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Short communication

Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma by liquid chromatography

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Abstract

A simple, selective, sensitive and precise high-performance liquid chromatographic plasma assay for the antihypertensive drugs, irbesartan and hydrochlorothiazide is described. Good chromatographic separation was achieved using a Supelcocil C_{18} (5 μm, 15 cm×4.6 mm) column and a mobile phase consisting of 10 m*M* potassium dihydrogen phosphate:methanol:acetonitrile (5:80:15 v/v/v) (pH:2.5) while at a flow-rate of 1.0 ml min^{-1}. Irbesartan and hydrochlorothiazide were detected at 275 nm and were eluted 5.8 and 7.8 min, respectively, after injection. No endogenous substances were found to interfere. The method utilizes protein precipitation with acetonitrile as the only sample preparation involved prior to reversed-phase high-performance liquid chromatography. No internal standard was required. Linearity range for irbesartan and hydrochlorothiazide was 10.0–60.0 μg ml^{-1} and 4.0–20.0 μg ml^{-1}, respectively. The determination of intra- and inter-day precision (RSD) was less than 2.5 and 3.5%, at all concentration levels, while the inter- and intra-day accuracy (% difference) was less than 4.9–6.2%. This method is being used in a therapeutic drug monitoring service to quantitate these therapeutic agents in patients for pharmacokinetic studies.

Keywords: Irbesartan; Hydrochlorothiazide

1. Introduction

The absolute risk of cardiovascular events is mainly determined by high blood pressure, although there are some other important contributors, such as age, race and presence of other cardiovascular risk factors. Hence, antihypertensive therapy enables to reduce considerably the risk of developing cardiovascular complications that cause a high mortality rate in the industrialized countries [1]. The trend in cardiovascular drug research has been to develop new compounds acting on very specific targets such as cell surface receptors. Angiotensin II receptor antagonists represent a new pharmacological class of antihypertensive drugs. They block selectively the AT_1 angiotensin II receptor, are long-acting and have a good tolerability profile.

Irbesartan, (2-butyl-3-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]1,3-diazaspiro[4,4]non-1-en-4-one), is the first member of a new chemical class of a non-peptide angiotensin II receptor antagonist. The first

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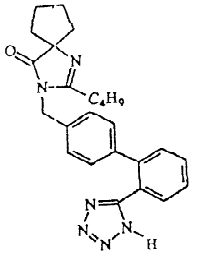
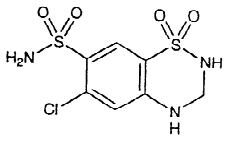
approved indication for irbesartan is for hypertension.

Hydrochlorothiazide, or 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide is a diuretic of the class of benzothiadiazines.

Irbesartan (**I**) and hydrochlorothiazide (**II**) have the following structural formulae:



(I)



(II)

A new combination dosage form of irbesartan and hydrochlorothiazide is indicated in the treatment and management of edema and hypertension. To knowledge, neither high-performance liquid chromatographic methods nor spectrophotometric methods have been described for the simultaneous determination of both drugs in biological fluids. Therefore has created a need for new analytical method for their simultaneous determination.

There are a number of reported methods for the determination of the two pharmaceuticals. Only a high-performance liquid chromatographic (HPLC) method has been developed for determination of irbesartan in human urine [2]. On the other hand, although various methods have been reported for the assay of hydrochlorothiazide, individually or in their combination with other drugs, including, have been in pharmaceutical formulations and biological fluids, there methods use voltammetry [3], capillary zone electrophoresis [4–6], spectrophotometry [7–15], HPLC [16–23].

As a crucial part of the drug development process, a rapid, sensitive and selective assay is required to measure drug concentrations in human plasma samples from clinical pharmacokinetic studies. I report an accurate and sensitive validated HPLC assay with spectrophotometric detection for the simultaneous determination of irbesartan and hydrochlorothiazide in human plasma samples.

2. Experimental

2.1. Apparatus

A chromatographic system consisted of a HP 1100 series mode quaternary pump with a HP 1100 series manual injector 20 μl fixed loop, equipped with a VWD variable wavelength UV/VIS detector. The detector was set at 275 nm (0.02 a.u.f.s.) and peak areas were integrated automatically by computer using the Hewlett–Packard Chem Station software program.

