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Fast screening method for the determination of angiotensin II receptor antagonists in human plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

A selective, accurate and precise high-performance liquid chromatographic assay coupled to fluorescence detection was developed for the detection of some angiotensin II receptor antagonists (ARA II): Losartan, Irbesartan, Valsartan, Candesartan cilexetil and its metabolite Candesartan M1. The analytes and the internal standard (bumetanide, a high-ceiling diuretic) were extracted from plasma under acidic conditions by means of solid-phase extraction using C_8 cartridges. This procedure allowed recoveries close to 80% for all these drugs excluding Candesartan cilexetil (70%) which presented adsorption processes on glass and plastic walls. The analytes and potential interferences were separated on a reversed-phase column, μBondapak C_{18}, at room temperature. A gradient elution mode was used to carry out the separation, the optimal mobile phase being composed of acetonitrile–5 m*M* acetate buffer, pH 4, at variable flow-rates (from 1.0 to 1.2 ml/min). Fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm. Intra- and inter-day relative standard deviations for all the compounds were lower than 8% except for Losartan (12%) and the method assesses a quite good accuracy (percentage of relative error ~6% in most of the cases). The limit of quantitation for these compounds was 3 ng/ml for Candesartan cilexetil and M1, 16 ng/ml for Losartan and 50 ng/ml for Irbesartan and Valsartan, which allows their determination at expected plasma concentration levels. This assay method has been successfully applied to plasma samples obtained from hypertensive patients under clinical studies after oral administration of a therapeutic dose of some of these ARA II compounds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: HPLC fluorescence; Plasma; Angiotensin II receptor antagonists

1. Introduction

High blood pressure is quantitatively the largest single risk factor for premature death and disability due to its extremely high prevalence in industrialised countries [1].

Angiotensin antagonists are the first major innovation in hypertension management in over a decade. Their specificity of action and excellent side-effect profile provide good conditions for patient compliance as well as high effectiveness [2,3]. Thus, the World Health Organisation (WHO) Guidelines have recommended the prescription of these drugs as a first-line treatment for essential hypertension [4].

Angiotensin II receptor antagonists (ARA II) have been developed to specifically and selectively block the AT1 receptor of the renin–angiotensin system by displacing angiotensin II from it [5].

This family of antagonists are a chemically heterogeneous group of compounds, but they have as a common moiety the imidazole ring. The existence

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of some other fluorescent functional groups in the molecular structure of ARA II compounds such as biphenyl, tetrazole, imidazole and benzimidazole [6,7] makes possible the development of a HPLC–fluorescence method for the determination of ARA II compounds in biological fluids such as urine and plasma.

They are rapidly absorbed after oral administration, but there are differences concerning their oral bioavailability related to distinctions in their presystemic (first-pass) metabolism. Their therapeutic dose ranges from 8 mg (Candesartan cilexetil) up to 300 mg (Irbesartan), consequently, maximum plasma concentration varies from ng/ml levels (Losartan and Candesartan) up to μg/ml (Irbesartan and Valsartan). Time to achieve maximum concentration in plasma is between 2 and 3 h after their intake [8–11]. The low levels of antihypertensive expected in human plasma for some of these drugs (ng/ml) led us to propose a high sensitive detection such as fluorescence. By means of this detection, it would be possible to apply the method for establishing pharmacokinetic profiles up to 24 or 36 h after intake of formulation, once concentrations have largely decreased.

Analytical methods developed for the determination of these antagonists mainly use the liquid chromatographic technique, with photometric [12–17], fluorimetric [18–24] or mass spectrometric detection [25–27]. In the literature, there has been no screening method for these drugs in plasma although there are two reported methodologies for screening them in urine [28,29].

The present study aimed to validate a screening method to simultaneously quantify some ARA II compounds (Losartan, Irbesartan, Valsartan, Candesartan cilexetil and its metabolite Candesartan M1) in human plasma by means of liquid chromatography coupled to fluorescence detection which would provide an efficient separation as well as sensitive detection.

The compounds under study were: Losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-{[2′-(1H-tetrazol-5-yl)biphenyl-4-yl] methyl}imidazole, potassium salt), Irbesartan (2-butyl-3-{[(2′-(1H-tetrazole-5-yl)-biphenyl-4-yl]methyl}-1,3diazaspiro[4,4]non-1-en-4 - one), Valsartan ((*S*)-*N*-valeryl-*N*-{[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-valine), Candesartan cilexetil (±-1-cyclohexyloxycarbonyloxy)ethyl 2-ethoxy1-{[2′ - (1H - tetrazol - 5 - yl)biphenyl - 4 - yl] - methyl} - 1H-benzimidazole-7-carboxylate) and its major metabolite Candesartan M1 (2-ethoxy-1-{[2′-(1H-tetrazol-5-yl) - biphenyl - yl]myl]methyl} - 1H - benzimidazole - 7-carboxylic acid). The formulae of these drugs are shown in Fig. 1.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of two Waters Model 510 HPLC pumps, a Waters Model 717 Plus autosampler and a Waters 474 scanning fluorescence detector (Barcelona, Spain).

The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm. Instrumental parameters as gain and attenuation were set at normal (no highest sensitivity) values (10 and 256, respectively). Chromatograms were recorded by means of a computer and were treated with the aid of the software MILLENNIUM 32 Chromatography Manager from Waters.

A Waters μBondapak C_{18}, 300×3.9 mm I.D., 10 μm column was used to perform separation. Prior to the analytical column, a Waters Novapak C_{18} guard column 20×3.9 mm I.D., 4 μm was placed to prevent column degradation. The column was maintained at room temperature during analysis.

