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Direct determination of verapamil in urine and serum samples by micellar liquid chromatography and fluorescence detection☆

M. Rambla-Alegre, M.T. Gil-Agustí, M.E. Capella-Peiró, S. Carda-Broch, J.S. Esteve-Romero *

Àrea de Química Analítica, CCEE, ESTCE, Universitat Jaume I, 12080 Castelló, Spain

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Abstract

Verapamil, a calcium channel antagonist, is one of the most commonly prescribed drugs in the treatment of hypertension. In this work, it was determined in serum and urine samples by a sensitive and precise chromatographic procedure without any pre-treatment step in a C18 column using a micellar mobile phase of 0.15 M sodium dodecyl sulfate and 5% pentanol at pH 7. Fluorescence detection set at 230 nm (excitation) and 312 nm (emission) was used. Verapamil is eluted at 12.5 min with no interference by the protein band or endogenous compounds. Linearities ($r > 0.998$), as well as intra- and inter-day precision, were studied in the validation of the method. LODs were also calculated to be 11.0, 18.5 and 20.2 ng/mL in micellar solution, serum and urine, respectively. Recoveries in the biological matrices were in the 97–99% range. Drug excretion in urine was studied in a volunteer receiving treatment for hypertension, and verapamil, as an unchanged drug, was separated from other metabolites. The procedure developed can be useful in the field of toxicology and clinical analysis.

Keywords: Verapamil; Micellar mobile phase; Direct injection; Serum; Urine

1. Introduction

Verapamil hydrochloride (Fig. 1) is a phenylalkylamine-derivate calcium-channel blocking agent. Chemically, it is a basic ($\log K = 9.1$) and highly hydrophobic compound ($\log P_{o/w} = 4.6$) [1]. Its principal physiological action is to inhibit the transmembrane influx of extracellular calcium ions across the membranes of myocardial cells and vascular smooth muscle cells, without changing serum calcium concentrations. It is used in the treatment of hypertension and angina pectoris [2]. Verapamil has also been shown to be effective treatment for supraventricular tachyarrhythmias, variant angina, and cardiomyopathy.

In medical practice verapamil is used in conventional tablet form with a minimal dose of 40 mg and a maximal dose of 180 mg, as well as in a slow release form in doses of 120 to 240 mg. Only 10–20% of the 90% of the dose absorbed from the digestive tract penetrates to the circulatory system in an unchanged form [3]. The remaining part of the verapamil dose undergoes a first-pass effect, mainly in the liver. In humans, verapamil is metabolized to more than six metabolites, according to a number of authors, that are excreted mainly in urine [4].

As verapamil is one of the frequently prescribed calcium channel blockers, results of evaluations of the therapy have led to reports of toxic effects and overdoses at concentrations above 1000 ng/mL in serum [5]. The potential for high morbidity and mortality rates with overdosage is significant. Ingestion of these agents should be suspected in any patient who presents in an overdose situation with unexplainable hypotension and conduction abnormalities. The potential for toxicity should be noted in patients with underlying hepatic or renal dysfunction who are receiving therapeutic doses. Hemodynamic collapses with bradyarrhythmias and heart block have been reported with intentional overdoses as well as with usual therapeutic doses of verapamil [6].

Methods of determination of verapamil in serum or urine samples have been developed which make use of conventional HPLC. Some of these methods focus on the determination of

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* Corresponding author. Tel.: +34 964 728093; fax: +34 964 728066.
E-mail address: estevej@exp.uji.es (J.S. Esteve-Romero).

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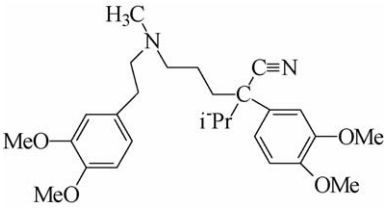

Fig. 1. Structure of verapamil.

the enantiomers [7], its pharmacokinetics [8,9], and the simultaneous analysis of verapamil and its metabolites [10–12]. Other studies based on toxicity and poisoning by verapamil have also been performed [13–15].

