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Short communication

Determination of the angiotensin-converting enzyme inhibitor lisinopril in urine using solid-phase extraction and reversed-phase high-performance liquid chromatography

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Abstract

A simple, accurate and precise high-performance liquid chromatographic method is described for assaying lisinopril in human urine. Urine (1 ml) containing lisinopril and enalaprilat (internal standard) was acidified with 10 μl of 6 *M* nitric acid, passed through a Sep-Pak C_{18} cartridge and eluted with 3 ml of 10% acetonitrile, followed by 6 ml of distilled water. The separations were carried out using a μBondapak C_{18} column with a mobile phase comprising acetonitrile (60 ml), methanol (10 ml) and tetrahydrofuran (10 ml) in 15 m*M* phosphate buffer (920 ml) at pH 2.90. Separations were performed at 40°C and detection was at 206 nm. Standard calibration plots of lisinopril in urine were linear ($r > 0.998$) and recovery was greater than 64%. The lowest quantifiable concentration was 0.5 μg/ml. Within-day and between-day imprecision (coefficient of variation) ranged from 2.51% to 9.26%, and inaccuracy was less than 8.3%.

1. Introduction

Lisinopril, a lysine analog of the nonsulfhydryl angiotensin-converting enzyme (ACE) inhibitor enalapril, is used for the treatment of hypertension and congestive heart failure in daily dosages of 10–80 mg [1]. The measurement of this drug in biological fluids is challenging since it has poor electromagnetic absorbance which is exacerbated by the low peak serum concentrations (< 75 ng/ml) produced during routine clinical use. Furthermore, lisinopril is an amphoteric, peptide-like molecule which cannot be extracted from biological fluids with organic solvents.

Previous analytical methods included measurement of the ACE activity in the presence of lisinopril [2], and a double antibody, in-house radioimmunoassay [3–5] which is sensitive but requires the synthesis of radio-labels and antisera. The published HPLC assays for bulk drug and solid dosage formulations [6] are simple but unsuitable for application in pharmacokinetic studies due to sensitivity limitations and interference from endogenous peaks in biological samples. We now describe a simple, accurate and precise HPLC method which employs solid-

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phase extraction for assaying lisinopril in urine at the detection wavelength of 206 nm.

2. Experimental

2.1. Materials

Lisinopril and enalaprilat were obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA). Acetonitrile, methanol, chloroform and tetrahydrofuran were HPLC grade. Water was freshly distilled. Anhydrous potassium dihydrogen orthophosphate, phosphoric acid, sodium hydroxide, nitric and hydrochloric acids were A.R. grade.

2.2. Instrumentation

Analyses were performed on a modular system consisting of a Model U6K universal sample injector (Waters Australia, Sydney, N.S.W., Australia), a Model 501 pump (Waters), a Model 484 Lambda-Max variable-wavelength detector (Waters) and a Model C-R6A recording integrator (Shimadzu, Tokyo, Japan).

2.3. Sample preparation

Stock solutions of 1 mg/ml of lisinopril and enalaprilat in 0.1 *M* HCl were prepared and stored at −4°C. A lisinopril standard, seeded control, or unknown urine sample (1 ml) was pipetted into an appropriately labelled tube, 80 μl of internal standard solution (containing 8 μg/ml) were added, then 10 μl of 6 *M* nitric acid. The mixture was agitated by vortex-mixing for 30 s, then passed through a Sep-Pak C_{18} cartridge (Waters) previously conditioned with 10 ml of methanol, 10 ml of distilled water and 20 ml of 0.1 *M* hydrochloric acid. The cartridges were washed with 20 ml of 0.1 *M* HCl and the washings discarded. Three millilitres of 10% (v/v) acetonitrile in water were added to the cartridge followed by 6 ml of distilled water, and the combined eluents saved. Acetonitrile in this solution was removed by exposure to air flow at 65°C and 25 μl of 6 *M* nitric acid added to the aqueous residue. This solution was reapplied to the Sep-Pak cartridge which was then washed with 10 ml of chloroform before elution of lisinopril and enalaprilat with 6 ml of methanol. The eluent was evaporated at 65°C under airflow, the residue washed with 1 ml of acetonitrile (removed by aspiration), and then reconstituted in 0.5 ml of 10% (v/v) methanol in chloroform by vortex-mixing for 30 s. This solution was transferred to another tube, evaporated, then reconstituted in 100 μl of mobile phase prior to injection.

2.4. Chromatography and quantitation

Chromatography was conducted using a mobile phase of acetonitrile (60 ml), methanol (10 ml), tetrahydrofuran (10 ml) and 15 m*M* potassium dihydrogen orthophosphate (920 ml, pH 2.9) pumped at 1.5 ml/min through a μBondapak C_{18} (Waters; 300 × 3.9 mm I.D., 10 μm) held in a thermostatted water bath (40 ± 1°C). The injection volume was 10 μl and peaks were detected at 206 nm, at a sensitivity of 0.05 a.u.f.s. (absorbance units full scale). The integrator attenuation was 16 and the chart speed was 0.25 cm/min. The total run time for an assay was approximately 12 min.

