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Validation of a solid phase extraction-high performance liquid chromatographic method for the determination of eprosartan in human plasma

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Abstract

In this work, a solid phase extraction-reversed phase high performance liquid chromatographic (SPE-RP-HPLC) method with photometric detection for monitoring the antihypertensive drug eprosartan has been validated in order to assure good quantitation of eprosartan in plasma samples obtained from patients under cardiovascular treatment. This analytical method was developed by using experimental design and quantitation was accomplished with the internal standard method. No interferences were observed from endogenous compounds of plasma and other drugs which are commonly co-administered in elderly patients. The recoveries of eprosartan from plasma samples, measured at three levels of the linear concentration range (150–4000 ng/mL) were found to be between 93.4 and 102.8%. The intraday and interday precision and accuracy (measured by relative standard deviation, RSD, and relative error, RE, respectively) were always lower than 13% (RSD) and 4% (RE). Stability studies showed that eprosartan stock solutions are stable for at least 3 months when stored at 8 °C and plasma samples containing the drug were stable at least during the whole analytical method.

Keywords: Eprosartan; Validation; Human plasma; Liquid chromatography; Solid phase extraction

1. Introduction

Some inadequate habits as fattening food and the sedentary lifestyle induce the development of a group of some related pathologies such as obesity, diabetes, hypertension, arteriosclerosis and hyperlipidaemia. These have more incidence in elderly patients, even though the hypertension is increasing in children and could become a problem of epidemic proportions [1].

The different kind of drugs which are mixed in patients under cardiovascular treatment [diuretics, angiotensin-II receptor antagonists (ARA-II), angiotensin conversor enzyme inhibitors (ACEI), β-blockers, calcium antagonists, statins, antidiabetics or anti-rheumatics drugs] make difficult avoiding interferences between them and achieving good quantitation goals. In addition to the intake drugs, their metabolites and the formulation excipients, endogenous compounds from biological samples [2] and dietary substances and supplements [3] must be considered as potential interferences.

Bioanalysis involves work with low analyte concentrations, and complex matrixes which may contain unknown and variable components. The industry consensus on bioanalytical validation, reflected in Food and Drug Administration (FDA) guidelines and other publications [4–7] has done much to improve the quality of bioanalysis and to establish generally accepted validation specifications. Almost all quantitative acceptance criteria applied to validation and quality control of bioanalytical methods rely on data generated using spiked control biofluid samples. The bioanalytical validation experiments have some limitations that appear to relate largely to the complexity and potential variability of biomatrix samples [8].

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical and, biopharmaceutics and clinical pharmacology studies. Bioanalytical method validation includes all the procedures that demonstrate that a particular method used for quantitative measurement of analytes in

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a given biological matrix, such as blood, plasma, serum or urine, is reliable and repeatable for the intended use. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. Moreover, the suitability of a technique may also be influenced by the final objective of the study. Specific validation criteria are needed for methods intended for analysis of each analyte (drug and/or metabolite). Unless a method is used on a regular basis that provides confidence in its continued validity, it is essential to document that the method is still valid prior to analysis of samples in a study. Therapeutic monitoring requires the availability of a single method that can be used for all the possibilities (co-administered drugs, disease states, patients' characteristics ...) in order to save time, cost and effort.

In this work, the antihypertensive drug eprosartan has been analysed in plasma obtained from patients with cardiovascular disease. This drug is a highly selective, non-peptide angiotensin-II antagonist. The compound has been showed to inhibit angiotensin-II induced vasoconstriction in preclinical species and cause reductions in systolic and diastolic blood pressure at peak effect after dosing in clinical patients [9,10]. It belongs to the ARA-II family. These angiotensin antagonists are safe and effective agents for the treatment of hypertension and heart failure, either alone, or in combination with diuretics. Because of this, they have been proposed as an alternative to the more traditional angiotensin-converting enzyme (ACE) inhibitors.

The correct separation and quantitation of eprosartan in human plasma samples is crucial to monitor the plasma concentration levels, which will inform us if the therapeutic levels are safe and effective during the whole inter-dose range.

