chemicals and Reagents

R,S-verapamil hydrochloride, its N-demethyl derivative (norverapamil) and gallopamil (cfr. Fig. 1), the internal standard, were all kindly supplied by S.M.B Pharmaceuticals (Brussels, Belgium) and were used without further purification.

Sodium acetate, glacial acetic acid, sodium hydroxide and potassium dihydrogen phosphate were of p.a. quality from Merck (Darmstad, FRG). 2-Aminoheptane was purchased from Aldrich (Gillingham, Great Britain) and was distilled two times before use.

Acetonitrile from Riedel-de Haen (Seelze, FRG) and methanol from Janssen (Geel, Belgium) were of HPLC grade.

The water used in all experiments was purified on a Milli-Q system from Millipore (Bedford, MA, USA).

Bond Elut DECs (1-mL capacity) packed with 50 mg of cyanopropyl modified silica (CN endcapped: CN^{EC}) with a mean particule size of 40 μm were obtained from Analytichem (Harbor City, CA, USA).

The stationary phase of the LiChroCART analytical column was Superspher 100 RP-18 (particule size : 4 μm) and the LiChroCART guard column was packed with LiChrospher 100 RP-18 (particule size : 5 μm) from Merck.

Chromatographic technique

The mobile phase (isocratic mode) was a mixture of pH 3.0 acetate buffer, acetonitrile and 2-aminoheptane (70:30:0.5, v/v/v) [26]. Before use, it was degassed for 15 min. in a ultrasonic bath. The flow-rate of the mobile phase was 1.1 mL/min.

The acetate buffer of pH 3.0 was obtained by mixing 0.01 N sodium acetate with 33 mL of glacial acetic acid per liter, the pH being adjusted if necessary. The buffer was filtered through a 0.45 μm nylon membrane filter from Schleicher & Schuell (Dassel, FRG) before use.

Standard solutions

Stock solutions of verapamil, norverapamil and gallopamil (internal standard) were prepared by dissolving 50 mg of each compound in 50 mL of methanol. A mixed verapamil/norverapamil solution (10 μg/mL for each compound) was made in water and diluted either with water (concentrations ranging from 0.03 to 3.00 μg/mL) when used to spike plasma samples (1.5 mL) for calibration curves or with a mixture of pH 3.0 buffer and methanol (70:30) when injected directly for recovery measurements. The stock solution of gallopamil was diluted with water to the concentration of 5 μg/mL.

The methanolic solutions were stored in a refrigerator at 4 °C while the aqueous solutions were prepared daily.

Automatic sample preparation

After centrifugation of the plasma sample at 6000 rpm for 10 min., a 1.5-mL volume of plasma was transferred manually into a vial which was then placed on the appropriate rack of the ASPEC system. All the other operations of the SPE procedure and the injection of the final extract into the HPLC system were performed automatically by the sample processor (ASPEC system). As outlined in Table 1, the automatic operations are performed in the following way (total cycle time : 20.4 min) :

- **Addition of internal standard solution** (flow rate : 3.0 mL/min; air volume : 1.0 mL) : after addition of 0.03 mL-volume of gallopamil solution, the plasma sample is homogenized by bubbling.
- **DEC conditioning** (flow rate : 6.0 mL/min; air volume : 0.3mL) : at the beginning of the cycle, the DEC holder is located above the drain

TABLE 1

Automatic Sample Preparation Procedure

Steps	Liquids	Volume (mL)	Dispensing flow rate (mL/min)
IS. Addition	IS Solution	0.03	3.00
Conditioning	Methanol	1.00	6.00
	B. pH 7.4	1.00	6.00
Sample loading	Plasma	1.00	0.18
Washing	B. pH 7.4	1.00	1.50
Elution	CH3OH+0.2% AH	0.24	1.50
Buffer addition	B. pH 3.0	0.41	1.50
Mixing	Plasma extract	0.65	3.00
Filling of the injection loop	Plasma extract	0.65	0.75

DEC : Bond Elut CN^{EC} (50 mg; 1 mL capacity)
B. : Buffer IS: Internal Standard AH: 2-Aminoheptane

cuvette (front position). The DEC (Bondelut CN^{EC}, 50 mg) is first washed with 1.0 mL of methanol , then with 1.0 mL of phosphate buffer (pH 7.4).