The clean-up procedure consisted of solid-phase extraction and was performed using Varian Bond Elut C_8 columns non-endcapped 1 ml/100 mg (Barcelona, Spain). These columns 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).

Blood samples and later, plasma samples were centrifuged in a 5804 R Eppendorf refrigerated centrifuge (Hamburg, Germany).

2.2. Chemicals

ARA II compounds were kindly provided by the

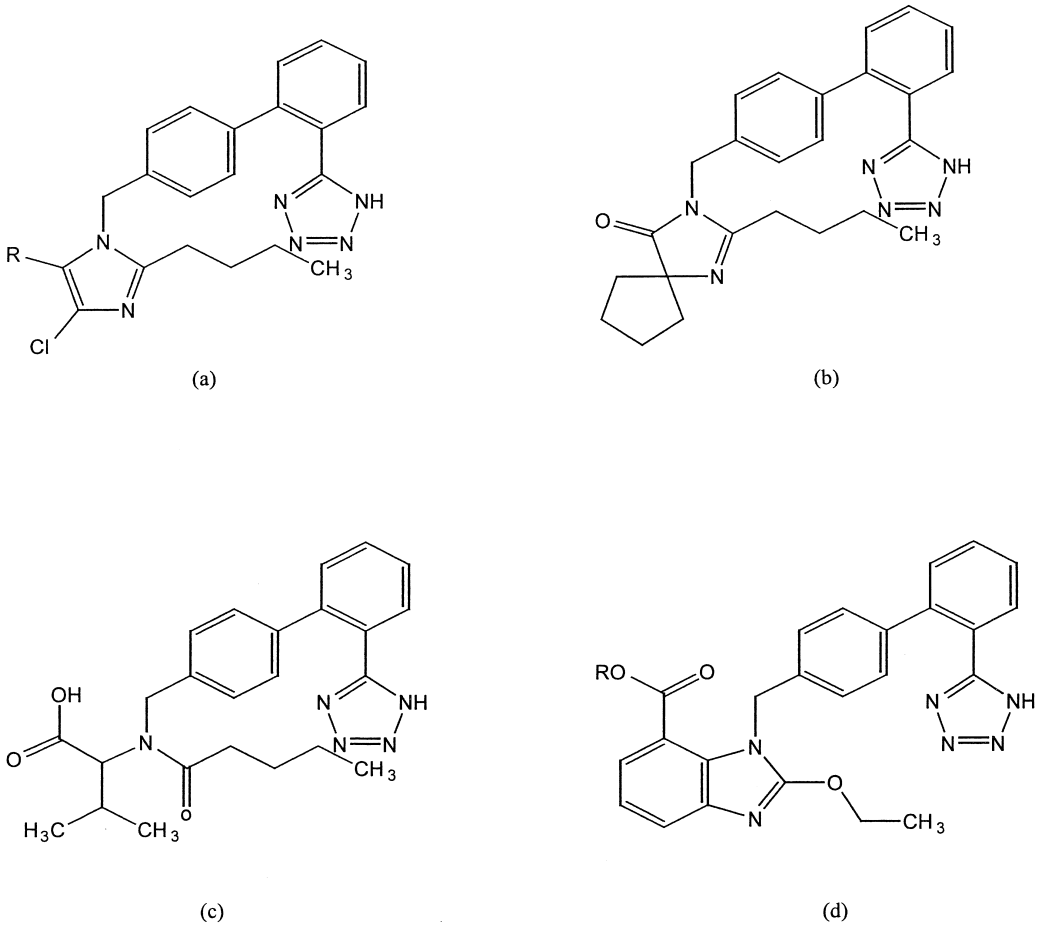

Fig. 1. Chemical structure of ARA II. (a) Losartan, (b) Irbesartan, (c) Valsartan, (d) Candesartan Cilexetil R = CH (CH_3) OOCO, Candesartan M1 R = H.

manufacturers: Losartan (Merck, New Jersey, USA), Irbesartan (Sanofi, Montpellier, France), Valsartan (Novartis Pharma, Basel, Switzerland) and Candesartan cilexetil and its metabolite Candesartan M1 (Astra, Mölndal, Sweden). The internal standard was a high-ceiling diuretic, Bumetanide, [3-(aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid] provided by Farmacusi (Barcelona, Spain).

Acetonitrile and methanol were HPLC grade and purchased from LabScan (Dublin, Ireland). All reagents used were Merck analytical reagent grade (Darmstadt, Germany). Ultrapure distilled and deionized water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA, USA). Buffer solutions were 0.1 *M* H_3PO_4–KH_2PO_4 (pH 2) and 0.5 *M* CH_3COOH–CH_3COONa (pH 4). Stock solutions of the antagonists (1000 μg/ml) were prepared in both methanol and acetonitrile and were stored at 4 °C in the dark to prevent degradation. Working solutions were prepared daily by dilution from stock solutions.

Drug-free plasma was obtained from several healthy female and male volunteers aged 20–50 years who were not under medical treatment (for detailed procedure see Section 2.4).

2.3. Chromatographic conditions

Separation of the analytes was carried out using a gradient elution mode at room temperature. The

optimal composition of the mobile phase was achieved by different mixtures of pure acetonitrile and 5 m*M* sodium acetate buffer, pH 4. The flow-rate was increased (up to 1.2 ml/min) during the chromatography in order to shorten the retention time of the late-eluting analyte, Candesartan cilexetil. The gradient is shown in Table 1. The effluent was monitored at excitation and emission wavelengths of 250 and 375 nm, respectively.

All the eluents were filtered through a 0.45-μm type HVLP Durapore membrane filters (Millipore, Dublin, Ireland) and the residual air was removed from them by bubbling helium through. The volume injected into the chromatographic system was 20 μl.