Conventional HPLC using aqueous-organic mobile phases for the determination of drugs in physiological fluids with direct injection present many problems. Drugs are often strongly bound to proteins and in a complex matrix where interference from numerous endogenous compounds is expected, so this technique is usually not feasible. Proteins tend to denature and precipitate in the injection valve or with the column heat thus producing obstruction or clogging of the system. To solve this problem the precipitation of proteins and extraction of drugs should be performed.

Micellar liquid chromatography (MLC) is an alternative to these methods for drug determination in physiological fluids. Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins, rather than precipitating into the column, are solubilized and washed harmlessly away, eluting with the solvent front. Another advantage is that the use of micellar mobile phases is non-toxic, non-flammable, biodegradable and relatively inexpensive, in comparison to aqueous-organic solvents [16]. Two selected studies are an example of using micellar mobile phases with direct injection applied to the determination of stimulants in urine [17] and barbiturates in serum [18].

The aim of this paper is to determine verapamil in biological fluids using a new MLC method which is simple and easy to use. The method was partially validated according to USFDA guidelines [19], and applied to the determination of verapamil in real urine samples of a volunteer under treatment for hypertension. The procedure developed here could also be useful in the area of clinical analysis and forensic sciences.

2. Experimental

2.1. Instrumentation

The pH of the solutions was measured with a Crison GLP 22 (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonification unit were from Selecta (Barcelona). The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, an autosampler and fluorescence detector. A Digicen 20-R centrifuge (Orto Alresa, Madrid, Spain) was utilized to centrifuge serum samples.

2.2. Chemicals and reagents

Verapamil was purchased from Sigma (St. Louis, MO, USA). Distilled-deionized water was used throughout. Sodium dodecyl sulfate, sodium dihydrogen phosphate and methanol were from Merck (Darmstadt, Germany). Sodium hydroxide was from Riedel-de Haën (Seelze, Germany). *n*-Amyl alcohol (pentanol) was from Sharlau (Barcelona).

2.3. Solutions and sample preparation

The micellar mobile phase was prepared using SDS, which was buffered with sodium dihydrogen phosphate 0.01M at pH 7 using 0.1 M of sodium hydroxide, and lastly pentanol was added to achieve the desired concentration.

A stock solution of 10 μg/mL of verapamil was prepared. Adequate amounts of solid standard were weight, dissolved with methanol, and finally filled up with micellar solution. Frozen biological samples were taken out before analysis. Several volumes of liquid serum or urine were mixed with an appropriate volume of stock solution to achieve desired concentration and 1:10 dilution before injection.

2.4. Chromatographic conditions

A reversed-phase Kromasil C18 column (250 mm × 4.6 mm, 5 μm particle size) and a pre-column (30 mm) of similar characteristics (Sharlab, Barcelona, Spain) were used. The mobile phase was 0.15 M SDS, 5% (v/v) pentanol at pH 7. The flow-rate and injection volume were 1 mL/min and 20 μL, respectively. Experiments were performed at 25 °C. Detection was performed at 230 nm (excitation) and 312 nm (emission) when using fluorescence detection. The retention time for verapamil was 12.5 min. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. A.10.01).

2.5. Serum and urine sampling

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK) and centrifuged for 5 min at 3000 rpm at 4 °C; serum was kept. Urine samples were collected in a Urine Collection Cup (BD Vacutainer Systems). Analyses were performed with 1 mL of serum or urine. Biological samples were injected directly into the chromatographic system after 1:10 dilution with the selected mobile phase. Before injection, solutions were filtered directly through 0.45 μm nylon membranes into the autosampler vials.

3. Results and discussion

3.1. Selection of mobile phase composition

Verapamil is a basic compound ($\log K = 9.1$) with an equilibrium between two forms, one molecular and another that is positively charged. In the normal pH working range of C18 columns (3–7), the positively charged form of verapamil is predominant from pH 3 to 7 and there will be no changes in the

retention behavior in this pH range. On the other hand, verapamil is a highly hydrophobic substance ($\log P_{o/w} = 4.6$) and this means that by using C18 columns and pure micellar mobile phases of SDS retention of this substance would be too high.

Experimentally, the high retentions of drug were confirmed using pure mobile phases of high concentration of surfactant. The addition of a short chain alcohol (butanol and pentanol) was needed to reduce the run time for this hydrophobic compound [20]. Two mobile phases with different pH (3 and 7) were tested to corroborate the behavior of verapamil in the system, being the retention the same in both cases. Finally, pH 7 was selected to extend the column life.