- **Loading with plasma sample** (flow rate : 0.18 mL/min; air volume : 1.0 mL) : 1.0 mL-volume of plasma is aspirated by the autosampler needle from the corresponding vial and dispensed on the DEC.
- **Washing** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : 1.0 mL of phosphate buffer pH 7.4 is dispensed on the DEC.
- **Elution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : the DEC holder is pushed by the needle over the collection rack. A 0.24-mL volume of methanol containing 0.2 % of 2-aminoheptane is applied on the DEC. The eluate is collected in the tube located under the DEC.

- **Dilution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : 0.41 mL of acetate buffer (pH 3.0) are dispensed on the DEC. The DEC holder is then replaced in its front position.
- **Mixing** : the diluted eluate is successively aspirated and dispensed in the collection tube by the needle. These operations are repeated three times.
- **Injection** : the whole volume of the final extract (0.65 mL) is aspirated by the needle from the collection tube and dispensed in the loop filler port of the injection valve. By switching of the injection valve, 0.25 mL of the final extract is injected into the HPLC column, the excess being directed to the waste.

In order to avoid detrimental effects of the strong binding of the analytes to plasma proteins on the absolute recoveries, the minimum dispensing flow rate available (0.18 mL/min) has been selected for the sample loading step [23].

The phosphate buffer of pH 7.4 which was used in the conditioning and washing steps, was prepared in a 1-L volumetric flask by mixing 250 mL of 0.1M potassium dihydrogen phosphate with 195.5 mL of 0.1 M sodium hydroxide and adding water to the volume.

Each plasma sample was prepared individually during the chromatographic analysis of the previous sample (concurrent mode).

Before the beginning of the first cycle and after each use in the SPE procedure, the needle of the autosampler was rinsed with water and a 40-μL segment of air was generated before pipetting the liquid to be transferred in order to avoid cross-contamination.

RESULTS AND DISCUSSION

Fluorometric detection

In the development of a bioanalytical procedure, a proper choice of the detection mode is particulary important. Due to their higher sensitivity and selectivity, fluorescence and electrochemical detection are usually preferred to UV-VIS detection, especially when no derivatization step is needed. Verapamil and its main metabolite, norverapamil, as well as gallopamil (I.S.) have native fluorescence properties, which makes fluorometry an obvious choice in this case. The main interest of this detection mode in the present method is the

substantial gain of sensitivity which can be obtained in comparison with UV detection, a high degree of selectivity being already provided by the SPE procedure and the HPLC separation [27].

The sensitivity of fluorescence detection could be further improved if the conventional xenon lamp (150 W) of the detector was replaced by a mercury-xenon lamp (200 W), particularly at excitation wavelengths lower than 300 nm. For the compounds studied, the fluorescence signal at 310 nm (excitation wavelength : 275 nm) was then seven times higher [28].

As shown in Fig. 2, 2-aminoheptane, added to the HPLC mobile phase to improve analyte peak symmetry and efficiency, gave rise to a significant increase of the background fluorescence. When the amine was used as supplied by the manufacturer, a constant baseline was obtained only after passage of more than 400 mL of mobile phase. By monitoring the HPLC eluate (cfr. Fig. 2), several breakthrough curves were observed, indicating the presence of at least five fluorescent impurities with widely different hydrophobic character. The equilibrium was reached more rapidly by use of the sulphate salt of the amine but the background fluorescence was only slightly diminished. The latter could be considerably reduced, however, by distillation of the amine, which eliminated mainly the most retained impurities and consequently also decreased the equilibrium time.

Memory effects

Table 2 shows the detrimental influence of memory effects on repeatability by direct injection of aqueous standard solutions of verapamil and norverapamil into the HPLC system. The use of a pH 7.4 phosphate buffer for the preparation of standard solutions of the analytes as well as for rinsing the dilutor needle and the external tubing of the injection valve gave rise to very high RSD values of about 10 %(n=5). Under these conditions, the total amount of the analytes was not introduced into the HPLC system, as it was demonstrated by injecting successively five samples of a aqueous standard solution and then a mixture of pH 3.0 acetate buffer and methanol (70:30, v/v) containing no analytes. On the chromatogram corresponding to the injection of this mixture, small residual peaks were observed at the retention times of the two analytes. The replacement of the 7.4 buffer by a pH 3.0 acetate buffer as rinsing liquid did not improve significantly the RSD values (cfr. Table 2).

