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Rapid and sensitive determination of indapamide in human blood by liquid chromatography with electrospray ionization mass spectrometric detection: application to a bioequivalence study

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A rapid and sensitive method using liquid chromatography with electrospray ionization mass spectrometric detection was developed and validated for the determination of indapamide in human blood. Blood samples were extracted with *n*-hexane-dichloromethane (1 : 1, v/v) and separation was performed on a Symmetry C_{18} column (150 × 3.9 mm i.d., 5μm) with the mobile phase consisting of acetonitrile-water (60 : 40, v/v). Indapamide and internal standard (propylparaben) were detected by negative electrospray ionization and selected ion recording (SIR) at m/z 364 for indapamide and m/z 179 for propylparaben. This method has a lower limit of quantification (LLOQ) 2.0 μg/L with a linear calibration range of 2.0 μg/L to 120 μg/L. The method showed excellent reproducibility with an inter- and intra-assay precision of <9.4% (% RSD), as well as excellent accuracy with an inter- and intra-assay accuracy of between 98.0 and 102%. Furthermore, the method was successfully applied to a bioequivalence study in which 20 healthy volunteers received a single oral dose of 3 mg reference and test sustained-release indapamide formulations, in an open, two-period, randomized crossover protocol. The maximum blood concentrations (C_{max}) were 60.3 ± 22.6 μg/L and 57.6 ± 18.7 μg/L at 13.1 ± 6.9 h and 18.3 ± 7.4 h, the times to reach the peak concentration (T_{max}), for the test and reference tablets, respectively. The relative bioavailability of the test tablets was 110.1 ± 34.5%, compared with the reference tablets. There were no statistically significant differences in the main pharmacokinetic parameters, and the two formulations were judged to be bioequivalent.

1. Introduction

Indapamide, 3-(aminosulfony)-4-chloro-*N*-(2,3-dihyodro-2-methyl-1*H*-indol-1-yl) benzamide, is a diuretic with antihypertensive effects.

It is up to 79% protein-bound, extensively metabolized and widely distributed throughout tissues (Legorburu et al. 1999). The analysis of indapamide was performed in blood because it is preferentially bound to red blood cells (Miller et al. 1993). Determination of indapamide in biological fluids has been carried out by a number of methods, including thin-layer chromatography (TLC) using both unlabeled and labeled drugs, fluorimetry and high-performance liquid chromatography (HPLC) with UV or with amperometric detection (Grebow et al. 1981; Zendelovska et al. 2003; Legorburu et al. 1999). However, the low sensitivity and complicated methods of preparation are inherent disadvantages of these methods.

In the present study, a rapid and sensitive HPLC method with electrospray ionization mass spectrometric detection was developed and validated for the determination of indapamide in human blood and successfully applied to a bioequivalence study of sustained-release indapamide tablets.

2. Investigations, results and discussion

2.1. Method validation tests

Indapamide and internal standard (propylparaben) were well separated under the experimental conditions, with retention times of 6.0 and 7.6 min, respectively (Fig. 1B). No endogenous interference was found at the corresponding retention times of indapamide or internal standard (Fig. 1A), and both compounds eluted as completely resolved peaks in the blood sample extracts after administration of a single 3 mg oral dose of indapamide to healthy volunteers (Fig. 1C).

The calibration curves for the blood assay obtained by plotting the peak-area ratio (Y) of indapamide and internal standard versus the drug concentration (X) were found to be linear over the concentration range of 2.0–120.0 μg/L. The linear regression equation of the calibration curve was $Y = -0.0756 + 0.0523\ X$, and the correlation coefficient was 0.998. The LLOQ, determined 2 μg/L, met the acceptance criteria with a precision of 13.6% and an accuracy of 106%.

The precision and accuracy of the assay were estimated by analyzing quality control (QC) samples containing low, medium and high concentrations of indapamide (Table 1).