Several mobile phases were checked with the aim of selecting the optimum one. Composition of SDS (M)-alcohol (%) of the mobile phases that were studied are the following: 0.05–2 butanol, 0.2–2 butanol, 0.15–5 butanol, and 0.15–5 pentanol all buffered at pH 7, and the results of retention factors, efficiencies and asymmetries were: 60.9, 450 and 1.1; 22.1, 915 and 1.1; 21.9, 1013, and 1.4; and 12.2, 995, and 1.3, respectively.

Using a medium concentration of SDS, 0.15M, and a high concentration of butanol, 5%, the retention achieved was similar than using an extreme concentration of surfactant and low concentration of butanol, moreover the efficiency in the first mobile phase was better. This is the usual behavior observed in MLC when the concentration of alcohol is increased, efficiencies are also increased. With the aim of reducing the retention time, the type of alcohol was changed to pentanol holding the same concentration. Retention time was decreased by 10 min, and the other chromatographic parameters were similar.

The effect of SDS concentration and the type and concentration of alcohol on the separation of verapamil from the protein band was studied. In the whole mobile phase studied the separation was achieved, while a second step to be taken into consideration was to obtain a minimum analysis time. The two mobile phases with the highest strength, 0.15 M SDS-5% butanol and 0.15 M SDS-5% pentanol, were compared. Using the same concentration of SDS and alcohol, the difference between them is only the nature of the organic modifier. The strength of the alcohol can be known by using butanol and pentanol, since their carbon chain increases by one unit. Pentanol was finally selected due to its reducing the analysis time by 50%. Efficiency is usually better using butanol, but in the drug under study here it proved to be similar for both alcohols, as was the asymmetry.

The mobile phase finally used to perform the analysis was 0.15 M SDS-5% pentanol at pH 7, which allows the drug to be quantified in an adequate analysis time without any interference by the proteins or endogenous compounds.

3.2. Serum and urine blanks behavior

To study the conditions of drug-free serum and urine samples, 6 different serum and urine samples from volunteers were checked, the profile of the protein band being similar to that shown in Fig. 2 in all cases. No other peaks were found in the matrices that were studied.

When serum and urine samples are injected directly into an MLC system with fluorescence detection, the chromatograms show a band of proteins and endogenous compounds placed in the first few minutes called the protein band. Fig. 2A and B show the chromatograms of blank serum and urine samples, respectively. The profile of the protein band is similar in both cases, the drug should be eluted after 3 min in the biological fluids tested. The protein band observed with direct injection is a drawback of the procedure when using less strongly retained hydrophilic substances because of possible overlaps between them. In this work, the high background signal at the beginning of the chromatogram consisting of proteins is not a problem due to the high hydrophobicity of the compound that was eluted far away from this band.

3.3. Method validation

3.3.1. Linearity

Calibration curves were constructed using the areas of the chromatographic peaks measured at eight increasing concentrations, in the range from 100 to 2000 ng/mL, in three different matrices: micellar solution, serum and urine. For the physiological fluids, freshly prepared solutions were spiked. To study the variability of the calibration parameter, the curves were obtained for 5 days over a period of two months for a different set of standards. Standard curves were calculated by the equation $y = mx + c$ using least square regression. A correlation of more than 0.99 was desirable for all the calibration curves. Table 1 shows the slopes, intercepts and regression coefficients of the calibration curves, and the calibration parameters for intra-day reproducibility using the same set of standards injected on the same day. Coefficients of regression were always $r > 0.998$.

3.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection (*3s criterion*) were evaluated by injecting a series of 10 solutions containing verapamil at the lowest concentration of the calibration curve. Results were based on the

Table 1
Day-to-day calibration parameters of verapamil in three different matrices

