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Short communication

Simple, sensitive and rapid LC–MS method for the quantitation of indapamide in human plasma—application to pharmacokinetic studies

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Abstract

A sensitive and specific method using liquid chromatography with electrospray ionization mass spectrometry (LC–ESI–MS) has been developed and validated for the identification and quantification of indapamide in human plasma. A simple liquid–liquid extraction procedure was followed by injection of the extracts on to a C_{18} column with gradient elution and detection using a single quadrupole mass spectrometer in selected ion monitoring (SIM) mode. The method was tested using six different plasma batches. Linearity was established for the concentration range 0.5–100.0 ng/ml, with a coefficient of determination (r) of 0.9998 and good back-calculated accuracy and precision. The intra- and inter-day precision (RSD%) was lower than 10%, and accuracy ranged from 85 to 115%. The lower limit of quantification was reproducible at 0.2 ng/ml with 0.2 ml plasma. The proposed method enables the unambiguous identification and quantification of indapamide for pre-clinical and clinical studies.
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Keywords: Indapamide; HPLC–ESI–MS; Human plasma; Pharmacokinetics

1. Introduction

Indapamide, 3-(aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1H-indol-1-yl)benzamide, is an antihypertensive agent administered to individuals with mild to moderate hypertension. The diuretic and natriuretic effects are mainly due to the structure of *O*-chlorobenzenesulfonamide, a molecule present in various diuretics. However, a varied side chain gives the drug characteristic properties. Indapamide presents an indolinyl ring and uniquely exhibits free-radical scavenging activity as well as a direct vasodilator action [1–3].

Although the assay of indapamide in pharmaceutical formulations and determination of the related substances profile is frequently referred in literature, using spectrometric and chromatographic [4–7] methods, only very few HPLC/UV methods were dedicated to its determination in biological fluids [8–10].

Isolation of indapamide from plasma is a tedious task, because of low concentration levels and matrix induced interferences. More often, liquid–liquid extraction procedures require two or three successive steps (extraction, backextraction, and re-extraction) [8,9] to eliminate matrix interferences. Moreover, ethyl acetate and diethyl ether were used as extracting phases. Solid phase extraction (SPE) was successfully used to isolate indapamide from plasma samples resulting after 5 mg active substance intake as an immediate release product [10,11]. However, recovery of indapamide strongly depends on the type of the hydrophobic adsorbent, variations from 30 to 100% being observed. Furthermore, costs related to SPE automation as well as cartridge consumption during complete BA/BE studies should be also considered somewhat limitative (note that the multiple use of a SPE cartridge is not recommended and for a study completion, including validation purposes, around 2000 samples are run). It is worthwhile to note that UV detection can be considered as not selective and sensitive enough to assay indapamide in biological fluids. Recently, a SPE/HPLC/(AP-ESI) MS2 method has been proposed for the assay of 35 diuretics (including indapamide) in urine [12]. In many cases, highly

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specific mass spectrometric detection, especially using tandem mass spectrometry (MS–MS) just requires minimum separation on column [13], but it is too expensive to use for the routine measurements in the pre-clinical laboratory.

Positive ion monitoring may bring higher sensitivity for analysis [14], but in this trial, negative ion monitoring was adopt during analysis for the less endogenous interferences, lower signal noise and to make more easier for separation. Chlorpropamide was adopted as internal standard because of its similarity of retention, ionization with the indapamide and the less endogenous interferences at chlorpropamide $[M\text{-H}]^-$, *m*/*z* 275.0.

Therefore, our aim was to combine high separation capabilities of liquid chromatography with the selective/sensitive characteristics of mass spectrometry for determination of indapamide in plasma samples at 0.5 ng/ml level. Isolation of indapamide was achieved by a single step liquid–liquid extraction in diethyl ether, followed by solvent evaporation, re-dissolution of the residue and injection onto the chromatographic column. The analytical procedure was fully validated and successfully used to assess bioequivalence of two marketed pharmaceutical formulations of 2.5 mg indapamide.

