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J. Biochem. Biophys. Methods 70 (2008) 1297–1303
JOURNAL OF biochemical and biophysical methods
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HPLC method for determination of verapamil in human plasma after solid-phase extraction ☆

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Received 22 June 2007; received in revised form 24 September 2007; accepted 30 September 2007

Abstract

A simple, rapid and precise HPLC method has been developed for the assay of verapamil in human plasma. The clean up of the plasma samples was tested using several adsorbents for solid-phase extraction and best recovery was obtained using mixed-mode cartridges (HLB - hydrophilic-lipophilic balance) ranging between 94.70 and 103.71%. HPLC separation was performed with isocratic elution on Lichrospher 60 RP-select B column (250 mm × 4 mm I.D., 5 μm particle size). The mobile phase was 40% acetonitrile and 0.025 mol/L KH_2PO_4 with pH 2.5 at flow rate of 1 mL/min. Diltiazem was used as internal standard and the detection wavelength was 200 nm. The calibration curves were linear in the range of 10–500 ng/mL. The developed method is convenient for routine analysis of verapamil in human plasma.

Keywords: Verapamil; Human plasma; Solid-phase extraction; HPLC

1. Introduction

Verapamil is a calcium blocker (Fig. 1) with a widespread use in management of supraventricular tachyarrhythmias, angina pectoris, hypertrophic cardiomyopathy and hypertension [1]. It is mostly used in a conventional tablet form in doses ranging from 40 to 180 mg and in a slow release form in doses of 120 and 240 mg.

About 90% of orally introduced verapamil is absorbed in the digestive tract, but only 10–20% of the dose enters the circulatory system unchanged. The biological half-time of verapamil is 3–7 h and the therapeutic concentrations of the drug in human plasma range from 20–500 ng/mL. Verapamil is metabolized mainly in human liver to six (according to some authors to twelve) metabolites, which are excreted through the kidneys. Only norverapamil, the *N*-demethylated metabolite, is pharmacologically active [2]. It shows about 20% of the efficacy of verapamil with regard to the vasodilatation effect, but has not antiarrhythmic activity.

Many investigations have been performed for determination of verapamil in human plasma using high-performance liquid chromatography (HPLC) with various detection systems. Some of them employ fluorescence detection [3–10], very few employ spectrophotometric detection in the UV range [11,12], whereas mass spectrometric detection is used in the recent developed methods [13–17].

As for the sample preparation procedures employed prior to the HPLC determination of verapamil, there is a diversity in the speed and complexity of the clean-up methods starting from direct determination of verapamil from rat plasma [7], through using only deproteinization of the plasma samples [11,13] to the more complex ones such as liquid–liquid extraction (LLE) using various organic solvents [3,4,6,9,10,15] and solid-phase extraction (SPE) with various adsorbents [4,8,9,17]. The most sophisticated sample preparation procedures employ automated column switching techniques for on-line sample preparation [5,7,8,12]. The efficiency of the liquid–liquid extraction and automated liquid–solid extraction has been compared by Hubert et al. [4] who found better sensitivity and precision of the liquid–solid extraction, but still recommended the liquid–liquid procedure as useful alternative. SPE using C_{18} SepPak cartridges was used for extraction of verapamil and its metabolites in serum samples (recovery: 87.7 ± 5.8 to 92.7 ± 4.0%)

☆ Dedicated to Academician Bojan Soptrajanov, the leader of the Spectroscopy Group in Macedonia, on the occasion of his 70th birthday.

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doi:10.1016/j.jbbm.2007.09.009

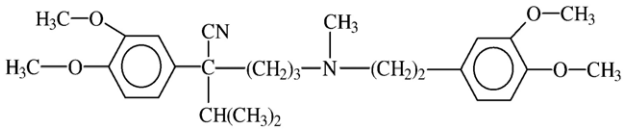

Fig. 1. Structure of verapamil.

and SPS C_8 pre-column for on-line SPE (recovery: 94.34 to 98.25%) [8]. Verapamil and norverapamil in blood, liver and kidney using HPLC with fluorescence detection with prior LLE and SPE with C_2 silica phases has been used and the LLE procedure proved to be simpler, less expensive and more versatile than SPE especially for complex biological specimens such as tissue samples [9]. A sample preparation procedure employing SPE with a mixed-mode sorbent (reversed phase and cation exchanger) has been developed for the determination of 14 drugs in whole blood including verapamil with high recoveries for most of them (> 96% fro verapamil) [17].

