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Short communication

Determination of indapamide in human serum using 96-well solid-phase extraction and high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

A sensitive and specific method using high-performance liquid chromatography (LC)–electrospray tandem mass spectrometry (ESI–MS/MS) for the determination of indapamide in human serum was developed and validated. Indapamide and an internal standard (4-diethylaminobenzoic acid) were isolated from serum samples by solid-phase extraction (SPE) with Oasis®HLB 96-well plates and determined by LC–MS/MS in multiple reaction monitoring (MRM) mode. The calibration curve of serum indapamide was linear in the range of 0.2–20 ng/ml with a correlation coefficient of 0.9999. The repeatability, intermediate precisions, and accuracies at 0.2, 5, and 20 ng/ml in serum were less than 15%. The absolute recoveries of indapamide and the internal standard were 79.4–81.5% and 87.5%, respectively, and the low limit of quantitation of serum indapamide was 0.2 ng/mL. The analytical method was applied to a bioequivalence study of KYD-041 (1 mg as film-coated tablets, test formulations) and Natrix®Tab.1 (1 mg as sugar-coated tablets, reference formulation). The 90% confidence interval of the ratios (test formulation/reference formulation) for $\log(C_{max})$ and $\log(AUC_t)$ were in the range log(0.80)–log(1.25), which supports the conclusion that KYD-041 is bioequivalent to Natrix®Tab.1 with respect to the rate and extent of indapamide absorption.

1. Introduction

Indapamide, 3-(aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-l-yl)benzamide, is a non-thiazide antihypertensive/diuretic agent which contains sulfamoyl chlorobenzamide and methylindoline groups. Indapamide possesses a mild and sustained antihypertensive effect when administered orally at low doses. It differs from other antihypertensive/diuretic agents in that it has a limited diuretic and vasodilatory effect.

In recent years, it has been suggested the administration of low doses of the diuretic agents, therefore, we developed the film-coated and scored tablets of indapamide. Then it was needed to confirm the bioequivalence of the developed formulations to the existing formulations.

There are several reports concerning the determination of indapamide in biological fluids by high-performance liquid chromatography (HPLC) using ultraviolet (UV) absorption detectors or electrochemical detectors (ECD) that limit quantification to 10–75 ng/ml [1–4] and 2 ng/ml [5], respectively.

Recently, there have been many reports of the use of LC–MS for the determination of drugs in biological fluids. LC–MS methods for the quantitation of indapamide in blood have been reported frequently in recent years [6–10]. The LC–MS method has high specificity and sensitivity, since the limit of quantification of indapamide was found to be 0.5–2 ng/ml in these reports. However, liquid–liquid extraction method was employed in many previous studies, required several steps for the sample preparation.

It is necessary that the limit of indapamide quantification is less than 0.5 ng/ml in order to determine sample indapamide concentrations for bioequivalence studies of 1 mg tablet formulations. We must also bear in mind the need for a simple procedure to analyze a large number of serum samples in a short period of time. To improve the sensitivity of LC–MS, sample clean-up is an effective tool to eliminate endogenous interference and prevent ion suppression. Therefore, we employed solid-phase extraction for sample preparation. In addition, solid-phase extraction enables simple and rapid sample preparation. In this study, we employed 96-well solid-phase extraction plates to obtain high sample throughput.

We have developed a rapid, simple, specific, and sensitive method for the determination of serum indapamide employing solid-phase extraction coupled to high-performance liquid chromatography with tandem mass spectrometry detection (LC–MS/MS). We also report the validation for the method and

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application to a bioequivalence study of two formulations of indapamide.

2. Experimental

2.1. Chemicals and reagents

Indapamide reference standard was supplied by Inabata & Co., Ltd. (Osaka, Japan). Internal standard 4-diethylaminobenzoic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Analytical grade ammonium acetate and phosphoric acid were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). HPLC grade formic acid was obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). HPLC grade methanol was obtained from Kishida Chemical Co., Ltd. (Osaka, Japan). HPLC grade acetonitrile was obtained from Merck (Dermstadt Germany). Water was distilled using an autostill WG220 distillation system (Yamato Scientific Co., Ltd (Tokyo, Japan)).

2.2. Instrumentation

HPLC was performed on a Waters 2795 separation module including an autosampler and solvent delivery manager. MS was performed on a Waters Micromass® Quattro micro API™ tandem quadrupole mass spectrometer. The parameters of LC–MS system were controlled by MassLynx™ 4.0 software with QuanLynx™ Application Manager.