This paper describes the development and validation of a highly sensitive LC–MS method for the quantitation of indapamide in human plasma and shown the main pharmacokinetic parameters.

2. Experimental

2.1. Chemicals and reagents

Indapamide reference standard (99.7% purity) and chlorpropamide reference standard (99.3% purity) were identified and supplied by National Institute for the Control of Pharmaceutical and Biological Produces (Beijing, China); methanol was chromatographic pure grade and purchased from Merck (Merck Company, Germany). Other chemicals were all of analytical grade. Deionized water was distilled before using. Other reagents were used as received.

2.2. Instrumentation and operating conditions

Liquid chromatography was performed using a Shimadzu LC-10AD HPLC system equipped with an autosampler (SIL-HTc). The HPLC was coupled to a Shimadzu LCMS-2010A quadrupole mass spectrometer with an electrospray ionization (ESI) interface. Data acquisition and processing were accomplished using Shimadzu LCMS solution Software for the LCMS-2010A system. Chromatographic separation was achieved on a Shim-pack stainless-steel column (C_{18}, 5 μM, 150 mm × 2.0 mm I.D., Shimadzu) at 40 °C. Mobile phase A consisted of 1 μM ammonium acetate and 0.4 μM triethylamine in water; mobile phase B was methanol. Each mobile phase was filtered through a 0.25 μM membrane and degassed under reduced pressure. Linear gradient elution was employed with a 7.5 min run time and its sequence was as follows: A–B (45:55) held for 1.5 min after injection, 20:80 at 3.0 min and held up to 4.0 min, and thereafter 45:55. Analyses were conducted at a flow rate of 0.2 ml/min. The ESI source was set at negative ionization mode. The $[M\text{-H}]^-$ ions of indapamide (*m*/*z* 364) and of chlorpropamide (*m*/*z* 275) for were selected as ions for selected ion monitoring (SIM) detection, respectively. The quantification was performed using peak areas. The MS operating conditions were optimized as follows: drying gas 1.5 l/min, CDL temperature 250 °C, block temperature 200 °C, probe voltage +4.0 kV.

2.3. Preparation of stock solutions

The stock solutions of indapamide and chlorpropamide (IS) were prepared and the concentrations calculated after correcting for purity. The primary stock solutions of indapamide was prepared by dissolving 10.0 mg of indapamide in 10 ml methanol to produce a concentration of 1.0 mg/ml and was stored at 4 °C. The internal standard stock solution was prepared by dissolving 10.0 mg of chlorpropamide in 10 ml methanol to produce concentration of 1.0 mg/ml, and was also stored at 4 °C. This solution was further diluted with methanol to prepare the internal standard working solution containing 0.2 μg/ml of chlorpropamide. Working solutions of indapamide at 10 ng/ml, 20 ng/ml, 0.2 μg/ml and 2.0 μg/ml were prepared daily in methanol by appropriate dilutions of the stock solution.

2.4. Calibration curves

Typical calibration curves were constructed with six blank plasma samples spiked with appropriate amounts of the standard solutions. The calibration range was 0.5–100.0 ng/ml of indapamide for plasma. Each sample also contained 4.0 ng (20 μl × 0.2 μg/ml) of the internal standard. In each calibration run a plasma blank sample (no IS) was also analyzed. Calibration curves were prepared by determining the best-fit of peak area ratios R (peak area of analyte/peak area of internal standard) versus concentration (x), and fitted to the equation $R = bx + a$ by unweighted least-squares regression.

2.5. Preparation of quality control samples

Quality control samples were prepared at three different concentration levels, low limit (1.0 ng/ml), middle level (10.0 ng/ml) and a high level (100.0 ng/ml). QC samples were prepared daily by spiking 0.2 ml plasma samples with appropriate volumes of a standard solution to produce the stated final concentrations of indapamide and 20.0 ng/ml (20 μl × 0.2 μg/ml) of internal standard. The extraction and analytical procedures were those described below.