The purpose of this study was to develop a solid-phase extraction method for extraction of verapamil from human plasma and develop and validate a chromatographic method with UV detection suitable for the determination of the investigated drug. The sample preparation procedure was developed by testing several types of adsorbents and HLB cartridges were found to give best results for separation and concentration of verapamil from plasma. This is a very convenient method for separation and concentration of the analyzed drug and elimination of plasma interferences, because of its efficiency and rapid sample preparation. The method was validated by evaluating the recovery, selectivity, linearity, precision and accuracy. Finally, the method was used for determination of verapamil in real plasma samples from patients.

2. Materials and methods

2.1. Reagents and materials

Verapamil and the internal standard (I.S.), diltiazem, were kindly provided from Knoll GmbH (Ludwigshafen, Germany) and De Graaf sa (Lugano, Switzerland), respectively. Acetonitrile, methanol, phosphoric acid and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). All reagents used were HPLC grade. The used HPLC column was Lichrospher 60 RP-select B (250mm × 4mm I.D., 5 μm particle size) from Merck and cartridges used for solid-phase extraction were LiChrolut RP-18 (Merck), TSC (Merck), ENVI-Carb (Supelco) and Oasis HLB (Waters).

2.2. Preparation of standard solutions and calibration

Stock solutions of verapamil and diltiazem were prepared at concentrations of 1 mg/mL by dissolving appropriate amounts of verapamil hydrochloride and diltiazem hydrochloride in water. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

Calibration curves were constructed with five blank plasma samples spiked with appropriate amounts of the standard solutions. Linear regression analysis was performed. The calibration range was 10–500 ng/mL of verapamil for plasma, while the three control standards contained 25, 150 and 300 ng/mL verapamil in blank plasma.

2.3. Instrumentation and HPLC analysis

Analysis of verapamil was carried out using a Varian HPLC system equipped with a ternary pump Model 9012 and UV Diode Array Detector Model 9065. Different column packing including C18 and RP-select B were tested. The final choice, giving satisfying peak shape, resolution and run time was a Lichrospher 60 RP-select B column (250mm × 4 mm I.D., 5 μm particle size) with the analogous guard column (4 mm × 4 mm, 5 μm particle size). The mobile phase consisting of 0.025 mol/L KH_2PO_4, pH 2.5 and acetonitrile in ratio 60:40 (*V*/*V*) was found to give best results. Isocratic elution was performed with a flow rate of 1 mL/min at ambient temperature. Elution was monitored in the whole UV region and the best detection was achieved at 200 nm. Separation of the analyzed drug was performed with the solid-phase extraction manifold Visiprep™ from Supelco.

2.4. Sample preparation

Human plasma was prepared from heparinized whole-blood samples. Blood samples were collected from healthy volunteers and stored at −20°C. After thawing, samples were spiked daily with working solutions of verapamil and internal standard, diltiazem.

A set of cartridges with different stationary phases for SPE were tested: LiChrolut RP-18 (Merck), TSC (Merck), ENVI-Carb (Supelco) and Oasis HLB (Waters). The best results for isolation of verapamil and I.S. were obtained when extraction was performed with Oasis HLB cartridges using the following procedure.

Before extraction, plasma samples were buffered with 1 mL of 0.1 mol/L KH_2PO_4 solution with pH 9 and 1 mL of prepared samples were spiked with 50 μL of internal standard, diltiazem. The cartridges were conditioned with 1 mL methanol and 1 mL water. The buffered plasma was introduced into the cartridge. 1 mL solution of 65% methanol with 2% NH_4OH was used to rinse the cartridge and remove the interferential components. Elution was performed with 1 mL solution of 65% methanol with 2% acetic acid. The eluate was evaporated to dryness under air for about 30 min at 40 - 45°C and the residue was dissolved in 200 μL mobile phase. 100 μL of the final solution was injected into the HPLC system.