Several HPLC methods have been previously reported for the determination of ARA-II in pharmaceuticals and biological samples. Hillaert et al. [11–13] have described some electrophoretic and chromatographic screening methods for eprosartan and other ARA-II alone or in combination with diuretics in pharmaceuticals. Other authors [14–17] have developed methods to separate ARA-II in biological fluids such as plasma and urine, but these do not include eprosartan. Only Lundberg et al. [18] determine eprosartan in human plasma by HPLC-UV. This author achieves a good quantitation in samples from healthy volunteers but the application of the method developed to samples from patients who are being administered more drugs than eprosartan is not included.

This article describes the validation of a SPE-HPLC-UV method to determine eprosartan in plasma samples from patients under cardiovascular treatment. To develop the extraction procedure and the HPLC method, experimental design and MultiSimplex program, respectively were used [19]. Although Lundberg et al. [18] reported good results with an isocratic method, it was observed that the high polarity of eprosartan makes difficult the separation of this drug from endogenous compounds of plasma at the detection wavelength used in the chrormatographic system (232 nm). Due to this fact, a gradient elution mode using a chromatographic column designed for the separation of polar compounds was chosen in this work.

2. Experimental

2.1. Chemicals and reagents

Eprosartan {(*E*)-3-[2-butyl-1-[(4-carboxy-phenyl)methyl]-1H-imidazol-5-yl]-2-[(2-thienyl)-methyl]propenoic acid} and the internal standard irbesartan {2-butyl-3-[[2′-(1H-tetrazole-5-yl)(1,1′-biphenyl)-4-yl]methyl]-1,3-diazaspiro[4,4]non-1-en-4-one} were kindly supplied by Solvay (Barcelona, Spain) and Sanofi-Synthelabo (Montpellier, France), respectively. Structures of both compounds are shown in Fig. 1.

Reagent grade trifluoroacetic acid (TFA), phosphoric acid and sodium monohydrogen and sodium dihydrogen phosphate were obtained from Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were obtained from Scharlab (Barcelona, Spain). Purified water from a Milli-Q Element A10 water system (Millipore, Bedford, MA, USA) was used in the preparation of buffer and reagent solutions. Drug-free control human plasma was purchased from Blood Bank of Galdakao Hospital (Bizkaia, Spain).

2.2. Instrumentation and chromatographic conditions

The chromatographic system consisted of two Waters (Milford, MA, USA) Model 510 HPLC pumps, a Waters Model 717 Plus Autosampler, and a Waters 490E programmable multiwave-

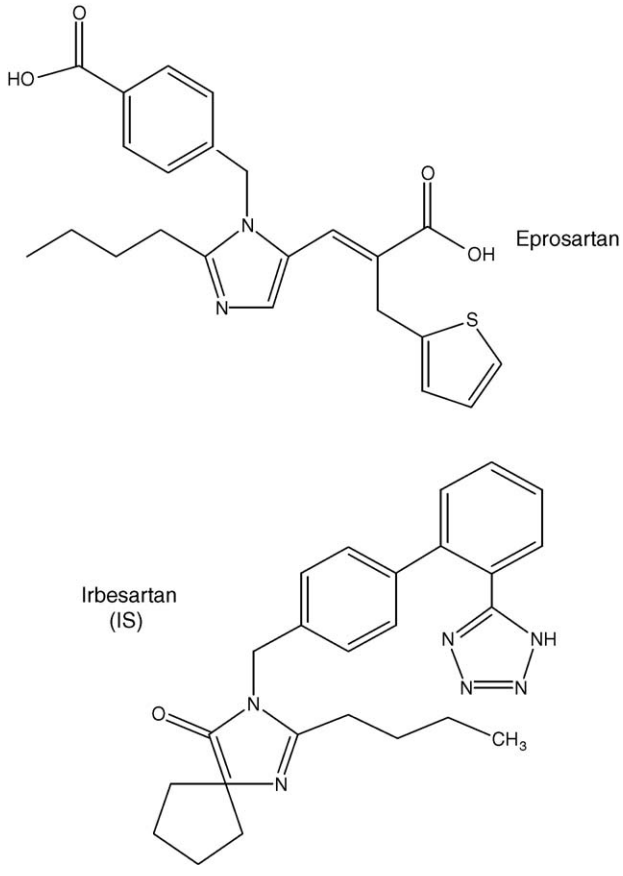

Fig. 1. Chemical structures of eprosartan and the internal standard irbesartan.

length detector. Chromatograms were recorded by means of a computer and were treated with the aid of the software Millenium 32 Chromatography Manager from Waters.

