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Achiral and chiral high-performance liquid chromatography of verapamil and its metabolites in serum samples

E. Brandšteterová[a,*], I.W. Wainer[b]

[a]*Faculty of Chemical Technology, Department of Analytical Chemistry, Slovak Technical University, Radlinského 9, 812 37 Bratislava, Slovak Republic*

[b]*Georgetown University, Medical Center, Room C305, Medical Dental Building, 3900 Reservoir Road A.W., Washington, DC 20007, USA*

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Abstract

Rapid and simple achiral and chiral HPLC assays have been developed for the determination of verapamil and its metabolites in serum samples. Two achiral reversed-phase columns, Hisep C_{18} (150×4.6 mm) and NovaPak C_{18} (150×3.9 mm) were used for the simultaneous separation of all analyzed compounds. An α_1-AGP column (100×4.0 mm) was recommended for successful chiral separations of verapamil and its seven metabolites. All analyses were realised with fluorescence detection at λ_{ex}=276 nm and λ_{em}=310 nm. Limits of quantitation were in the range 1.0 to 5 ng/ml for all compounds. Both off-line SPE (SepPak C_{18} cartridges) and the on-line SPE with a semipermeable surface SDS C_8 pre-column, (10×4.6 mm) were used for the clean-up and sample preconcentration. Extraction recoveries for all analyzed compounds were 87.7±5.8 to 92.7±4.0% for off-line SPE and 94.3±4.2 to 98.2±5.1% for on-line SPE. The complete assay could be applied for achiral and chiral monitoring verapamil and all its metabolites in serum samples.

Keywords: Column switching; Verapamil; Norverapamil

1. Introduction

Verapamil, 5[(3,4-dimethoxyphenylethyl)methylamino] - 2 - (3, 4 - dimethoxyphenyl) - 2 - isopropylvaleronitrile, is a calcium channel antagonist widely applied in a variety of cardiovascular disorders. Pharmacokinetic studies have shown that verapamil undergoes extensive biotransformation and several *N*-dealkylated and O-demethylated metabolites have been described [1,2] (Fig. 1). It is known that individual stereoisomers, especially the enantiomers of biologically active chiral molecules may differ in their metabolism, toxicity, plasma disposition and urine excretion kinetics [3]. Verapamil is also a chiral compound that is administered as a racemic mixture of *R*-(+) and *S*-(−) enantiomers which show different pharmacokinetic and pharmacodynamic properties. For example, *S*-(−)-verapamil is 10–20-times more potent that *R*-(+)-verapamil [4]. After intravenous administration, plasma clearance and apparent volume of distribution of *S*-(−)-verapamil are almost twice as high as those of *R*-(+)-verapamil [5]. After oral administration *S*-(−)-verapamil undergoes extensive first-pass metabolism, resulting in the

*Corresponding author.