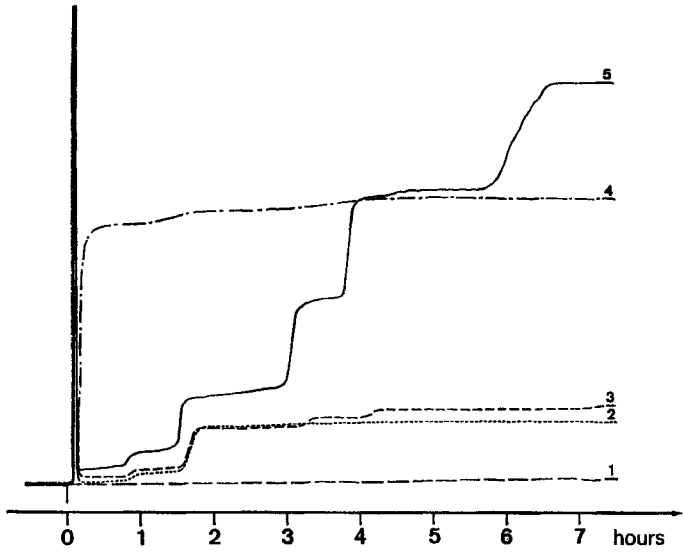


FIGURE 2

Fluorescence monitoring of the HPLC eluate
0.5 % 2-Aminoheptane in phosphate buffer pH 3-Acetonitrile (70/30)
- (1) : mobile phase without the amine
- (2) : amine distilled twice
- (3) : amine distilled once
- (4) : sulphate salt of the amine
- (5) : unpurified amine

As can be seen in Table 2, the best results with respect to repeatability were obtained with standard solutions of the analytes prepared in a mixture of pH 3.0 acetate buffer and methanol (70:30, v/v). In spite of the particularly good results obtained with pH 3.0 buffer as rinsing liquid, water was finally given the preference due to the unfavourable effect of this buffer on the recovery of verapamil and norverapamil (cfr. Table 4).

It should be noted that plasma samples spiked with the same amounts of analytes and treated successively according to the SPE procedure described in Materials and Methods gave reproducible results (cfr. Table 7) by use of water

TABLE 2

Memory Effects Obtained by Direct Injection

Aqueous standard solution of drugs	Dilutor/ pipettor rinsing liquid	R.S.D. (%; n=5)		Residual peak
		NV	V	
B. pH 7.4	B. pH 7.4	10.3	9.2	+
B. pH 7.4	B. pH 3.0	4.1	6.5	+
B. pH 3.0 /CH3OH	B. pH 3.0	0.8	0.4	-
B. pH 3.0 /CH3OH	Water	1.4	1.5	-

NV : Norverapamil V : Verapamil B. : Buffer
B. pH 3.0/CH3OH : Acetate buffer pH 3.0 - Methanol (70:30)
Analyte concentration : 100 ng/mL

as rinsing liquid and that no residual peaks were observed when a blank plasma sample was analyzed immediately afterwards under the same conditions.

Dispensing mode for the liquids in the elution step

In the development of the SPE procedure by use of aqueous standard solutions of the analytes, the elution of these compounds from the extraction cartridges was the first step to be studied. At this stage, the different SPE steps had not yet been optimized. As selected previously in a similar fully automated method for the bioanalysis of another basic drug, diltiazem [24,27], DECs filled with ordinary cyanopropyl silica were used, as well as a pH 7.4 phosphate buffer for the conditioning and washing steps. The elution was first performed with a 0.3-mL volume of methanol (cfr. Fig. 3) and 0.7 mL of acetate buffer