A Waters Atlantis dC18, 100 × 3.9 mm I.D., 3 μm, 100 Å column was used to perform separation. It was thermostatted at 35 ± 0.2 °C in a column oven controlled by a Waters Temperature Control Mode. Prior to the analytical column, a Waters μBondapak C18 guard column 10 μm was placed to prevent column degradation.

The clean-up procedure consisted of a solid-phase extraction and was performed using Varian Bond Elut C8 cartridges non-endcapped 100 mg/1 mL (Barcelona, Spain). These cartridges were placed in a vacuum manifold system from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Bedford, MA, USA).

Plasma extracted samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

Plasma samples were centrifuged in a 5904R Eppendorf (Hamburg, Germany) refrigerated centrifuge.

2.3. Preparation of calibration standards and quality control samples

A 232 μg/mL stock solution of eprosartan was prepared in 100% methanol from white powder of eprosartan mesylate by using an analytical balance. A 174 μg/mL stock solution of irbesartan was also prepared in 100% methanol. This last solution was diluted to produce a 20 μg/mL working solution with methanol. All solutions were stored at refrigerator temperature (4–8 °C) and protected from light.

Calibration curves were prepared from 1 mL aliquots of human plasma by spiking drug free control plasma using the stock and working solutions. Ten calibration standards were used, nominally ranging from 150 to 5000 ng/mL.

For the validation of the assay, three pools of quality control (QC) plasma samples were prepared containing 150, 600 and 4000 ng/mL of eprosartan. Parts of the three pools of QC samples were stored at −20 °C to study the stability after thaw–freeze cycles and in long term.

2.4. Extraction procedure

Calibration standards and 1 mL aliquots of the control blanks and validation QC samples were spiked with the working solution of irbesartan to provide an internal standard concentration of 300 ng/mL. An aliquot (1 mL) of 1 M phosphoric acid was added in all samples, which was followed by brief vortex mixing and centrifugation for 5 min in a high-speed centrifuge (10,621 × *g*) refrigerated at 4 °C.

The SPE cartridges were conditioned with 2 mL of methanol, followed by 2 mL of phosphate buffer (50 mM, pH 2). The samples (1 mL of plasma + 1 mL of phosphoric acid) were applied to the cartridges manually and washed with 1 mL methanol–50 mM phosphate buffer, pH 2, 20:80 (v/v) and 1 mL of phosphate buffer (50 mM, pH 6.8), followed by a 10 min drying period at high vacuum. The cartridges were then eluted with 2 mL of methanol.

Table 1
Gradient elution conditions

Time (min)	ACN 0.026% TFA	H_2O 0.031% TFA	Flow rate (mL/min)
0	20	80	1.25
5	45	55	1.25
10	45	55	1.25
14	20	80	1.25

The eluent was evaporated to dryness under nitrogen at 60 °C. The residue was reconstituted with 100 μL of acetonitrile, vortex mixed and transferred to autosampler vials by filtering them with a 0.45 μm syringe filter. Twenty microliters of aliquots were injected onto the HPLC system for analyzing.

2.5. Chromatographic conditions

The mobile phase consisted of a mixture of water 0.031% TFA (pump A) und acetonitrile 0.026% TFA (pump B), low pressure mixed, and delivered in gradient mode at a flow rate of 1.25 mL/min. The gradient is shown in Table 1. Previously to use, the mobile phase was passed through a 0.45 μm membrane filter from Millipore (Bedford, MA, USA) and degassed in an ultrasonic bath. The chromatographic separation was performed at 35 ± 0.2 °C and the analyte was monitorized photometrically at 232 nm.

3. Results and conclusions

The validation of SPE-RP-HPLC method developed in our laboratory [19] for the determination of eprosartan in plasma samples was made following the International Conference Harmonisation and FDA guidelines [4–6] for biovalidation.

3.1. Recovery

To calculate the absolute recovery of the SPE procedure, six replicates of spiked plasma samples at three different concentration levels (150, 600 and 4000 ng/mL) of eprosartan were used. These samples were compared with blank plasma samples which had been extracted following the same SPE procedure but spiked just before the evaporation step. In all cases the IS (300 ng/mL) was added just before the evaporation step.