2.4. Plasma samples collection

Venous blood samples were collected from healthy volunteers or patients by syringe, they were immediately transferred into heparinized tubes (containing tripotassium EDTA) and gently mixed. Blood samples were centrifuged at 3500 rpm for 10 min under controlled temperature (4 °C) to avoid either decomposition or biological activity. The plasma supernatant was carefully separated from blood cells and collected in polypropylene tubes to be frozen at −20 °C until the analysis.

The same working procedure was followed for blank plasma samples from healthy volunteers and plasma obtained from hypertensive patients under treatment with some of these ARA II compounds.

Each blood sample was taken approximately 2 h (time interval which is supposed to include maximum plasma concentration) after the administration of the daily dose of the ARA II compound.

Table 1
Gradient elution mode for ARA II compounds in human plasma

Time (min)	5 m*M* acetate buffer, pH 4 (%)	Acetonitrile (%)	Flow rate (ml/min)
0	70	30	1.0
15	40	60	1.2
21	5	95	1.2
24	70	30	1.0
25	70	30	1.0

2.5. Solid-phase extraction of plasma samples

Frozen plasma samples were thawed and brought to room temperature. They were mixed thoroughly by inversion and allowed to sit 2 min for particulates to settle out. A 0.25-ml aliquot of plasma was spiked with the appropriate amount of stock solution of the ARA II compound and the internal standard, bumetanide. Afterwards, it was acidified with 0.25 ml of a 1 *M* H_3PO_4 solution in order to precipitate proteins and clarify the plasma sample by eliminating fibrinous material. The mixture was properly shaken and centrifuged for 5 min at 10 000 *g* at 4 °C.

The Bond Elut C_8 cartridge was conditioned with 2 ml of methanol followed by 1 ml 0.1 *M* phosphate buffer, pH 2. The cartridge was not allowed to dry before the application of 0.5 ml of the plasma sample previously treated. The sample was slowly passed through the cartridge, at a very low vacuum ($P<5$ mmHg; 1 mmHg=133.322 Pa). The column was then washed with 0.5 ml of methanol–0.1 *M* phosphate buffer, pH 2 (50:50, v/v) and dried at full vacuum ($P>20$ mmHg) for 20 min. The analytes of interest and the internal standard were eluted with 0.5 ml of methanol. Finally, 0.1 ml of 10% (v/v) ethylenglycol solution in methanol was added to the eluate to prevent adsorption processes of Candesartan cilexetil [23].

After vortex mixing the eluate, it was dried under a stream of nitrogen at 40 °C. The remaining residue was dissolved in 0.25 ml of the starting mobile phase and 20 μl of this solution was injected into the chromatographic system under the optimised chromatographic conditions.

2.6. Measurements and calculations

Chromatographic data management was automated using a computer and handled by means of the software MILLENNIUM 32 Chromatography Manager from Waters. The quantitation of the ARA II concentration level from human plasma was based on the chromatographic peak area ratios (ARA II to internal standard bumetanide). The standard calibration curves were fitted to a linear regression equation of the peak area ratio versus the analyte:internal standard concentration ratio. ARA II concentrations in the clinical samples were then

determined from the regression equation obtained from spiked human plasma samples.

2.7. Extraction recovery

Three concentration levels of plasma samples (6, 110 and 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml, 1.7 and 4 μg/ml for Irbesartan and Valsartan) were used to calculate recovery of the proposed extraction procedure. Three replicate ($n=3$) spiked plasma samples at these different concentration levels were extracted to calculate mean recovery. All spiked plasma samples contained the internal standard (I.S.) Bumetanide at 2 μg/ml concentration level.

The mean recoveries were estimated by measuring the peak areas obtained from extracting spiked plasma samples and comparing them with the areas of non-extracted standard solutions of the same concentration level.

2.8. Assay validation

The validation was performed in accordance to the summary report of the conference on Analytical Methods Validation: Bioavailabilty, Bioequivalence and Pharmacokinetic Studies, which has provided guidelines for pharmacokinetic studies in humans and animals [30].

Calibration data were generated by spiking blank plasma samples with the appropriate volume of stock solution of these ARA II compounds yielding concentrations of 3, 6, 16, 50, 110, 170 and 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 50, 110, 260, 800 ng/ml and 1.7, 2.7 and 4 μg/ml for Irbesartan and Valsartan. Calibration curves were represented by plots of the peak areas ratios (ARA II:I.S.) versus the same concentration ratios and were fitted to the linear regression $y = a + bx$. Calibration standards were spiked with 2 μg/ml of the internal standard Bumetanide.

The limit of quantitation was defined as the lowest concentration in the calibration curve that can be measured with acceptable accuracy, precision and variability. A maximum intra-day relative standard deviation (RSD) of 20% and a maximum deviation from the nominal value of 20% were allowed. It was determined by using at least five plasma samples spiked with appropriate volume of stock solution of the ARA II compound.

The intra- and inter-day accuracy and precision of the method were evaluated using quality control (QC) samples of different known concentration levels (6, 110 and 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml, 1.7 and 4 μg/ml for Irbesartan and Valsartan). Intra-day accuracy and precision were determined by assaying five replicates of each concentration level of the above mentioned QC samples in a single run. Inter-day precision and accuracy was established over a 3-month period. Precision was characterised by the RSD,%, whereas accuracy was expressed as a percentage error of nominal versus measured concentration (RE,%). The limits of acceptable variability were set at 15% except for the limit of quantitation concentration level which allows up to 20%.

At least six different lots of plasma donations from male and female volunteers were carefully evaluated for interference in the assay.

