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Short communication

Optimization of a solid-phase extraction method for determination of indapamide in biological fluids using high-performance liquid chromatography

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Abstract

A new simple and rapid high-performance liquid chromatographic (HPLC) method with UV detection for the determination of indapamide in biological fluids has been developed. Indapamide and internal standard were isolated from serum and whole blood samples by solid-phase extraction with RP select B cartridges. The chromatographic separation was accomplished on a reversed-phase C_8 column with a mobile phase composed of 0.1% (v/v) triethylamine in water (pH 3.5) and acetonitrile (63:37, v/v). UV detection was set at 240 nm. The calibration curves were linear in the concentration range of 10.0–100.0 ng/ml for serum, and 50.0–500.0 ng/ml for whole blood, and the limits of quantification were 10.0 and 50.0 ng/ml, respectively.
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1. Introduction

Indapamide, 3-(aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-1-yl)benzamide, is an antihypertensive agent administered to individuals with mild to moderate hypertension. Indapamide is an extensively metabolized drug with only ~7% of the total dose administered, recovered in urine as unchanged during the first 48 h [1]. There are several investigations concerning the determination of indapamide in pharmaceutical preparations by high-performance liquid chromatography (HPLC) with UV detection [2,3] and with amperometric detection [4]. A number of assay methods for indapamide in biological fluids have been reported [4–9]. Indapamide is preferentially bound to red blood cells [10], which requires separation and concentration of indapamide prior to its determination in whole blood samples. In the published methods, liquid–liquid extraction with diethyl ether as solvent has been used for sample preparation [5,6]. The determination of indapamide in human serum [7], in plasma [6,8] and urine [4,6,9] after liquid extraction using ethyl

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acetate has also been investigated. The disadvantage of these methods employing liquid–liquid extraction (with much chemical consumption) of indapamide from biological fluids is that they involve several steps yielding poor separation from the blood and serum endogenous interferences. Also, there are problems in the dissolution of the residue after liquid extraction and evaporation of organic layer under a gentle stream of nitrogen. All these methods are time consuming (usually up to 1 h) because of multiple steps of extraction, drying, etc.

Therefore, we have developed a new HPLC method suitable for the determination of indapamide in biological fluids employing solid-phase extraction for sample preparation, which enables simple and rapid isolation and concentration of the analysed drug. For the purpose of minimizing the variability caused by sample pretreatment we suggest a method of internal standardization for the quantification of indapamide.

2. Experimental

2.1. Materials

Indapamide working standard was supplied by Laboratori Alchemia, Italy, and the internal standard diazepam by Select Chemie, Switzerland. HPLC grade acetonitrile and methanol were purchased from Across Organics, Belgium. Triethylamine, *o*-phosphoric acid, sodium carbonate and columns for solid-phase extraction were obtained from Merck (Germany).

2.2. Instrument and chromatographic conditions

A series of parameters, including composition and pH of mobile phase, column packing, flow rate and detection wavelength, were tested with respect to the location and shape of the peaks of indapamide and of the internal standard in the corresponding chromatograms. The final choice of the stationary phase giving satisfying resolution and run time was a reversed-phase Supelcosyl LC-8-DB column, 250× 4.6 mm I.D. (5 μm, particle size), protected by a Supelguard™ LC-8-DB guard column (2 cm). A mobile phase consisting of 0.1% (v/v) triethylamine in water, pH 3.5, and acetonitrile (63:37, v/v) delivered by a Perkin-Elmer LC series 200 pump was found to give the best results. The triethylamine solution was prepared by adding 100 μl triethylamine to 100 ml H_2O and adjusting the pH of this solution (3.5) with concentrated *o*-phosphoric acid. The mobile phase was filtered and degassed with helium. Isocratic elution was performed with changes in the flow rate as follows: 1.2 ml/min from 0 to 7.5 min and 1.5 ml/min from 7.5 to 17.0 min. Chromatographic separations were performed at 37 °C. An ultraviolet diode array detector (Perkin-Elmer LC 235 C) was used for detection and 240 nm was chosen as optimal for determination of indapamide. The samples were introduced in the column using a Perkin-Elmer LC ISS Series 200 autosampler; injection volume was 130 μl. The chromatographic system was controlled by the software package Turbochrom Version 4.1 plus and UV spectrometric data were produced by TurboScan Version 2.0.