Other apparatus used included a Radiometer NEL pH 890 pH meter digital equipped with a combined glass– calamol electrode and ultrasound generator.

2.2. Chemicals used

Irbesartan and hydrochlorothiazide were kindly supplied by Sanofi. Analytical grade potassium dihydrogen phosphate, phosphoric acid and HPLC grade methanol, and acetonitrile were purchased from Merck.

2.3. Procedure for high-performance liquid chromatography

2.3.1. Chromatographic conditions

Solutions and mobile phases were prepared at the time of use. The mobile phases used were 10 m*M* potassium dihydrogen phosphate:methanol:acetonitrile (5:80:15 v/v/v) (pH 2.5). The analytical column was a Supelcocil C_{18} (5 μm, 15 cm×4.6 mm) column. All analysis were done under isocratic conditions at a flow-rate of 1.0 ml min^{-1} and at room temperature.

2.3.2. Standards

Stock solutions of irbesartan and hydrochlorothiazide were made daily by dissolving the appropriate amount of drug pure substance in methanol to yield a final drug concentration of 1.0 mg ml^{-1}, respectively. Separate solutions were prepared for the calibration standards and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 2% of the plasma volume.

2.3.3. Preparation of plasma sample

The plasma samples were stored in the freezer at −17 °C and allowed to thaw at room temperature before processing. The plasma samples were centrifuged at 4000 *g* for 10 min. An aliquot (1.0 ml) was pipetted into a 10 ml polypropylene tube and acetonitrile (2.0 ml). The mixture was vortex mixed briefly and after standing for 5 min at room temperature the mixture was centrifuged at 4000 *g* for 20 min. Then the supernatant was injected into the HPLC system.

2.3.4. Calibration and linearity

Calibration curves were constructed in the range 10.0–60.0 $\mu g ml^{-1}$ for irbesartan and 4.0–20.0 $\mu g ml^{-1}$ for hydrochlorothiazide to encompass the expected concentrations in measured samples. Curves were obtained daily for 3 days by plotting the peak area of these drugs against concentrations of these drugs. Linear calibration curves were generated by weighted $(1/y^2)$ linear regression analysis and obtained over the respective standard concentrations ranges. The suitability of the calibration models were confirmed by back-calculating the concentrations of the calibration standards.

2.3.5. Analytical recovery

Absolute recoveries of 10 different concentrations of irbesartan (10.0–60.0 $\mu g ml^{-1}$) and hydrochlorothiazide (4.0–20.0 $\mu g ml^{-1}$) in plasma were determined by assaying the samples as described above and comparing the peak areas of both drugs with those obtained from direct injection of the compounds dissolved in the processed blank plasma.

2.3.6. Precision and accuracy

The precision and accuracy of the assay was ascertained based on analysis of plasma quality control samples. Plasma quality control sample concentrations for irbesartan and hydrochlorothiazide were 10.0–60.0 $\mu g\ ml^{-1}$ and 4.0–20.0 $\mu g\ ml^{-1}$, respectively. Five replicate quality control samples at each concentration were analyzed on 3 consecutive days and five replicate samples were analyzed on a third day after which intra-day and inter-day means, standard deviations (SD) and coefficients of variation (C.V.) were calculated by standard methods.

2.3.7. Application to plasma sample

The assay was applied to an open-label, single-dose (150.0 mg irbesartan, 12.5 mg hydrochlorothiazide) pharmacokinetic study in three young healthy volunteers. After drug administration, blood samples for analytical determinations were collected in heparinized tubes. Within 30 min after blood collection, blood plasma was separated by centrifuging at 4000 *g* for 10 min. The sample were stored at −17 °C until analysis.

3. Results and discussion

The aim of this work, a new, simpler, more accurate, reproducible and sensitive HPLC method has been developed for the simultaneous determination of irbesartan and hydrochlorothiazide in human plasma after administration of the drug. A satisfactory separation of each drug from biological endogenous components in human plasma was obtained. To find the appropriate HPLC conditions for separation of the examined drugs, various reversed-phase columns, isocratic and gradient mobile phase systems were tired. The optimum wavelength for detection was 275 nm at which much better detector responses for the two drugs were obtained. The mobile phases used were 10 m*M* potassium dihydrogen phosphate:methanol:acetonitrile (5:80:15 v/v/v) (pH 2.5). The mobile phase was found to be essential to improve the sharpness and thinness of the irbesartan and hydrochlorothiazide peak. Fig. 1a shows the chromatogram of a pure standard mixture. The retention times for the investigated drugs were found to be 5.8 min (irbesartan) and 7.8 min (hydrochlorothiazide). No internal standard was required.