The stability of ARA II compounds was investigated during one sample run in the autosampler at room temperature after several hours of reconstitution following extraction from plasma.

Stability of these drugs in plasma at −20 °C was determined by periodic analysis of spiked samples over 4 months. In addition, selected clinical plasma samples from hypertensive patients were also assayed repeatedly over this period to guarantee stability in this medium.

The stability of these five ARA II compounds through several freeze–thaw cycles was also investigated.

3. Results and discussion

3.1. Chromatographic conditions

A screening method was previously developed in our laboratory for these five ARA II compounds in human urine using HPLC with photometric detection, so optimal conditions for the separation were already investigated [28]. The same chromatographic conditions were intended to be applied to the separation of Losartan, Irbesartan, Valsartan, Candesartan

cilexetil and its metabolite Candesartan M1 in human plasma.

Octadecylsilane (C_{18}) was chosen as packing of the analytical column that accomplished an efficient separation, pH 4 was chosen as optimal pH value in the aqueous component of the mobile phase (sodium acetate buffer) and acetonitrile was chosen as the best option for organic modifier of mobile phase. Using these conditions, slightly differential retention of the five analytes was achieved on the column support with a gradient elution mode.

There were small differences with the previous HPLC–photometric detection method developed for these antagonists in urine. The internal standard was different (Bumetanide and not Nimodipine) a fact that made us introduce some variations in gradient sequence. Initially, the urine gradient program was attempted but overlap of some analytes occurred in the case of plasma so it was discarded.

The fluorescence excitation and emission wavelengths were optimised considering the fluorescence spectra of these compounds and some published analytical methods dealing with fluorescence detection to quantitate these antagonists [18–24]. All the ARA II compounds studied showed maximum fluorescence response in a narrow wavelength range, 250–270 nm for excitation and 370–390 nm for emission (Table 2). Optimal wavelengths for their simultaneous determination were set at 250 and 375 nm, for excitation and emission, respectively. The acidic characteristics of these antihypertensive drugs (pK_a values from 3 to 6) confirmed a high dependence of fluorescent properties on pH. Basic pH values reduced significantly the intrinsic fluorescence of all these molecules, though all the ARA II compounds did not exhibit the same fluorescence at acidic pH values. There was a selection to be made based on the plots $I_{F,rel}$ versus pH for these ARA II compounds aiming to ensure a stable value of fluorescent signal at pH selected (Fig. 2) [31]. Some of them, Valsartan and Candesartan cilexetil, have slight variations of fluorescence at pH 2 and 3 making fluorescence detection unfeasible at this pH value. The acid pH value with a constant fluorescent signal and a good separation for all the ARA II compounds was 4.0. This is not the optimal pH value for fluorescence of Losartan as reflected in some of the references for its single determination which use lower pH values [18,19], but taking into account that the aim of this work is the simultaneous determination of these five ARA II compounds, loss of some sensitivity was preferred to provide a good performance for the majority of the compounds.

Table 2
Excitation and emission wavelengths for angiotensin II receptor antagonists

	Excitation wavelength (nm)	Emission wavelength (nm)
Losartan	247	387
Irbesartan	259	385
Valsartan	259	399
Candesartan cilexetil	272	384
Candesartan M1	259	392

3.2. Plasma samples treatment

Plasma composition is much more complex than that of urine though plasma has not the same inter-individual variability as urine and it assesses a good homogeneity in a normal population (pH value, proteins and salt concentration being practically constant). Plasma treatment include some complementary steps prior to the isolation of the analytes of interest from the matrix.

Precipitation of plasma proteins is often the first step in plasma treatment and has to be carried out to clean plasma in some way. It serves to cleave the union between protein and drug in order to determine the total drug concentration in this fluid. Precipitant agents such as strong acids or organic solvents for analytes unstable at acid pH values are commonly used. Several of these precipitant agents, such as 1 *M* phosphoric acid, 1 *M* perchloric acid, 0.1 *M* hydrogen chloride, methanol, ethanol and acetonitrile were investigated in different proportions (1:1, 2:1 and 3:1) precipitant to plasma volume. Some of these (mainly phosphoric acid, hydrogen chloride and acetonitrile) were found in the literature for cleaning-up of plasma samples of these ARA II compounds [14,17–24,26]. Precipitant agents must have two characteristics—no loss of analyte and maximum clean-up must be achieved following this process [32]. Phosphoric acid, perchloric acid and hydrogen chloride as well as acetonitrile gave the cleanest