3. Results

Several HPLC method variables with respect to their effect on the place and shape of the peaks of verapamil and internal standard, diltiazem, were tested: HPLC columns with different bonded phases (C18 and RP-select B), series of mobile phases

with different amount of organic modifier acetonitrile in the mobile phase, the effect of buffer concentration, the effect of mobile phase pH, the effect of *iso*-propanol in the buffer solution and the effect of temperature on retention and resolution of analyzed component and internal standard. According to the obtained data, satisfactory resolution and relatively short analysis time were obtained when separation was performed on Lichrospher 60 RP-select B column (250 mm × 4 mm I.D., 5 μm particle size) protected with appropriate guard column at ambient temperature, using the optimal mobile phase consisting of 40% (*V*/*V*) acetonitrile in 0.025 mol/L KH_2PO_4 buffer, pH 2.5. The flow rate on the mobile phase was 1 mL/min and detection of verapamil and internal standard diltiazem was performed on wavelength of 200 nm. The retention times of verapamil and internal standard, diltiazem were 4.2 and 5.6 min respectively.

Several types of cartridges for solid-phase extraction [TSC (Merck), LiChrolut RP-18 (Merck), ENVI-CARB (Supelco) и Oasis HLB (Waters)] were tested in order to obtain satisfactory values for the recovery of verapamil and the internal standard (diltiazem). In order to achieve good separation of verapamil and I.S. from the plasma matrix, different parameters were checked: dilution and buffering of the samples, washing solutions, pH and the composition of the elution solvents. The best purification and satisfied recovery values for verapamil were obtained using HLB columns, preparing the samples with 0.1 mol/L KH_2PO_4 (pH 9). Extraction recoveries for verapamil using HLB columns were satisfactory ranging from 94.70–103.71% and these cartridges were used for all investigations in this study.

3.1. Method development

In order to develop a convenient, simple and accurate HPLC method for quantitative determination of verapamil in human plasma with internal standard, diltiazem, several investigations were performed. Diltiazem was chosen as internal standard because of its good response at the working wavelength and not interfering in the analysis of verapamil. It is chemically stable and similar with the analyzed component; verapamil and the reverse combination (diltiazem, as analyzed substance and verapamil, as internal standard) has already been used in a validated method [18].

A set of HPLC columns with different bonded phases (C18 and RP-select B) were tested in our preliminary experiments. Symmetrical peak shapes, satisfactory resolution and relatively short analysis time were obtained when separation was performed on Lichrospher 60 RP-select B column (250 mm × 4 mm I.D., 5 μm particle size) protected with appropriate guard column. All investigations were performed with 1 mL/min flow rate on the mobile phase. The elution was monitored in the whole UV region and the wavelength of 200 nm was chosen for detection of verapamil and internal standard diltiazem because of the highest sensitivity.

Series of mobile phases with different amount of organic modifier acetonitrile present in the mobile phase were tested to investigate its effect on separation of verapamil and diltiazem. In Fig. 2a the results obtained by elution with acetonitrile present in the range from 30 to 60% are presented. Satisfactory resolution and analysis time were obtained when using 40% (*V*/*V*) acetonitrile in the mobile phase.

