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High-performance liquid chromatographic assay for amiloride in plasma and urine

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ABSTRACT

A sensitive and simplified high-performance liquid chromatographic procedure has been developed for quantification of amiloride in rabbit plasma, as well as human plasma and urine. Following protein precipitation with perchloric acid, the supernatant was directly injected into a C_{18} Nucleosil column. The mobile phase consisted of methanol–water (45:55) containing 0.1 *M* perchloric acid, and the compound was quantitated using a fluorescence detector at excitation and emission wavelengths of 286 and 418 nm, respectively. The average recovery was 97.6%. The calibration curve was linear over the range 2.0–20.0 ng/ml. The limit of detection was 0.5 ng/ml.

INTRODUCTION

Amiloride (N-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide) is a potassium-conserving drug with natriuretic, diuretic and antihypertensive activity. It is used to restore normal serum potassium levels in patients who develop hypokalemia, and in patients who would be exposed to a particular risk if hypokalemia were to develop.

The pharmacokinetics and bioavailability of amiloride in plasma and/or urine have been investigated by determining its levels by fluorometry [1], radioactivity measurement [2] and densitofluorometry [3]. High-performance liquid chromatographic (HPLC) methods for determining amiloride in plasma and/or urine have also been reported [4–6]. These methods are selective and sensitive, but they require time-consuming extractions or the mobile phase being saturated with stationary phase. Recently, more simplified HPLC techniques employing solid-phase extraction and pre-column enrichment procedures have been published [7,8].

This paper described a sensitive, rapid and simplified reversed-phase HPLC method for determining the drug in rabbit plasma, as well as human plasma and

urine. The method involves protein precipitation with 6% perchloric acid, and separation of the supernatant on a reversed-phase C_{18} column. It has been used to determine amiloride in the plasma and urine of six volunteers who had taken amiloride tablets, and to provide data on the pharmacokinetics and bioavailability of the drug.

EXPERIMENTAL

Reagents and standard

Pure authentic amiloride was supplied by China Pharmaceutical University (Nanjing, China). Perchloric acid used was of analytical reagent grade and obtained from Nanjing Chemistry Reagent Plant. HPLC-grade methanol was obtained from Wujing Chemical Plant (Shanghai, China).

Apparatus and chromatographic conditions

The high-performance liquid chromatographic pump (Waters Assoc., Milford, MA, U.S.A.) was fitted with a 50-μl loop injector (Shanghai Scientific Instrumental Factory, Shanghai, China) and a HP1046A fluorescence detector (Hewlett-Packard, Germany). The signal was recorded on a recorder (Yokogawa Hokushin Electric, Tokyo, Japan). A reversed-phase C_{18} Nucleosil column (25 cm × 4.6 mm I.D.; particle size 7 μm) (Dalian Institute of Chemical Physics, Dalian, China) was employed at ambient temperature.

The mobile phase was prepared by mixing an aqueous solution of 0.10 *M* perchloric acid with HPLC-grade methanol in a ratio of 55:45. This solution was degassed ultrasonically prior to use. The wavelengths of excitation and emission of the fluorescence detector were set at 286 and 418 nm, respectively. The recorder was set at a speed of 20 cm/h.

Sample preparation

A 0.2-ml volume of a plasma sample was placed in a clean, dry test-tube, and protein precipitation was accomplished by the addition of 0.2 ml of 6% perchloric acid solution followed by vortex-mixing for 30 s and centrifugation at 3000 *g* for 10 min. The supernatant was used for analysis. Urine samples were diluted 1:100 with 6% perchloric acid and then used for analysis.

Preparation of the standard solution and the calibration curve

An aqueous stock solution of amiloride was prepared by accurately weighing 10 mg of authentic amiloride and dissolving it in 1% (v/v) lactic acid solution in a 100-ml volumetric flask.

Plasma standards were produced by spiking 0.2-ml aliquots of fresh human plasma to give the following range of amiloride concentrations: 0, 2.0, 4.0, 8.0, 16.0 and 20.0 ng/ml. These standards were vortex-mixed for 30 s and then allowed to stand at ambient temperature for 20 min to equilibrate. Similarly, urine

calibration standards were produced by spiking 50-μl aliquots of fresh human urine to give the same concentrations of amiloride.

