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A High-Performance Liquid Chromatographic Assay for the Determination of Losartan in Plasma

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ABSTRACT

A rapid and sensitive HPLC method was developed for determination of losartan in plasma. Losartan was extracted from plasma by a two-step extraction procedure using chloroform as extracting solvent in acidic medium. HPLC analysis was performed on a cyano reversed-phase column using phosphate buffer (pH 4.3), acetonitrile (750:250, v/v) as mobile phase with a flow rate of 0.9 mL/min. Sodium diclofenac was selected as internal standard. Excellent linearity between the peak area ratios and losartan concentrations over the range of 2-200 ng/mL of plasma was observed. The limit of determination with UV detection at 225 nm, with a CV < 5% was 2 ng/mL in 500 µL of plasma sample. The assay was rapid, safe and reliable for use in pharmacokinetic studies of losartan in human being.

Keywords: *Losartan, Plasma, Extraction, HPLC analysis*

Losartan, the potassium salt of 2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2-(1H-tetrazol-5-yl)-biphenyl-4-yl) methyl] (Fig 1) is a potent orally active and highly selective AT_1-subtype, non-peptide angiotensin II (AII) receptor antagonist and antihypertensive agent [1-3].

Having a sensitive and reliable technique is crucial for determination of losartan in biological fluids and studying its pharmacokinetic investigation in man. Several HPLC techniques, mostly based on UV detection are reported for extraction and determination of losartan in biological matrices [4-7]. These methods mostly need extracting steps that are sophisticated and time consuming with some interfering materials that could affect the overall determination of losartan. Carrying out some of these methods [4, 5] we encountered difficulties during extraction of losartan from plasma, including interfering materials that isolation of them was complex, time consuming and affected obtaining a clean extraction and reasonable peaks.

The aim of this study was to establish a simple, rapid and sensitive HPLC method to overcome the above mentioned problems and allowed determination of the range of concentrations of losartan in biological matrices applicable to pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals and reagents. Losartan and sodium diclofenac as Internal Standard (Fig 1) were purchased from Merck Sharp & Dohme Research Laboratory (Rahway, NJ, USA). HPLC grade acetonitrile and analytical grade chloroform were from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical reagent grade and used without any further purification.

Solutions and buffers. Stock solution of losartan was prepared by dissolving 20 mg losartan in 100 mL distilled water to give a final concentration of 200 µg/mL. Standard solutions were obtained by diluting this solution with distilled water to give concentrations over the range of 20-2000 ng/mL for preparation of the standard curve.

The solution of internal standard (IS) was prepared by dissolving 20 mg sodium diclofenac in distilled water to a final concentration of 200 µg/mL.

Phosphate (0.3 M) solution was prepared by dissolving 5.22 g potassium phosphate dibasic in 100 mL distilled water. A mixture of 4.5 mL of this solution with 0.5 mL of concentrated orthophosphoric acid was used for acidifying and precipitating of plasma proteins. All solutions were stored at 4°C.

Phosphate buffer (0.05 M) was prepared by dissolving 7.1 g dibasic sodium phosphate in approximately 800 mL distilled water. The pH was adjusted to 4.3 with 1 M orthophosphoric acid and water was added to 1000 mL.

Chromatographic conditions. The HPLC system consisted of a series 510 pump, a 717 plus Autosampler