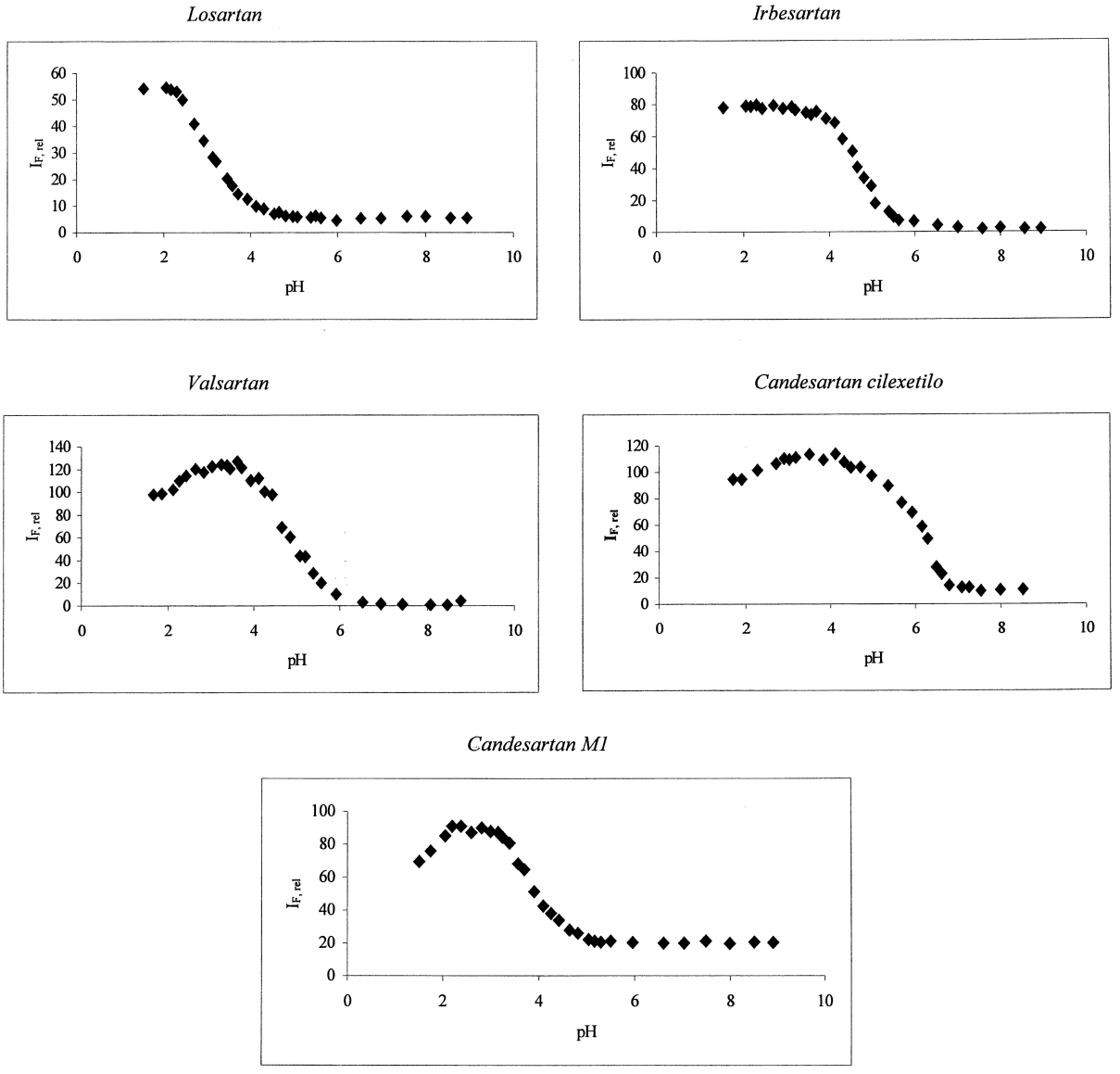

Fig. 2. Relative fluorescent intensity ($I_{F,\ rel}$)–pH data at 0.5 *M* ionic strength for ARA II studied.

extracts but good recoveries were only obtained when phosphoric acid and hydrogen chloride were used. Coprecipitation processes can occur in the cases where losses of the analyte are found. Hydrogen chloride gave very low recoveries for Candesartan cilexetil, thus we rejected this solvent. Phosphoric acid (1 *M*) was chosen as the best precipitant agent. A 1:1 (v/v) relation was considered optimal because higher proportions did not imply either cleaner extracts or higher recoveries.

After precipitation, usually additional extraction steps have to be developed to isolate analyte from the matrix. Direct injection of the plasma, just after precipitation, was attempted but the extract was not clean enough to be free of interferences for the analysis. Solid-phase extraction was selected as the

secondary plasma treatment procedure. The transference of the validated method for the extraction of these angiotensin II receptor antagonists from human urine [28] to plasma was examined. The critical step was found to be the washing/eluting one. Interferences were still found when washing with methanol–0.1 *M* phosphate buffer, pH 2 (40:60, v/v) and eluting with pure methanol. Other washing and eluting solvents were studied to get higher selectivity in plasma extraction for these ARA II compounds. A secondary washing step with dichloromethane, acetone, chloroform, hexane and diethyl ether was made but recoveries were too low, indicating that partial elution was achieved by means of these solvents. Alternative elution with ethyl acetate, THF or acetonitrile instead of methanol did not guarantee lack of interferences. On seeing the elution profiles obtained with methanol–0.1 *M* phosphate buffer, pH 2, we assayed the washing of the samples with a mixture somewhat enhanced in organic percentage, 50:50. This provided the cleanest extracts with a low loss of recovery for Irbesartan. The rest of steps and variables of SPE were not modified from the reported screening method for ARA II compounds in human urine [28].

3.3. Internal standard

Fluorescence detection is highly selective since only a minimal fraction of the analytes exhibit intrinsic fluorescence. This point complicates the selection of the internal standard (I.S.) for the determination of ARA II compounds in human plasma by HPLC–fluorescence detection. The properties of several antihypertensive drugs (including diuretics, β-blockers and calcium-channel blockers) were studied but with the separation conditions used only atenolol, triamterene and bumetanide were fluorescent. Bumetanide, a high-ceiling diuretic, was chosen as the most appropriate I.S. because of its good recovery and separation under the SPE-HPLC fluorescence method developed.