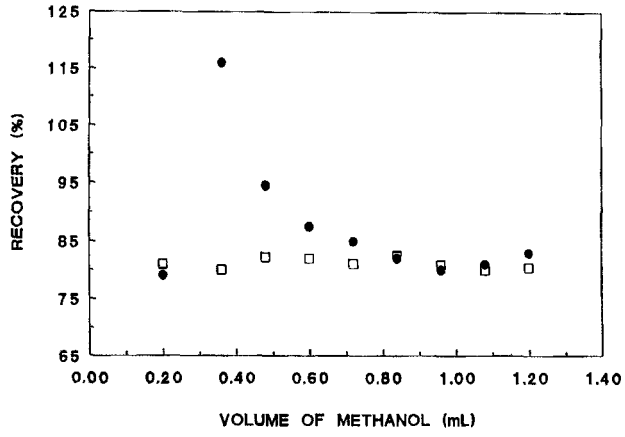


FIGURE 3

Influence of the dispensing mode of phosphate buffer (pH 7.4) on the recovery of verapamil

DEC : Bond Elut cyanopropyl-bonded phase (CN; 50 mg)
Verapamil concentration : 100 ng/mL
Conditioning liquids : 1. Methanol 2. Phosphate buffer pH 7.4
Washing liquid : Phosphate buffer pH 7.4
Buffer added to the methanolic eluate : pH 3.0 acetate buffer
(●) : dispensed in the collection tube
(□) : dispensed on the DEC

(pH 3.0) was subsequently delivered into the corresponding collection tube located under the extraction cartridge. The aim of this buffer addition is to reduce the eluting strength of the final extract to such an extent that it becomes equivalent or preferably lower in comparison with that of the HPLC mobile phase [27].

Fig. 3 shows that under these preliminary conditions anomalously high (more than 110%) and poorly reproducible recoveries were obtained for verapamil at the 100 ng/mL concentration level. On the other hand, the use of methanol volumes higher than 0.7 mL gave rise to lower (around 80 %) but more reproducible recoveries.

It can also be seen in Fig. 3 that when acetate buffer was passed through the DEC just after the application of methanol, this unfavourable effect was eliminated and a fairly constant recovery of about 80 % was obtained for all methanol volumes tested. In all these experiments, the amount of buffer added always corresponded to 70 % of the final extract, i.e. the same proportion as in the HPLC mobile phase.

These results can be explained by the fact that if only methanol is applied on the DEC and acetate buffer is introduced directly in the collection tube, the volume of eluate collected is lower than the methanol volume dispensed. This is particularly obvious when the latter is small. The passage of the buffer through the DEC is thus necessary, not to improve the elution of the analytes, but to obtain a constant volume of eluate, equivalent to the volume of methanol dispensed. Even small volumes of methanol could be restored under these conditions (cfr. Fig. 4), so that this dispensing mode was selected for further experiments.

Composition of the eluent

As can be seen in Table 3, solvents often used in reversed-phase chromatography such as acetonitrile and methanol were first tested as eluents. With acetonitrile, particularly low recoveries of about 20 % were obtained for verapamil and norverapamil. Methanol gave, however, significantly higher recoveries than acetonitrile. In contrast to what is usually observed in reversed-phase HPLC, acetonitrile seems to have a lower eluting strength than methanol under these conditions. Similar observations have been reported previously by solid phase extraction of basic compounds [29-31]. The results obtained here with acetonitrile are probably to be related to the strong tendency of basic compounds to interact with free silanol groups, particularly when the solid phase consists of ordinary cyanopropyl silica.

Still the recoveries of about 80 % obtained with methanol as eluting solvent are not quite satisfactory. The addition to methanol of 2-aminoheptane, i.e. the same competing amine as used in the HPLC mobile phase, gave rise to a significant increase in the recoveries of the two analytes, at a concentration to 0.2 % (cfr. Table 3). No further increase in recovery were obtained, however, by adding higher amounts of 2-aminoheptane to methanol (e.g. 0.4 %). The favourable influence of the competing amine on the elution of verapamil and