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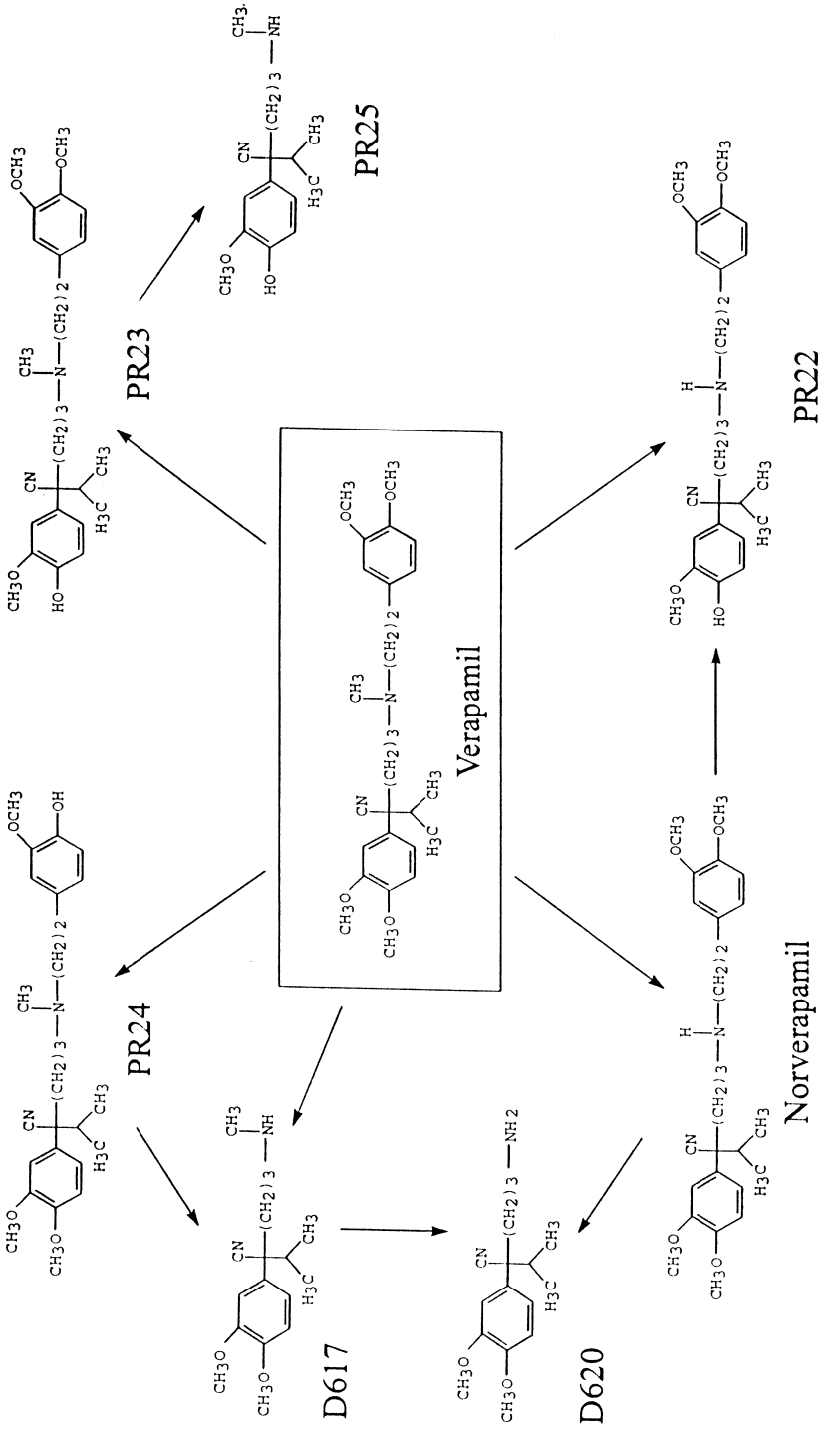

Fig. 1. Metabolic scheme of verapamil and its metabolites.

predominance of the *R*-(+)-verapamil in plasma [6] and the protein binding is enantioselective with the free fraction of *S*-(−)-verapamil greater than that of *R*-(+)-verapamil [7]. Several structural analogs of verapamil were studied for their ability to reverse multi-drug resistance (MDR) in human cell lines [8]. One less cardiotoxic compound is *R*-(+)-verapamil which has equivalent activity in the modification of MDR [9,10]. The major metabolite of verapamil – norverapamil has 20% of the coronary vasodilator potency of verapamil. Some papers reported a simple high-performance liquid chromatography (HPLC) method for the determination of verapamil and its metabolites combined with the liquid extraction of analytes from plasma samples [11–13] but in many cases all metabolites were not analyzed or their separation was not sufficient for quantitative analysis [11,12]. Some preparation procedures have very low extraction recoveries [13]. A simple HPLC determination of verapamil and norverapamil in plasma using on-line solid-phase extraction (SPE) has also been published [14]. The detection limit was 1 ng/ml for verapamil.

The determination of enantiomers of verapamil and norverapamil in serum in the achiral–chiral HPLC system was published for α_1-acid glycoprotein (AGP) chiral stationary phase coupled to a shielded hydrophobic phase (Hisep) column. The system has been reported as the first one for the analysis of serum concentrations of some verapamil enantiomers after the clinical administration of the drug and chiral separation of norverapamil in clinical samples [15].

Simultaneous direct determination of enantiomers of verapamil and norverapamil in plasma using a derivatized amylose chiral stationary phase has also been reported [16]. A Chiralpak AD column was connected in series with a diol silica column (Lichrocart DIOL) and both compounds were separated using the same mobile phase.

The determination of the enantiomeric ratio of verapamil, norverapamil and gallopamil in plasma by the chiral HPLC assay with AGP and Chiralpak AD columns was reported by Fieger and Blaschke [17]. Verapamil was separated on an AGP column after the acetylation of the main metabolite – norverapamil. Both drugs were determined on a Chiralpak AD column. The simultaneous analysis of verapamil and norverapamil enantiomers in human plasma was presented using butanol–hexane extraction and norverapamil acetylation step before the chiral separation on the AGP column. The derivatizing agent (acetic anhydride) was added directly to the extraction solvent [18]. The use of a chiral ovomucoid column to determine the stereochemical composition of the major metabolites of verapamil in dog urine samples after the oral administration has also been studied [19]. The structure identification of the isolated verapamil metabolites was confirmed by HPLC–mass spectrometry (MS) and fast atom bombardment (FAB) MS–MS techniques. The same chiral column was used for on-line determination and resolution of verapamil enantiomers by HPLC with column switching [20]. Aging effects on stereoselective pharmacokinetics and pharmacodynamics of verapamil have also been studied [21–24]. Total verapamil concentrations were determined by achiral HPLC, verapamil and norverapamil (*R*) and (*S*) enantiomers were separated using an amylose chiral column.