Assay validation

A series of plasma samples containing known amounts of amiloride at five different concentrations was prepared by adding standard solutions of amiloride to the blank plasma. The samples were analysed on seven separate occasions for the within-day assay and five separate occasions for the between-day assay. Absolute recoveries of amiloride were calculated by comparing the peak heights of the drug added to the blank plasma with the peak heights of the drug added to the final 6% perchloric acid.

Human experiment

Six adult subjects took two tablets of amiloride (each contained 5 mg of amiloride, made in China). Venous blood was samples at 0, 1, 2, 3, 4, 5, 7, 10, 12 and 24 h and centrifuged, and 0.2 ml of plasma was removed for treatment according to *Sample preparation,* and assay. After a two-week interval, the same six volunteers took two imported 5-mg tablets of amiloride. The same procedure was carried out.

RESULTS

Chromatography

The chromatographic conditions described were implemented in order to provide the best compromise between chromatographic resolution and peak symmetry and relatively short retention times. Under these HPLC conditions, amilo-

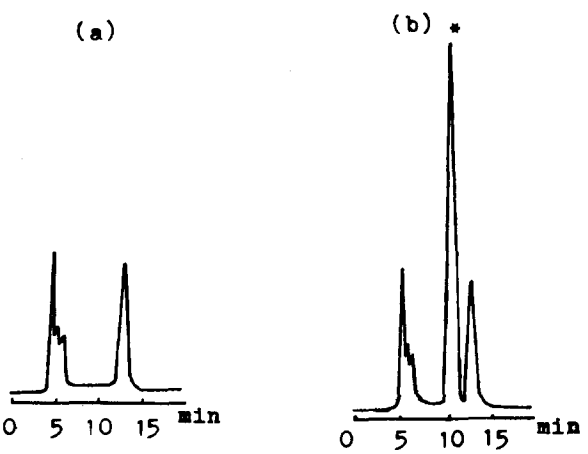

Fig. 1. Typical chromatograms of amiloride in rabbit plasma. (a) Blank plasma; (b) a plasma sample containing 0.628 μg/ml amiloride 5 h after intravenous bolus of 5 mg of amiloride. Peak * = amiloride.

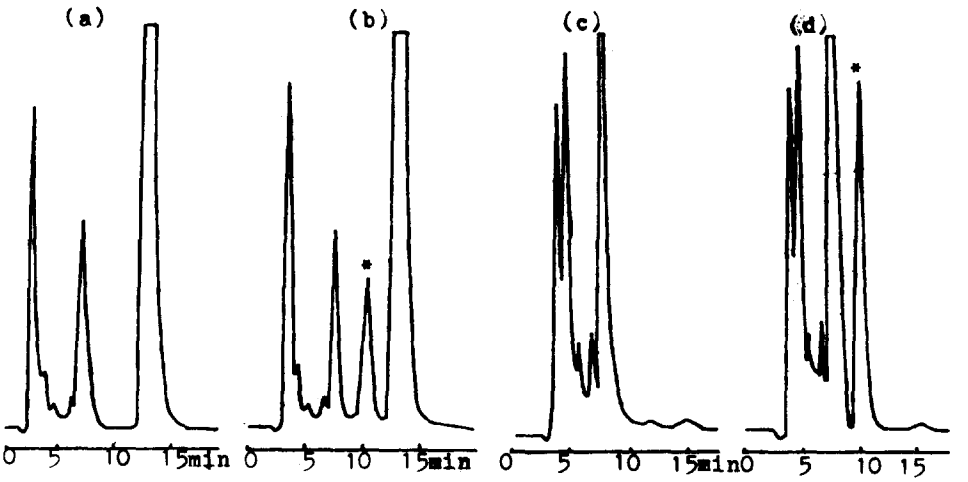

Fig. 2. Representative chromatograms of amiloride in human plasma and urine. (a) Blank plasma; (b) subject plasma containing 15.58 ng/ml amiloride 3 h after oral administration of 10 mg of amiloride; (c) blank urine; (d) subject urine containing 1.194 μg/ml amiloride 12 h after ingestion of 10 mg of amiloride. Peak * = amiloride.

ride had a retention time of 11 min, with a time between injections of 15 min. Fig. 1 illustrates typical rabbit plasma chromatograms. Fig. 2 illustrates typical human plasma and urine chromatograms.

Linearity

Standard calibration lines were constructed by plotting the peak heights of amiloride against the concentrations of the compound in the spiked standard. These plots were linear over the range 2.0–20.0 ng/ml for plasma or urine. The equations of linear regression were $y = 0.5104x - 1.9312$ ($r = 0.9997$) for plasma and $y = 0.4994x - 0.6193$ ($r = 0.9999$) for urine.

