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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMILORIDE IN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic method has been developed for amiloride in rabbit plasma and urine which uses a reversed-phase C_{18} column, a mobile phase (flow-rate 2 ml/min) consisting of 32% acetonitrile in 0.15 *M* perchloric acid, pH 2.2, and spectrofluorometric detection via excitation at 286 nm. A simple extraction step with ethyl acetate eliminates interfering peaks. Short retention times of about 2.3 and 3.8 min are observed for amiloride and the internal standard, triamterene, respectively. The method can measure 4 ng/ml amiloride in plasma. This assay has been used to explore the pharmacokinetics of amiloride in rabbits. The plasma disposition profile is biexponential after a 50-mg intravenous bolus dose and there is no evidence for saturable elimination at zero-order infusion rates of 1.8, 3.6 and 7.2 mg/h.

INTRODUCTION

Amiloride, a pyrazinecarbonylguanidine, is a potassium-sparing diuretic which exerts its effect by blocking distal tubular exchange of sodium, potassium and hydrogen, in the presence or absence of aldosterone [1, 2].

Amiloride is usually administered orally and from 15 to 26% of the dose is reportedly absorbed [3]. However, in two studies using ^{14}C-labeled drug, urinary and fecal collections for 72 h accounted for 90% of the administered compound [4, 5]. Reports on the distribution of amiloride as well as its elimination profile are few in number and also filled with controversy. Better studies are needed to clarify the disposition of this drug, particularly in patients with impaired renal function, who will undoubtedly exhibit a decreased renal

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clearance of the diuretic and thus may be disposed to adverse reactions unless properly dosed.

The primary reason for the lack of satisfactory pharmacokinetic data on amiloride is the absence of a suitable method for measuring amiloride in biological fluids. To this point the assays have included a fluorescence method [6] and measurement of radioactivity [5].

This report describes the development of a high-performance liquid chromatographic (HPLC) assay which has permitted us to examine the pharmacokinetics of amiloride in rabbits. The method has the required sensitivity and specificity to be used in human studies.

EXPERIMENTAL

Materials

Amiloride · HCl was supplied by Merck Sharp and Dohme (Rahway, NJ, U.S.A.). Triamterene, internal standard, was obtained from Smith, Kline & French (Montreal, Quebec, Canada). All chromatographic solvents were HPLC grade, and all other chemicals were analytical reagent grade.

Apparatus

An Altex Model 100A liquid chromatographic pump (Aviation Electric, Montreal, Canada) and a Rheodyne valve-loop injector fitted with a 175-μl loop were employed. A Spherisorb ODS 250 × 4.1 mm I.D. column (Altech, 10-μm particle size) was connected to a Schoeffel FS-970 fluorescence detector (Westwood, NJ, U.S.A.) whose excitation wavelength was set at 286 nm and which contained a KV370 emission cut-off filter. This filter transmits more than 99% and less than 1% of the incident light for wavelengths over 395 nm and under 345 nm, respectively. The mobile phase consisted of 32% acetonitrile in 0.15 *M* perchloric acid, pH 2.2 and was pumped at a constant flow-rate of 2.0 ml/min. The mobile phase was degassed by ultrasonication prior to use. The chromatograms were recorded on a potentiometric recorder (Westronics - MT 21, Fort Worth, TX, U.S.A.). All chromatography was carried out at ambient temperature.

Preparation of standard curve

Stock solutions of amiloride and the internal standard, triamterene, were prepared by dissolving the compounds in 1% (v/v) lactic acid. The plasma amiloride standard curve was prepared by diluting the above standard (300 μg/ml) with drug-free rabbit plasma so that 100 μl of plasma would correspond to a concentration range of 0.5—20 μg/ml. The urine amiloride standard curve was prepared similarly, yielding a concentration range of 10—50 μg/ml.

Sample preparation

After delivering 100 μl of a plasma sample to a clean, dry test tube, 1 ml ethyl acetate and 70 ng triamterene (10 μl) were added. While vortexing, 50 μl sodium hydroxide (5.0 *M*) were added. After vortexing a further 30 sec, the mixture was centrifuged at 3000 *g* for 2 min. Thereafter 0.8 ml of the upper organic layer was transferred to a second tube containing 0.5 ml hydro-

chloric acid (0.1 *M*). After vortexing again for 30 sec, the solution was subjected to a gentle nitrogen flow at 60°C in order to evaporate residual ethyl acetate and excess hydrochloric acid. An aliquot of the remaining solution (10—50 μl) was injected into the HPLC instrument.

Before being prepared for chromatography, all urine samples were first diluted ten-fold. Thereafter they were treated in an identical fashion to the plasma samples except that only 50 μl of the diluted urine was processed in conjunction with 105 ng triamterene.