2.6. Extraction procedure

QC samples, calibration standards, and clinical plasma samples, were extracted employing a liquid–liquid extraction technique. To each tube containing 0.2 ml plasma, 20.0 ng/ml (20 μl × 0.2 μg/ml) of internal standard, 100 μl of 0.1 M sodium hydroxide solution and 5 ml diethyl ether were added, and then

were vortexed for 2 min. Afterwards, samples were centrifuged for 10 min at 40000 × *g*. The organic layer was removed and evaporated under a stream of nitrogen at 40 °C. The residue was re-dissolved in 200 μl methanol. An aliquot of 5 μl was injected into the LC–MS system.

2.7. Method validation

The method validation assays were performed according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guide. The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in the SIM channels using the proposed extraction procedure and chromatographic/MS conditions, and the results were compared with those obtained for an aqueous solution of the analyte at a concentration near to the LLOQ.

The matrix effect on the ionization efficiency of analyte and IS was evaluated by comparing the peak areas of analyte and IS dissolved in blank sample extract (i.e., the final solution obtained from blank plasma after extraction and reconstitution) with those for indapamide and IS dissolved to the same concentrations in methanol. Three different concentration levels of indapamide (1.0, 10.0 and 100.0 ng/ml) with 20 ng/ml of internal standard were evaluated by analyzing five samples at each concentration level. The blank plasma used in this study was obtained as six different batches. If the peak area ratios for the plasma extracts versus clean methanol solutions were <85 or >115%, a matrix effect was implied.

Linearity was tested for the concentration range 0.5–100.0 ng/ml. For the determination of linearity, standard calibration curves containing at least nine points (non-zero standards) were used. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. For a calibration run to be accepted, three out of nine non-zero standards including the LLOQ and ULOQ were required to meet the following acceptance criteria: no more than 15% deviation at LLOQ and no more than 10% deviation for standards above the LLOQ. The acceptance criterion for the correlation coefficient was ≥0.998, otherwise the calibration curve was rejected. Five replicate analyses were performed on each calibration standard. The samples were run in order from low to high concentration.

The intra-day precision and accuracy of the assay were measured by analyzing five spiked samples of indapamide at each QC level (1.0, 10.0 and 100.0 ng/ml). The inter-day precision and accuracy was determined over three days by analyzing 15 QC samples ($n = 5$ for each concentration level) each day. The precision was within 15%, and accuracies (deviation values) were required to be within 15% of the actual values.

The extraction yield (absolute recovery) was determined by comparing the indapamide/IS peak areas obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post-extraction to the same nominal concentrations (1.0, 10.0 and 100.0 ng/m). The recovery of IS was also determined similarly.

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantification (LLOQ), and was required to meet the following criteria: LLOQ response should be 10 times that of the average noise level in the SIM chromatogram, and be identifiable, discrete and reproducible within a precision of 20%. Samples at the concentration of 0.2 ng/ml were investigated as the LLOQ, and the reproducibility and precision were determined at this concentration.

2.7.1. Short-term temperature stability

Stored spiked plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (around 6 h). These samples were then analyzed as described above.

2.7.2. Post-preparative stability

The autosampler stability was conducted by re-analyzing extracted QC samples kept under the autosampler conditions (4 °C) for 24 h.

2.7.3. Freeze–thaw stability

QC plasma samples containing indapamide were tested after three freeze (−20 °C) and thaw (room temperature) cycles. Long-term stability of indapamide in human plasma was studied for a period of 10 days employing QC samples at the three different levels. If in this stability study the analyte was found to be unstable at −20 °C it should be stored at −70 °C. The stability of the indapamide and internal standard working solutions were evaluated by testing their validity over 6 h at room temperature. This stability of working solutions was expressed as for percentage recovery.