Recovery, precision and accuracy

The results of the absolute recovery experiments are illustrated in Table I. The average (± S.D.) recovery of amiloride was 97.6 ± 1.78% in plasma. The coefficients of variation (C.V.) indicate the overall variability of the assay to be excellent, with all C.V. values less than 6% for within-day and less than 7% for between-day. Levels as low as 0.5 ng/ml can be seen over a baseline signal-to-noise rate of 2.0.

Application

Fig. 3 illustrates a plasma concentration *versus* time profile for amiloride in the rabbit following a 5-mg intravenous bolus of amiloride.

Mean plasma concentration–time curves six volunteers ingesting Chinese ami-

TABLE I

WITHIN- AND BETWEEN-ASSAY PRECISION, ACCURACY AND ABSOLUTE RECOVERY DATA FOR AMILORIDE IN PLASMA

Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)	Absolute recovery (mean ± S.D.) (%)
Within-day (n = 7)				
10	10.12 ± 0.46	4.5	101.2	95.7 ± 4.1
25	24.46 ± 1.42	5.7	97.8	98.0 ± 4.0
50	49.42 ± 2.57	5.2	98.8	99.2 ± 4.9
Between-day (n = 5)				
10	9.21 ± 0.57	6.2	92.1	
20	18.70 ± 0.55	2.9	93.5	
80	72.96 ± 2.94	4.0	91.2	

loride tablets and imported ones are presented in Fig. 4 and Fig. 5, respectively. At 4 h, peak concentrations are seen for both the Chinese and the imported products, at 27.76 and 23.17 ng/ml, respectively. At 24 h, plasma levels decreased to 5.76 and 3.57 ng/ml, respectively.

The urine samples were collected and the volumes recorded at 12-h intervals after ingestion. Total urinary amiloride was measured by treating 0.2 ml of urine according to *Sample preparation*. The results are shown in Table II. Analysis of

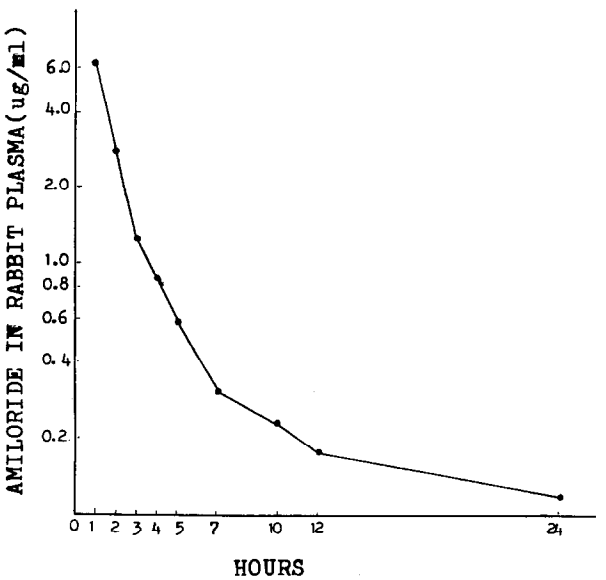

Fig. 3. Plasma concentration–time curve for amiloride following a 5-mg intravenous bolus to a rabbit.

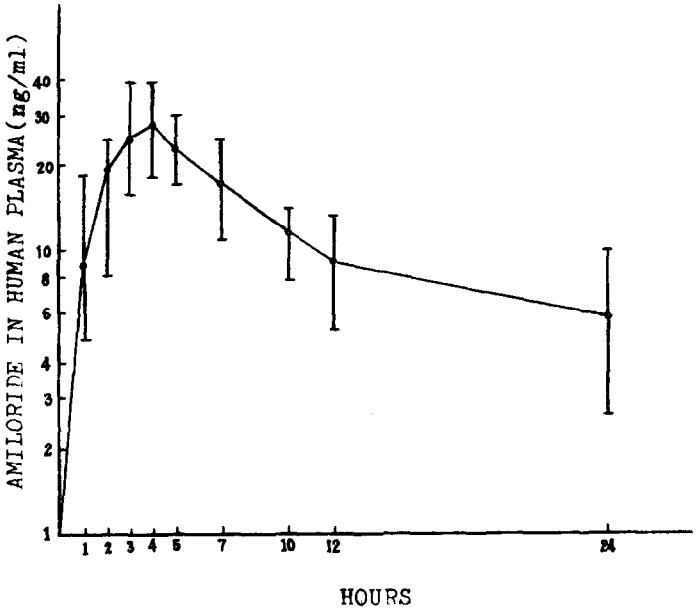

Fig. 4. Mean plasma levels of amiloride after oral administration of 10 mg of Chinese-produced amiloride to six human volunteers.