Solid-phase extraction was performed on a Waters 96-Well plate manifold.

2.3. HPLC conditions

The HPLC separation was performed on a Waters Atlantis® dC_{18} column (5 μm, 2.1 mm × 100 mm) at 40 °C. A flow rate of 0.2 ml/min was employed. The mobile phases were (A) to 500 ml of 0.01 mol/L ammonium acetate add 0.25 ml of formic acid and (B) acetonitrile. The analytes were eluted with an isocratic elution using 40%(B) for 8 min, followed by a linear gradient from 40% (B) to 80% (B) over 1 min, then hold for 2 min, followed by a linear gradient from 80% (B) to 40% (B) over 1 min, then hold for 4 min before returning to initial conditions.

2.4. MS conditions

The mass spectrometer, equipped with an electrospray ionization (ESI) source, was run in positive ion mode (ES+) and set up in multiple reaction monitoring (MRM) mode. For MRM data collection during the LC experiments, the capillary voltage was 1.5 kV, the source temperature was 120 °C, the desolvation temperature was 400 °C, the desolvation gas flow was 800 L/h, and the cone gas flow was 50 L/h.

The MRM transitions of precursors to product ions were as follows: m/z 366.0 → 132.0 for indapamide and m/z194.1 → 150.0 for 4-diethylaminobenzoic acid.

The cone voltage was 20 V and the collision energy was 15 eV for both compounds. The inter-scan delay and the inter-channel delay were both 50 ms. The dwell time for each MRM channel was 0.2 s.

2.5. Preparation of standard solutions

A stock solution of 200 μg/ml indapamide was prepared by dissolving 20 mg of indapamide reference standard in methanol. The stock solution was diluted with water to get an intermediate concentration of 5 μg/ml. Working solutions of indapamide (2, 5, 10, 20, 50, 100 and 200 ng/ml) were prepared by further dilution with appropriate volumes of water.

An internal standard (I.S.) stock solution of 800 μg/ml was prepared by dissolving 40 mg of 4-diethylamino benzoic acid in methanol. The I.S. stock solution was diluted with water to get an intermediate concentration 40 μg/ml for I.S. A working solution of 4 μg/ml of I.S. was prepared by further dilution with an appropriate volume of water.

2.6. Sample preparation

Solid-phase extraction using Oasis®HLB 96-Well plates (10 mg, Waters) was performed on a vacuum manifold. 0.5 ml of serum sample or 0.45 ml of serum blank spiked with 50 μl of the reference standard of indapamide (calibration and QC samples) were transferred into a centrifuge tube, and 25 μl I.S. working solution and 10 μl phosphoric acid then added. The 96-well plates were conditioned with 1 ml methanol, followed by 1 ml water. Each serum sample was vortex-mixed and applied to a 96-well plate. After washing with 1 ml of 5% methanol in water, elution was performed with 0.3 ml of methanol and then with an additional 0.2 ml of methanol.

2.7. Method validation

For the specificity test, three blank samples were tested for interference using the proposed method. The results were compared with those obtained from an aqueous solution of indapamide and the I.S.

The calibration curves (consisting of seven points) were determined by least-squares linear regression analysis. The linearity of the method was confirmed by comparing the slopes, the intercepts and the correlation coefficients. Moreover, the residual errors from the regression lines were also determined.

The precision and accuracy were determined over 6 days by analyzing six spiked samples of indapamide at three concentration levels. The precision was expressed as the relative standard deviation (RSD, %), and the accuracy was measured according to the following equation: Accuracy (%) = (measured concentration − nominal concentration)/nominal concentration × 100.

The limit of quantification (LLOQ) was the lowest concentration of the analyte measured with acceptable accuracy and precision (<20%).

2.8. Stability

Indapamide stability in serum (0.5 and 20 ng/ml) was assessed by long-term (−20 °C, 3 months), short-term (room temperature, 24 h), and freeze-thaw (three cycles) stability tests.

Standard solutions, I.S. solutions, and stock solutions of indapamide and I.S. were also tested for stability at room temperature for 14 days or 5 °C for 4 weeks.

Moreover, the stability of sample solutions on an autosampler at 20 °C for 24 h was studied.