Recovery and assay precision

The absolute recoveries of amiloride and triamterene from rabbit plasma and water were determined by extracting the agents using the aforementioned procedure and comparing the chromatograms to those obtained from an unextracted aqueous solution.

To determine the inter-assay precision for the plasma amiloride, six replicates at levels of 1.10 and 17.56 μg/ml were analyzed on different days. The inter-assay precision for urinary amiloride was determined in a similar way at concentrations of 1.61 and 18.25 μg/ml. These tests of precision were based upon calibration curves prepared on each day of the test.

The intra-assay precision was determined for the plasma and urine procedure by using six replicates of each of the above solutions. This test was based upon a single calibration curve constructed on the day of the test.

Quantitation

Standard curves for amiloride were constructed using peak height ratios obtained for the drug to the internal standard, versus the concentration of the drug. The concentration of amiloride in biological samples was determined by interpolation following linear regression of the standard curve.

Rabbit experiments

Intravenous amiloride was prepared by dissolving the drug in minimum 1% lactic acid with heating at 70°C and sonication. The solution was then adjusted to pH 3.5 with 5 *M* sodium hydroxide. Just prior to administration the solution was filtered (Swinex-25) and a portion retained to establish the exact dose administered.

New Zealand White male rabbits were weighed and placed in a restraining cage. Catheters (Angiocath, Deseret, Sandy, UT, U.S.A.) were placed in the marginal veins of each ear. The dose was administered in one ear while blood samples were obtained from the other. Urine samples were collected through a Foley catheter (No. 8 Fr., Acmi, Norcross, GA, U.S.A.) inserted into the bladder via the urethra.

Data analysis

The compartmental characteristics of the amiloride disposition in rabbits were established using standard pharmacokinetic procedures [7]. All computer fitting of data was performed on a Dec-10 Model 1090 computer using a nonlinear computer fitting program originally described by D'Argenio and Schumitzky [8].

RESULTS AND DISCUSSION

Reversed-phase HPLC has become a popular tool for measuring drugs in biological fluids. Since amiloride is a weak base (p*K*a = 8.7) [9] it appeared logical that ion-pairing would be needed to achieve satisfactory chromatography using C_{18} columns. It was found that perchloric acid retarded the elution of amiloride such that potential interfering components in the solvent front would be avoided. By experimentation it was found that the mobile phase described previously yielded sharp, well resolved peaks.

Among eleven compounds tested as potential internal standards, only 4-bromoantipyrine and triamterene appeared to be suitable. Triamterene was chosen as the internal standard because its properties were quite similar to amiloride and therefore small fluctuations in extraction technique or chromatography would not lead to noticeable changes in peak height ratios.

In the preparation of plasma samples, acetonitrile or acetone were employed initially to precipitate proteins. However, the resulting supernatant gave rise to numerous undesirable peaks which in part interfered with amiloride quantitation. Therefore an extraction method was developed. Of the ten organic solvents investigated, ethyl acetate provided superior recovery of amiloride while excluding interfering components. The volume and concentration of alkalinizing agent was also selected to optimize amiloride recovery. Finally, it was found that in the absence of hydrochloric acid in the evaporation step, amiloride concentrations less than 1 μg/ml yielded a large variation in recoveries. The evaporation step was needed to remove a volatile component which otherwise interfered with amiloride fluorescence detection.

Figs. 1 and 2 exhibit chromatograms which reflect the analysis of amiloride in rabbit plasma and urine, respectively. The retention times of amiloride and triamterene were about 2.3 and 3.8 min, respectively. No interfering peaks

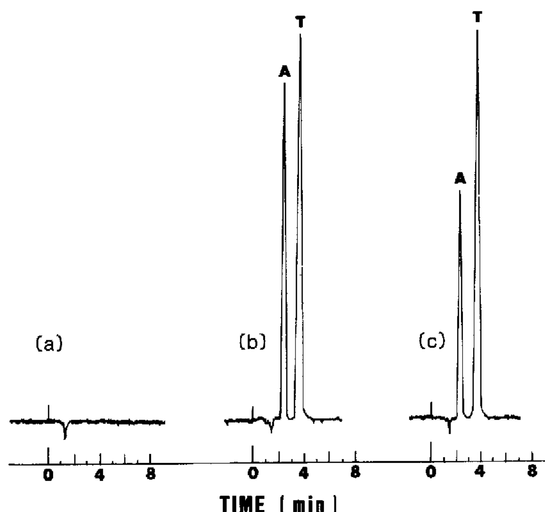

Fig. 1. Chromatograms obtained in the assay of plasma amiloride (A) using triamterene (T) as the internal standard. (a) Blank rabbit plasma, 0.05 ml injected; (b) blank rabbit plasma containing amiloride (2 mg/l) and triamterene (0.35 mg/l), 0.05 ml injected; and (c) plasma collected from a rabbit at 280 min during an intravenous infusion of 7.2 mg/h.