3.4. Linearity

The linear ranges of the standard curves for the validated assayed in plasma were 50 ng/ml–4 μg/ml for Irbesartan and Valsartan, 3–250 ng/ml for Candesartan cilexetil and its metabolite M1 and 16–250 ng/ml for Losartan. The mean correlation coefficients of calibration curves were 0.999. These ranges include the expected maximum plasma concentration (C_{max}) of these ARA II at therapeutic doses according to pharmacokinetic data (Losartan 200 ng/ml (50 mg), Irbesartan 2 μg/ml (150 mg), Valsartan 2 μg/ml (80 mg), Candesartan M1 150 ng/ml (16 mg Candesartan cilexetil) [8–11]. The application of this screening method to the determination of Valsartan and Candesartan in clinical samples from patients undergoing treatment with these antagonists confirmed the appropriate concentration range of the calibration curves. The limits of quantitation (LOQs) are 3 ng/ml for Candesartan cilexetil and its metabolite M1, 16 ng/ml for Losartan and 50 ng/ml for Irbesartan and Valsartan.

The significant data of the assay validation are shown in Table 3.

3.5. Precision and accuracy

Statistical evaluation of the results established

Table 3
Assay validation of Losartan, Irbesartan, Valsartan, Candesartan cilexetil and its metabolite Candesartan M1 by HPLC with fluorimetric detection in human plasma

ARA II	Retention time (min)	Linear range (ng/ml)	Slope ± *t S*	Linear regression coefficient, *r*	Limit of quantitation LOQ (ng/ml)
Losartan	11.5±0.06	16–250	0.1±0.05	0.99	16.3
Irbesartan	12.6±0.05	50–4000	4.2±0.2	0.999	50.7
Valsartan	14.4±0.04	50–4000	4.2±0.1	0.999	51.5
Candesartan cilexetil	22.6±0.01	3–250	3.9±0.1	0.999	3.3
Candesartan M1	9.7±0.15	3–250	4.4±0.8	0.999	3.2

Table 4
Intra-day precision and accuracy in the assay

ARA II	Low concentration level[a]		Medium concentration level[b]		High concentration level[c]	
	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
Losartan	–	–	10.0	9.7	9.8	8.7
Irbesartan	1.9	5.7	2.4	−4.9	2.6	2.1
Valsartan	5.4	3.7	3.9	−2.6	3.4	0.9
Candesartan cilexetil	3.0	1.9	2.8	0.4	2.8	0.7
Candesartan M1	2.8	−2.3	3.0	−3.5	3.0	1.4

[a] 6 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml for Irbesartan and Valsartan.
[b] 110 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 1.7 μg/ml for Irbesartan and Valsartan.
[c] 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 4 μg/ml for Irbesartan and Valsartan.

good accuracy and precision of the method according to the validation guides mentioned [30].

Intra- and inter-day mean found concentrations did not deviate from the nominal concentrations by more than ±6% for all the ARA II compounds except for Losartan (12%). The mean accuracy was consequently 94% in all the cases except for Losartan with a mean value of 88%.

Intra- and inter-day precision did not exceed the 8% RSD including the LOQ concentration level, except in the case of Losartan (up to 12%). Intra- and inter-day precision and accuracy values are shown in Tables 4 and 5, respectively.

3.6. Recovery

Quantitative recoveries calculated from spiked plasma samples at QC concentration levels (6, 110 and 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml, 1.7 and 4 μg/ml for Irbesartan and Valsartan) after the SPE procedure are collected in Table 6.

The mean absolute recoveries (after comparison of values at the three concentration level studied) do not depend on concentration level and were at least 70% for all the analytes and the I.S.

3.7. Selectivity

After evaluation of several plasma lots from different sources, no interfering components were detected. Thus, sample treatment and chromatographic analysis produced a selective assay for the analytes. Representative chromatograms obtained from control human plasma and plasma spiked with 110 ng/ml of Losartan, Candesartan cilexetil and Candesartan M1 and 1.7 μg/ml of Irbesartan and Valsartan and 2 μg/ml of Bumetanide (I.S.) are shown in Fig. 3.

Table 5
Inter-day precision and accuracy in the assay

ARA II	Low concentration level[a]		Medium concentration level[b]		High concentration level[c]	
	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
Losartan	–	–	12.1	12.4	11.4	10.1
Irbesartan	3.4	8.9	3.8	−6.7	3.7	2.3
Valsartan	8.0	6.7	5.0	−4.6	5.5	1.3
Candesartan cilexetil	5.0	2.8	4.5	1.1	4.1	1.2
Candesartan M1	3.6	−4.6	4.1	−4.9	3.6	1.75

[a] 6 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml for Irbesartan and Valsartan.
[b] 110 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 1.7 μg/ml for Irbesartan and Valsartan.
[c] 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 4 μg/ml for Irbesartan and Valsartan.

Table 6
Absolute recoveries of ARA II compounds from human plasma at QC concentration levels

ARA II	Recovery (%)		
	Low concentration level[a]	Medium concentration level[b]	High concentration level[c]
Losartan	–	67.2	71.2
Irbesartan	87.6	84.3	84.8
Valsartan	83.2	82.2	85.7
Candesartan cilexetil	68.3	70.3	71.5
Candesartan M1	86.5	90.7	90.5

[a] 6 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1 and 110 ng/ml for Irbesartan and Valsartan.
[b] 110 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 1.7 μg/ml for Irbesartan and Valsartan.
[c] 250 ng/ml for Losartan, Candesartan cilexetil and Candesartan M1and 4 μg/ml for Irbesartan and Valsartan.

3.8. Stability

The stability of stock solutions of these ARA II compounds in methanol was checked and proved to be stable for at least 1 year at 4 °C when protected from light. The analytes reconstituted in the starting mobile phase were also stable at ambient conditions (no control of temperature in the autosampler) for at least 24 h, thus allowing us to automate the procedure. No significant degradation in plasma samples (spiked or clinical) during 4 months at −20 °C was detected. Furthermore, these five ARA II compounds were stable for at least at four freeze–thaw cycles.