2.9. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of indapamide: KYD-041 (1 mg as film-coated tablets, test formulations from Kyoto Pharmaceutical Industries, Ltd.) and Natrix®Tab.1 (1 mg as sugar-coated tablets, reference formulations from Kyoto Pharmaceutical Industries, Ltd./Nihon Servier Co., Ltd./Dainippon Sumitomo Pharma Co., Ltd.). The study consisted of an open, single-dose, two-period crossover protocol in 10 healthy men. Each volunteer was orally administered one tablet

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

Compound	Concentration	Recovery
Indapamide	1	81.5 ± 1.2 (n = 3)
	5	80.7 ± 0.9 (n = 3)
	20	79.4 ± 1.8 (n = 3)
Internal standard	200	87.5 ± 1.4 (n = 9)

(1 mg) of indapamide as a reference or test formulation. Venous blood samples (5 ml) were collected before and 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24, 36 and 48 h after dosing. After 1 h at room temperature, serum was separated by centrifugation (3000 r.p.m. at 4 °C) for 10 min, and was stored at −20 °C until assayed for indapamide.

The geometric means and 90% confidence interval (CI) of ratio (test formulation/reference formulation) of the log-transformed values of AUC_t and C_{max} were used for bioequivalence assessment following previously described guidelines. SAS® (Release 8.2 for Windows) software was used for calculation of pharmacokinetics parameters.

3. Results and discussion

3.1. HPLC conditions

Reverse phase liquid chromatography with C18 columns have been mainly used to determine indapamide in biological fluids. Among the available brands of columns, the Atlantis dC_{18} column was chosen for this study because it provided a stronger signal for indapamide. The mobile phase was selected for this assay so as to provide a short retention time and high sensitivity. The addition of ammonium acetate to the mobile phase provided an increase in sensitivity, as it favored the protonation of the indapamide molecules. Acetonitrile was selected as an organic modifier since acetonitrile gave a better peak shape for indapamide and the back pressure was lower than that for methanol. The addition of formic acid to the mobile phase resulted in delayed elution of the I.S. (4-diethylamino benzoic acid) and also prevented ion suppression from endogenous serum components. After detection of the analyte peak, linear gradient elution was employed with increasing amounts of acetonitrile in order to elute endogenous serum components earlier.