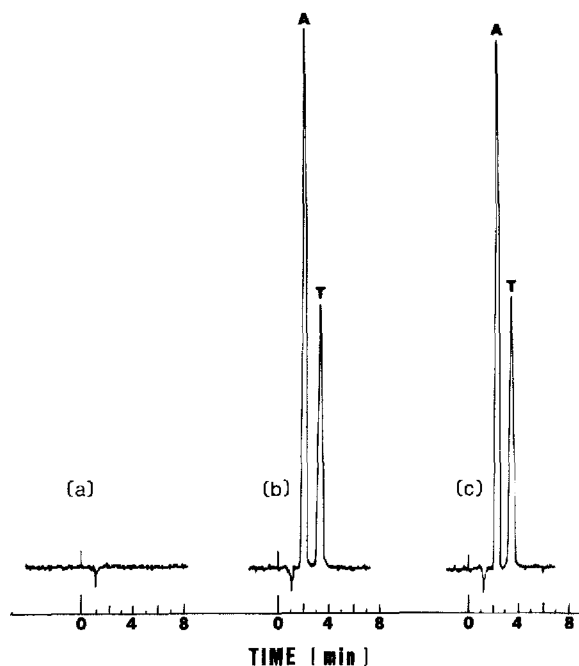

Fig. 2. Chromatograms obtained in the assay of urinary amiloride (A) using triamterene (T) as the internal standard. (a) Blank rabbit urine, 0.03 ml injected; (b) blank rabbit urine containing amiloride (40 mg/l) and triamterene (2.8 mg/l), 0.03 ml injected; and (c) urine collected from a rabbit during an intravenous infusion of 7.2 mg/h.

were observed using the aforementioned procedure for preparing biological samples. Calibration curves were linear over the concentration range used.

For the determination of amiloride in plasma (0.5—20 μg/ml) the average absolute recovery was found to be 65.9% with a 7.6% coefficient of variation. The corresponding results for the absolute recovery of amiloride from water were 71.5% and 7.0%, respectively. The absolute recoveries of triamterene at the standard concentration for plasma and water were 89.0% and 87.3%, respectively. Tables I and II present information on the inter- and intra-assay variability for the procedure. The small coefficients of variation confirm the reproducibility of the assay method.

The amiloride assay has been used to explore the pharmacokinetics of this

TABLE I

AMILORIDE INTER-ASSAY PRECISION (n = 6)

Sample	Theoretical concentration (mg/l)	Mean measured concentration (mg/l)	C.V. (%)
Plasma	1.10	1.19	5.43
	17.56	17.72	3.93
Urine	1.61	1.84	4.31
	18.25	18.39	1.79

TABLE II

AMILORIDE INTRA-ASSAY PRECISION (n = 6)

Sample	Theoretical concentration (mg/l)	Mean measured concentration (mg/l)	C.V. (%)
Plasma	1.10	1.18	2.46
	17.56	17.02	2.09
Urine	1.61	1.80	3.16
	18.25	17.88	2.64

agent in rabbits. The central purpose of these studies has been to examine drug interactions involving the renal disposition of selected compounds. The following preliminary data substantiate the applicability of the analytical method.

Rabbits receiving a 50-mg intravenous bolus of amiloride exhibited plasma disposition profiles as illustrated in Fig. 3. The biexponential disappearance curves yielded pharmacokinetic constants indicative of a two-compartment model as summarized in Table III. The distribution spaces observed were greater than the total blood volume (58—70 ml/kg [10]) in the rabbit, thereby indicating that the greater part of amiloride was located in extravascular sites. This finding is consistent with that noted in humans [4] and the dog [6]. From the urinary excretion of amiloride it was found that about 60% of the drug is removed renally by the rabbit. In order to test whether the two-compartment model adequately described the amiloride pharmacokinetics in rabbits, and to establish whether dose-dependent factors might become important, the agent was given as an infusion. On different days, three infusions (1.8, 3.6, and 7.2 mg/h) were preceded by initial loading doses of 7.5, 15, and 30 mg, respectively. The outcome of such experiments, as illustrated in Fig. 4, established the appropriateness of the two-compartment model, and the absence of saturable eliminating routes at the doses employed [7].