Matrix	Day	Slope	Intercept	r
Micellar media	1	0.249 ± 0.003	3.3 ± 0.7	0.9992
	2	0.277 ± 0.003	5.1 ± 1.2	0.9993
	3	0.265 ± 0.003	3.8 ± 2.4	0.9998
	4	0.273 ± 0.002	5.2 ± 1.3	0.9994
	5	0.271 ± 0.001	6.1 ± 0.7	0.9997
	Mean	0.267 ± 0.011	4.7 ± 1.1	
Serum	1	0.251 ± 0.004	4.8 ± 0.8	0.9990
	2	0.293 ± 0.004	1.7 ± 0.4	0.9986
	3	0.274 ± 0.005	3.7 ± 0.8	0.9991
	4	0.291 ± 0.008	2.6 ± 0.6	0.9994
	5	0.266 ± 0.002	6.5 ± 1.5	0.9997
	Mean	0.275 ± 0.018	3.9 ± 1.9	
Urine	1	0.257 ± 0.001	3.0 ± 1.7	0.9991
	2	0.309 ± 0.001	1.9 ± 0.5	0.9984
	3	0.279 ± 0.002	3.2 ± 0.7	0.9993
	4	0.293 ± 0.001	4.0 ± 0.6	0.9994
	5	0.287 ± 0.003	3.0 ± 0.5	0.9998
	Mean	0.285 ± 0.019	3.0 ± 0.8	