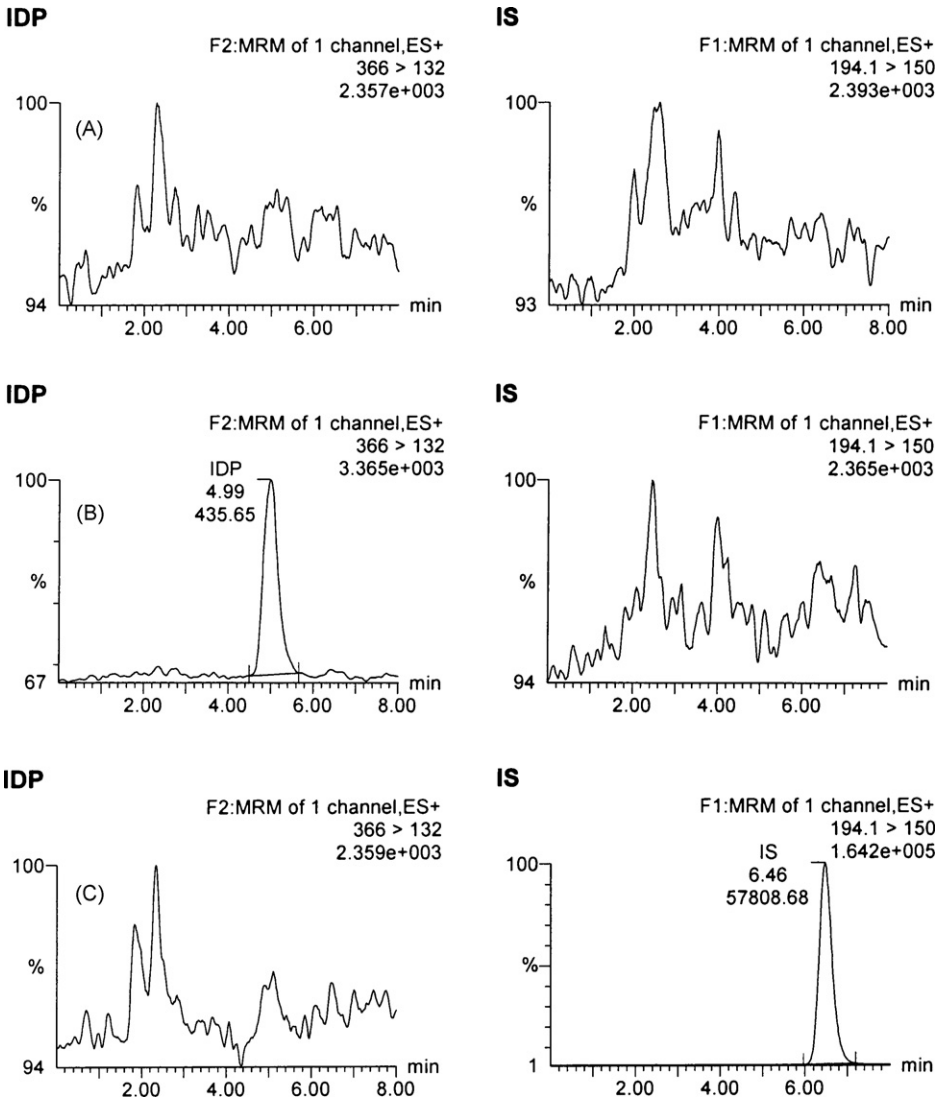

Fig. 1. MRM chromatograms for indapamide and internal standard (A) blank serum, (B) blank serum spiked with indapamide (0.2 ng/ml) and (C) blank serum spiked with internal standard.

3.2. MS conditions

Indapamide is a weak acid, therefore, we selected electrospray ionization for the study to obtain high sensitivity. Flow-injection analysis of standard solution found that the positive mode gave a stronger signal for indapamide than the negative mode. Full-scan mass spectra of indapamide and 4-diethylamino benzoic acid (I.S.) showed the protonated molecules ($[M+H]^+$) at m/z 366.0 and 194.1, respectively. Fragmentation of the protonated $[M+H]^+$ precursors found that the most abundant ion in the full-scan MS/MS spectrum of indapamide was at m/z 132.0, and for 4-diethylamino benzoic acid (I.S.) it was at 150.0.

In order to optimize the MS parameters, a solution of indamapide was injected into the LC–MS spectrometer and the peak area was measured.

3.3. Sample preparation

As indapamide and 4-diethylamino benzoic acid are acidic compounds, reversed-phase or ion-exchange modes are suitable for solid-phase extraction. Indapamide binds strongly to matrix proteins, so to break these interactions, it is necessary to acidify samples through the addition of phosphoric acid. In ion-exchange mode, however, the binding force to acidic compounds is decreased. We therefore selected the Oasis® HLB sorbent, which is a copolymer of hydrophilic *N*-vinylpyrroridone and lipophilic divinylbenzene and provides higher retention capacity than silica-based reversed-phase sorbents. Solid-phase extraction was performed according to generic procedure [11].

The extraction yield (absolute recovery) was determined for three levels of spiked serum samples (1, 5, 20 ng/ml of indapamide and 200 ng/ml of I.S.). The mean recovery was 79.4–81.5% for indapamide and 87.5% for I.S. as shown in Table 1. No significant difference was observed between the levels of indapamide in the samples, and the consistency in recovery of indapamide and I.S. was ensured. The recovery was higher when the elution was carried out in two steps (0.3 ml and 0.2 ml) than one step (0.5 ml).

3.4. Method validation

3.4.1. Separation and specificity

The specificity of the method was measured in three different batches of blank serum.

As shown in Fig. 1, no interfering peaks in the retention times of indapamide and I.S. were observed in blank serum. Additionally, no interfering peaks in the retention times of the compound of interest were observed in serum spiked with indapamide or I.S.

3.4.2. Linearity

Calibration curves were drawn on three different days by analyzing the spiked samples of indapamide (0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml). The calibration curves were linear with a correlation coefficient of 0.9999 and the residual errors from the regression lines were from −7.6% to 6.5% as calculated from the simple linear equation using a weighting factor ($1/y$).