The absolute recovery from human plasma ranged from 93.4 to 102.8% (Table 2). The recovery did not appear to be concentration dependent.

Table 2
Absolute recovery of eprosartan from 1 mL of human plasma ($n = 6$)

	Nominal concentration (ng/mL)		
	150	600	4000
% Recovery	93.4	102.8	93.9
RSD	8.8	2.8	2.6

3.2. Repeatability of the extraction procedure

This parameter was measured by using six replicates of plasma samples spiked at three concentration levels in the work range: high, medium and low. The repeatability of the extraction, in terms of RSD did not exceed the 10%, as it is collected in Table 2.

3.3. Determination of linearity and work range

The linearity of the analytical method was determined by means of different calibration curves. For each series of analyses, a regression line was fitted by applying the linear regression model based on the least square method. A blank plasma sample was spiked with the appropriate volume of the eprosartan stock solution to achieve a concentration of 5000 ng/mL of the drug. The rest of the concentration levels were prepared by diluting this sample till: 3000, 1000, 800, 600, 400, 300, 250, 200 and 150 ng/mL, covering the whole work range. The IS concentration was 300 ng/mL in all solutions. The work range was defined considering the normal therapeutic doses (300–600 mg/day) and the time to achieve the maximum plasmatic levels (1–3 h after dose). The expected range was extended in order to detect potential overdoses.

All samples were treated and analyzed as it is described in Section 2. Calibration curves were obtained plotting the corrected area (ratio analyte area/IS area) for each concentration level. At least, six concentration levels with two replicates were used in all calibration curves. The correlation coefficients, slope and intercept values are given in Table 3.

Calibration samples did not exceed the limit value (RE > 15%) for the interpolated concentration with regard to nominal concentration, so the proposed model was accepted for eprosartan in the studied range from 150 to 5000 ng/mL.

3.4. Evaluation of method precision and accuracy

In this work, the precision was calculated for three concentration levels: 150, 600 and 4000 ng/mL of eprosartan and 300 ng/mL of IS. Precision was measured using six spiked plasma samples for each concentration level and each day. The evaluation of method precision was carried out in a day (intraday precision) and in three different days (interday precision) and evaluated by means of the RSD.

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. It was tested by using six replicates for each concentration level of eprosartan (150, 600 and 4000 ng/mL) and IS (300 ng/mL) intra- and interday (in three different days). To evaluate this parameter relative error (RE) was used.

As it can be seen in Table 4, the obtained values for precision (RSD < 15%) and accuracy (RE < 15%) follow the FDA proposed validation rules [4].

3.5. Selectivity

Selectivity towards interferences from endogenous components present in biological fluids is established by processing some independent sources of the same matrix.

In the present study, the selectivity has been studied by using independent plasma samples from six different healthy volunteers. It has been proved to be sufficient for the routine determination of eprosartan in human plasma samples. The blank samples showed neither significative differences between them nor area values higher than the 20% of the LOQ's area at the analyte retention time or higher than 5% of the internal standard area at its corresponding retention time.

Although the selectivity has been confirmed by this way, if it is possible, it would be convenient to take blood samples from patients before starting the anthypertensive treatment to evaluate the suitability of this method in each patient, due to the high interindividual variability and the intake of other drugs simultaneously.

3.6. Limit of quantitation

The limit of quantitation (LOQ) was calculated from a relationship S/N equal 10. The eprosartan concentration obtained

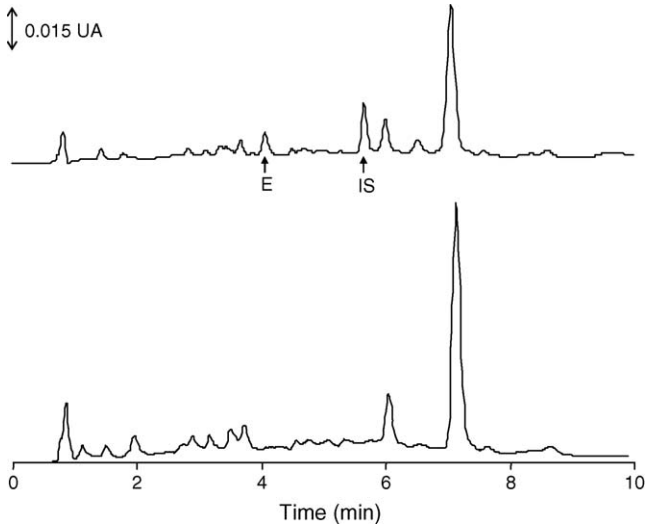

Fig. 2. Chromatograms of a plasma sample spiked with eprosartan (E) 150 ng/mL and irbesartan (IS) 300 ng/mL (above) and a blank plasma sample (below). Chromatographic conditions given in Section 2.