3.9. Application to real samples

The developed method was applied to the determination of Valsartan and Candesartan in plasma samples obtained from hypertensive patients. Fig. 4 shows the chromatograms of plasma samples collected in a 2-h period after the intake of the ARA II formulation and treated as reported previously. Concentration values found for clinical samples were interpolated from the daily calibration curves of these ARA II compounds.

Patients were under treatment with Vals 80 mg (Valsartan) and Atacand 16 mg (Candesartan cilex-

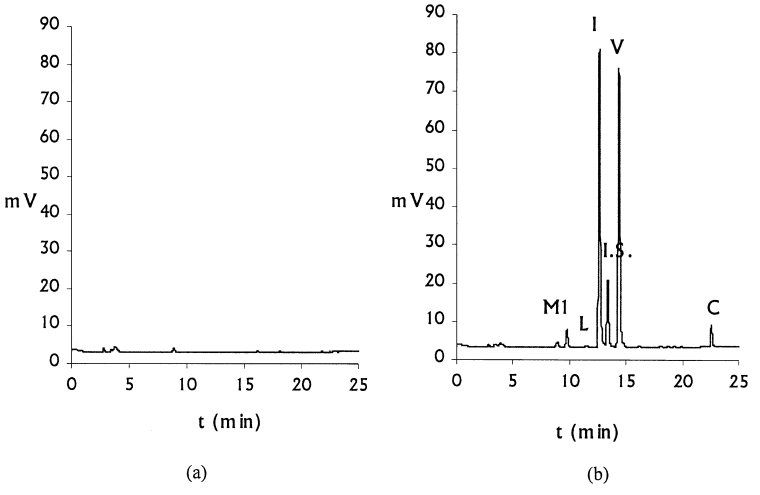

Fig. 3. (a) Chromatogram of a control plasma sample, (b) Chromatogram of a plasma sample spiked yielding concentrations of 100 ng/ml for Losartan, Candesartan cilexetil and M1 and 2 μg/ml for Irbesartan and Valsartan. Bumetanide is used as internal standard (2 μg/ml). Chromatographic conditions as described in Section 2.3.

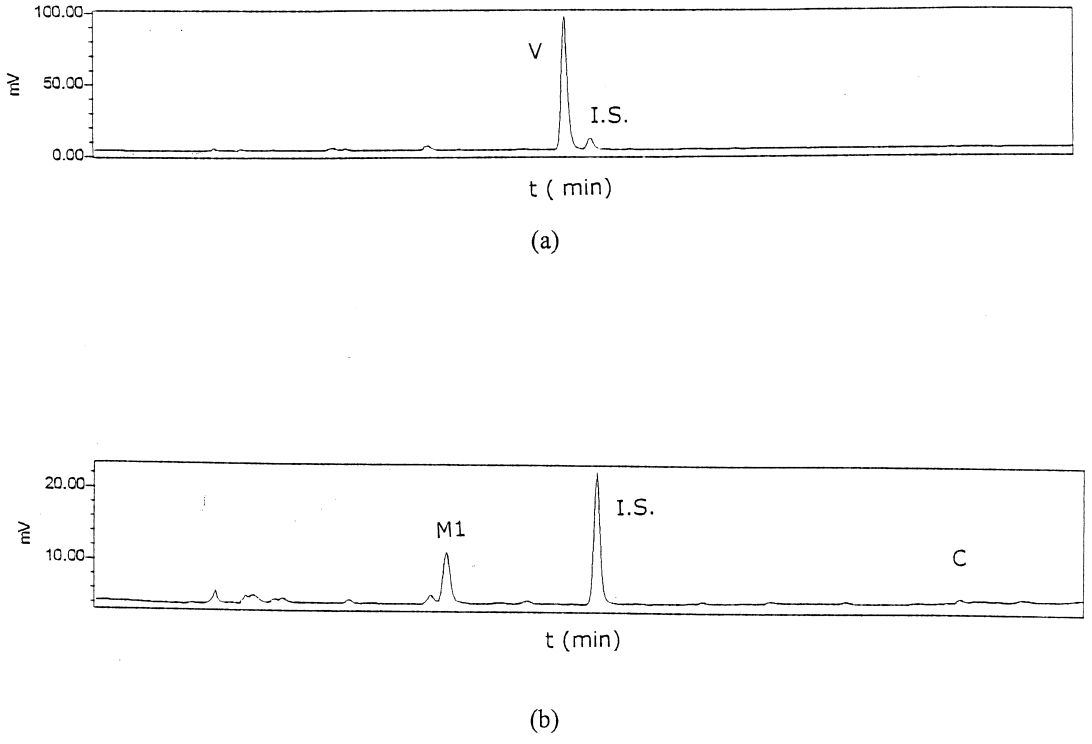

Fig. 4. (a) Chromatogram of a plasma sample obtained from a hypertensive patient 0–2 h following an oral dose of Vals Valsartan 80 mg. (b) Chromatogram of a plasma sample obtained from a hypertensive patient 0–2 h following an oral dose of Atacand Candesartan 16 mg. Chromatographic conditions as described in Section 2.3.

etil). Concentration values were 3.2 μg/ml for Valsartan and 9.6 ng/ml for Candesartan cilexetil and 123 ng/ml for Candesartan M1. Candesartan cilexetil is rapidly metabolised to M1 after ingestion of a therapeutic dose, thus no high plasma concentration is expected (see Fig. 4b). Found values for concentrations are in accordance to pharmacokinetic data [C_{max} Valsartan 2 μg/ml (from 80 mg) at t_{max} 2–3 h, C_{max} Candesartan M1 150 ng/ml (from 16 mg Candesartan cilexetil) at t_{max} 2–3 h] though, it would be advisable to apply this method to a wider range of hypertensive population to verify these data and carefully control sample collection time to measure real peak plasma concentration.