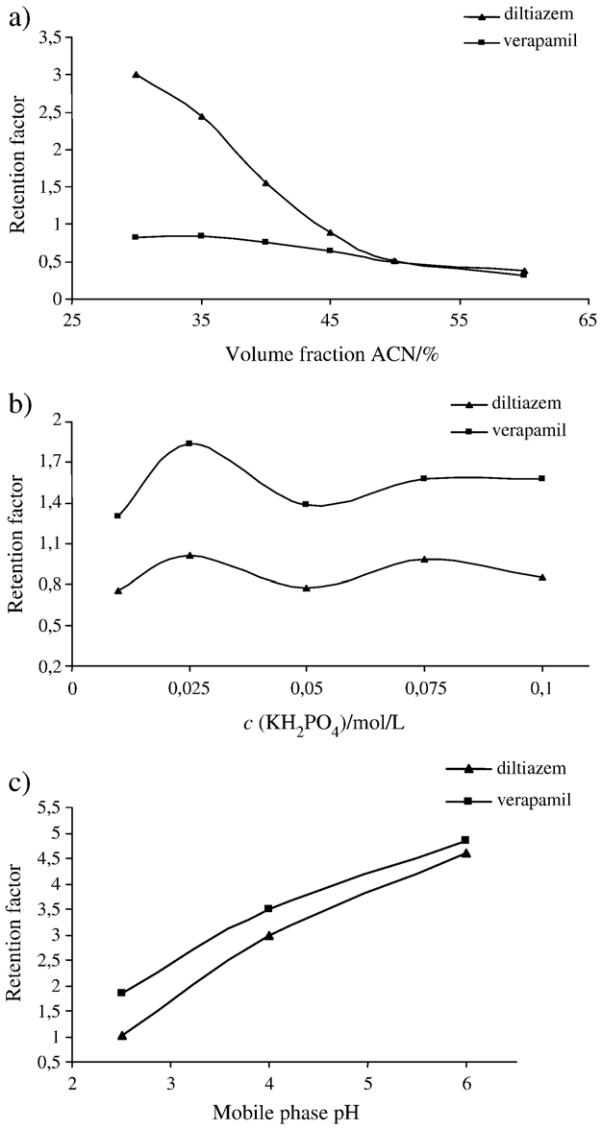

Fig. 2. Effect of: a) acetonitrile content, b) buffer concentration, and c) pH of the mobile phase on retention.

The effect of buffer concentration on analyte retention and separation was also tested in the concentration range from 0.01 to 0.1 mol/L. The buffer concentration of 0.025 mol/L KH_2PO_4 (Fig. 2b) was chosen because the subsequent increasing of concentration did not cause significant changes on retention times of the components and peak shapes.

The effect of mobile phase pH on analyte retention and the shape of analyte peaks was tested as well. The obtained results showed a relatively long analysis time and poor separation when using a mobile phase with pH = 6.0. When decreasing the pH value, separation improved and best resolution between two peaks and short analysis time was obtained with a mobile phase with pH = 2.5. The results are shown in Fig. 2c.

Additionally, the effect of *iso*-propanol added in the buffer solution was tested in the range from 0.5 to 4%. This test was

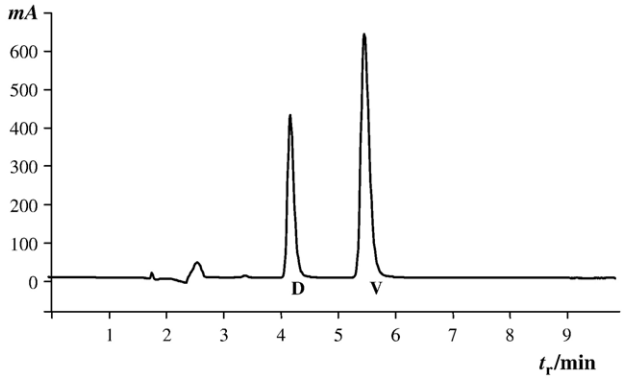

Fig. 3. Chromatogram of standard solutions of verapamil (10 μg/mL) and internal standard diltiazem (10 μg/mL), (D-diltiazem, V-verapamil).

performed because of the fact that it has been used as an organic modifier in the method for determination of diltiazem in human plasma using verapamil as internal standard [18]. The presence of *iso*-propanol in the mobile phase did not improve the analyte resolution and peak shape, but on the contrary, it caused distortion and tailing of the analyte peaks. Therefore, the further analyses were performed using mobile phase without *iso*-propanol.

Finally, the effect of temperature on retention and resolution of analyzed component and internal standard in range from 20 to 50°C was tested. The obtained results showed that temperature does not have a significant effect on peaks shape and the analysis time is negligible shorten. So, ambient temperature was chosen for all further investigations.

