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SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF AMILORIDE IN BODY FLUIDS

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SUMMARY

A simplified rapid high-performance liquid chromatographic (HPLC) procedure has been developed for the measurement of amiloride in plasma or urine. Solid-phase extraction columns provide quick, clean and simple sample preparation, allowing ten samples to be processed in less than 5 min. The HPLC system uses a standard reversed-phase (C_{18}) column with detection by ultraviolet absorption at 365 nm. The assay has been used to define plasma amiloride concentration–time profiles and to quantitate urine amiloride recovery in healthy men after repeated administration at two doses.

INTRODUCTION

Amiloride is a potassium-sparing agent which acts at distal tubular sites in the kidney to promote natriuresis and attenuate kaliuresis [1]. Little is known of its pharmacokinetics since reliable and convenient methods for measurement of the drug in body fluids have not hitherto been available. Existing procedures using ^{14}C-labelled compound [2,3], fluorometric analysis [4] or high-performance liquid chromatography (HPLC) [5] have lacked the sensitivity or specificity for precise estimation or the capacity to handle multiple samples. Recently, more

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practical HPLC techniques employing fluorescence detectors have been published [6,7].

This report describes a simple yet sensitive HPLC method using ultraviolet detection for the assay of amiloride in plasma and urine. We also present preliminary pharmacokinetic data in normal men.

EXPERIMENTAL

Materials and reagents

Amiloride and triamterene were provided by Ciba-Geigy Labs. (Horsham, U.K.). HPLC-grade acetonitrile was obtained from May & Baker (Dagenham, U.K.) and triethylamine from Sigma Labs. (Poole, U.K.). All other solvents were of analytical reagent grade. The Vac-Elut system and Bond Elut C_8 1-ml columns were supplied by Jones Chromatography (Llanbradach, U.K.). The centrifugal filters were obtained from Scotlab Instrument Sales (Bellshill, U.K.).

High-performance liquid chromatography

The solvent system was a 12% solution of acetonitrile containing 4 ml l^{-1} glacial acetic acid, with pH adjusted to 4.5 with triethylamine. This mobile phase was delivered by a Gilson 302 HPLC pump (Scotlab Instrument Sales) at a flow-rate of 2 ml min^{-1} via a Waters WISP autosampler. A standard reversed-phase stainless-steel column, 160 mm × 5 mm I.D., packed with Waters C_{18} 10-μm material slurry on a Shandon column packer (Shandon Southern Products, Runcorn, U.K.) was employed. Detection was by ultraviolet absorption at 365 nm using a Kontron UVICON 730 LS C spectrophotometer and the chromatogram was plotted on a Shimadzu CR 1 B recording integrator. The volume injected was 180 μl for plasma or 50 μl for urine.

Sample preparation

The solid-phase columns, one per sample or standard, were primed by washing through 2 ml methanol followed by 2 ml deionised water. Plasma (1 ml) (plus working standard) was added to the column reservoir followed by 300 μl (75 ng) internal standard. After drawing through to waste, the columns were rinsed with 2 ml water and dried by suction. Elution of the drug and internal standard was achieved by adding 200 μl of a solvent containing 50% mobile phase, 45% methanol and 5% glacial acetic acid to the reservoir. The eluent was drawn through under suction and collected for transfer to autosampler vials. Plasma samples stored for several weeks at −20 °C sometimes required brief centrifugation before assay.

Urine samples were injected directly after filtration through Bioanalytic systems 1-μm (MFI) regenerated cellulose centrifugal filters. This was possible because levels were sufficiently high to be measured without sample concentration and there were no interfering peaks.

Preparation of standard curve

Amiloride stock solution was 100 mg l^{-1} in methanol–water (50:50). The internal standard, 250 mg l^{-1} triamterene stock solution, was prepared similarly. Both were stored at −20 °C. Since amiloride is light-sensitive, working standard and internal standard solutions were prepared daily by diluting the stock solution 1:1000 with water in subdued light to give 100 and 250 ng ml^{-1}, respectively.