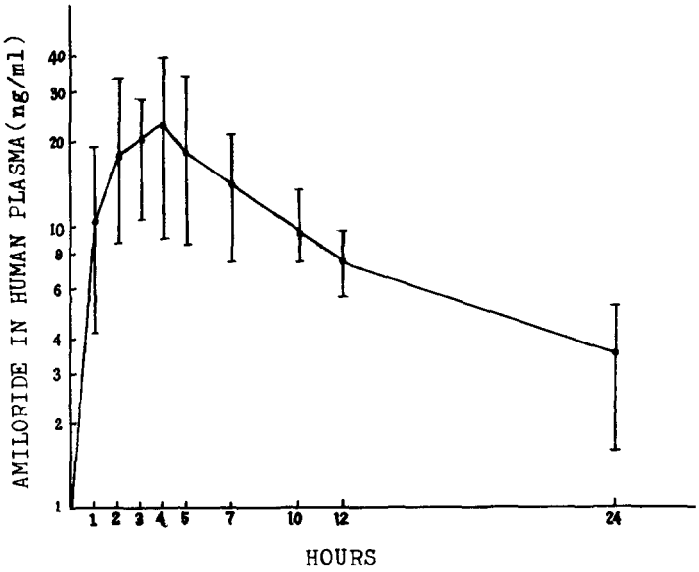

Fig. 5. Mean plasma levels of amiloride after oral administration of 10 mg of imported amiloride to six human volunteers.

TABLE II

EXCRETED AMOUNT OF AMILORIDE AFTER ADMINISTRATION TO SIX VOLUNTEERS

Subject	Chinese product			Imported product		
	0–12 h (mg)	12–24 h (mg)	24-h Excretion (%)	0–12 h (mg)	12–24 h (mg)	24-h Excretion (%)
1	3.63	0.88	45.1	4.04	0.94	52.4
2	4.68	1.42	61.0	1.97	1.46	34.3
3	0.65	0.08	7.3	1.08	0.78	18.7
4	4.89	1.46	63.5	1.95	1.55	35.0
5	0.96	0.13	10.9	1.45	2.10	23.4
6	4.79	1.06	58.5	1.71	1.70	34.1

the urinary samples from the six volunteers indicated that some amiloride was excreted unchanged. The average excretion rates in urine were 41.1% (Chinese products) and 33.0% (imported) of the oral amiloride dose in 24 h.

DISCUSSION

HPLC methods employing liquid–liquid extraction have been reported for determining amiloride levels in animal plasma and/or urine [4,6], but it has lower recovery and sensitivity because of the necessity for extraction and drying procedures. An HPLC method using a silica gel column has also been reported [5], but in this the aqueous mobile phase had to be saturated with silica and maintained strictly at pH 7 in order to prevent silica dissolving out of the column. Thus, this method lacked stability and was not easy to control.

The present method employs a reversed-phase C_{18} column and methanol–water as the mobile phase. Perchloric acid was added to mobile phase as an ion-pair reagent so that a sharp and well resolved peak of the drug was obtained, otherwise the drug could not be analysed on this column. In the preparation of plasma samples, acetonitrile or methanol was employed initially to precipitate proteins, but the resulting supernatant gave rise to numerous undesirable peaks, which interfered in part with amiloride quantitation. However, when perchloric acid was used as the deproteinizing, agent there were no interfering substances and the supernatant could be directly injected.

A fluorescence detector was used because of the lower concentration of the drug in plasma and because amiloride has native fluorescence. The excitation and emission wavelengths of amiloride were scanned, and values of 286 and 418 nm, respectively, were selected. It is an advantage of the method that the supernatant can be directly injected into the column with no concentration procedure. The analytical column has successfully been used to analyse *ca.* 200 samples of plasma and urine.

CONCLUSION

The method provided superior recovery and good precision, and it is simple and reliable in both its chromatographic conditions and sample preparation procedure. It has been used satisfactorily to analyse amiloride concentrations in rabbit plasma, as well as human plasma and urine.

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