Table 2
Results of assay accuracy and precision for indapamide

Concentration added (ng/ml)			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0.2	Assay	1	0.1742	0.1875	0.2009	0.2115	0.2342	0.1940
	(ng/ml)	2	0.1712	0.1819	0.1887	0.2080	0.2290	0.1957
		3	0.1765	0.1781	0.1806	0.2261	0.2264	0.1967
		4	0.1737	0.1724	0.2019	0.2036	0.2284	0.1759
		5	0.1698	0.1692	0.2036	0.2075	0.2307	0.1892
		6	0.1878	0.1784	0.1946	0.2170	0.2161	0.2009
	Mean (ng/ml)		0.1755	0.1779	0.1951	0.2123	0.2275	0.1921
	S.D. (ng/ml)		0.0065	0.0065	0.0090	0.0081	0.0062	0.0088
	Accuracy (%)		−12.6	−11.3	−2.8	5.5	13.4	−4.0
			Estimate of precision			90% Confidence intervals		
	Repeatability		$s_r = 0.0076$		(3.8%)	$0.0063 \leq \sigma_r \leq 0.0097$		
	Intermediate precision		$s_{Rw} = 0.0212$		(10.6%)	$0.0147 \leq \sigma_{Rw} \leq 0.0407$		
5	Assay	1	4.863	4.861	5.148	5.185	4.977	5.155
	(ng/ml)	2	4.931	4.729	5.112	5.164	5.091	5.080
		3	4.798	4.763	5.070	5.018	5.117	5.212
		4	4.881	4.758	5.169	5.211	5.279	5.237
		5	4.780	4.587	5.127	5.160	5.161	5.142
		6	4.823	4.667	5.080	5.228	5.109	5.177
	Mean (ng/ml)		4.846	4.728	5.118	5.161	5.122	5.167
	S.D. (ng/ml)		0.056	0.093	0.038	0.075	0.098	0.055
	Accuracy (%)		−3.5	−5.7	2.0	2.5	2.1	3.2
			Estimate of precision			90% Confidence intervals		
	Repeatability		$s_r = 0.073$		(1.5%)	$0.060 \leq \sigma_r \leq 0.093$		
	Intermediate precision		$s_{Rw} = 0.200$		(4.0%)	$0.138 \leq \sigma_{Rw} \leq 0.382$		
20	Assay	1	19.21	19.24	20.44	20.55	19.90	20.08
	(ng/ml)	2	19.07	19.01	20.07	20.88	19.81	19.96
		3	18.91	19.19	20.43	20.82	19.70	20.01
		4	18.78	19.39	20.22	20.56	19.40	19.93
		5	18.90	19.20	20.46	20.18	19.32	19.91
		6	18.77	19.20	20.20	20.13	19.94	19.97
	Mean (ng/ml)		18.94	19.21	20.30	20.52	19.68	19.98
	S.D. (ng/ml)		0.17	0.12	0.16	0.31	0.26	0.06
	Accuracy (%)		−5.7	−4.2	1.1	1.9	−1.9	−0.2
			Estimate of precision			90% Confidence intervals		
	Repeatability		$s_r = 0.20$		(1.0%)	$0.17 \leq \sigma_r \leq 0.25$		
	Intermediate precision		$s_{Rw} = 0.64$		(3.2%)	$0.44 \leq \sigma_{Rw} \leq 1.23$		

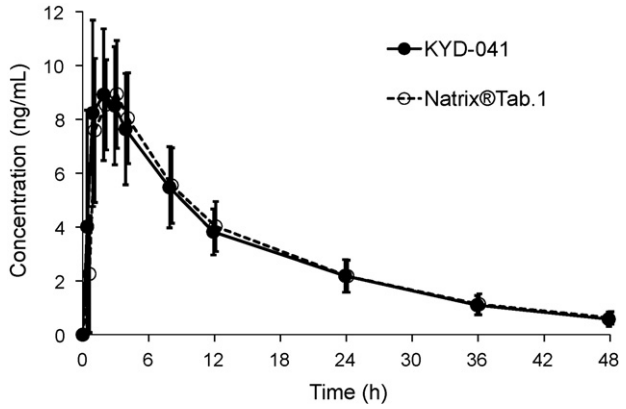

Fig. 2. Mean concentration of indapamide in serum after the administration of test formulations (KYD-041) and reference formulations (Natrix®Tab.1) to 10 healthy volunteers.

3.4.3. Precision and accuracy

Precision and accuracy were determined over 6 days by analyzing six spiked samples of indapamide at low, middle, and high concentration levels (0.2, 5, and 20 ng/ml).

Assay data for each concentration level, estimates of precision and 90% confidence intervals for repeatability or intermediate precision are presented in Table 2.

The results satisfactorily met the acceptance criteria: mean accuracies were less than ±15% and estimates of precision for repeatability and intermediate precisions were less than 15%.