Drug-free control plasma was spiked with working standard to provide concentrations of 1–50 ng ml^{-1} (10 μl = 1 ng), and these were prepared as samples on the Bond Elut columns. A standard curve of the peak height versus concentration was drawn.

Drug-free urine was spiked with stock standard solution in the range 0.2–5 μl^{-1}, i.e. 100 μl in 50 ml to 2 ml urine. A standard curve was prepared in a similar manner to that for plasma.

Recovery and assay precision

The absolute recoveries of amiloride and triamterene from plasma were determined by comparing peak areas of assayed plasma to those of unextracted standard solution of the drugs at six concentrations (1–50 ng ml^{-1}). Ten samples of plasma at each concentration (1, 2, 5, 15, 30 and 50 ng ml^{-1}) and of urine at each concentration (1, 2 and 4 μg ml^{-1}) were assayed to determine the precision of the assay. This test was based on a single calibration curve.

Quantitation

Concentrations of amiloride in plasma were computed on the recording integrator. This was calibrated on a single mid-range standard using peak-height ratios of drug to internal standard concentration. Linearity was verified by testing a range of higher and lower standards, and comparing the computed results with the known values before any assay was undertaken. Every fifth sample was a known standard prepared in the same batch to control for any drift in detector sensitivity. As the urine samples were injected directly with no extraction, no internal standard was required. Therefore, the integrator was calibrated to measure absolute height rather than relative heights. Again standards were analysed at frequent intervals during each run of urine samples.

Human experiment

Twelve healthy male subjects were treated with oral amiloride, 5 mg and 20 mg in combination with 100 mg hydrochlorothiazide, once daily for eight days in a randomised, balanced, cross-over study. Treatments were separated by drug-free intervals of at least two weeks. Drugs were administered at 09:00 h with 150 ml water after a standard breakfast. Venous blood was taken from a forearm vein immediately prior to drug administration on day 1 and 8, and hourly from 10:00 h to 17:00 h on the eighth day. Samples were transferred immediately to lithium heparin tubes and plasma was extracted as soon as possible. Timed 24-h urine collections into acid (30 ml of 1 *M* hydrochloric acid) were made on the day before

drug ingestion and daily throughout the treatment periods. Aliquots were taken after measurement of volume. Plasma and urine samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Of the methods currently available for measuring amiloride, few are suitable for pharmacokinetic studies. The use of radiolabelled drug [3] is sensitive but lacks capacity. Yip et al. [5] employed a multiple-step solvent extraction with fluorescence detection for amiloride determinations; in experiments in rabbits, levels were about 500-fold higher than would be expected in man at therapeutic dose [2,3,5]. The methods described by Shi et al. [6] and Vincek et al. [7] represent advances but both employ fluorescence detectors which are not widely available in drug assay laboratories and a time-consuming evaporation step. Our starting point was an assay using more readily accessible ultraviolet detection and a column-switching trace-enrichment technique. This chromatographic system was suitable, but the column-switching device was time-consuming to operate manually and the direct injection of serum caused frequent blocking of both trace enrichment and analytical columns.

Solid-phase extraction techniques perform a similar function to trace-enrichment columns. They use sorbents of the same types as are used in analytical columns, but with a much greater particle size (usually 40 μm instead of 3–10 μm). The sorbent is held at the bottom of a syringe-like column between two inert frits. A sorbent is chosen which has a high affinity for the compound of interest – in this case, C_8 material was chosen. The plasma passes through the material, and the drug is bound to the sorbent.

The sorbent may the be washed with buffer or solvent to remove any impurities – in the case of amiloride, deionised water was sufficient. The drug is eluted from the sorbent in a very small volume (less than 500 μl for a 1-ml column) thus eliminating the laborious and costly task of evaporating off large volumes of organic solvent. The eluting solvent first tried was the mobile phase of the HPLC assay which eliminated the solvent front problems found in some assays. However, we found that triamterene (internal standard) was eluted in a smaller volume if the acetic acid concentration was increased. Polar interactions, particularly with unbonded silanol groups, are characteristic of silica-containing solid-phase extraction columns raising the possibility of a secondary mode of retention with triamterene. Blocking of the silanol groups with triethylamine and pH regulation with buffers led to no difference in recoveries.