2.3. Preparation of standards

Stock solutions of 100 μg/ml of indapamide and 200 μg/ml of the internal standard diazepam were prepared monthly in methanol and stored at 4 °C. No change in stability over a period of 1 month was observed. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

2.4. Sample preparation

Human serum 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 stock solutions of indapamide and internal standard.

A solid-phase extraction vacuum manifold (Merck) was used for sample preparation. Satisfactory values for recovery of indapamide were obtained with a single extraction with RP select B solid-phase cartridge (200 mg) for isolation of the drug and the internal standard from blood and serum samples. The cartridge was conditioned sequentially by 2 ml methanol and 2 ml water, followed by 0.1 ml of 0.1 *M* sodium carbonate solution, and, only for

blood samples, conditioning of the columns was finished with an additional 0.05 ml of heparin (1000 U/ml). The spiked sample (total volume 2.5 ml) was introduced into the cartridge under vacuum at 5 p.s.i. Water (2 ml) was used to rinse the cartridge. The clean-up was accomplished with an additional 0.05 ml of methanol through the cartridge. Elution was then performed in two steps: first with 0.2 ml of methanol and this eluate was collected in a clean tube without vacuum, and then with an additional 0.1 ml methanol. The tube with eluate and cartridge were centrifuged at 3500 rpm for 5 min. After centrifugation the sample was filtered using a filter with pore size of 0.45 μm and 130 μl volume was injected into the HPLC system.

2.5. Calibration curves

Typical calibration curves were constructed with six blank blood and serum samples spiked with appropriate amounts of the standard solutions. The calibration range was 10.0–100.0 ng/ml of indapamide for serum and 50.0–500.0 ng/ml for blood. The standard samples were prepared according to the procedure as unknown samples. The calibration curves were obtained by plotting the peak area ratio of indapamide to internal standard versus concentration of indapamide in ng/ml. The regression equations were calculated by the least-squares method.

3. Results and discussion

3.1. Method development

A series of studies was conducted in order to develop a convenient and easy-to-use method for quantitative analysis of indapamide in biological fluids. Several HPLC method variables with respect to their effect on the separation of indapamide and internal standard (diazepam) from the matrix were investigated. Indapamide is not easily extracted from biological samples and so the measurement of the samples without an internal standard should produce a large variation of data. Also the internal standard method can be beneficial when transfers, evaporations and solid-phase extraction, or other experimental operations that can cause losses, are used since the ratio of analyte to internal standard remains constant. Diazepam was chosen as the internal standard because it gives a good response and working wavelength and did not interfere in the analysis of indapamide. Also the selection of diazepam was based on its chromatographic and extraction behavior.

In our extensive preliminary experiments a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, 2-propanol and triethylamine with different volume fractions were tested. The results were most satisfactory when mobile phase consisted of 0.1% (v/v) triethylamine in water with pH 3.5 and acetonitrile in volume fractions 63:37. A set of column packing including C_8, C_{18} and LC-8-DB with different lengths and particle sizes was tested and the LC-8-DB packing showed best separation. Among several flow-rates tested (0.8–2 ml/min) the rate of 1.2 ml/min from 0 to 7.5 min and then 1.5 ml/min from 7.5 to 17.0 min was the best with respect to location and resolution of the peaks of indapamide and internal standard from the interfering peaks. The elution was monitored in the whole UV region and the wavelength of 240 nm exhibited the best detection. A typical chromatogram of standard solutions of indapamide (1000 ng/ml) and internal standard (1000 ng/ml) produced by the developed HPLC method is shown in Fig. 1. Retention times of indapamide and internal standard are 7.3 and 14.2 min, respectively.

In addition, different cartridges for solid-phase extraction (C_{18}, TSC (toxicology screening cartridge) and RP-select B) were tested in order to obtain satisfactory values for recovery of indapamide. The extraction recoveries were calculated by comparing the peak height of indapamide obtained for low, medium and high level quality control samples ($n=3$ for each level for indapamide, $n=9$ for internal standard) and those resulting from the direct injection ($n=3$, working solutions) of the theoretical amount of either indapamide or internal standard (=100% recovery). Results of this investigation are presented in Table 1.