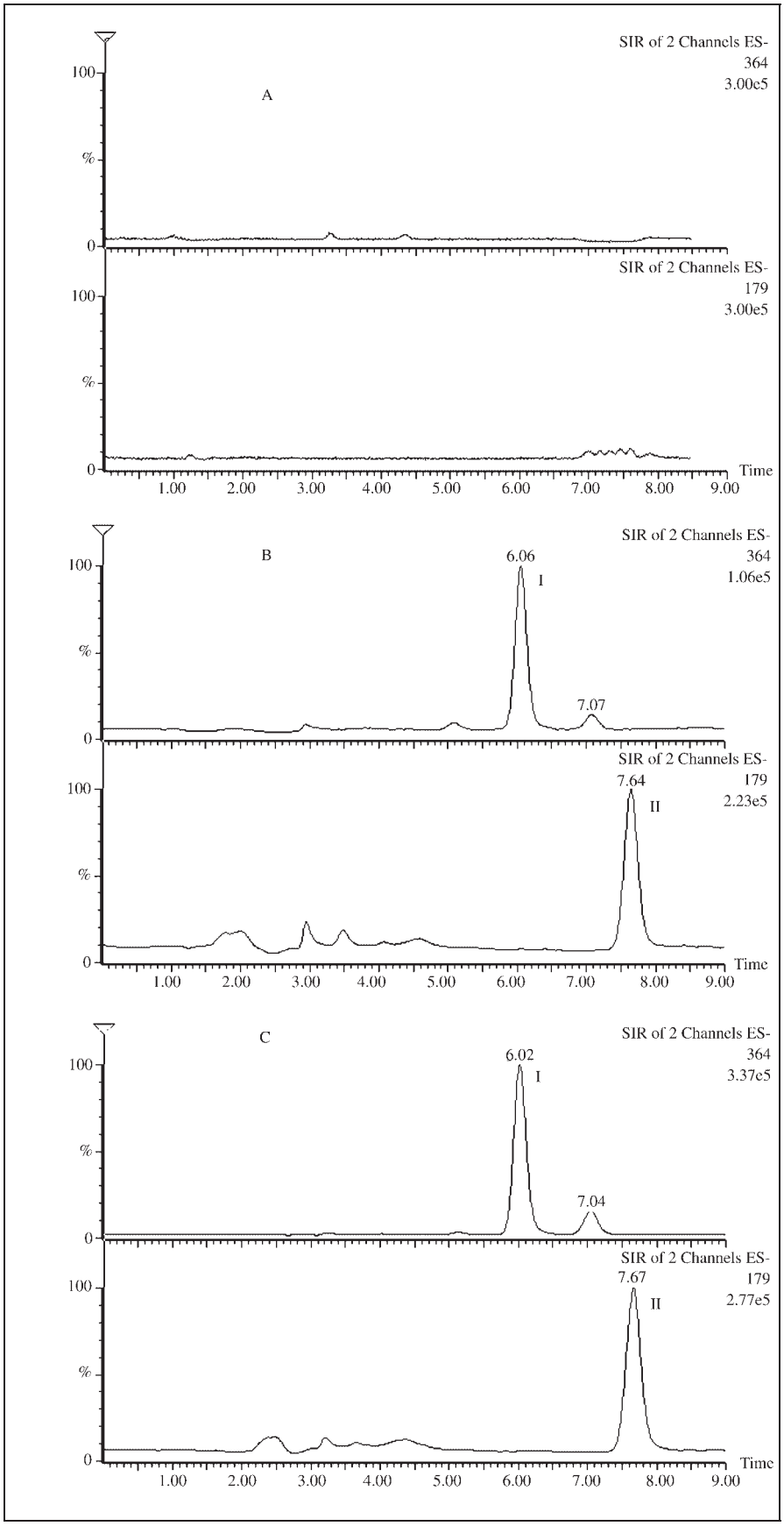

Fig. 1:
SIR chromatograms of indapamide (I) and internal standard (propylparaben, II) in blood samples. (A) blood blank; (B) blood spiked with 20 μg/L indapamide and 40 μg/L internal standard; (C) blood of a volunteer 24 h after a 3 mg oral dose of sustained-release indapamide tablets

The intra- and inter-day precision and accuracy for the determination of indapamide were less than 9.4% (Table 1), falling well within the limits of acceptability.

The extraction recovery was determined from the ratio of the mean peak area of the extracted samples to the mean peak area of the unextracted samples. The relative recovery was $68 \pm 5.3\%$ for indapamide and $80 \pm 7.1\%$ for propylparaben. Extraction recoveries for indapamide and propylparaben were consistent, precise and reproducible throughout the validation experiments and were within the acceptance criteria.