The aim of presented work was to develop an effective achiral HPLC assay for the simultaneous determination of verapamil and all its metabolites with both off-line and on-line SPE. The second goal deals with the successful chiral separation of all metabolite fractions on the same chiral column with the chromatographic resolution values sufficient for the quantitative analysis of all metabolite enantiomers.

2. Experimental

2.1. Chromatographic equipment and conditions

Two HPLC modular systems (I, II) were used for all separations. System I: a SP 2000 binary pump, an AS 3000 autosampler, a FL 2000 fluorescence detector, all from Spectra-Physics (Santa Clara, CA, USA), and a six-port Rheodyne 7125 injection valve (column switching). System II: two 501 pumps, a U6K injector from Waters (Milford, MA, USA) and a RF 535 fluorescence detector from Shimadzu (Kyoto, Japan).

Two chromatographic columns were used for

achiral HPLC separations: Hisep C_{18} (150×4.6 mm) (Regis, Morton Grove, IL, USA) and NovaPak C_{18} (150×3.9 mm) (Waters, Milford, MA, USA). A chiral α_1-AGP column (100×4 mm) (ChromTech, Norsborg, Sweden) was applied for chiral separations.

A semipermeable surface (SPS) C_8 pre-column (10×4 mm) from Regis was used for the on-line direct sample analysis, and SepPak C_{18} SPE cartridges (100 mg, 3 ml) (Waters) for off-line SPE preparations.

The mobile phases for achiral HPLC analyses consisted of 35–37% acetonitrile in 0.3% triethylamine (TEA) (phosphoric acid was used to adjust the pH to pH 4.0), with a flow-rate 1 ml/min for the Hisep C_{18} column and 35% acetonitrile in 0.03% TEA, pH 3.8 for the NovaPak C_{18} column. Flow-rate was 0.9 ml/min. Fluorescence detection was realised at excitation, λ_{ex}=276 nm and emission, λ_{em}=310 nm.

Three mobile phases were tested for chiral separation using an α_1-AGP column: (1) 0.01 *M* Na_2HPO_4 (pH 7)–CH_3CN (90:10); (2) 0.01 *M* Na_2HPO_4 (pH 6.6)–CH_3CN (90:10) and (3) 0.01 *M* Na_2HPO_4 (pH 7)–CH_3CN (92:8). Flow-rate was 0.9 ml/min for all used chiral mobile phases.

2.2. Chemicals

Standards of verapamil hydrochloride, norverapamil hydrochloride, verapamil *R*-(+) enantiomer, verapamil *S*-(−) enantiomer, norverapamil *R*-(+) enantiomer, norverapamil *S*-(−) enantiomer, D 617, D 620, PR 22, PR 23, PR 24 and PR 25 were kindly supplied by Knoll, Ludwigshafen, Germany. Methanol, acetonitrile (gradient grade); triethylamine, phosphoric acid and disodium hydrogensulphate (analytical grade) were supplied by Merck (Darmstadt, Germany).

2.3. Serum samples

Model lyophylized serum samples Sevatest Exapat were obtained from Imuna, Šarišské Michalany, Slovak Republic and blank human serum and the volunteer serum samples from Faculty Hospital, Comenius University, Bratislava, Slovak Republic.

2.4. Procedures

SPE in off-line mode was carried out using SepPak C_{18} cartridges and the procedure consisted of the following steps: wash with 3 ml methanol, 3 ml water, load 0.5 ml of serum diluted with 0.5 ml water, wash with 2 ml water, elute with 1 ml methanol (with 0.3% TEA).

On-line preseparation was realised using the SPS C_8 pre-column. The following on-line procedure was recommended: wash pre-column with 5 ml water (1 ml/min), load sample (serum diluted with water 1:1, 100 μl), wash pre-column with water (2 min, 1 ml/min), wash pre-column with the mobile phase (1.7 min, 1 ml/min) and elute the analyte to the analytical column with the mobile phase (1 ml/min).

3. Results and discussion

3.1. Achiral chromatographic separation

As was discussed in the Introduction, the complete simultaneous achiral HPLC separation of verapamil and its described metabolites presented in Fig. 1 with the sufficient chromatographic resolution values has not been published until now. For this reason the chromatographic conditions for the achiral simultaneous separation have also been studied. Two columns (Hisep C_{18} and NovaPak C_{18}) were tested for achiral separations. The HPLC chromatogram of the model mixture of all analyzed compounds on the NovaPak C_{18} column is shown in Fig. 2.