A new calibration curve was generated to assay samples in each analytical run, and was used to calculate the concentration of indapamide in the unknown samples in that run. The calibration was obtained halfway through each run. In order to monitor the accuracy and precision of the analytical method, a number of QC samples were prepared to ensure that the method continued to perform satisfactorily. In this case the QC samples were prepared in duplicate at each of the three concentrations (1.0, 10.0 and 100.0 ng/ml), and were analyzed together with the processed test samples at intervals based on the total number of samples per batch.

3. Clinical study design

This was an open randomized, balanced, two-period crossover study in 20 Chinese healthy men. Each volunteer received in random order, single oral dose of 2.5 mg indapamide test tablets or reference tablets in cycle. Blood samples (5 ml) for assay of plasma concentration of indapamide were collected at the time of 0, 0.5, 1, 1.5, 2, 3.0, 4.0, 6.0, 8.0, 12, 24, 48 and 72 h after oral administration of the medicals. They were put into lithium heparin tubes and immediately were centrifuged at 3000 × *g* for 10 min. The plasma obtained was frozen at −20 °C in coded polypropylene tubs until analysis.

4. Results and discussion

4.1. Selection of IS

It is necessary to use an IS to obtain high accuracy when a mass spectrometer is used as the HPLC detector. Chlorpropamide was adopted as IS because of its similarity in its retention and ionization characteristics with those of the analyte, and because of the minimal endogenous interferences in the SIM channel for chlorpropamide ($[M\text{-H}]^-$ at m/z 275).

4.2. Sample preparation

Liquid–liquid extraction was advantageous because this technique not only extracted the analyte and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Ethyl acetate, trichloromethane and diethyl ether were all tested as extraction solvent, and diethyl ether was finally adopted because of its high extraction efficiency. Sodium hydroxide (0.1 ml × 0.1 M) was added to the plasma in order to accelerate the drugs' dissociation from the plasma and reduce interference since most endogenous are of acidic nature.

4.3. Separation and specificity

Negative ion electrospray mass scan spectra of indapamide and chlorpropamide are shown in Fig. 1. The major ions observed $[M\text{-H}]^-$ (m/z 364) for indapamide and $[M\text{-H}]^-$ (m/z 275) for chlorpropamide were selected for the SIM. Examples of SIM chromatograms for extracts of spiked blank plasma are shown in Fig. 2. The retention times of indapamide and the IS were 7.1 and 5.7 min, respectively.

The total HPLC–MS analysis time was 7.5 min per sample. No interferences were observed for indapamide or IS in blank plasma samples because of the high selectivity of the SIM mode, and no ionization suppression effects were found under the developed sample preparation and chromatographic conditions. The SIM chromatograms obtained for an extracted plasma sample of a healthy volunteer who participated in a pharmacokinetics study conducted on 20 persons are depicted in Fig. 3.

The purpose of the investigation was to develop a specific and sensitive procedure for the determination of indapamide used as an antihypertensive agent. Liquid chromatography with electrospray ionization mass spectrometry (HPLC–ESI–MS) has several advantages for the analysis of indapamide. The combination of HPLC with ESI–MS leads to a short run time and yields both high selectivity and sensitivity. ESI is a "gentle" ionization technique that produces high mass-to-charge $[M+\text{H}]^+$ or $[M\text{-H}]^-$precursor ions with minimal fragmentation of the analyte.

4.4. Method validation

The method exhibited a good linear response for the range of concentrations from 0.5 to 100.0 ng/ml with a coefficient of determination of 0.9998. Data for intra- and inter-day precision of the method for indapamide as determined from the QC samples runs at the concentrations of 1.0, 10.0 and 100.0 ng/ml are presented in Table 1. The lower limit of quantitation (LLOQ) for indapamide was found to be 0.50 ng/ml and the lower limit of detection (LLOD) was 0.20 ng/ml. The extraction recoveries determined for indapamide and IS were about 90% and the results were shown to be consistent, precise and reproducible.