Table 3
Parameters corresponding to linear regressions obtained from the calibration curves

	Intercept (estimated ± SD)	Slope (estimated ± SD)	Correlation coefficient	Linear range (ng/mL)
Curve 1	−0.26 ± 0.06	$(2.81 \pm 0.04) \times 10^{-4}$	0.998	150–5000
Curve 2	0.006 ± 0.003	$(3.02 \pm 0.01) \times 10^{-4}$	0.999	150–5000
Curve 3	0.017 ± 0.005	$(3.82 \pm 0.04) \times 10^{-4}$	0.999	150–5000

was 150 ng/mL. This concentration corresponds to the first point of calibration curve. A representative chromatogram for this level of concentration is showed in Fig. 2. The precision and accuracy of the LOQ are acceptable since the RSD and RE values are lower than 15% (Table 4).

3.7. Stability

The stability of eprosartan was studied at two different concentration levels and at different experimental conditions: after three thaw–freeze cycles, at −20 °C stored for 2 months, at room temperature for 4 h and during 24 h in the autosampler. The mean values and standard deviations of the ratios between the concentration found and initial concentration was used for the stability

Table 4
Precision and accuracy obtained for three different eprosartan concentration levels ($n = 6$)

	Concentration level					
	150 ng/mL		600 ng/mL		4000 ng/mL	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
Precision RSD	8.5	12.2	3.6	4.4	4.1	5.9
Accuracy RE	2.8	3.4	1.4	1.8	1.9	0.4