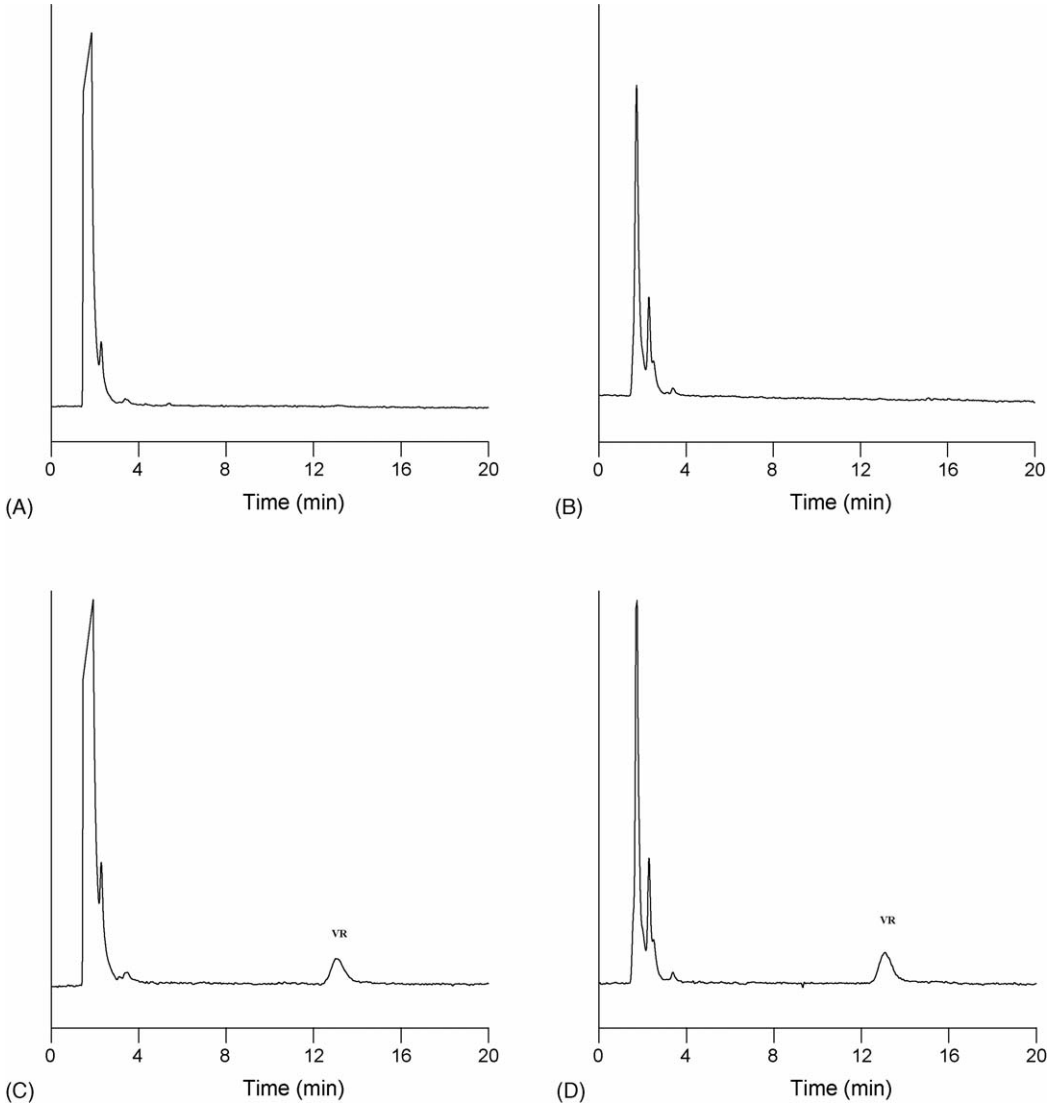

Fig. 2. Chromatograms of serum (A) and urine (B) blanks and spiked LOQ concentrations (100 ng/mL) in serum (C) and urine (D).

standard deviation of the response and on the slope of a specific calibration curve containing the drug in a range of low concentrations. Results of LODs in ng/mL were 11, 19, and 21 in micellar media, serum and urine, respectively. Limit of quantification was accepted as the lowest standard on the calibration curve. A concentration of 100 ng/mL was qualitatively determined with good precision and accuracy, being this concentration the lower limit of quantification (LLOQ), according to the FDA guideline.

3.3.3. Selectivity

Six randomly selected control drug-free human serum and urine samples were processed directly into the chromatographic system and analyzed to determine the extent to which endogenous components may contribute to interfere with the retention time of the drug. No interference for endogenous compounds was found in the physiological matrices that were studied.

3.3.4. Accuracy and precision

The accuracy and precision of the analyses were evaluated in assays performed during the same day and on consecutive days. The values showed in Table 2 correspond to intra-day and inter-day analyses for five determinations at three concentrations for the three matrices under study.

The intra-day precision of the method was determined by preparing spiked serum and urine samples at three different concentrations of verapamil within the calibration range. Five replicates were performed at each concentration (low, mid and high quantification concentration sample). Inter-day precision was checked with the same concentrations as in the intra-day assay and the determination was repeated daily for 5 days. The results are shown in Table 2.

3.4. Analysis of serum and urine samples

The applicability of the method developed to determine verapamil was verified by its analysis in spiked serum and urine samples. The recovery was calculated by comparing the peak areas of the spiked samples with those obtained using a micellar media at the same concentration. The results shown in Table 3 indicate that determination in biological samples is possible

Table 2
Inter-day and intra-day accuracy, and precision of verapamil in three different matrices ($n = 5$)

	Added concentration (ng/mL)	Mean concentration found (ng/mL)	CV (%)	Accuracy (%)
Micellar media[a]				
Intra-day[b]				
Low	300	319.7	1.4	106.6
Mid	800	831.8	1.2	103.9
High	1500	1556.9	1.2	103.8
Inter-day[b]				
Low	300	315.4	2.2	105.1
Mid	800	836.9	1.3	104.6
High	1500	1527.9	0.89	101.9
Serum[a]				
Intra-day[b]				
Low	300	288.3	3.9	96.1
Mid	800	802.5	0.56	100.3
High	1500	1551.1	0.62	103.4
Inter-day[b]				
Low	300	313.3	2.5	104.4
Mid	800	813.1	1.3	101.6
High	1500	1517.3	1.0	101.2
Urine[a]				
Intra-day[b]				
Low	300	323.9	3.8	107.9
Mid	800	815.3	2.2	107.9
High	1500	1505.1	1.2	101.9
Inter-day[b]				
Low	300	313.8	1.6	104.6
Mid	800	791.2	1.0	98.9
High	1500	1489.1	0.56	99.3

[a] Matrix
[b] QC samples

with no matrix interference. Results were consistent, precise and reproducible, and recoveries obtained were in the range from 97 to 99%, which represented the total amount of the tested drug.

Fig. 2C and D show the chromatograms for verapamil in spiked serum and urine samples at LLOQ concentration, respectively. No interference is observed and the verapamil peak is separated from the protein band and large enough to be quantified.

3.5. Drug control in real urine samples

The pharmaceutical formulation contained a mixture of a calcium channel blocker (verapamil) and angiotensin-converting enzyme inhibitor (trandolapril) for use as drugs to treat hypertension. Following oral administration of a single conventional tablet containing 180 mg of verapamil to volunteer, urinary studies were conducted. Samples of urine were taken 6 times a day, approximately every 2 h, and the volume was measured. Dilution of urine samples by 1:10 in 0.05 M SDS was performed and the solution was injected directly into the chromatograph without any other treatment than filtration. No interference from trandolapril was observed, since this compound is not fluorescent at the selected wavelength.