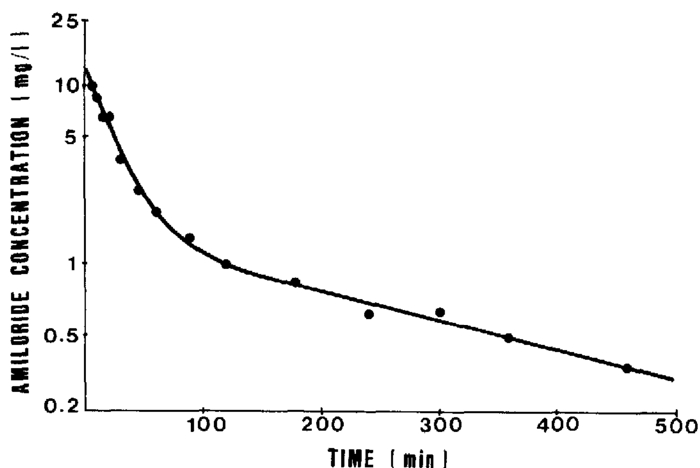

Fig. 3. Plasma amiloride concentrations observed in a rabbit (3.93 kg) following an intravenous bolus dose of 50 mg. The solid line represents a computer fit resulting in an equation, $C = 12.12e^{-0.0447t} + 1.83e^{-0.0036t}$.

TABLE III

FITTED AMILORIDE PHARMACOKINETIC PARAMETERS OBTAINED FROM FOUR RABBITS RECEIVING A 50-mg BOLUS DOSE

Parameter	Mean ± S.D.		Range	
Vc (ml/kg)	815.4	± 157.9	610.1	–954.4
α (min^{-1})	0.046	± 0.009	0.038	–0.058
β (min^{-1})	0.0040	± 0.0007	0.0036	–0.0050
k_{10} (min^{-1})	0.0184	± 0.0015	0.0165	–0.0200
k_{12} (min^{-1})	0.0218	± 0.0067	0.0166	–0.0313
k_{21} (min^{-1})	0.0099	± 0.0016	0.0082	–0.0116
$t½\,\alpha$ (min)	15.41	± 2.67	11.91	–18.38
$t½\,\beta$ (min)	176.5	± 25.7	138.6	–192.5
TBC (ml/min/kg)	15.01	± 3.43	11.65	–19.09

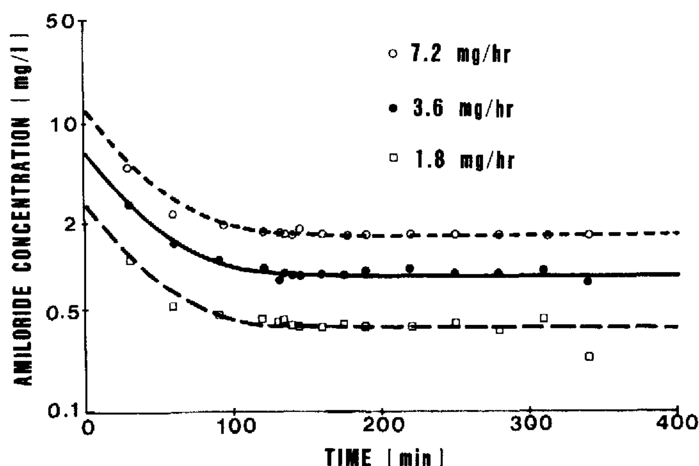

Fig. 4. Plasma amiloride concentrations observed in a rabbit on three different occasions following infusion of 1.8 (□ — — □), 3.6 (•—•), and 7.2 (○ — — ○) mg/h, preceded by loading dose of 7.5, 15 and 30 mg, respectively. The solid line is a computer fit while the other lines represent simulation based upon the fitted parameters of a two-compartment model (V_1 = 724.6 ml/kg, α = 0.0345 min^{-1}, β = 0.00149 min^{-1}, k_{21} = 0.00185 min^{-1}, weight = 3.31 kg).

The sensitivity of the HPLC assay was examined to determine its utility for human studies. In contrast to the aforementioned sample preparation method, 1 ml plasma, 6 ng triamterene, 5 ml ethyl acetate, and 0.5 ml sodium hydroxide were used. The final aqueous hydrochloric acid solution was reduced to about 100 μl. By injecting 50 μl, a measurable peak three times the baseline noise was observed for a plasma amiloride concentration of 4 ng/ml. Thus the assay could follow the pharmacokinetics of amiloride in man even after a 10-mg dose [11].

Clinically, amiloride has been used with other diuretics and beta-blockers. Some of these drugs have been tested with this assay to determine if they would interfere with the quantitation. Those tested were chlorothiazide, hydrochlorothiazide, furosemide, spironolactone, propranolol, and metoprolol. In each case, peaks for these drugs, if observed at all, did not interfere with

those of amiloride or triamterene. Further tests with metabolites of these compounds are yet to be done.

In conclusion, a new simple HPLC method has been developed for amiloride which is rapid, specific, and of requisite sensitivity to be used even in human studies.

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