The method was validated with regard to linearity, limit of detection and qualitation, recovery, precision, accuracy and specificity.

Peak areas of irbesartan and hydrochlorothiazide of calibration standards were proportional to the concentration of irbesartan and hydrochlorothiazide in plasma over the range tested 10.0–60.0 $\mu g\ ml^{-1}$ and 4.0–20.0 $\mu g\ ml^{-1}$, respectively. Each concentration was tested in triplicate. The results of the linearity of the method are presented in Table 1. The calibration curves were fitted by linear least-square

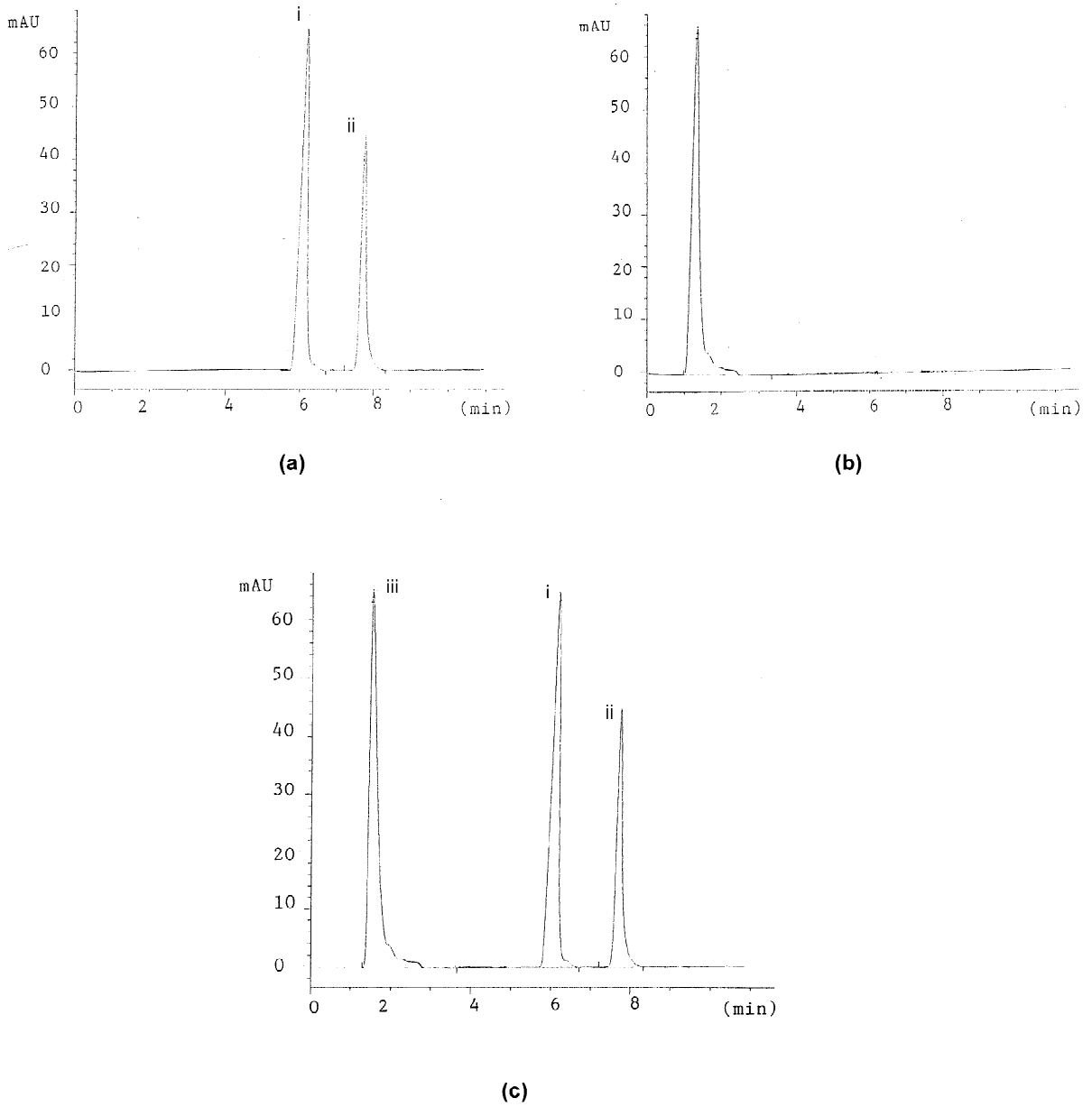


Fig. 1. (a) HPLC chromatogram of a 20 μl injection containing (i) 15.0 μg ml^{-1} of irbesartan; (ii) 10.5 μg ml^{-1} of hydrochlorothiazide. (b) HPLC chromatogram of drug-free plasma. (c) Plasma sample obtained from a volunteer 6 h after a single oral dose of 150.0 mg irbesartan, 12.5 mg hydrochlorothiazide containing; (i) irbesartan; (ii) hydrochlorothiazide; (iii) human serum.

regression and showed coefficients of determination greater than 0.9996.

Individual specificity, in relation to endogenous plasma components, was demonstrated by analysis of a series of randomly selected drug-free plasma samples. Typical chromatograms obtained from a drug-free plasma and a plasma sample obtained from a volunteer 6 h after a single oral dose of 150.0 mg irbesartan, 12.5 mg hydrochlorothiazide are presented in Fig. 1b and c. The retention times for the

Table 1
Statistical analysis of calibration graphs in the determination of irbesartan and hydrochlorothiazide by using high-performance liquid chromatography

Parameters	Irbesartan	Hydrochlorothiazide
Range (μg ml^{-1})	10.0–60.0	4.0–20.0
Detection limits (μg ml^{-1})	0.98	0.43
Regression equation (Y)[a]		
Slope (b)	2.67×10^{-3}	3.95×10^{-3}
SD on slope (S_b)	1.61×10^{-4}	1.15×10^{-4}
Intercept (a)	1.20×10^{-3}	7.91×10^{-3}
SD on intercept (S_a)	6.63×10^{-4}	5.41×10^{-4}