3.2. SPE procedures

A simple off-line SPE has been used for serum samples preparation and clean-up. The SPE procedure described in Experimental has been recommended and extraction recoveries and relative standard deviation (RSD) values at two concentration levels for all analyzed compounds were determined. Extraction recoveries of an off-line SPE mode for six independent extraction procedures are listed in Table 1. The HPLC chromatogram of blank and control serum spiked with verapamil, its metabolites and internal standard (I.S.) after off-line SPE are shown in Fig. 3a and b.

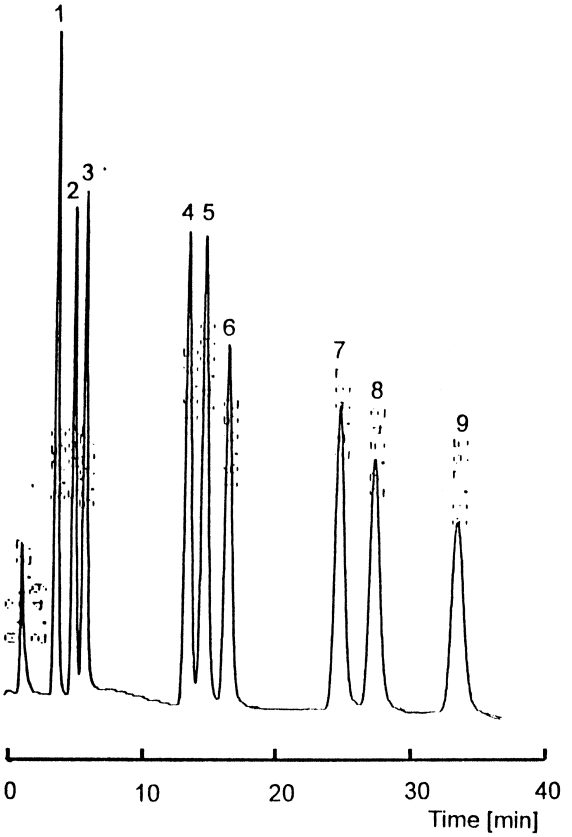

Fig. 2. HPLC chromatogram of model mixture of verapamil, its seven metabolites and gallopamil (c=100 ng/ml). HPLC system II; column: NovaPak C_{18} (150×3.9 mm); mobile phase: 35% acetonitrile in 0.03% TEA, pH 3.8; flow-rate: 0.9 ml/min. Peaks: 1=PR 25, 2=D 620, 3=D 617, 4=PR 22, 5=PR 23, 6=PR 24, 7=norverapamil, 8=verapamil, 9=gallopamil.

The column switching system was used for the direct serum sample injection. The switching system program is described in detail in Experimental. Extraction recoveries for on-line SPE have also been calculated from six independent analyses and were in the range 94.3±4.2 to 98.2±5.1% for verapamil and all its metabolites. On-line SPE using the SPS pre-column combined to the used HPLC system with the Rheodyne 7125 valve is illustrated in Fig. 4. The HPLC chromatograms of serum sample spiked with verapamil, its five metabolites, the internal standard and the volunteer serum sample after direct injection onto the HPLC system are illustrated in Fig. 5a and b. Verapamil and its three metabolites (norverapamil, D 617 and D 620) were determined in the volunteer sample (30 min), Fig. 5b.

Table 1
Extraction recoveries and relative standard deviations for off-line SPE

Compound	Concentration (ng/ml)	Recovery (%)	RSD (%)
Verapamil	10	91.1	5.2
	100	94.3	4.4
Norverapamil	10	90.8	4.3
	100	92.7	3.3
D 617	10	89.7	5.8
	100	91.7	4.6
D 620	10	88.8	5.4
	100	90.6	4.9
PR 22	10	91.4	5.1
	100	92.1	4.3
PR 23	10	90.1	5.4
	100	93.4	4.2
PR 24	10	91.2	5.1
	100	92.7	4.0
PR 25	10	87.7	5.8
	100	91.9	4.2

3.3. Method validation

3.3.1. Repeatability, precision, accuracy

The HPLC assay is robust, the repeatability of k' values determined from 10 independent injections of standards and control serum samples at two concentration levels (10 ng/ml and 100 ng/ml) show a small between-run variability. Mean repeatability of all k' values was 0.72%. As can be seen in Fig. 3a, no interfering compounds were found in blank plasma samples and the rest of proteins eluted in less than 3 min, which is sufficient for the first eluting peak separation (PR 25). All compounds are separated in 35 min with Rij values from Rij=1.1 (PR 22 and PR 23) to Rij=4.3 (D 617 and PR 22).