From these data, the following optimal mobile phase was established: 40% (*V*/*V*) acetonitrile in 0.025 mol/L KH_2PO_4 buffer, pH 2.5. The retention times of verapamil and internal standard, diltiazem were 4.2 and 5.6 min respectively. Typical chromatograms obtained from standard solution of verapamil and diltiazem produced by the developed HPLC method are shown in Fig. 3. These conditions were applied on the plasma samples after the SPE procedure and the peaks of verapamil and diltiazem were well separated from the matrix components remained after the sample preparation procedure.

For determination of the analytical recovery of the sample preparation procedure, a calibration diagram was constructed using aqueous standard solutions. The obtained linear dependence peak area of verapamil/peak area of diltiazem with correlation coefficient (0.9987) is the following:

$$\text{Area(V)/Area(D)} = 0.008721 \cdot \gamma(\text{D})[\text{ng/mL}] + 0.166907$$

The extraction recoveries were calculated by comparing the peak area of verapamil and internal standard obtained after solid-

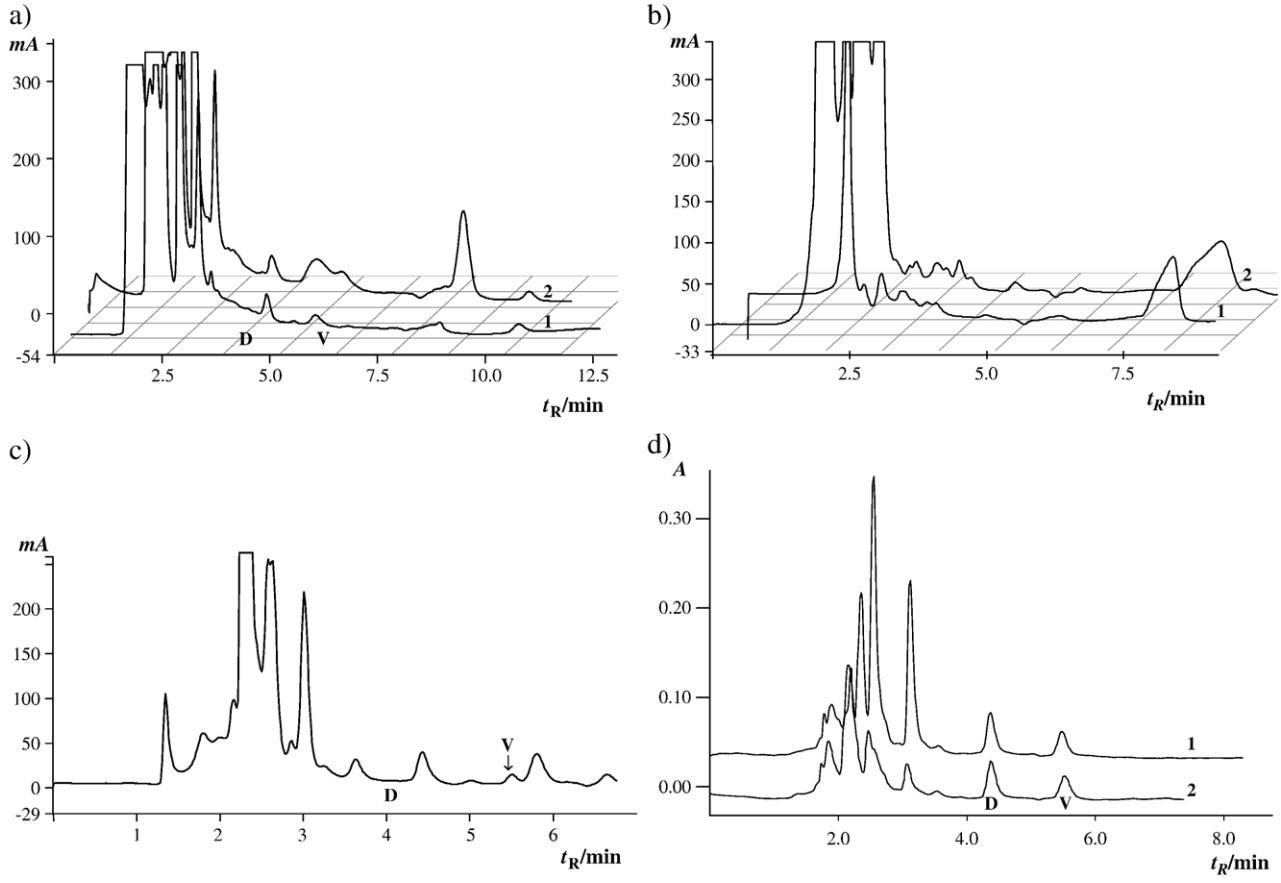

Fig. 4. Chromatograms of plasma samples prepared with different solvents and obtained after SPE on different cartridges. a. Samples prepared on TSC with: 1-H_3BO_3 and 2-KH_2PO_4 (pH 9). b. Samples prepared on ENVI-Carb with 0.01mol/L KH_2PO_4: 1-pH 6 and 2-pH 3. c. Samples prepared on RP-18 with KH_2PO_4 (pH 9). d. Samples prepared on HLB with: 1-H_3PO_4 and 2-KH_2PO_4 (pH 9).

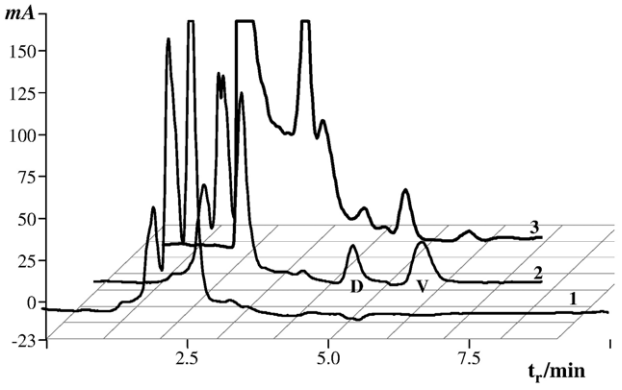

Fig. 5. Chromatograms of 1-blank plasma, 2 - spiked plasma containing 300 ng/mL verapamil and 250 ng/mL diltiazem, and 3 -blood sample containing 23 ng/mL verapamil (D-diltiazem, V-verapamil).

phase extraction and those resulting from the direct injection of aqueous standard solutions of verapamil and internal standard.