SE of estimation (S_e)	2.47×10^{-3}	3.65×10^{-5}
Correlation coefficient (r)	0.9999	0.9996
RSD (%)[b]	1.35	0.99
% Range of error[b]		
(95% Confidence limit)	1.10	0.95

[a] $Y = a + bC$ where C is concentration in μg ml^{-1} and Y in absorbance units.
[b] Five replicate samples.

investigated drugs were found to be 5.8 min (irbesartan) and 7.8 min (hydrochlorothiazide). No endogenous plasma components elute at the retention time irbesartan and hydrochlorothiazide. Besides, it can be seen from the chromatogram that in plasma, irbesartan and hydrochlorothiazide are separated as two peak without their metabolites.

Other antihypertensive drugs (valsartan, lisinopril, losartan and hydroflumethiazide) are extracted by this procedure. Nevertheless, these compounds elute at retention times different from those of irbesartan and hydrochlorothiazide and therefore do not interfere with the analysis. Potentially co-administered antihypertensive drugs tested were detected at different retention times (valsartan 3.1 min, lisinopril 9.1 min, losartan 3.5 min and hydroflumethiazide 9.0 min).

The limit of detection (LOD) and the limit of quantification (LOQ) of irbesartan and hydrochlorothiazide were calculated on the peak area using the following equations:

$$\mathrm{LOD} = 3 \times N/B \qquad \mathrm{LOQ} = 10 \times N/B$$

where N, the noise estimate, is the SD of the peak areas (three injections) of the drugs, B is the slope of the corresponding calibration curve. The limit of quantification and the limit of detection of irbesartan and hydrochlorothiazide were found to be 2.98 μg ml^{-1} and 1.86 μg ml^{-1} and 0.98 μg ml^{-1} and 0.43 μg ml^{-1}, respectively.

The absolute recovery was calculated by comparing the areas under the peaks obtained from standard working solutions with the peak-areas from standard samples (Table 2). The recoveries of irbesartan and hydrochlorothiazide were 98.2±1.14 and 99.8±2.5 in plasma, respectively.