As can be seen, satisfactory values for recovery of indapamide and internal standard were obtained when solid-phase extraction was performed on RP-

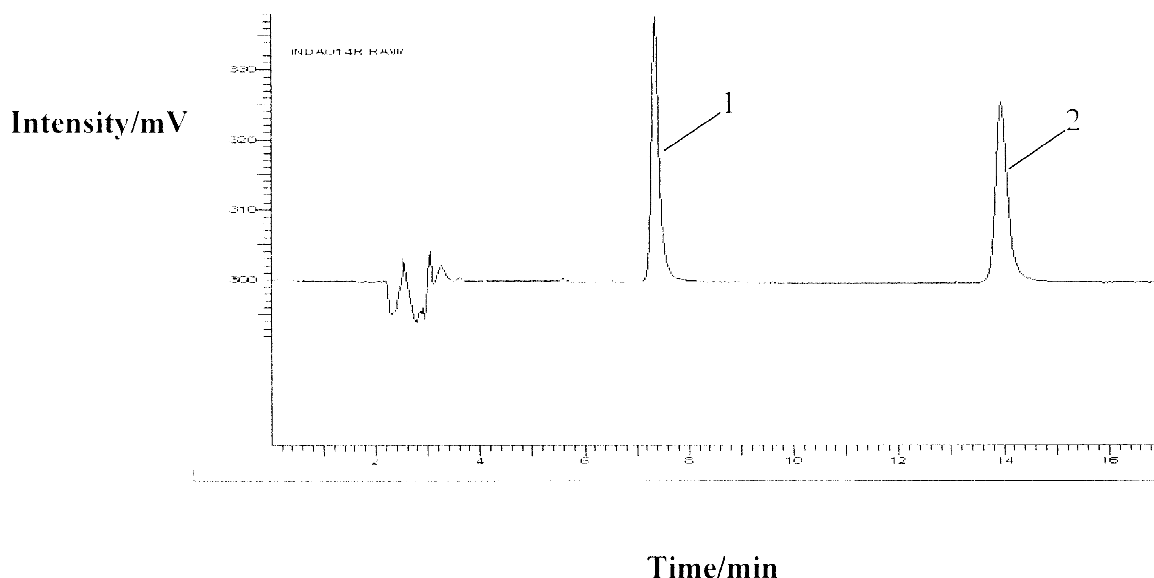

Fig. 1. Chromatogram of standard solutions of indapamide (1000 ng/ml) and internal standard (1000 ng/ml); 1, indapamide; 2, internal standard.

select B cartridges, especially for serum samples. The complexity of the whole blood matrix produces a low, but reproducible recovery value for internal standard. The best results were obtained using RP-select B because these cartridges are more suitable for enrichment of the somewhat stronger polar compounds, whereas C_{18} cartridges are more suitable for non-polar molecules using the same matrix.

Table 1
Absolute recoveries of indapamide and internal standard from spiked serum and blood samples

	Mean recovery (%)		
	TSC	C_{18}	RP select B
Serum			
γ(indapamide)/ng/ml			
15.0	48.8	31.2	96.9
50.0	52.2	34.4	97.2
90.0	55.1	39.5	100.7
γ(internal standard)/ng/ml			
60.0	21.2	80.8	99.7
Whole blood			
γ(indapamide)/ng/ml			
70.0	35.2	29.1	80.1
180.0	39.7	31.1	81.1
400.0	41.0	35.5	83.5
γ(internal standard)/ng/ml			
200.0	35.2	50.1	59.2

γ, mass concentration.