The results of intra- and inter-day precision values (RSDs) and accuracy (recovery) were also calculated for the same concentration levels. In all cases

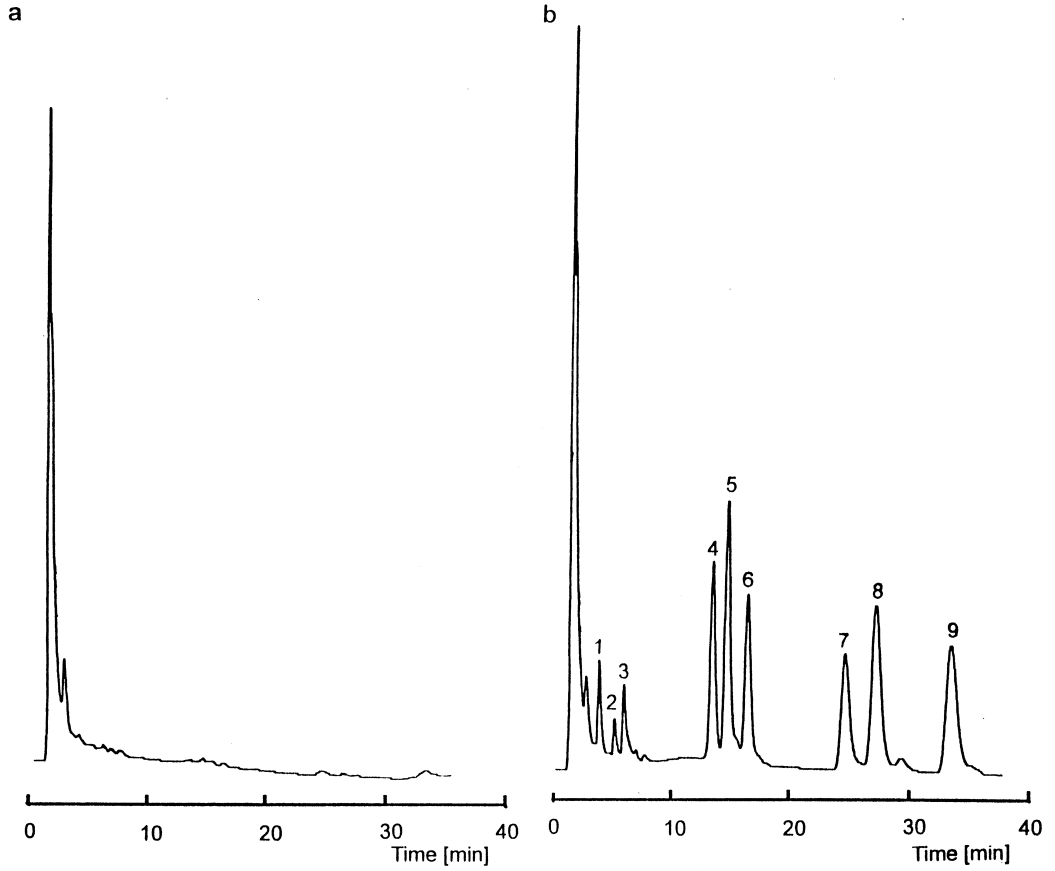

Fig. 3. HPLC chromatogram of off-line SPE extracts of serum blank (a) and control serum spiked with verapamil, its seven metabolites and I.S. (b). Separation conditions as in Fig. 2. Peaks as in Fig. 2.