Standard calibrations were based on the measurements of lisinopril at concentrations of 16, 10, 4, 2, 1 and 0.5 μg/ml and the calibration equation was constructed by least-squares linear regression of the peak-height ratio of lisinopril to enalaprilat on urinary lisinopril concentration. Unknown lisinopril concentrations were calculated by inverse prediction. The absolute recovery from drug-free urine and distilled water was assessed at 10 μg/ml and 1 μg/ml for lisinopril, and at 8 μg/ml for enalaprilat.

3. Results and discussion

Fig. 1 shows chromatograms of: (A) drug-free urine; (B) a urinary standard lisinopril concentration of 12 μg/ml; and (C) a urine sample containing 4.71 μg/ml collected 3 h after a healthy subject took a 20-mg lisinopril tablet.

Table 1
Within-day and between-day imprecision and inaccuracy of lisinopril assay in urine

Target concentration (μg/ml)	Found concentration		Inaccuracy (%)
	Mean ± S.D. (μg/ml)	C.V. (%)	
Within-day (n = 5)			
1.20	1.22 ± 0.07	5.8	1.7
12.0	12.4 ± 0.31	2.5	3.3
Between-day (n = 8)			
1.20	1.30 ± 0.12	9.2	8.3
12.0	12.3 ± 0.66	5.4	2.3

Lisinopril and enalaprilat were eluted in 7.5 and 10.5 min, respectively. The lowest quantifiable concentration was approximately 0.5 μg/ml based on the maximum tolerable C.V. of 15%. Within-day and between-day inaccuracy was 8.3%, or less, at concentrations of 1.2 μg/ml and 12 μg/ml (Table 1). The absolute recovery of lisinopril was greater than 64% in either water or drug-free urine, while the recovery of internal standard was greater than 56% (Table 2). Calibration data pooled from 16 replicate standard curves ($y = 0.177x + 0.0268$) showed high linearity with $r > 0.998$ in all cases. Enalaprilat, the active metabolite of the pro-drug enalapril, was employed as a suitable internal standard in the assay since it has very similar chemical and chromatographic properties to lisinopril, and enalapril is not co-prescribed with lisinopril to patients.

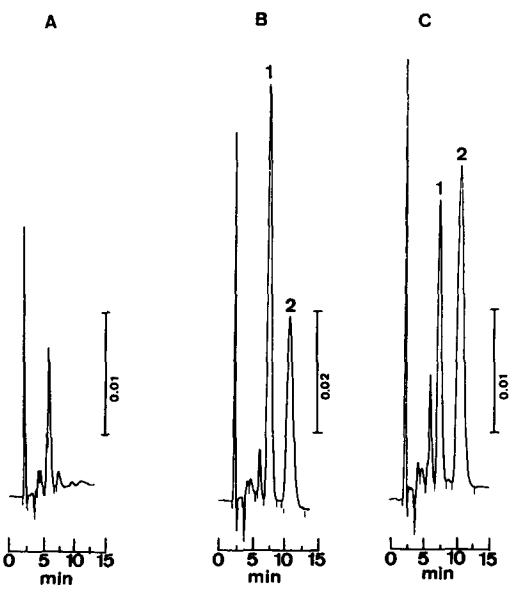

Fig. 1. Chromatograms of the analysis of: (A) drug-free urine; (B) a standard containing 12 μg/ml lisinopril and 8 μg/ml enalaprilat in urine; and (C) a urine sample containing 4.71 μg/ml lisinopril collected 3 h after administration of a lisinopril tablet (20 mg) to a healthy male subject. Peaks: 1 = lisinopril; 2 = enalaprilat (internal standard).

The lack of chromophores on lisinopril (and enalaprilat) meant that detection had to be performed at 206 nm where endogenous substances gave numerous interfering peaks in diluted, unprocessed urine samples. Accordingly, much effort was invested in developing an extensive clean-up procedure which eliminated these

Table 2
Absolute recovery of lisinopril and enalaprilat in water and drug-free urine

Concentration (μg/ml)	Absolute recovery (mean ± S.D. (%)[a]	
	Water	Urine
Lisinopril		
1.0	72.2 ± 7.0 (9.7)	72.4 ± 6.0 (8.3)
10.0	65.8 ± 3.9 (5.9)	64.1 ± 4.5 (7.0)
Enalaprilat		
8.0	55.6 ± 5.9 (10.6)	58.2 ± 2.7 (4.6)

[a] Values in parentheses are coefficients of variation (%).

intrinsic interferences. The hydrophilic nature of lisinopril [7] and enalaprilat severely limited the usefulness of ordinary liquid–liquid extraction. Although enalapril reportedly forms ion-pairs with sulphophthalein dyes such as bromothymol blue [8], the corresponding enalaprilat ion-pair was not extracted in dichloromethane because of the two polar carboxylate groups in the molecule. A similar situation was expected for lisinopril, and attempts to extract lisinopril into organic solvents by ion-pairing with either heptanesulphonic acid, perchloric acid, or tetrabutylammonium bromide were unsuccessful.