4. Discussion

4.1. SPE optimization

The testing of the SPE cartridges [TSC (Merck), LiChrolut RP-18 (Merck), ENVI-CARB (Supelco) and Oasis HLB (Waters)] was performed for establishing the conditions for obtaining satisfactory values for the recovery of verapamil and the internal standard, diltiazem. The influence of the dilution and buffering of samples, washing solutions, pH and the composition of the elution solvents was checked for all cartridges and Oasis HLB with mixed-mode stationary phase gave most satisfactory results.

TSC columns were found to be not convenient for extraction of verapamil from plasma and poor results were obtained when this type of cartridges was used for solid-phase extraction. Peaks of verapamil and I.S. had tailing and were not suitable for quantification (Fig. 4a). ENVI-Carb columns were also not convenient for efficient extraction of verapamil. Despite all efforts, the loss of analyte and internal standard was noticed during the washing step (Fig. 4b).

One of the most commonly used cartridges for SPE, RP-18 columns, were also tested for extraction of verapamil. Brandsteterova and Wainer [8] have used C_{18} SepPak cartridges for extraction of verapamil and its metabolites in serum samples (recovery: 87.7 ± 5.8 to 92.7 ± 4.0%). However, in our case, purification of the plasma samples and complete separation of verapamil with RP-18 columns was not attained because the interferences were adsorbed and coeluted with the analyte and good chromatographic separation of the peak of verapamil and the abutting peaks of the interferential components was not achieved (Fig. 4c). This may explain the poor data in the literature for employing this most universally used adsorbent for preparation of biological samples for determination of verapamil.