Two hours after oral administration, the concentration of verapamil in urine started to rise (1.8 μg/mL). The maximum

Table 3
Recovery of verapamil spiked in serum and urine samples ($n = 5$)

Matrix	Added (ng/mL)	Recovery (%)	CV (%)
Serum	100	98.3	2.9
	500	99.0	0.82
	1000	97.2	1.4
Urine	100	97.8	2.1
	500	98.9	1.2
	1000	98.3	1.1

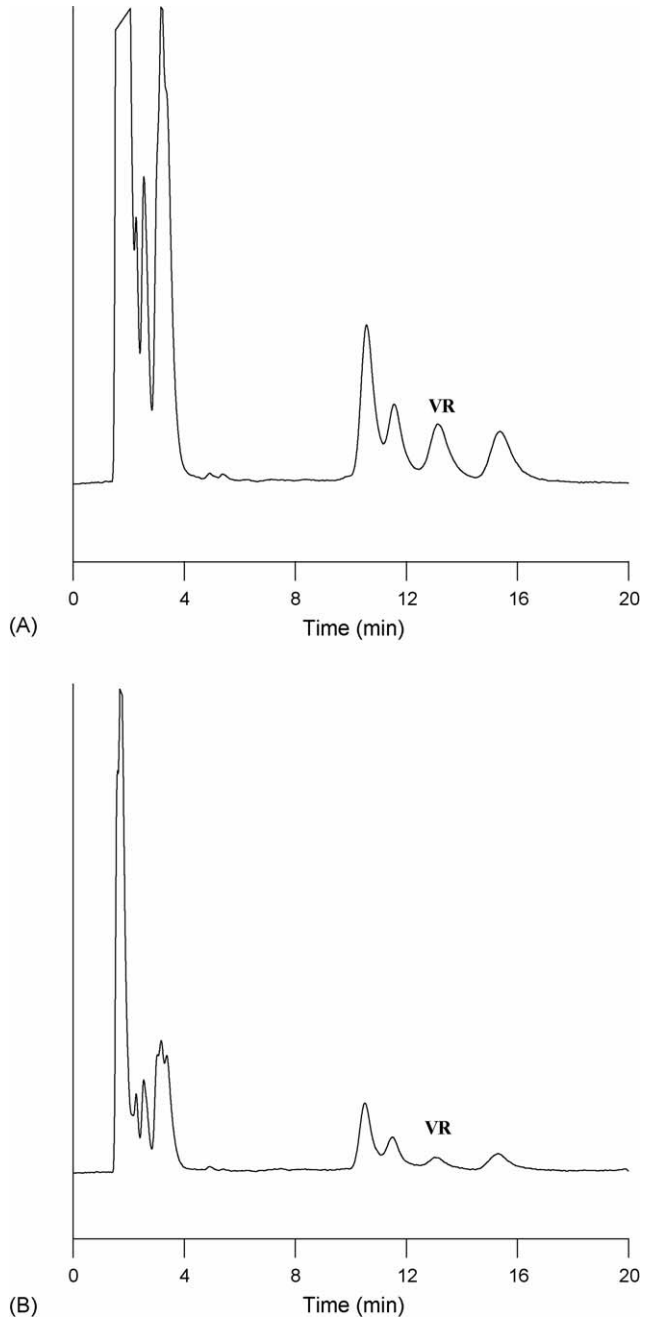

Fig. 3. Chromatograms of verapamil excreted in urine as unchanged drug 4 h after oral ingestion (A), and residual verapamil (B) (VR, verapamil).

concentration excreted in urine was found at 4 h after oral administration, this concentration being 7.1 μg/mL. It has to be noted that in all the intervals studied there is a residual concentration of verapamil in the range from 0.69 to 1.2 μg/mL due to the long period of treatment.