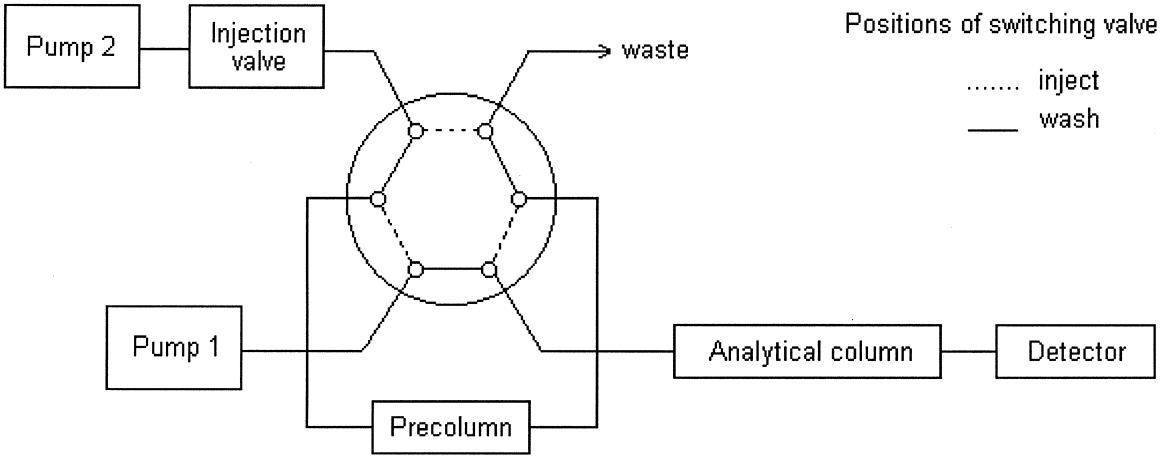

Fig. 4. Column switching system for on-line SPE procedure.

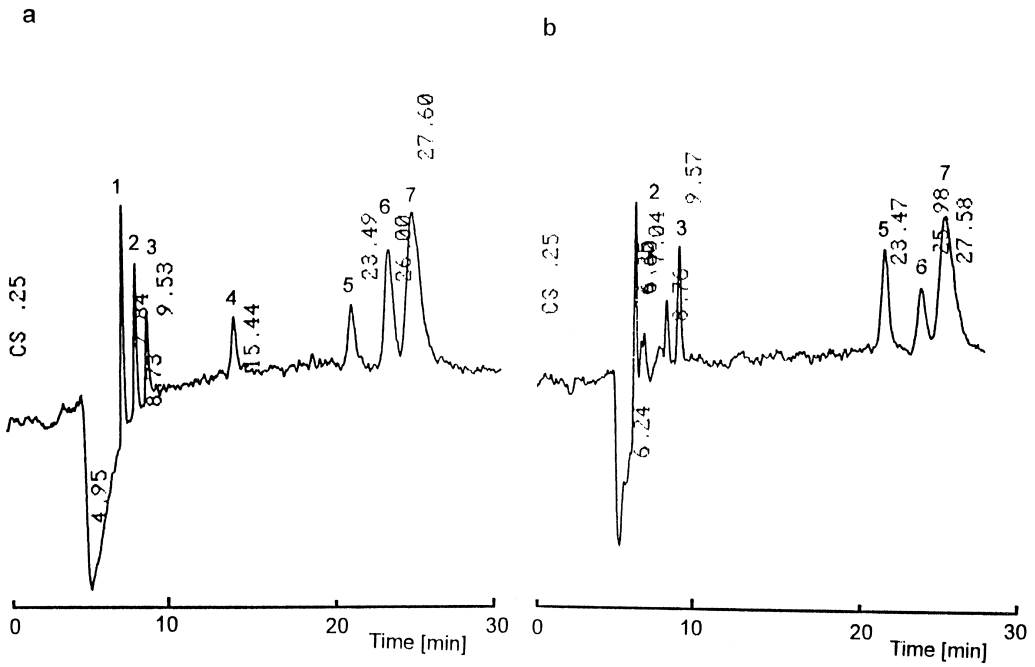

Fig. 5. HPLC chromatogram of on-line SPE extracts of control serum spiked with verapamil, its five metabolites and I.S., c=100 mg/ml (a) and the patient serum sample, t=30 min (b). HPLC system I; column: Hisep C_{18} (150×4.6 mm); mobile phase: 35% acetonitrile in 0.3% TEA, pH 4.0; flow-rate: 1 ml/min. Peaks: 1=PR 25, 2=D 620, 3=D 617, 4=PR 22, 5=norverapamil, 6=verapamil, 7=gallopamil.

accuracy was less than 13% for 10 ng/ml and 10% for 100 ng/ml. Mean RSD was less than 6% for concentration levels of 10 ng/ml and 5% for 100 ng/ml. Off-line SPE was used for the method validation.

3.3.2. Limit of quantitation

The limits of quantitation (LOQs) calculated from the peak area based on a signal-to-noise ratio of 10 were: verapamil, 3 ng/ml; norverapamil, 5 ng/ml; D 617, 2 ng/ml; D 620, 4 ng/ml; PR 22, 1.5 ng/ml; PR 23, 2 ng/ml; PR 24, 3 ng/ml and PR 25, 1 ng/ml.

3.3.3. Linearity

The calibration curves constructed by plotting peak area and standard concentration values were linear in the following ranges: verapamil, 20–400 ng/ml; norverapamil, 15–300 ng/ml; D 617, 2.5–30 ng/ml; D 620, 5–50 ng/ml; PR 22, 1.5–40 ng/ml; PR 23, 3–30 ng/ml; PR 24, 4–40 ng/ml and PR 25, 1–10 ng/ml.