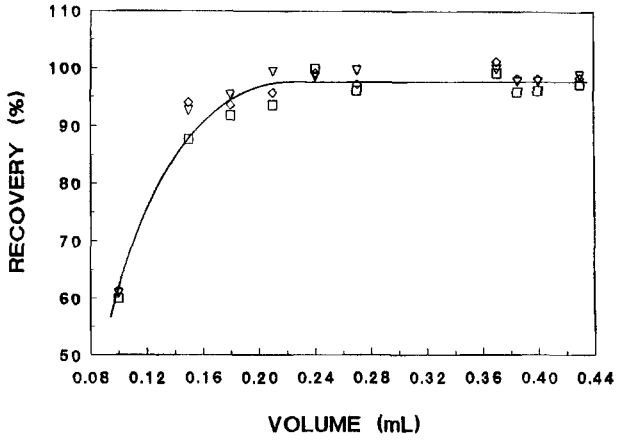


FIGURE 4

Minimum volume of eluent for the elution step

DEC : Bond Elut CN endcapped (CN^{EC}; 50 mg)
Eluent : methanol containing 0.2 % of 2-aminoheptane
Analytes concentration : 100 ng/mL
(▽) : norverapamil
(◇) : verapamil
(□) : gallopamil
Other conditions as described in Materials and Methods.

norverapamil seems to confirm that these compounds have strong interactions with the residual silanol groups at the surface of the solid phase.

Composition of the washing liquid

Table 4 shows the influence of the pH of the buffer used as washing liquid on the recovery of verapamil and norverapamil. In all experiments, the same buffer was used for the conditioning and washing steps.

As can be seen in Table 4, the washing of the DECs with pH 3.0 buffer causes the recoveries of the two analytes to decrease drastically. Lower recoveries were also obtained by using water instead of pH 7.4 buffer as

TABLE 3

Optimization of the Eluent Composition

Eluting liquid (0.3 mL)	Absolute recovery (%) NV	V
ACN	22.4	18.2
CH3OH	80.1	81.1
CH3OH+ 0.1% AH	81.2	81.6
CH3OH+ 0.2% AH	87.3	88.3
CH3OH+ 0.4% AH	86.6	88.4

NV : Norverapamil V : Verapamil AH : 2-Aminoheptane
DEC : Bond Elut CN (50 mg)
Buffer added to the eluent: acetate buffer pH 3.0 (0.7 mL)
Analyte concentrations : 100 ng/mL

washing liquid. This can hardly be explained only by an increase of the ionization of the analytes since verapamil (pKa : 8.6) is already present essentially in protonated form at pH 7.4. The higher retention of the cationic analytes at pH 7.4 is probably related to an increased ionization of the free silanol groups at the surface of the cyanopropyl silica support. No significant changes in the analyte recoveries were obtained by use of a pH 9.2 borate buffer, which indicates that the analytes are not more strongly adsorbed in uncharged form than in cationic form on this kind of solid phase. Finally, the pH 7.4 phosphate buffer which had been used in preliminary experiments and in a similar automated SPE method [27], was selected as washing liquid.

TABLE 4

Optimization of the Washing Step

Washing liquid	Absolute recovery (%)	
	NV	V
B. pH 7.4 (phosphate)	88.1	88.5
B. pH 9.2 (borate)	87.5	81.3
B. pH 3.0 (acetate)	46.6	39.3
Water	49.0	70.4

NV. : Norverapamil V. : Verapamil B : Buffer
DEC : Bond Elut CN (50 mg)
Concentration of the drugs : 100 ng/mL
Conditioning liquids : 1. Methanol 2. Same as washing liquid
Eluents : 1. Methanol (0.3 mL) 2. pH 3.0 buffer (0.7 mL)

Type of solid phase

Table 5 shows the recoveries of verapamil and norverapamil obtained with aqueous solutions of the drugs by using different kinds of solid phases in the DECs.

Due to the difficulties to obtain a quantitative elution of the two analytes, i.e. a recovery greater than 90 %, from DECs filled with ordinary cyanopropyl silica, other kinds of sorbents were tested. As observed previously [27], an increase of the amount of sorbent in the cartridge did not give rise to significant changes in recoveries. However, as can be seen in Table 5, a slight increase in the recoveries of verapamil and norverapamil was obtained by using DECs packed with endcapped cyanopropyl silica (CN^{EC}), probably due to a better elution of these compounds from the DECs by reduction of their interactions

TABLE 5

Type of Sorbent Used in the DECs

Sorbent (mg)	Absolute recovery (%)	
	NV	V
CN 50	86.1	84.8
CN 100	83.1	79.8
CN 50 Endcapped(EC)	93.7	92.6
C18 50	93.0	89.6

NV: Norverapamil V : Verapamil
Concentration of the drugs: 100 ng/mL
Eluent: CH3OH + 0.2% 2-Aminoheptane

with silanol groups. The analyte recoveries on C18 cartridges were about the same as those obtained with the endcapped cyano phase but the preference was given to the more polar phase, due its higher extraction selectivity.