Intra-assay precision of the method is illustrated in Table 3. It was estimated by assaying the quality control samples five times in the same analytical run. The precision was better than 3.5% and accuracy did not exceed 7% at all levels.

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (five levels analyzed three times, results averaged for statistical evaluation) in the same analytical runs (Table 4). The samples were prepared in advance and stored at +5 °C. The precision was better than 3.2% and accuracy did not exceed 7% at all levels.

Slight variations in retention times were observed using mobile phases prepared on different days. The column-to-column reproducibility was evaluated injecting the samples on two columns from different manufacturers and containing the same brand of packing material. The elution order and the res-

Table 2
Recovery of irbesartan (IRB) and hydrochlorothiazide (HYD) in spiked human plasma

Amount added (μg ml^{-1})		Amount found (μg ml^{-1})		Recovery (%)	
IRB	HYD	IRB	HYD	IRB	HYD
150.0	7.5	145.6		97.1	
150.0	10.0	149.8		99.9	
150.0	12.5	147.4		98.2	
150.0	15.0	148.9		99.2	
150.0	17.5	145.5		97.0	
100.0	12.5		12.3		98.4
125.0	12.5		12.3		98.4
175.0	12.5		12.9		103.2
200.0	12.5		12.8		102.4
225.0	12.5		12.1		96.8
	$\bar{X}$ (%)		RSD (%)		
IRB	98.2		1.14		
HYD	99.8		2.50		

Table 3
Intra-assay precision for the determination of irbesartan and hydrochlorothiazide

n	Irbesartan				Hydrochlorothiazide			
	Added	Measured	RSD (%)	Bias (%)	Added	Measured	RSD(%)	Bias(%)
5	100.0	97.8	1.7	2.5	15.0	14.8	3.5	3.5
5	150.0	149.7	0.4	3.8	12.5	12.6	1.1	5.3
5	200.0	199.5	2.5	1.4	10.0	9.9	0.9	4.2
5	100.0	99.9	1.9	0.9	10.0	10.1	0.7	1.8
5	200.0	200.8	0.9	2.9	15.0	15.4	1.9	2.9

olution of compounds were not affected and only slight variations in retention times were observed.

The stability of irbesartan and hydrochlorothiazide in solution containing the mobile phase was determined for the samples stored at +5 °C. The samples were checked after 3 successive days of storage and the data were compared with freshly prepared samples. In each case the RSD values of assays were found to be below 2.0% RSD. This indicates that the irbesartan and hydrochlorothiazide are stable in the solutions for at least 3 days.

The stability of irbesartan and hydrochlorothiazide in plasma was determined by periodic analysis of spiked samples at +5 °C. The results indicated that no degradation occurred. In addition, select clinical plasma samples, which were assayed repeatedly for 3 successive days, showed good stability of irbesartan and hydrochlorothiazide at −17 °C. Irbesartan and hydrochlorothiazide were also shown to be stable in plasma for at least 3 days. Furthermore, irbesartan and hydrochlorothiazide were stable through at least two freeze–thaw cycles.

The proposed method was applied to the determination of irbesartan and hydrochlorothiazide in plasma samples from the bioequivalence study. Plasma samples were periodically collected up to 24 h after oral administration of one tablet to three young man healthy volunteers. Fig. 2 illustrates the mean±SEM plasma concentration time profile of

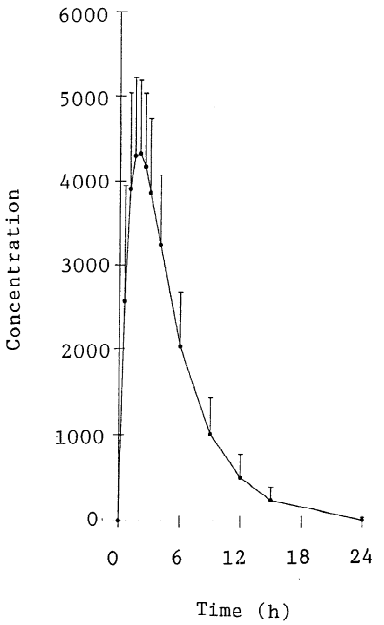


Fig. 2. Mean plasma concentration of irbesartan and hydrochlorothiazide in binary mixture after single oral dose (150.0 mg irbesartan, 12.5 mg hydrochlorothiazide).