Therefore, solid-phase extraction offered a potentially promising solution to the non-extractability of lisinopril. In order to retain lisinopril during sample cleanup it was necessary to add acidified urine to the Sep-Pak cartridge which had been preconditioned with 0.1 *M* HCl. While lisinopril is an amphoteric compound, it appears that suppression of the ionisation of the carboxylate moiety at low pH outweighs protonation of the amino group in terms of decreasing the polarity of the molecule, and therefore its retention on a lipophilic column. Lisinopril and enalaprilat were stable in urine adjusted to pH < 2 for at least 1 month storage at −4°C, and no degradation products were detected during the stability tests. The optimum elution solvent was 10% acetonitrile in water (3 ml) followed by 6 ml of water which, compared with other solvents, eluted lisinopril and enalaprilat with fewer endogenous peaks. It was necessary to completely remove acetonitrile from the eluent, otherwise significant elution of the two analytes occurred during the second passage through the Sep-Pak cartridges. An intermediate chloroform wash facilitated removal of traces of water in the methanolic eluent before it was concentrated under airflow. Washing of the dried extract by acetonitrile in the final stages of solid-phase extraction removed many polar interfering substances, but only a negligible amount of lisinopril was lost due to its very low solubility in this solvent. However, care must be taken to ensure that all traces of water are removed prior to the step, otherwise low recoveries are obtained. In some samples a very small peak coeluted with the leading edge of the larger lisinopril peaks, but was resolved from smaller peaks with no detrimental effect on assay performance.

The peak shapes of lisinopril and enalaprilat were related to the temperature of the analytical column, presumably because of the temperature-dependent kinetics of isomerisation around the proline-amide bond [9]. The effect of a range of temperatures (19–45°C) on peak resolution was investigated and the column efficiency at 45°C improved 86% for lisinopril, and 132% for enalaprilat, compared with that at room temperature (19°C); other conditions remaining unchanged. The life-span of bonded stationary phases tends to deteriorate rapidly above 40°C and, therefore, a working temperature of 40°C was chosen as a suitable compromise of column stability and performance; column efficiency was increased by 62% and 105%, respectively, for lisinopril and enalaprilat. The peak shapes of both compounds were satisfactory with the asymmetry factor decreasing from 2.25 to 1.20 for lisinopril, and from 2.32 to 1.33 for enalaprilat. Chromatography remained satisfactory after 500 injections and column life was adequately maintained by flushing the column with methanol (30 ml), followed by dimethylsulphoxide (1 ml) then 50% (v/v) methanol in dichloromethane (30 ml), whenever peak broadening or shifting was observed [10].

The application of the method in pharmacokinetic studies was demonstrated when a healthy, male volunteer (weight 53 kg, age 33 yr) took a single lisinopril tablet (20 mg) following which 13 urine samples were collected periodically. Maximum lisinopril excretion (0.609 mg/h; 3% of the dose) occurred 6–7 h after dosing, while the total amount of lisinopril excreted was 4.45 mg (22.3% of the dose) over 24 h (4.3 half-lives) which agrees with previous findings [11]. The lisinopril urinary excretion profile is shown in Fig. 2. The amount of accumulated lisinopril excreted unchanged was modelled as a function of time after drug administration [12] using PCNONLIN (version 4.2, Statistical Consultants, Lexington, KY, USA). First-order rate constants for absorption (K_a) and elimination (K_e) of 0.205 h^{-1} and 0.125 h^{-1}, respectively,

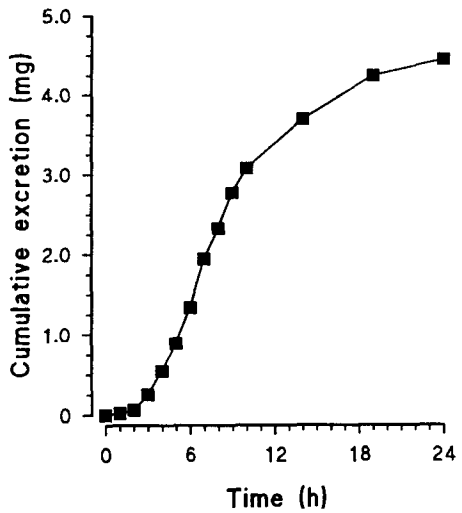

Fig. 2. Plot of cumulative amount of lisinopril excreted in urine versus time after administration of a lisinopril tablet (20 mg) to a healthy male subject.

were obtained from which an absorption half-life of 3.4 h and a terminal elimination half-life of 5.6 h were calculated. A lag time of 0.46 h was obtained.

In conclusion, we have developed a new method for assaying lisinopril in urine which is potentially useful for pharmacokinetic and bioavailability studies, provided that a sufficient number of samples are collected. While the extraction procedure described herein involves a number of steps, it is the first reported HPLC assay for application to biological fluids. Further studies are planned to develop the method for other clinical use including its modification for analysing lisinopril in plasma.

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