Linear dependences of peak areas on compound concentrations ($y=ax+b$) were as follows: verapamil: $y=8.2x+15.9$, $r=0.9998$; norverapamil: $y=7.8x-59.6$, $r=0.9990$; D 617: $y=38.6x-3.2$, $r=0.9999$; D 620: $y=30.9x+20.0$, $r=0.9994$; PR 22: $y=11.0x-8.1$, $r=0.9998$; PR 23: $y=20.1x-19.7$, $r=0.9996$; PR 24: $y=15.2x+12.4$, $r=0.9991$; PR 25: $y=6.9x+16.8$, $r=0.9992$.

3.4. Chiral separation of verapamil and its metabolites

As was discussed in the Introduction, verapamil and all its metabolites are chiral compounds. For this reason the HPLC chiral separations of analyzed compounds have also been studied. According to the literature and our own experience with some other columns (Cellulose OD-R and AD-R) when no successful chiral separations for PR 25, D 620 and D

Table 2
Rij and α values for verapamil and its metabolite chiral separations[a]

Compound	Mobile phase					
	I		II		III	
	Rij	α	Rij	α	Rij	α
PR 25	1.57	1.44	1.50	1.44	2.30	1.53
D 620	0.98	1.22	0.88	1.23	1.13	1.30
D 617	0.93	1.20	0.84	1.22	1.08	1.27
PR 22	1.08	1.20	0.94	1.26	1.12	1.18
PR 23	0.93	1.17	0.85	1.18	0.95	1.15
PR 24	1.15	1.29	1.09	1.33	1.34	1.33
NOR	0.92	1.16	0.85	1.18	0.92	1.14
VER	1.05	1.24	0.97	1.26	1.08	1.23

[a] Mobile phases: I, 0.01 *M* Na_2HPO_4 (pH 7)–CH_3CN (90:10); II, 0.01 *M* Na_2HPO_4 (pH 6.6)–CH_3CN (90:10); III, 0.01 *M* Na_2HPO_4 (pH 7)–CH_3CN (92:8). Flow-rate: 0.9 ml/min.

617 have been achieved, we used an α_1-AGP column for the chiral separations of all verapamil metabolites. Three mobile phases were tested and α and chromatographic resolution (Rij) values for all separated pairs are listed in Table 2.

The mobile phase with lower content of organic modifier (8% acetonitrile) provided high Rij values for PR 25, D 620 and D 617 but the analysis time was greatly increased (e.g., 72 min for PR 22). Therefore the mobile phase with 10% acetonitrile and pH 7 (mobile phase I) was chosen for the chiral separation of all metabolites. Chiral separations of verapamil and all its metabolites on the α_1-AGP column are shown in Fig. 6. *R*-(+) enantiomers are eluted as the first peaks and *S*-(−) enantiomers as the second. Maximum separation time is about 30 min.

As is obvious, only some of metabolites could be separated simultaneously. In some cases two compounds (e.g., verapamil–D 617; norverapamil–D 620; verapamil–PR 25) or three compounds (verapamil–PR 23–D 617; verapamil–PR 24–D 617) could be separated simultaneously on the chiral α_1-AGP column using the same mobile phase.

3.5. Serum analyses

SPE in the off-line and on-line modes was applied for monitoring verapamil in a volunteer serum sample. After a 120-mg oral verapamil application, concentrations of verapamil and its metabolites in serum in different time intervals were determined as follows: 3 h: verapamil – 130.4 ng/ml, norverapamil – 138.4 ng/ml, D 617 – 58.7 ng/ml, D 620 – 55.3 ng/ml, PR 22 – 0 ng/ml, PR 23 – 4.0 ng/ml, PR 24 – 6.7 ng/ml; 12 h: verapamil – 108.7 ng/ml, norverapamil – 149.3 ng/ml, D 617 – 69.2 ng/ml, D 620 – 77.2 ng/ml, PR 22 – 3.2 ng/ml, PR 23 – 5.7 ng/ml, PR 24 – 8.7 ng/ml; 24 h: verapamil – 53.5 ng/ml, norverapamil – 85.9 ng/ml, D 617 – 48.9 ng/ml, D 620 – 64.5 ng/ml, PR 22 – 1.5 ng/ml, PR 23 – 4.2 ng/ml, PR 24 – 6.5 ng/ml

The ratios *R*-(+)/*S*-(−)-verapamil=4.3 and *R*-(+)/*S*-(−)-norverapamil=1.8 for verapamil and norverapamil in serum sample were obtained after 12 h.