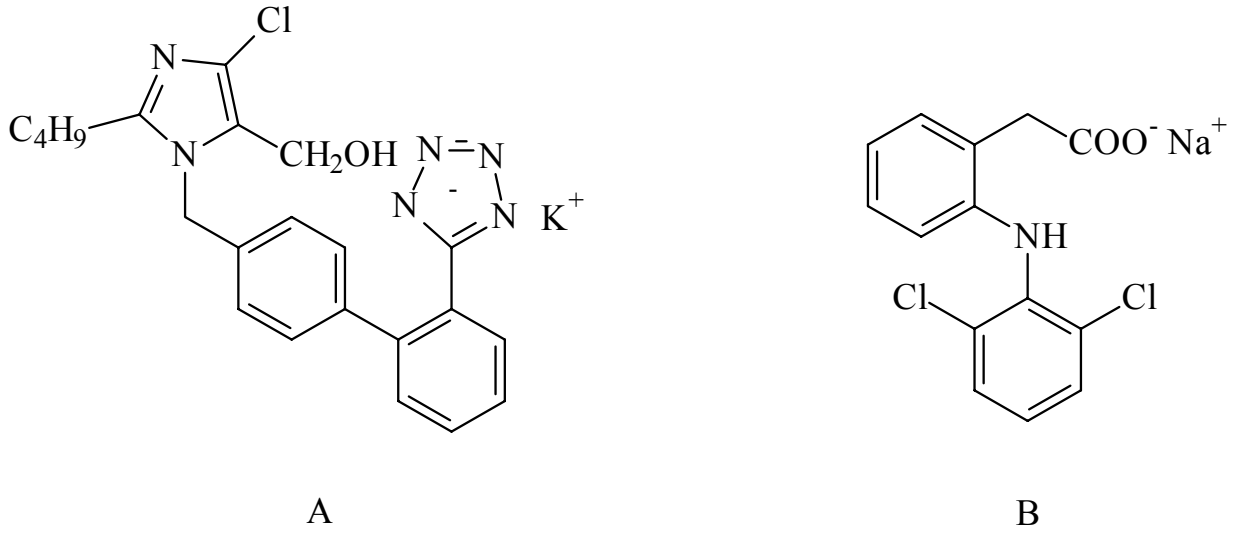

Fig 1. Chemical structure of losartan (A) and diclofenac sodium (B)(IS)

and a variable 480 UV detector and a 476 recorder-integrator all from Waters (Milford, MA, USA). Separation was achieved using a Nucleosil 100-5 CN analytical column (250 mm × 4.6 mm I.D., Macherey-Nagel, USA). The isocratic mobile phase consisted of phosphate buffer (pH 4.3), acetonitrile (750:250, v/v) prepared daily and degassed by passing through a 0.45 µm filter. All chromatographic separations were performed at room temperature. The flow rate was set to 0.9 mL/min. UV detection was performed at 225 nm.

Sample preparation. Frozen plasma samples were obtained from Blood Transfusion Center (Red Crescent Organization), thawed and allowed to reach room temperature. A 500 µL aliquot of plasma was placed into a test tube, 20 µL of IS solution and 50 µL standard solution of losartan were added. The tube was vortex-mixed for 10 sec and kept at room temperature for 10 min. After adding 2 mL of chloroform, the tube was vortex-mixed for 30 sec, then centrifuged at 1500 g for 10 min. The aqueous layer was completely removed and transferred to a clean test tube. 50 µL phosphate precipitation solution was added to the test tube and mixed. After the addition of 2 mL chloroform the tubes were vortex mixed for 30 sec and centrifuged for 3 min at 1500 g. The chloroform layer was completely removed and transferred to a clean test tube and evaporated to dryness at 50°C in a water bath under nitrogen. The residue was reconstituted in 200 µL of 0.05 M sodium hydroxide and 100 µL of the solution was injected into the HPLC system.

Extraction yield. Aliquots of 50 µL of losartan standard solutions (20-2000 ng/mL) and 20 µL of IS solution were added separately to two sets of five test tubes. To one set, 500 µL plasma was added and was extracted according to the sample preparation method. The other set was adjusted to the same volume by 0.05 M sodium hydroxide. 100 µL of each solution was injected into the HPLC system. The peak areas of the extracted samples and unextracted samples were compared. The experiment was repeated on three consecutive days.

Quantification. Calibration standards of losartan were prepared by spiking 50 µL of losartan standard solutions and 20 µL of IS solution to 500 µL of blank human plasma to give final concentrations over the range of 2-200 ng/mL. The sample extraction and HPLC analysis were performed as described above. Calibration curves were constructed by plotting the measured peak area ratios of losartan to the IS vs. concentrations of standard samples. The intraday (within-run) and interday (between-run) accuracy and precision of the method was determined by measuring four replicate samples of losartan standard solutions (20, 500, 1000, 2000 ng/mL) on three separate days.

RESULTS

Optimization was achieved by monitoring varying reversed-phase columns, mobile systems, flow rate, wavelength and using a simple two-step extraction method. The proposed method showed no interfering peaks of endogenous substances or late-eluting peaks with losartan and internal standard in plasma. Under the proposed chromatographic condition the retention times of losartan and internal standard were approximately 12.6 and 16.7 min respectively. Representative chromatograms are shown in Fig 2.

The mean recovery of losartan in different concentrations, determined by comparing peak areas from extracted standard samples with those of unextracted samples, was about 76%.

Sodium diclofenac was selected as internal standard due to its acceptable precision and accuracy for losartan determination. The recovery of IS was about 85%.