In order to improve the extraction procedure, cartridges for solid-phase extraction were conditioned with 0.1 *M* sodium carbonate solution before introducing the spiked samples. The columns can be preconditioned with an appropriate pH buffer and in our studies pH~10 was found to be most appropriate for adsorption of indapamide and internal standard. During the initial development of the procedure, a total of 0.5 ml of methanol was used to elute indapamide in two 0.25-ml aliquots. Lower eluent volume was advantageous to avoid the need for evaporation at low serum and blood drug levels. It was found that the first 0.2 ml eluent removed 60–65% of indapamide from the column, and the additional 0.1 ml of methanol was sufficient to achieve almost complete recovery. The minimum volume required to achieve recovery higher than 80% was 0.3 ml. When elution of indapamide was carried out in one step with 0.3 ml of methanol unsatisfactory values for recovery were obtained. The investigations show that the best results for

recovery of indapamide were obtained with the addition of 0.2 ml of methanol followed by another portion of 0.1 ml.

Under the chromatographic conditions described, indapamide and the internal standard peaks were well resolved. Endogenous serum and blood components did not give any interfering peaks. Typical chromatograms of blank serum and blood in comparison to spiked samples are shown in Fig. 2. Indeed, the method described in this report has sufficient sensitivity and reproducibility to permit pharmacokinetic studies. The developed HPLC method can be used for analysis of serum and blood samples from healthy volunteers after oral administration of 5 mg indapamide. Typical chromatograms of serum and blood extracts of a patient after administration of 5 mg indapamide are shown in Fig. 3. The chromatograms showed no interfering peak at the indapamide and internal standard peak positions. Applying the developed method it was found that the real concentrations of indapamide in serum and blood samples collected from healthy volunteers after administration of 5 mg indapamide were up to 64.3 and 275.5 ng/ml, respectively.

3.2. Method validation

3.2.1. Linearity

Linearity was tested on 3 different days at six concentration points ranging from 10.0 to 100.0 ng/ml of indapamide and 60.0 ng/ml of internal standard in serum samples, and from 50.0 to 500.0 ng/ml of indapamide and 200 ng/ml of internal standard in blood samples. Respective regression equations were: $y = 0.0143\gamma + 0.057$ for serum and $y = 0.0019\gamma + 0.0032$ for blood samples. The correlation coefficients were 0.9962 and 0.9971, respectively.

3.2.2. Precision

On 1 day and on 3 different days, spiked samples from each concentration used for construction of calibration curves were prepared in triplicate and analyzed by the proposed HPLC method. Then, the corresponding coefficients of variation were calculated. The intra- and inter-day variations of the method throughout the linear range of concentrations are shown in Table 2. As can be seen from results presented in Table 2, for intra-day precision, RSDs ranged from 2.9 to 6.0% for serum samples and from 4.8 to 7.1% for blood samples. For inter-day precision, RSDs ranged from 2.9 to 6.5% for serum samples and from 3.2 to 8.2% for blood samples. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

3.2.3. Accuracy

Intra- and inter-day accuracy was determined by measuring blood and serum quality control samples at low, middle and high concentration levels. An indication of accuracy was based on calculation of the relative error of the mean observed concentration as compared to the nominal concentration. Accuracy data are presented in Table 2. Relative errors at all three concentrations studied for serum and blood samples are less than 2.7% and it is obvious that the method is remarkably accurate which ensures reliable results are obtained.

3.2.4. Limit of detection and quantification

The limit of detection of this method was calculated as 3:1 signal-to-noise ratio and it was found to be 4.0 ng/ml for serum samples and 20.0 ng/ml for blood samples. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% ($n = 5$) and an accuracy of $\pm 15\%$ ($n = 5$). The limits of quantification were found to be 10.0 ng/ml for serum samples and 50.0 ng/ml for blood samples.

3.2.5. Stability of indapamide in serum and blood samples

The stability of indapamide in serum and blood was investigated using spiked samples at two different concentration levels prepared in duplicate. Spiked samples were analysed after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 h, after one and two freeze/thaw cycles and after 1 month stored at -20 °C. The results from this investigation show that indapamide added to serum or blood samples is stable under the different storage conditions.