Efficient purification and satisfied recovery values for verapamil were obtained using HLB columns, preparing the samples with concentrated H_3PO_4 (20 μL were added in 1ml sample) and 0.1mol/L KH_2PO_4 (pH 9). The obtained chromatographic peaks of verapamil and diltiazem are sharp, symmetrical and well separated from the matrix components present in the plasma (Fig. 4d). Extraction recoveries for verapamil using HLB columns were satisfactory ranging from 94.70–103.71% and these cartridges were used for all investigations in this study.

The developed HPLC method was used for analysis of patient plasma samples after oral administration of verapamil. Typical chromatogram obtained from plasma samples of patients prepared according to the developed procedure for sample preparation after a single oral dose of verapamil is shown in Fig. 5.

Table 1
Results for intercept, slope and R^2 of the calibration curve for the linearity test

Day	Intercept	Slope	R^2
1	0.00456	0.18847	0.9996
2	0.00494	0.19746	0.9995
3	0.01006	0.22193	0.9997

Table 2
Intra- and Inter- day accuracy and precision data for verapamil in human plasma determined after solid-phase extraction

Sample	25 ng/mL		150 ng/mL		300 ng/mL	
	Found concentration	e_r (%)	Found concentration	e_r (%)	Found concentration	e_r (%)
Intra-day accuracy and precision data for verapamil						
1	26.31	5.24	147.34	−1.77	310.91	3.64
2	24.52	−1.92	145.78	−2.81	295.34	−1.55
3	24.38	−2.48	153.67	2.45	312.18	4.06
<x>	25.07	0.28	148.93	−0.71	306.14	2.05
s_d	1.07		4.18		9.38	
RSD (%)	4.29		2.81		3.06	
Inter-day accuracy and precision data for verapamil						
I/1	24.23	−3.08	157.11	4.74	316.8	5.60
I/2	25.57	2.28	145.78	−2.81	276.76	−7.74
II/1	24.82	−0.72	146.25	−2.50	309.47	3.16
II/2	23.15	−7.40	151.40	0.93	288.44	−3.85
III/1	26.06	4.24	156.65	4.43	306.07	2.02
III/2	24.36	−2.56	150.93	0.62	293.83	−2.06
<x>	24.69	−1.21	151.35	0.90	298.56	−0.48
s_d	1.04		4.87		14.89	
RSD (%)	4.19		3.22		4.98	

Table 3
Limit of quantification of verapamil

Analysis	Found concentration (ng/mL)	Relative error (%)
1	9.4	−6.00
2	10.1	1.00
3	10.9	9.00
4	11.4	14.00
5	10.3	3.00
<x>	10.42	4.20
s_d	0.76	
RSD (%)	7.35	

Table 4
Stability of verapamil in human plasma

Analyzed samples	25.0 ng/mL	e_r (%)	300.0 ng/mL	e_r (%)
Immediately	26.29	5.16	301.74	0.58
After 2 h	27.18	3.38	313.38	3.86
After 24 h	25.74	−2.09	293.14	−2.85
Cycle 1	27.45	4.41	309.61	2.61
Cycle 2	24.97	−5.02	284.15	−5.82
After 1 month	26.38	0.34	308.09	2.10

4.2. Method validation

4.2.1. Linearity

Linearity was tested in 3 days at five concentration levels ranging from 10–500 ng/mL of verapamil and concentration of 250 ng/mL of internal standard, diltiazem in plasma samples. Slope, intercept, and correlation coefficient were calculated and the results are presented in Table 1.

4.2.2. Accuracy and precision

Intra- and inter-day accuracy was determined by measuring plasma quality control samples at low (25 ng/mL), medium (150 ng/mL) and high (300 ng/mL) concentration levels of verapamil. Concentration of internal standard in spiked plasma was 250 ng/mL. An indication of accuracy was based on calculation of the relative error of the mean observed concentration compared to the nominal concentration. Precision was expressed as relative standard deviation (RSD). Obtained results are presented in Table 2.

Relative errors were found to be between 0.72 and 7.74%, and relative standard deviation between 2.81 to 4.98%. The obtained results indicated satisfactory precision and accuracy of the proposed method.