Analyte-sorbent interaction can be affected by the sample matrix which may compete for the active sites of the support material and cause the adsorption of the analytes to decrease [32,33]. Therefore recoveries from plasma samples spiked with verapamil and norverapamil were studied immediately afterwards. Such plasma samples treated by the automated SPE procedure under the operational conditions selected above and applied on the DECs with the minimum dispensing flow rate (0.18 mL/min) [23] gave similar recoveries to those obtained with aqueous standard solutions. This seems to indicate that there are no significant effects from the matrix components on the adsorption and consequently on the recoveries of the analytes.The different SPE parameters optimized with aqueous solutions as described above can thus be used without modification for handling plasma samples.

TABLE 6

Absolute Recoveries of the Analytes

Conc. (ng/mL)	NV (%)	V (%)	G (%)
500	89	86	-
200	92	89	-
100	99	97	95
50	96	98	-
20	101	103	-
10	88	96	-
Mean	94	95	95

NV. : Norverapamil V. : Verapamil G. : Gallopamil

Volume of eluent

The last parameter to be optimized in the development of such an automated SPE procedure is the volume of eluent. In order to improve the detectability (LOD/LOQ) for the analytes in the present method without including any evaporation step, the minimum volume of solvent which still gives a satisfactory elution of these compounds was determined. Smaller volumes for the elution step could already be used with DECs containing 50 mg of sorbent instead of 100 mg [27], similar recoveries being obtained with both kinds of cartridges (cfr. Table 5).

Fig. 4 shows that a significant decrease of the recovery of verapamil was only obtained when the volume of eluent was lower than 0.20 mL. A volume of 0.24 mL of methanol containing 0.2 % of 2-aminoheptane was finally selected for the elution of the two analytes, norverapamil and verapamil having a similar behaviour. 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, giving the extract a total volume of 0.65 mL (concentration factor : 1.54).

Validation of the automated procedure

A typical chromatographic trace of a plasma extract containing verapamil and norverapamil (concentration : 10 ng/mL) is shown in Fig. 5B. Under the conditions selected for the HPLC separation, the mean capacity ratios (k') of norverapamil, verapamil and gallopamil were 3.4, 3.9 and 4.8, respectively (n =20).

Absolute recovery

Table 6 gives the absolute recoveries of the analytes at six different concentrations ranging from 10 to 500 ng/mL. The mean absolute recoveries for the two analytes were around 95 %. These absolute recoveries were calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions at the same concentration [34], using the same autosampler.

Linearity

The calibration curves were constructed in the range 3-500 ng/mL (n=8), the therapeutic plasma levels of verapamil exceeding not 500 ng/mL [1]. Linear regression analysis made by plotting the analyte/internal standard peak area ratio (y) versus the concentration (x) in ng/mL gave the following equations :

norverapamil : $y = 0.0154\ x + 0.0135$ $r^2 = 0.99999$

verapamil : $y = 0.0149\ x + 0.0453$ $r^2 = 0.99996$

The linearity of the calibration curves is demonstrated by the good determination coefficients (r^2) obtained for the regression lines.

Reproducibility

As shown in Table 7, the precision of the bioanalytical method was estimated by measuring the within-day and between-day reproducibilities of the analytes at four concentration levels ranging from 10 to 200 ng/mL. Mean values around 2.3 % and 3.7 % were obtained, respectively.