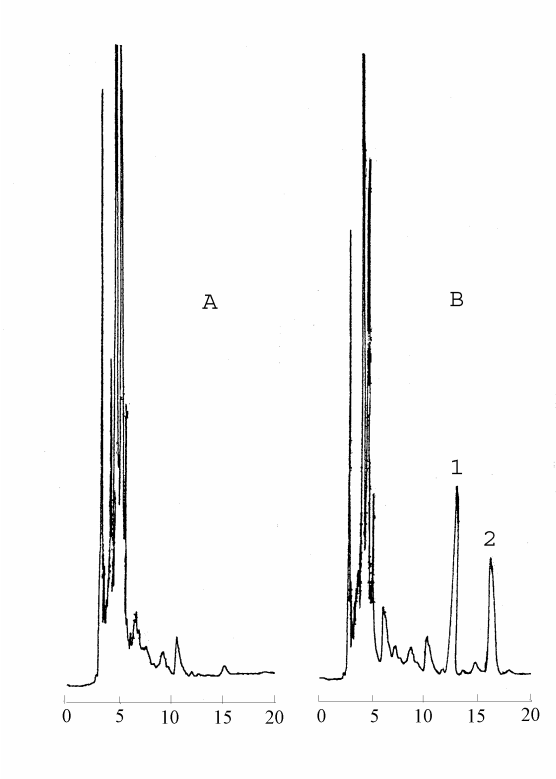

Fig 2. HPLC chromatograms of losartan and IS. (A) Drug free plasma; (B) Plasma spiked with losartan (50 ng/mL) and IS (20 µg/mL). Peaks: 1, losartan; 2, IS

Calibration, accuracy and precision. A standard curve for losartan in different range of concentrations (2, 5, 30, 50, 80, 100 and 200 ng/mL) in plasma was prepared. The calibration curve displayed excellent linearity ($r^2 > 0.99$) over the concentration range investigated. Typical calibration curve obtained in plasma is described by Y = 0.0077 X + 0.045, where Y is the peak-area ratio of losartan/IS and X is the drug concentration.

The accuracy and precision were determined by preparing and assaying five replicate samples of losartan at concentrations of 2, 50, 100 and 200 ng/mL in plasma on three separate days. Concentrations were determined using a calibration standard curve for each day. According to the intraday (within-run) and interday (between-run) data good accuracy and precision were observed over the entire concentration range. The results are presented in Table 1. The within-run and between-run variabilities showed CV values less than 3.07 in all four selected concentrations. The limit of determination of the method, defined as the minimum concentration that could be measured with a CV < 5% was found to be 2 ng/mL in 500 µL of plasma sample. The limit of detection with a S/N ratio of 3:1 was 0.5 ng/mL in plasma.

DISCUSSION

In this study the most important reported HPLC techniques for determination of losartan in biological fluids were revised. The proposed technique suggests a rapid, reliable and simple extraction method. The HPLC procedure is based partly on the modification of the chromatographic condition reported by Furtek and Lo [4]. Losartan is a weak acid and could be extracted from an acidic aqueous medium into an organic solvent [4]. Several extraction methods have been used to accomplish extraction of losartan and its metabolite from biological fluids [4-7]. The reported extraction methods for losartan and its major metabolite from plasma with methyl tertiary butyl ether, hexane and other reagents [4, 5] are sophisticated and time-consuming. Performing this procedure we encountered with some interfering endogenous substances that prevented us to obtain a clean extraction product and a clear overall peaks.

In the present study a two-step extraction procedure is described using chloroform as extracting solvent. The accuracy and precision data obtained for this simple and rapid procedure support the reliability of the assay for the determination of losartan in biological fluids and for pharmacokinetics studies.

CONCLUSION

A two-step extraction procedure for losartan from plasma and an improved method for determination of losartan are reported. Compared to previously published methods, the suggested extraction procedure is considerably more simple, rapid, reliable and sensitive. The HPLC technique based on UV detection is suitable for determination of small amounts of losartan with good accuracy and reproducibility comparable to the method performed by fluorescence detection [5]. Simple sample preparation procedure and a relatively short chromatographic time make this method suitable for processing of multiple samples in a limited amount of time for pharmacokinetic studies.

Table 1. Accuracy and precision in spiked plasma (n=15; five sets for three days)

Concentration added (ng/mL)	Concentration found (ng/mL)	% CV	% Error
Intra-day (n=5)			
2	1.95±0.03	1.38	-2.50
50	49.62±1.05	2.11	-0.75
100	100.86±3.06	3.07	0.86
200	200.18±3.48	1.74	0.09
Inter-day (n=15)			
2	1.95±0.02	2.67	-2.30
50	49.82±0.98	3.01	-0.35
100	101.16±3.05	1.97	1.16
200	200.32±5.34	1.23	0.16

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