Table 4
Inter-assay precision and accuracy for the determination of irbesartan and hydrochlorothiazide

n	Irbesartan				Hydrochlorothiazide			
	Added	Measured	RSD (%)	Bias (%)	Added	Measured	RSD (%)	Bias (%)
5	100.0	99.9	0.9	1.5	15.0	15.3	2.1	1.5
5	150.0	149.9	1.2	0.8	12.5	12.1	0.6	3.3
5	200.0	200.5	2.0	3.9	10.0	12.9	3.9	5.2
5	100.0	98.1	1.3	3.3	10.0	9.8	2.3	4.8
5	200.0	198.1	3.2	4.9	15.0	16.4	3.4	6.2

irbesartan and hydrochlorothiazide, following an oral dose containing 150.0 mg irbesartan and 12.5 mg hydrochlorothiazide. The plasma level of the mixture reached a maximum 2.3 h after the administration.

4. Conclusion

The procedure was successfully applied to the simultaneous determination of the studied compounds in human plasma without any interference from the additives and endogenous substances. It is a simple and accurate procedure requiring inexpensive reagents that could be used for rapid and reliable clinical and pharmacokinetic studies of irbesartan and hydrochlorothiazide.

References

[1] G.E. McVeigh, J. Flack, R. Grimm, Drugs 49 (1995) 161.
[2] L. Gonzalez, R.M. Alonso, R.M. Jimenez, Chromatographia 52 (2000) 735.
[3] M.A. Ghandour, E.A. Kasým, M.T. El-Haty, Anal. Lett. 35 (2002) 239.
[4] J.A. Prieto, R.M. Alonso, R.M. Jimenez, Electrophoresis 23 (2002) 102.
[5] A.A. Al-Majed, F. Belal, A.A. Al-Warthan, Spectrosc. Lett. 34 (2001) 211.
[6] J.A. Prieto, U. Akesolo, R.M. Jimenez, J. Chromatogr. A 916 (2001) 279.
[7] K. Kargosha, A.H.M. Sarrafi, J. Pharm. Biomed. Anal. 26 (2001) 273.
[8] A. El-Gindy, A. Ashour, L. Abdel-Fattah et al., J. Pharm. Biomed. Anal. 25 (2001) 923.
[9] N. Erk, Anal. Lett. 35 (2002) 283.
[10] A. El-Gindy, A. Ashour, L. Abdel-Fattah et al., J. Pharm. Biomed. Anal. 25 (2001) 171.
[11] A. El-Gindy, A. Ashour, L. Abdel-Fattah et al., J. Pharm. Biomed. Anal. 25 (2001) 299.
[12] N. Erk, J. Pharmaceut. Biomed. Anal. 24 (2001) 603.
[13] M.L. Luis, J.M.G. Fraga, F. Jimenez et al., Talanta 53 (2001) 761.
[14] S. Saðlýk, O. Saðýrlý, S. Atmaca, L. Ersoy, Anal. Chim. Acta 427 (2001) 253.
[15] R.A.S. Lapa, J.L.F.C. Lima, J.L.M. Santos, Anal. Chim. Acta 407 (2001) 225.
[16] L. Manna, L. Valvo, S. Alimonti, Chromatographia 53 (2001) 271.
[17] O. Atay, U. Tamer, D. Arýkan, Anal. Lett. 34 (2001) 1153.
[18] E. Banoðlu, Y. Özkan, O. Atay, Farmaco 55 (2000) 477.
[19] G. Carlucci, V. Di Carlo, P. Mazzeo, Anal. Lett. 33 (2000) 2491.
[20] G. Carlucci, G. Palumbo, P. Mazzeo et al., J. Pharm. Biomed. Anal. 23 (2000) 185.
[21] A.P. Argekar, J.G. Sawant, Anal. Lett. 33 (2000) 869.
[22] F. Belal, I.A. Al-Zaagi, E.A. Gadkariem et al., J. Pharm. Biomed. Anal. 24 (2001) 335.
[23] S.T. Hassib, Z.A. El-Sherif, R.I. El-Bagary et al., Anal. Lett. 33 (2000) 3225.