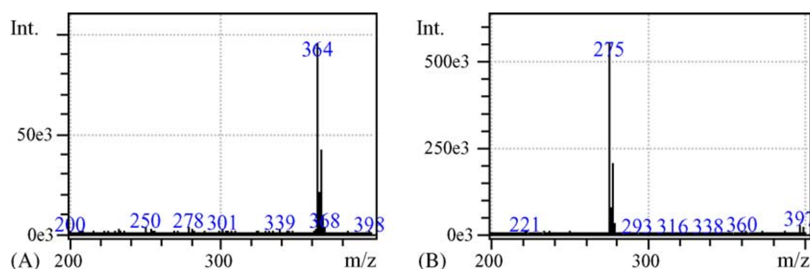

Fig. 1. Negative ion electrospray mass scan spectrum of Indapamide (A) and Chlorpropamide (B, internal standard).

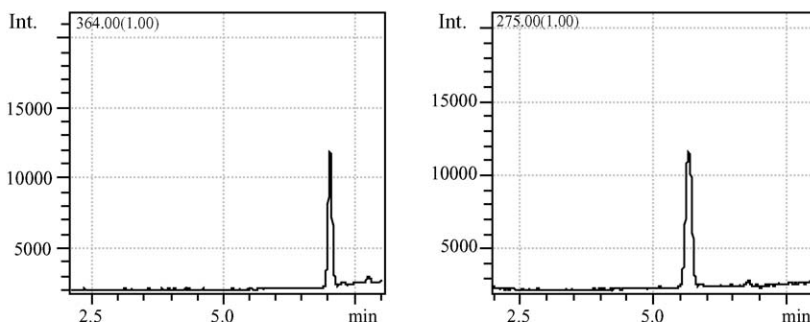

Fig. 2. The SIM (−) chromatograms extracted from blank plasma spiked with with indapamide at 50 ng/ml. The retention times of indapamide and the IS were 7.1 and 5.7 min, respectively.

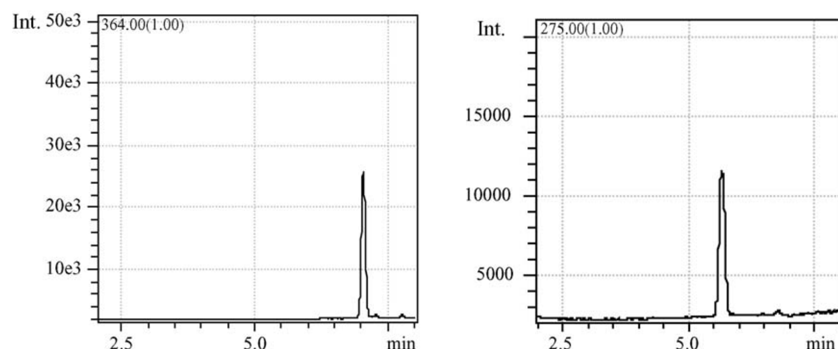

Fig. 3. The SIM (−) chromatogram for plasma sample of a healthy volunteer at 3 h after oral administration 2.5 mg indapamide. The retention times of indapamide and the IS were 7.1 and 5.7 min, respectively.

Table 1
The inter- and intra-day precision, accuracy of the method with determination of indapamide ($n = 5$)

Added concentration (ng/ml)	Inter-day			Intra-day								
				1 day			2 day			3 day		
	1.0	10.0	100.0	1.0	10.0	100.0	1.0	10.0	100.0	1.0	10.0	100.0
Back-caculated concentration (ng/ml)	1.10	9.86	99.18	0.90	9.23	100.00	0.96	10.30	99.64	1.00	10.58	98.96
	0.99	10.36	98.18	0.95	9.70	104.52	0.98	10.19	102.00	1.03	9.83	100.12
	1.08	10.28	97.84	0.94	9.52	99.06	1.07	9.42	97.44	1.01	10.21	95.74
	1.06	10.59	102.33	1.13	11.02	101.84	0.96	10.34	103.26	1.08	10.15	100.82
	0.99	10.65	97.89	0.95	10.62	110.02	1.14	10.06	103.64	1.01	10.35	100.76
Mean RSD%	1.04	10.35	99.08	0.97	10.02	103.09	1.02	10.06	101.20	1.03	10.23	99.28
	5.04	3.01	1.91	9.25	7.62	4.27	7.81	3.72	2.59	3.03	2.70	2.13
Mean accuracy (%)	104.39	103.49	99.08	97.18	100.19	103.09	102.31	100.64	101.20	102.56	102.26	99.28