4. Conclusions

The initial method development focused on the HPLC–photometric detection method validated in our laboratory for these antagonists in urine, but plasma concentration levels forced us to choose a more sensitive detection, so fluorescence detection was looked to as an alternative.

The chromatographic method described is adequate for quantitation in human plasma samples of five ARA II compounds, Losartan, Irbesartan, Valsartan, Candesartan cilexetil and its metabolite Candesartan M1 at different concentration levels (ng/ml and μg/ml) using Bumetanide as internal standard.

The SPE procedure is very simple and effective and provided no interference peaks for endogenous components, then the screening method developed for these ARA II compounds in human plasma is selective.

The separation of these five compounds takes place in <25 min and plasma treatment can be accomplished in 25 min for a maximum of 12

samples. In summary, the total analysis time can be estimated at around 50 min.

In spite of the complex matrix analyzed, acceptable values of precision and accuracy have been obtained by this method regarding the guidelines for assay validation in the bioanalytical laboratory.

This method can be successfully applied to plasma samples from obtained hypertensive patients, providing a unique method for the determination of five ARA II compounds.

This fluorescence detection method provided higher sensitivity (LOQ 5–60 ng/ml fluoresence detection versus ~500 ng/ml photometric detection) than the photometric one developed in our laboratory for these antagonists in urine, so this method offers an excellent way to quantify ARA II compounds in plasma as well as in urine with slight modifications in the SPE step.

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References

[1] G.E. McVeigh, J. Flack, R. Grimm, Drugs 49 (1995) 161.
[2] V. Regitz-Zagrosek, M. Neuss, J. Holzmeister, E. Fleck, J. Hypertens. 13 (Suppl. 1) (1995) S63.
[3] E.D. Freis, V. Papademetriou, Drugs 52 (1) (1996) 1.
[4] Guidelines Subcommittee of the World Health Organization-International Society of Hypertension (WHO-ISH), J. Hypertens. 17 (1999) 151.
[5] J.H. Bauer, G.P. Reams, Arch. Intern. Med. 155 (1995) 1361.
[6] T. Eicher, S. Hauptmann, The Chemistry of Heterocycles. Structure, Reactions, Synthesis and Applications, Georg Thieme Verlag, Stuttgart, New York, 1995.
[7] G.G. Guibault, Practical Fluorescence, 2nd ed., Marcel Dekker, New York, 1990.
[8] K.L. Goa, A.J. Wagstaff, Drugs 51 (5) (1996) 820.
[9] J.C. Gillis, A. Markham, Drugs 54 (6) (1997) 885.
[10] A. Markham, K.L. Goa, Drugs 54 (2) (1997) 299.
[11] K.J. McClellan, K.L. Goa, Drugs 56 (5) (1998) 847.
[12] A. Soldner, H. Spahn-Langguth, E. Mutschler, J. Pharm. Biomed. Anal. 16 (1998) 863.
[13] H. Lee, H.O. Shim, H.S. Lee, Chromatographia 42 (1/2) (1996) 39.
[14] C.I. Furtek, M.W. Lo, J. Chromatogr. B 573 (2) (1992) 295.
[15] E. Francotte, A. Davatz, P. Richert, J. Chromatogr. B 686 (1996) 77.
[16] T. Miyabayashi, M. Motohashi, K. Izawa, T. Yashiki, J. Chromatogr. B 677 (1996) 123.
[17] D.E. Lundberg, C.R. Person, S. Knox, M.J. Cyronak, J. Chromatogr. B 707 (1998) 328.
[18] D. Farthing, D. Sica, I. Fakhry, A. Pedro, T.W.B. Gehr, J. Chromatogr. B 704 (1997) 374.
[19] M.A. Ritter, C.I. Furtek, M.W. Lo, J. Pharm. Biomed. Anal. 15 (1997) 1021.
[20] S.Y. Chang, D.B. Whigan, N.N. Vachharajani, R. Patel, J. Chromatogr. B 702 (1997) 149.
[21] L.A. Brunner, M.L. Powell, P. Degen, G. Flesch, Lab. Robotic. Autom. 6 (1994) 171.
[22] A. Sioufi, F. Marfil, J. Godbillon, J. Liq. Chromatogr. 17 (10) (1994) 2179.
[23] T. Miyabayashi, T. Okuda, M. Motohashi, K. Izawa, T. Yashiki, J. Chromatogr. B 677 (1996) 123.
[24] H. Stenhoff, P.O. Lagerström, C. Andersen, J. Chromatogr. B 731 (1999) 411.
[25] Z. Zhao, Q. Wang, E.W. Tsai, X.Z. Qin, D. Ip, J. Pharma. Biomed. Anal. 20 (1999) 129.
[26] T. Iwasa, T. Takano, K. Hara, T. Kamei, J. Chromatogr. 734 (1999) 325.
[27] T. Kondo, K. Yoshida, Y. Yoshimura, M. Motohashi, S. Tanayama, J. Mass Spectros. 31 (1996) 873.
[28] L. González, R.M. Alonso, R.M. Jiménez, Chromatographia 52 (11/12) (2000) 735.
[29] H.H. Maurer, T. Kraemer, J.W. Arlt, Ther. Drug Monit. 20 (1998) 706.
[30] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.
[31] E. Cagigal, L. González, R.M. Alonso, R.M. Jiménez, J. Pharm. Biomed. Anal. 26 (2001) 477.
[32] J. Chamberlain, Analysis of Drugs in Biological Fluids, 4th ed., CRC Press, Boca Raton, FL, 1987.