Table 1: Accuracy and precision for the determination of indapamide in human blood

Added (μg/L)	Intra-day (n = 6)			Inter-day (n = 18)		
	Found (μg/L)	RSD (%)	Accuracy (%)	Found (μg/L)	RSD (%)	Accuracy (%)
4.00	3.97	9.4	99.3	4.06	7.3	102
20.0	19.6	5.8	98.0	20.1	6.7	101
80.0	80.6	3.6	101	79.5	3.9	99.4

Table 2: Main pharmacokinetic parameters of indapamide after administration of a single 3 mg oral dose of sustained-release indapamide tablets to healthy volunteers

Parameters	Test	Reference
T_{max} (h)	13.1 ± 6.9	18.3 ± 7.4
C_{max} (μg/L)	60.3 ± 22.6	57.6 ± 18.6
K_e (1/h)	0.0349 ± 0.0075	0.0333 ± 0.0062
$t_{1/2}$ (h)	21.1 ± 6.2	21.6 ± 5.1
AUC_{0-t} (μg h/L)	1964 ± 554	1863 ± 469

(mean ± SD, n = 20)

Indapamide is a basic compound, so weak alkali was added to blood samples in order to improve its extraction recovery. Several alkalization reagents were investigated, including sodium hydroxide, sodium carbonate and sodium bicarbonate at different concentrations. Finally, sodium bicarbonate (0.5 mol/L) was chosen because of its appropriate alkaline nature and ion intensity.

Indapamide added to blood samples (20 μg/L) was found to be stable for 24 h at 37 °C (stability defined as >90% of initial amount remaining). When stored at −20 °C, indapamide added to blood was found to be stable for at least 2 months.

2.2. Pharmacokinetics

The mean blood concentration-time profiles of indapamide after a single 3 mg oral dose of sustained-release indapamide test and reference tablets are shown in Fig. 2. The maximum blood concentrations (C_{max}) were 60.3 ± 22.6 μg/L and 57.6 ± 18.7 μg/L for test and reference preparations, respectively. Moreover, the times to reach the peak concentrations (T_{max}) of two preparations were found to be 13.1 ± 6.9 h and 18.3 ± 7.4 h for test and reference tablets, respectively. The relative bioavailability of the test tablets was 110.1 ± 34.5%, relative to the reference preparation. There were no statistically significant differences in the main pharmacokinetic parameters, and the two formulations were judged to be bioequivalent.

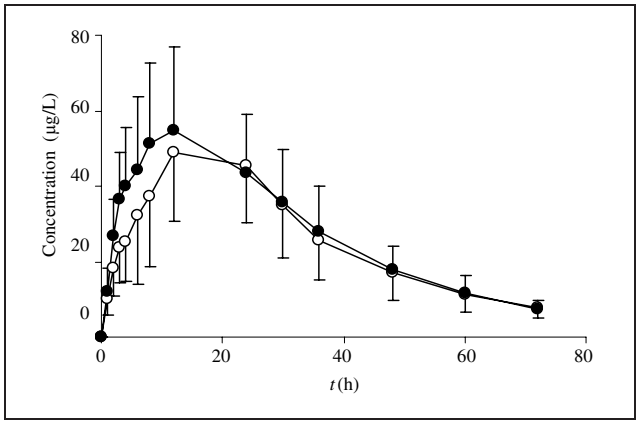

Fig. 2: Mean blood concentration-time profiles of indapamide for either test tablets or reference tablets following oral administration of a single dose of 3 mg sustained-release indapamide to healthy volunteers. Test tablets (●) and reference tablets (○). Each value represents the mean ± SD (n = 20)

3. Experimental

3.1. Chemicals and reagents

Test tablets (indapamide sustained-release tablets) were supplied by Ningxia Yuankang Pharmaceutical Co. Ltd. (each contains 1.5 mg indapamide, Yinchuan, China). Reference tablets (Natrilix®) were purchased from Laboratoires Servier (each contains 1.5 mg indapamide, Gidy, France). Indapamide was supplied by Ningxia Yuankang Pharmaceutical Co. Ltd. (Yinchuan, China) and the internal standard, propylparaben, by Shenyang Dongxing (Shenyang, China). HPLC-grade acetonitrile and methanol were purchased from Dima Technology Inc (Richmond Hill, USA). HPLC-grade *n*-hexane and dichloromethane were from Concord Tech. Co. (Tianjin, China). All other reagents were of analytical grade. Distilled water, prepared from deionized water, was used throughout the study.