4.2.3. Limit of quantification (LOQ)

Limit of quantification was accepted as the lowest concentration of verapamil which can be measured with an error less than 20%. It was determined using the lowest calibration standard in five different analytical days. Verapamil concentration of 10 ng/mL was accepted as LOQ. Results are presented in Table 3.

4.2.4. Stability of verapamil in plasma samples

Stability of verapamil in plasma samples was investigated with spiked samples at two different concentration levels prepared in duplicate. Spiked samples were analyzed after different storage conditions: immediately, after staying for 2 and 24 hours at room temperature, after one and two freeze/thaw cycles and after 1 month stored at −20°C. The obtained results are shown in Table 4 and show that relative errors are between 0.58 and 5.82%, which confirm the stability of verapamil in plasma samples under different storage conditions.

4.2.5. Ruggedness of the method

Ruggedness of the method was checked by applying the proposed HPLC method with mobile phases with different pH values and detection at different wavelengths. Relative error was calculated by comparing pH value of the mobile phase from 2.2 to 2.8 or to those obtained by changing the detection wavelength from 195 to 205 nm. Standard solution with medium concentration of the analyzed component (500 ng/mL verapamil spiked with 250 μg/mL internal standard) was directly injected into the HPLC system. Results presented in Table 5 show relative errors less than 4.7% indicating good ruggedness of the developed HPLC method at different values of pH of the mobile phase. On the other hand, changing of wavelengths in the range of 195–205 nm produces high relative errors for verapamil because of the considerable change of the absorbance with wavelength in this narrow region. These results clearly indicate that analysis of the investigated drug must be performed every time at wavelength of 200 nm. The relative errors for the internal standard, diltiazem, are in permissive range between 0.07–4.8%.

5. Conclusion

The developed HPLC method including solid-phase extraction for sample preparation is simple, rapid and convenient for determination of verapamil in plasma samples. Different cartridges for SPE were tested and the best results were

Table 5
Ruggedness of the method for verapamil determination using mobile phases with different pH values and determination at different detection wavelengths

Sample	pH 2.5	pH 2.2		pH 2.8		200 nm	195 nm		205 nm	
	Area	Area	e_r (%)	Area	e_r (%)	Area	Area	e_r (%)	Area	e_r (%)
Verapamil										
1	60485	62540	3.39	62564	3.44	60485	51713	−14.50	40954	−32.29
2	61019	61774	1.24	63672	4.35	61019	53962	11.56	39865	−34.67
3	60596	65166	7.54	63598	4.95	62596	45197	−27.79	40132	−35.88
4	61619	64176	4.15	64142	4.09	61619	52932	−14.09	38231	−37.95
<X>	61429.7	63414	4.08	63494	4.21	61429.7	50951	−16.99	39795.5	−34.20
Diltiazem										
1	42338	44158	4.29	43241	2.13	42338	44104	4.17	41425	−2.15
2	45073	42954	−4.70	46136	2.36	45073	47105	4.51	45112	0.08
3	44831	46475	3.67	45767	2.08	44831	47005	4.85	44866	0.07
4	45204	45498	0.65	46512	2.89	45204	47179	4.37	45173	−0.06
<X>	44361.5	44771.2	0.98	45414	2.37	44361.5	46348.2	4.47	44144	−0.52

achieved using HLB columns. The optimized SPE conditions for separation of verapamil with HLB cartridges provide efficient clean up of the complex biological matrix and high recovery of the investigated drug. Parameters of validation demonstrate good precision and accuracy, which proves the reliability of the proposed method. Finally, this method is suitable for identification and quantification of verapamil in human plasma and it can be useful in pharmaceutical studies.

Acknowledgements

The authors gratefully acknowledge the support given by the CEEPUS Program realized through the CEEPUS network (CII-HU-0010-01-0607 - Teaching and learning bioanalysis) for presenting the results at the Summer School 2007 - University of Pecz, Hungary. The financial support provided by the Ministry of Education and Science of the Republic of Macedonia (Project No. 13-800/3-05) is also acknowledged.

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