3.4.4. Range and limit of quantification

From the results of the studies on linearity, accuracy and precision, the range was defined as the lowest and highest concentration (0.2–20 ng/ml) in the calibration curves that met the acceptance criteria, and the limit of detection was defined as the lowest concentration (0.2 ng/ml) as described above.

3.4.5. Stability

The stability of indapamide in serum was investigated using spiked samples at low and high concentration levels (0.2 and 20 ng/ml).

Indapamide at each concentration level was stable at room temperature for 24 h and at −20 °C for 3 months. And after three freeze (−20 °C) and thaw (room temperature) cycles indapamide in serum was stable.

Stock solution of indapamide and I.S. were stable for 4 weeks at 5 °C. Standard solution and I.S. solution were stable at least 14 days at room temperature.

Sample solutions extracted from spiked serum were stable on the autosampler at 20 °C for 24 h.

3.4.6. Bioequivalence study

Fig. 2shows the content mean serum concentration-time profiles of 10 volunteers for test formulations (KYD-041) and reference formulations (Natrix®Tab.1), and the pharmacokinetic parameters derived from these profiles are presented in Table 3. There were no significant differences between the two formulations except for $t_{1/2}$, MRT and kel between time periods.

Table 3
Pharmacokinetic parameters of indapamide in serum samples

Parameter	Indapamide formulations	
	KYD-041	Natrix®Tab1
C_{max} (ng/ml)	9.87 ± 2.24	9.85 ± 1.96
AUC_t (ng h/ml)	140.1 ± 33.6	142.3 ± 31.2
AUC_∞ (ng h/ml)	152.2 ± 36.4	154.6 ± 35.4
t_{max} (h)	1.7 ± 0.9	2.3 ± 1.1
$t_{1/2}$ (h)	13.20 ± 2.13	13.00 ± 1.74
MRT (h)	13.80 ± 1.51	13.87 ± 1.02
kel (/h)	0.0536 ± 0.0084	0.0541 ± 0.0068

The 90% confidence interval of difference in the average value of logarithmic AUC_t and C_{max} between test and reference formulations was within the acceptable range of log(0.8)–log(1.25).

The test formulations (KYD-041) and reference formulations (Natrix®Tab.1) are therefore considered to be bioequivalent.

4. Conclusion

A method using high-performance liquid chromatography (LC)–electrospray tandem mass spectrometry (ESI–MS/MS) for the determination of indapamide in human serum employing solid-phase extraction with Oasis®HLB96-Well plate was developed. The method is rapid, simple, specific, and sensitive and additionally demonstrates good accuracy and precision. The limit of quantitation of indapamide was 0.2 ng/ml in serum. We believe that this method could provide a useful tool for the determination of indapamide in serum.

We applied the method to a bioequivalence study of two formulations of indapamide and demonstrated that test formulations were bioequivalent to reference formulations.

References

[1] R.L. Choi, M. Rosenberg, P.E. Grebow, J. Chromatogr. 230 (1982) 181.
[2] E. Gaetani, C.F. Laureri, M. Vitto, L. Borghi, G.F. Elia, A. Novarini, Boll. Chim. Farm. 125 (1986) 35.
[3] D. Zendelovska, T. Stafilov, M. Stefova, J. Chromatogr. B 788 (2003) 199.
[4] T.J. Hang, W. Zhao, J. Liu, M. Song, Y. Xie, Z. Zhang, J. Shen, J. Pharm. Biomed. Anal. 40 (2006) 202.
[5] P. Schiavi, R. Jochemsen, D. Guez, Fundam. Clin. Pharmacol. 14 (2000) 139.
[6] J. Tang, J. Li, J. Sun, J. Yin, Z. He, Pharmazie 60 (2005) 819.
[7] F. Albu, C. Georgita, V. David, A. Medvedovici, J. Chromatogr. B 816 (2005) 35.
[8] D.S. Jain, G. Subbaiah, M. Sanyal, U.C. Pande, P. Shrivastav, J. Chromatogr. B 834 (2006) 149.
[9] W.D. Chen, Y. Ling, H. Zhang, H. Li, Y. Xiong, G.J. Wang, L. Xie, J. Chromatogr. B 842 (2006) 58.
[10] L. Ding, L. Yang, F. Liu, W. Ju, N. Xiong, J. Pharm. Biomed. Anal. 42 (2006) 213.
[11] Oasis®HLB Extraction plate Instruction sheet (Waters).