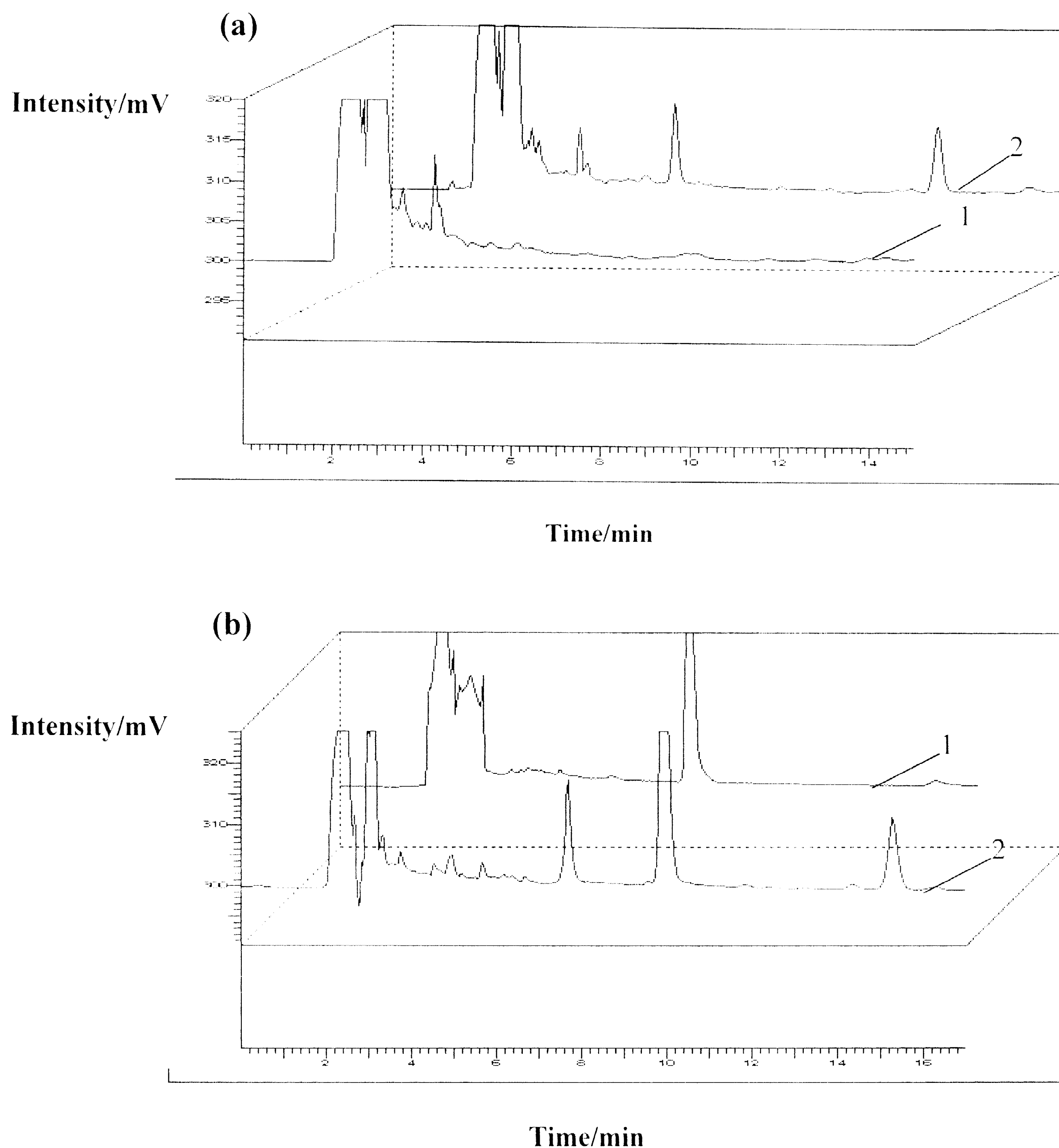

Fig. 2. Chromatograms of blank (1) and spiked (2) serum (a) and blood (b) samples. Concentrations of indapamide and internal standard in serum sample are 60 ng/ml and in blood sample are 200 ng/ml.