3.2. Instrument and LC-MS conditions

The HPLC system consisted of a Waters 1525 pump, ZQ2000 micromass spectrometer equipped with an electrospray ionization (ESI) source and Masslynx 4.0 Software (Waters Corporation, USA). A Symmetry C_{18} column (150 × 3.9 mm, 5 μm; Waters Corporation, USA) was used with a C_{18} guard column (10 × 4.6 mm, 5 μm). The column was maintained at 25 °C. The mobile phase consisted of acetonitrile-water (60 : 40, v/v) at a flow rate of 0.3 ml/min. Ionization conditions for LC-MS were as follows: capillary voltage 3.5 kV, cone voltage 45 V, source temperature 105 °C, desolvation temperature 200 °C. Quantification was performed using selected ion recording (SIR) of m/z 364 for indapamide and m/z 179 for the internal standard, propylparaben.

3.3. Study design

The open, two-period, randomized crossover protocol was approved by the Ethical Committee of People's Hospital of Liaoning Province. All volunteers were fully informed about the study and signed the informed consent form. The mean age of the twenty healthy volunteers was 23 y (range 20–25 y) and mean body weight was 66 kg (range 56–75 kg). Blood samples (approximately 3 mL) were withdrawn via an arm vein and transferred to heparinized tubes at 0, 1, 2, 4, 6, 8, 12, 24, 30, 36, 48, 60 and 72 h after drug administration and stored at −20 °C until analysis. Blood samples were thawed and allowed to reach room temperature before analysis.

3.4. Sample preparation

An aliquot of 100 μL mobile phase and 100 μL propylparaben (40 μg/L in mobile phase) were added to 0.50 mL blood samples in screw-cap glass tubes. After making alkaline with 100 μL 0.5 mol/L sodium bicarbonate, the mixture was vortexed for 1 min and extracted with 4 mL *n*-hexane-dichloromethane (1 : 1, v/v). After vortex-mixing for 2 min and centrifugation at 3500 rpm for 10 min, the organic layer was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase followed by vortex-mixing. Then, 20 μL of an aliquot of supernatant was injected onto the LC-MS system.

3.5. Method validation tests

3.5.1. Linearity and the lower limit of quantification

Blank blood samples spiked with a series of concentrations (2.0, 4.0, 8.0, 12.0, 20.0, 40.0, 80.0 and 120.0 μg/L) of indapamide were processed as described in the sample preparation section. Calibration curves for the blood assay were constructed using the peak-area ratio (Y) of indapamide to internal standard versus drug concentration (X). All calibration curves of indapamide were constructed prior to the experiments with correlation values of at least 0.995. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters). Six replicates of LLOQ were prepared independently of the standards used in the standard curve.

3.5.2. Precision and accuracy

The intra-day and inter-day variabilities were assayed (six replicates) at three concentrations (4, 20, 100 μg/L) of indapamide on the same day and on three sequential days, respectively. Accuracy and precision values of within 15% covering the range of actual experimental concentrations were considered acceptable.

3.5.3. Extraction recovery

The efficiency of the extraction process was determined at three concentrations (4, 20, 100 μg/L) of indapamide in triplicate and at one concentration (40 μg/L) of propylparaben in triplicate. The recovery was determined from the percentage ratio of the mean peak area of extracted samples to the mean peak area of unextracted samples at the respective concentrations.

3.5.4. Stability of blood samples

The stability of indapamide added to blood was evaluated at 37 °C and −20 °C, respectively. Blood samples containing indapamide (20 μg/L) were prepared and placed in capped tubes. Three samples were kept at −20 °C and others were maintained (in triplicate) at 37 °C for 4, 8, 12, 24 h, and then stored at −20 °C. Analyses were performed on the day following freezing. In separate experiments, the storage stability at −20 °C was determined by analysis of indapamide in blood after several storage periods up to 60 days.

3.6. Data analysis

The chromatographic data were automatically processed to obtain the peak-area ratios of indapamide to the internal standard and fitted to a weighted (1/C) linear regression relationship. The maximum blood concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were determined by visual inspection of the experiment data. The elimination rate constant was calculated by applying the least-squares regression technique to the data for the last four points of the blood concentration-time curve. The area under the blood concentration-time curve from time zero to the last measurable blood concentration point ($AUC_{0-72\,h}$) was calculated by the linear trapezoidal rule. The relative bioavailability (F%) was calculated by the following equation, $F(\%) = AUC_{Test}/AUC_{Reference}$.

All the means are presented with their SDs (mean ± SD) and an unpaired Student's t-test was used to determine any significant differences between the test and the reference formulations. Differences were considered to be significant at $P < 0.05$.

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