4.5. Stability

Table 2 summarizes the data from the short-term, freeze–thaw, and long-term stability, as well as for the post-preparative test of indapamide. The short-term stability indicated reliable stability behavior under the experimental conditions of the analytical runs. The results of the freeze–thaw stability test indicated that the analyte was stable in human plasma for three cycles when stored at −20 °C and thawed to room temperature. The post-preparative stability of QC samples showed that indapamide was stable in the sample extract when kept at 4 °C in the autosampler for 24 h. The findings from the long-term test indicate that storage of plasma samples containing indapamide at 20 °C is adequate when maintained for 10 days. Thus, no stability-related problems are expected during the routine analyses for the pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the working solutions was tested at room temperature. Based on the results obtained, these working solutions were stable over 6 h.

4.6. Results of pharmacokinetic study

The method described above was successfully used to analyze plasma samples obtained from 20 healthy volunteers which received a single dose of 2.5 mg indapamide tablets each in the pharmacokinetics study. The procedure developed was sensitive enough to permit the quantitative analysis of indapamide in plasma with acceptable accuracy and precision over a period of 72 h after a single oral administration. The mean

Table 2
Data showing stability of indapamide in human plasma at different QC levels ($n = 5$)

	1.0 (ng/ml)		10.0 (ng/ml)		100.0 (ng/ml)	
	Recovery (mean ± SD) (%)	RSD (%)	Recovery (mean ± SD) (%)	RSD (%)	Recovery (mean ± SD) (%)	RSD (%)
Short-term stability	94.08 ± 6.94	7.38	98.23 ± 4.05	4.12	96.63 ± 5.22	5.41
Freeze and thaw stability	90.03 ± 8.45	9.39	99.82 ± 3.98	3.99	92.08 ± 9.48	10.3
Long-term stability	87.58 ± 5.27	6.02	90.43 ± 2.39	2.64	88.51 ± 3.33	3.76
Post-preparative stability	98.91 ± 7.42	7.5	103.65 ± 11.26	10.86	94.94 ± 11.68	12.3

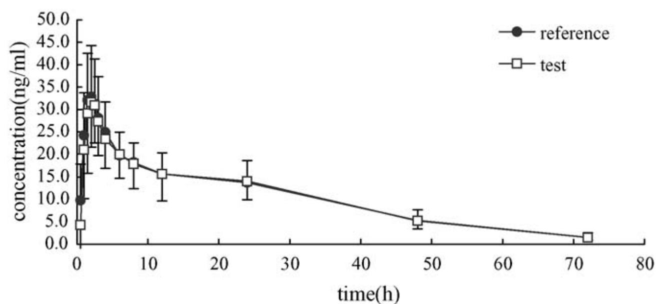

Fig. 4. Mean drug plasma concentration–time curve of indapamide in 20 volunteers after oral administration 2.5 mg indapamide.

plasma concentration–time profiles of volunteers is represented in Fig. 4. The test tablets were found to be bioequivalent to the reference one.

5. Conclusion

The proposed analytical method provided a rapid, sensitive and specific assay for indapamide determination in human plasma. A simple liquid–liquid extraction procedure and a short run time limited the total anaylysis time, and this is important for large sample batches. It was shown that this method is suitable for the analysis of indapamide in human plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies in humans.

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