Detectability

Limits of detection (LOD) and limits of quantitation (LOQ) were calculated from regression lines [35] obtained with calibration curves ranging from 2 to 100 ng/mL. The LOD for verapamil was equal to 1.0 ng/mL and its

TABLE 7

Reproducibility of the Automated Method

Within-day reproducibility			
Concentration (ng/mL)	n	Norverapamil R.S.D.(%)	Verapamil R.S.D.(%)
200	5	2.0	2.2
100	6	1.9	1.4
50	5	1.5	2.4
10	5	4.2	3.0
Mean :		2.4	2.3
S.D. :		1.2	0.7

Between-day reproducibility			
Concentration (ng/mL)	n	Norverapamil R.S.D.(%)	Verapamil R.S.D.(%)
200	5	2.4	2.2
100	5	3.0	1.9
50	5	3.9	4.1
10	5	5.6	6.3
Mean :		3.7	3.6
S.D. :		1.4	2.0

NV. : Norverapamil V. : Verapamil G. : Gallopamil

LOQ to 3.3 ng/mL. The LOD and LOQ for norverapamil were equal to 1.7 ng/mL and 5.8 ng/mL, respectively.

Selectivity

No endogenous sources of interference were observed at the retention times of the analytes. Typical chromatograms obtained with a blank plasma (A) and with a spiked plasma containing with 10 ng/mL of each drug (B) are presented in Fig. 5.

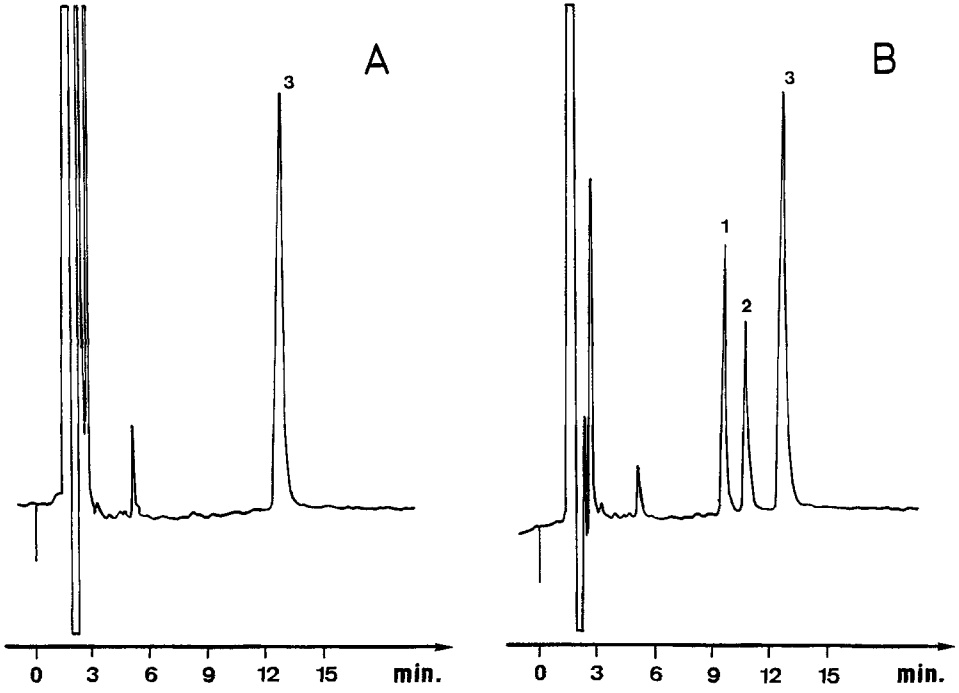


FIGURE 5

Typical chromatograms obtained by using SPE on DEC coupled to HPLC
Mobile phase : pH 3.0 acetate buffer : 70
Acetonitrile : 30
2-Aminoheptane : 0.5
Solid phase : Superspher 100 RP-18 (4 μm)
Fluorescence detection : excitation, 275 nm; emission, 310 nm
DEC : Bond Elut CN endcapped (CN^{EC}; 50 mg).
Sample : A : blank plasma
B : spiked plasma
Peaks : 1 : Norverapamil : 5.9 ng (10 ng/mL)
2 : Verapamil : 5.9 ng (10 ng/mL)
3 : Gallopamil [IS] : 57.8 ng (98 ng/mL)

Application of the automated method

The fully automated method developed for the determination concentrations of both verapamil and norverapamil in human plasma has been applied successfully to more than 4600 analyses in the framework of bioavailability studies and has proved to be sensitive and rugged.

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