4. Conclusion

The developed HPLC method employing solid-phase extraction for sample preparation is simple and convenient for the determination of indapamide in serum and blood samples. The previously reported

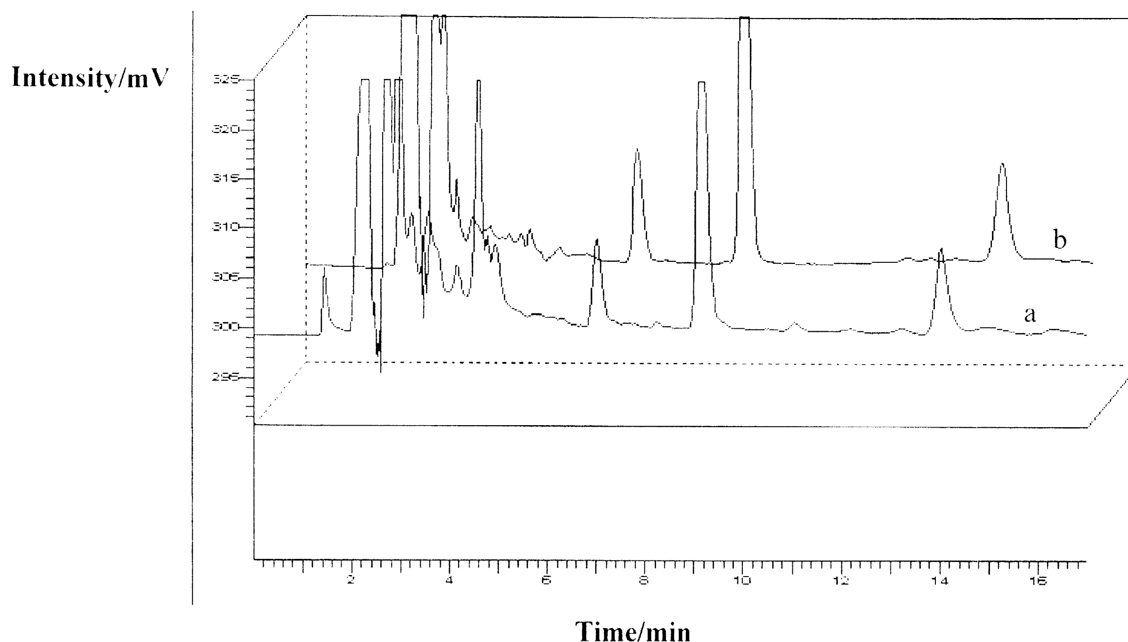

Fig. 3. Chromatograms of serum (a) and blood (b) samples from a healthy volunteer dosed with 5 mg indapamide (2 h post-dose and spiked with 60 ng/ml diazepam for serum and 200 ng/ml diazepam for blood sample).

Table 2
Intra- and inter-day precision and accuracy data

Indapamide nominal concentration (ng/ml)	Intra-day		Inter-day	
	Mean ($n=3$) observed concentration (ng/ml)	Relative standard deviation (%)	Mean ($n=9$) observed concentration (ng/ml)	Relative standard deviation (%)
Precision				
Serum				
10.0	9.9	6.0	9.4	6.5
60.0	59.1	3.1	60.2	2.9
100.0	99.3	3.7	98.5	3.3
Whole blood				
50.0	48.2	7.1	49.3	8.2
200.0	198.4	5.8	201.5	5.3
500.0	497.2	4.8	498.7	3.2
		Relative error (%)		Relative error (%)
Accuracy				
Serum				
15.0	15.3	2.0	15.3	2.0
50.0	50.6	1.2	51.3	2.6
90.0	89.1	−1.0	88.7	−1.4
Whole blood				
70.0	68.5	−2.1	68.1	−2.7
180.0	177.4	−1.4	176.7	−1.8
400.0	394.5	−1.4	396.2	−0.95

methods for the analysis of indapamide in biological fluids [5–9] were unsatisfactory because most of them are time consuming (usually up to 1 h), involving multiple steps of extraction yielding endogenous interferences with separation from the blood and serum . This method is advantageous compared to the methods for the determination of indapamide in biological fluids by liquid–liquid extraction because of its simplicity, efficient clean-up of the complex biological matrix, shorter time of analysis (~25 min) and high recovery of indapamide. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. In conclusion, this paper describes a very simple and sensitive HPLC method for the determination of indapamide suitable to monitor serum and blood concentrations during clinical pharmacokinetic studies in humans.

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