4. Conclusion

A rapid and simple achiral HPLC assay was developed for the simultaneous HPLC analysis of verapamil and its seven metabolites in serum samples. SPE preseparation steps in off-line and on-line modes have been recommended as effective clean-up and preconcentration procedures. Limits of quantitation, linearity, precision and recoveries for the complete assay were evaluated and the optimal separation conditions were described for routine monitoring of all analyzed compounds. Chromatographic conditions for the chiral separation of all verapamil metabolites using a single chiral column and the same mobile phase has also been recommended.

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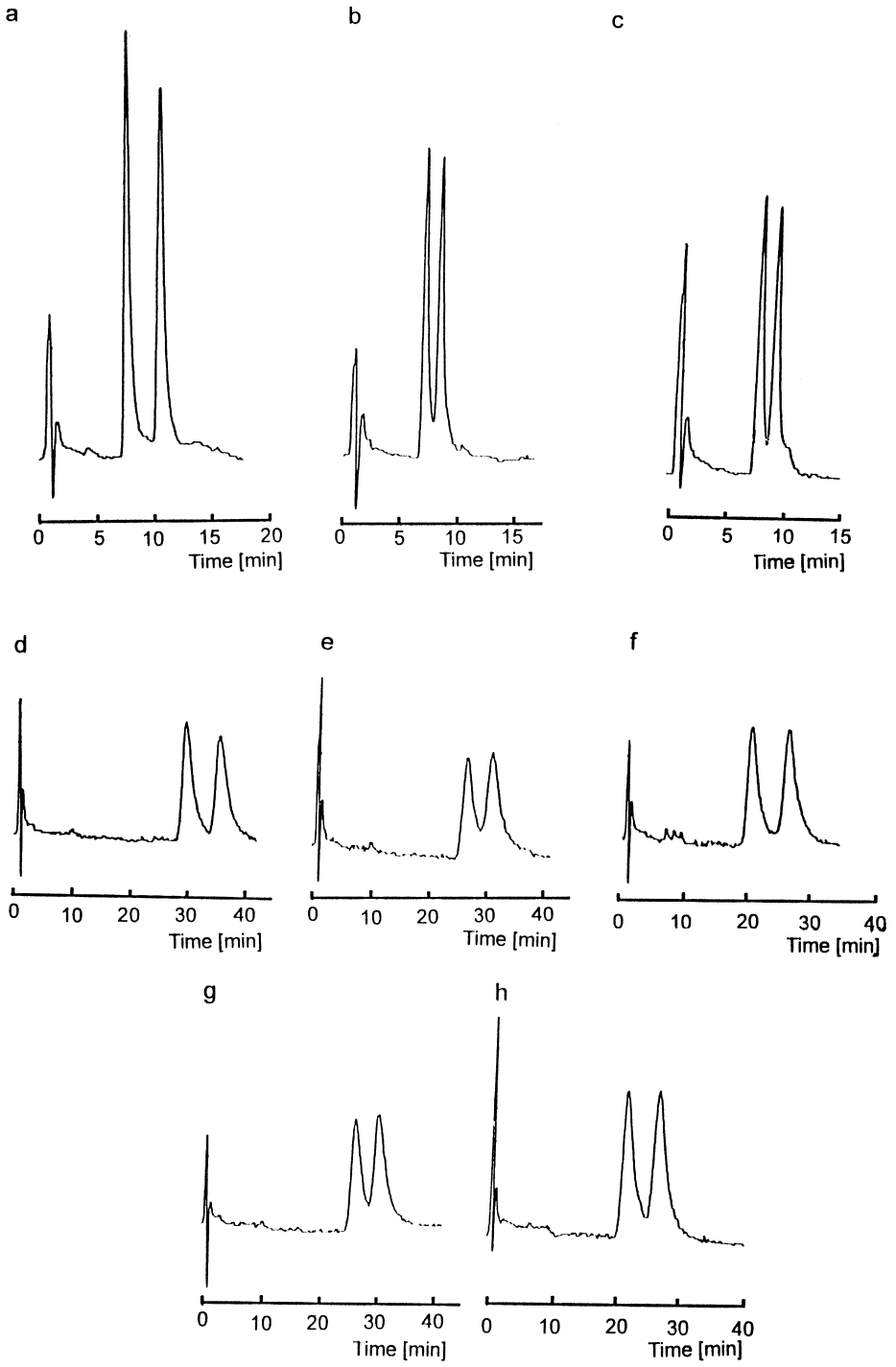

Fig. 6. HPLC chiral separation of verapamil and its seven metabolites. Column: α_1-AGP (100×4 mm); mobile phase: 0.01 *M* Na_2HPO_4 (pH 7)–acetonitrile (90:10); flow-rate: 0.9 ml/min. Peaks: a=PR 25, b=D 620, c=D 617, d=PR 22, e=PR 23, f=PR 24, g=norverapamil, h=verapamil.

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