Fig. 3 depicts a chromatogram of verapamil in unchanged form excreted at the maximum concentration; the urine sample was taken 4 h after oral administration (A), and the residual concentration (B) is an example of the minimum level excreted during the day. As can be seen in the figure, there are three additional peaks corresponding to dealkylated or demethylated metabolites, and it is well known that verapamil is rapidly metabolized in the liver. The final quantity of verapamil eliminated was 5.04 mg, that is to say 2.8% of the dose taken.

4. Conclusions

The main advantage of the procedure developed is the possibility of direct injection of biological (serum and urine) samples due to the fact that proteins and other components in the matrix are solubilized by the surfactant. The limit of quantification achieved using the fluorescence detector is sensitive enough to check levels of toxicity in serum and can be used in the area of clinical or forensic sciences. The use of a mobile phase consisting of 0.15 M SDS-5% pentanol at pH 7 allows the elution of verapamil in 12.5 min.

Results of calibration curves, repeatability, reproducibility and accuracy grant the method to be useful for quality control in the determination of verapamil in pharmaceutical formulations, since results are shown to be good for analyses performed in micellar media. To validate the method in biological fluids, it was applied to serum and urine. Results of spiked samples were in accordance with the injected quantity, and recoveries were close to the concentration that was added.

Another further advantage of the method is the use of hybrid-micellar mobile phases, which are less toxic and flammable and have a lower environmental impact in comparison with conventional HPLC that uses organic solvents.

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References

[1] C.A.S. Bergström, K. Luthman, P. Arturson, Eur. J. Pharm. Sci. 22 (2004) 387.
[2] American Hospital Formulary Service, Drug Information, American Society of Health-System Pharmacists, Bethesda, MD, 1988.
[3] R. Kirsten, K. Nelson, D. Kirsten, B. Heintz, Clin. Pharmacokinet. 34 (1998) 457.
[4] S.K. Gupta, S. Hwang, I. Atkinson, J. Longstreth, J. Clin. Pharmacol. 36 (1996) 25.
[5] F. Musshoff, S. Padosch, S. Steinborn, B. Madea, Forensic Sci. Int. 142 (2004) 161.
[6] R. Gay, S. Algeo, R. Lee, M. Olajos, E. Morkin, S. Goldman, J. Clin. Invest. 77 (1986) 1805.
[7] E. Brandsteterová, I.W. Wainer, J. Chromatogr. B 732 (1999) 395.
[8] W. Sawicki, Eur. J. Pharm. Biopharm. 53 (2002) 29.
[9] W. Sawicki, S. Janicki, Int. J. Pharm. 238 (2002) 181.
[10] W. Sawicki, J. Pharm. Biomed. Anal. 25 (2001) 689.
[11] M. Walles, W.M. Mullett, K. Levsen, J. Borlak, G. Wünsch, J. Pawliszyn, J. Pharm. Biomed. Anal. 30 (2002) 307.
[12] O.H. Jhee, J.W. Hong, A.S. Om, M.H. Lee, W.S. Lee, L.M. Shaw, J.W. Lee, J.S. Kang, J. Pharm. Biomed. Anal. 37 (2005) 405.
[13] H. Kinoshita, T. Taniguchi, M. Nishiguchi, H. Ouchi, T. Minami, T. Utsumi, H. Motomura, T. Tsuda, T. Ohta, S. Aoki, et al., Forensic Sci. Int. 133 (2003) 107.
[14] C.A. Hofer, J.K. Smith, M.F. Tenholder, Am. J. Med. 95 (1993) 431.
[15] P. Immonen, A. Linkola, E. Waris, Int. J. Cardiol. 1 (1981) 101.
[16] J. Esteve Romero, S. Carda Broch, M. Gil Agustí, M.E. Capella-Peiró, D. Bose, Trends Anal. Chem. 24 (2005) 75.
[17] M. Gil-Agusti, M.E. Capella-Peiro, A. Martinavarro-Dominguez, J. Esteve-Romero, Chromatographia 57 (2003) 51.
[18] M.E. Capella-Peiro, M. Gil-Agusti, A. Martinavarro-Dominguez, J. Esteve-Romero, Anal. Biochem. 309 (2002) 261.
[19] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001 BP, website: http://www.fda-gov/ceder/guidance/index.htm.
[20] J.R. Torres-Lapasio, J.J. Baeza-Baeza, M.C. Garcia-Alvarez-Coque, Anal. Chem. 69 (1997) 3822.