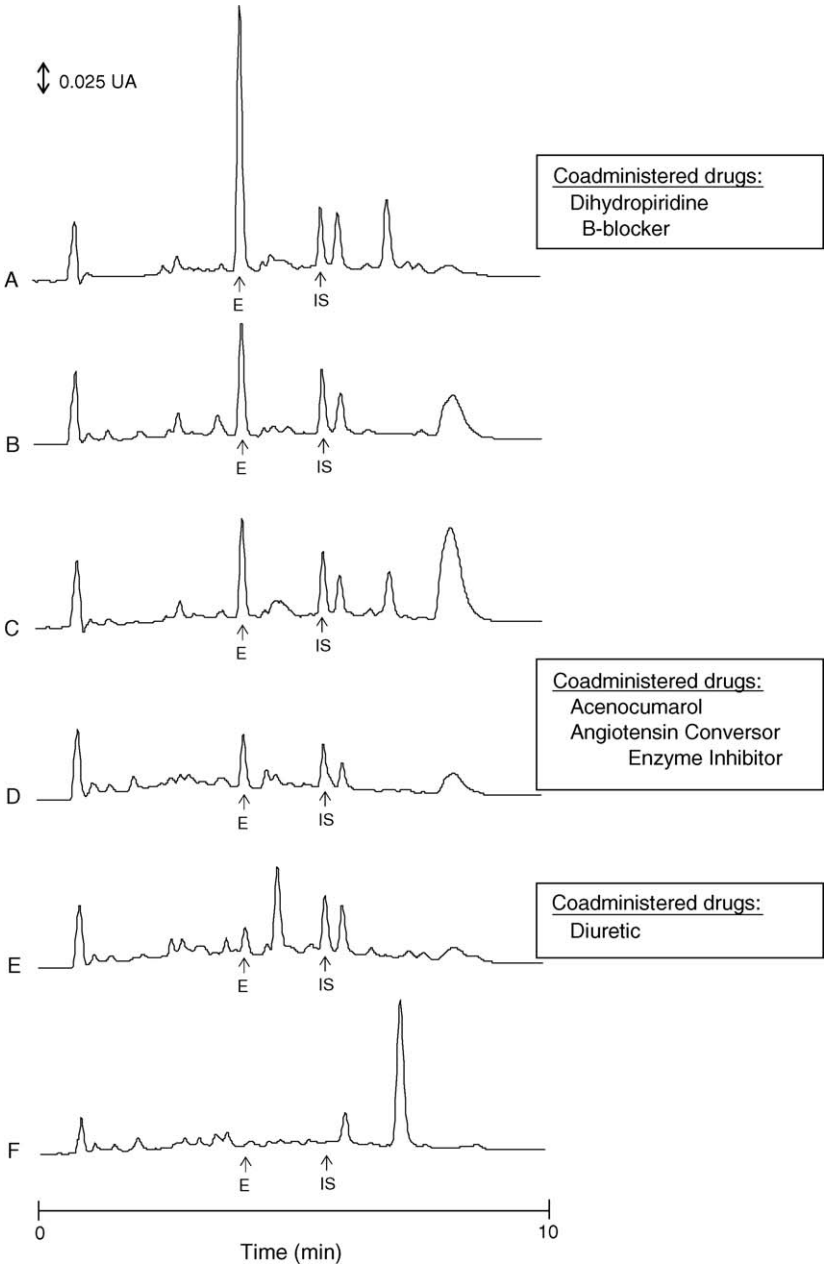

Fig. 3. Chromatograms corresponding to plasma samples obtained from healthy volunteers (B and C) 2 h after the dose intake, from patients (A, D and E) 1 h and 45 min, 12 and 24 h after the dose intake, respectively, and a blank plasma sample (F). The chromatographic conditions are given in Section 2.

Table 5
Mean values and standard deviations of the ratios concentration found ($n = 3$)/initial concentration obtained at two different concentration levels and at different experimental conditions in the eprosartan stability study

Stability	Mean ± SD	
	600 ng/mL	4000 ng/mL
At room temperature		
1 h	0.96 ± 0.05	0.98 ± 0.02
2 h	1.01 ± 0.08	0.95 ± 0.03
4 h	0.98 ± 0.08	1.05 ± 0.06
After thaw–freeze cycles		
Cycle 1	0.97 ± 0.05	1.01 ± 0.02
Cycle 2	0.99 ± 0.07	1.05 ± 0.03
Cycle 3	0.9 ± 0.1	1.04 ± 0.06
After 2 months at −20 °C	0.9 ± 0.2	0.98 ± 0.01

evaluation. As can be seen in Table 5, eprosartan was stable in the experimental conditions assayed.

The stability of eprosartan in methanolic solutions kept at refrigerator temperature was also found to be acceptable for 3 months.

3.8. Application to plasma samples

The developed method has been successfully applied to plasma samples obtained from healthy volunteers and patients under cardiovascular treatment with eprosartan 600 mg/day and co-administered drugs, such as β-blockers, dihydropiridines, statins, ACE inhibitors, diuretics or non-steroids anti-inflammatories.

The samples were taken between 1 and 24 h after the oral administration of eprosartan 600 mg. In all cases, no interferences were observed at the analyte or internal standard retention times as can be seen in Fig. 3. Plasma concentration levels (expressed as mean ± SD in ng/mL) were obtained interpolating from the calibration curve: 1951 ± 44 (1 h 45 min after dose), 689 ± 31 (1 h 40 min after dose), 618 ± 32 (2 h 10 min after dose), 375 ± 36 (12 h after dose), 153 ± 39 and 152 ± 39 (24 h after dose). The eprosartan plasma concentration levels found in healthy volunteers were lower than those obtained in patients under cardiovascular treatment.

4. Conclusions

The SPE-HPLC-UV developed procedure is a suitable and valid method for the determination of eprosartan in plasma samples obtained from patients under cardiovascular treatment. It has proved to be a sensitive, accurate, precise and repeatable method for the quantitative determination of eprosartan in human plasma, without interferences from the endogenous compounds and co-administered drugs.

The use of this method can save effort when monitoring patients who take several medications, specially when polar drugs are mixed. There is no need to have more than one HPLC system or to change the HPLC column to measure plasma from patients on different medication treatments, which are very common in elderly patients.

The validity, LOQ and the linearity range of the method make it acceptable for eprosartan monitorization during 24 h after dose intake. This is necessary to assure that antihypertensive drug plasma levels are included in the therapeutic range during all the interdose range to decrease the incidence of cardiovascular events.

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