Chromatograms which illustrate the analysis of amiloride in human plasma and urine are shown in Figs. 1 and 2, respectively. The retention times were 1.7 min for amiloride and 3.2 min for triamterene. There were no interfering peaks. Calibration curves were linear over the concentration ranges.

To assess the precision and accuracy of the assay, ten samples for each of six levels to embrace plasma amiloride concentrations of 1–50 ng ml^{-1} were analysed. Ten samples of standard and internal standard were also assayed for recoveries. The results are presented in Table I. The small coefficients of variation for the estimates of precision of the assay indicate its reproducibility. The average

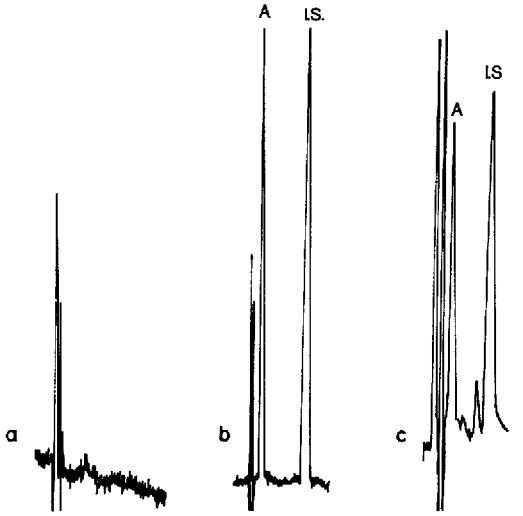

Fig. 1. Chromatograms obtained after a 180-μl injection of (a) blank plasma; (b) blank plasma containing 30 ng ml^{-1} amiloride (A) and 75 ng ml^{-1} triamterene (internal standard, I.S.); and (c) plasma 4 h after administration of 5 mg amiloride (A); consecutive daily dosing for eight days.

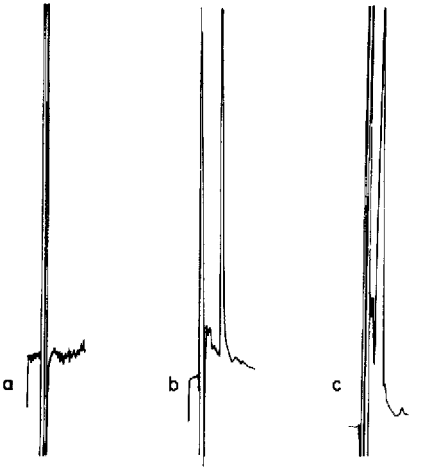

Fig. 2. Chromatograms obtained after a 50-μl injection of (a) blank urine; (b) blank urine containing 5 μg ml^{-1} amiloride; and (c) urine on day 4 of treatment with amiloride, 20 mg daily.

recovery of amiloride was 100.9% with a coefficient of variation of 4.6%. For triamterene, recovery averaged 64.9% with a coefficient of variation of 4.9%. The absolute recovery of amiloride from plasma was considerably greater than that reported in another recently described but much more complex HPLC method [5]. Recoveries of the internal standard were similar to those previously reported.

TABLE I

PRECISION OF ASSAY FOR PLASMA AMILORIDE CONCENTRATION

Also shown are results of recoveries from plasma.

Predicted concentration (ng ml^{-1})	Actual concentration (mean ± S.D., $n=10$) (ng ml^{-1})	Coefficient of variation (%)
1	1.02 ± 0.12	11.87
2	2.01 ± 0.11	5.63
5	5.05 ± 0.09	1.84
15	15.00 ± 0.19	1.26
30	30.52 ± 0.89	2.91
50	49.73 ± 1.72	3.45
Standard recovery (%)	100.9 ± 4.50	4.46
Internal standard recovery (%)	64.9 ± 3.19	4.92

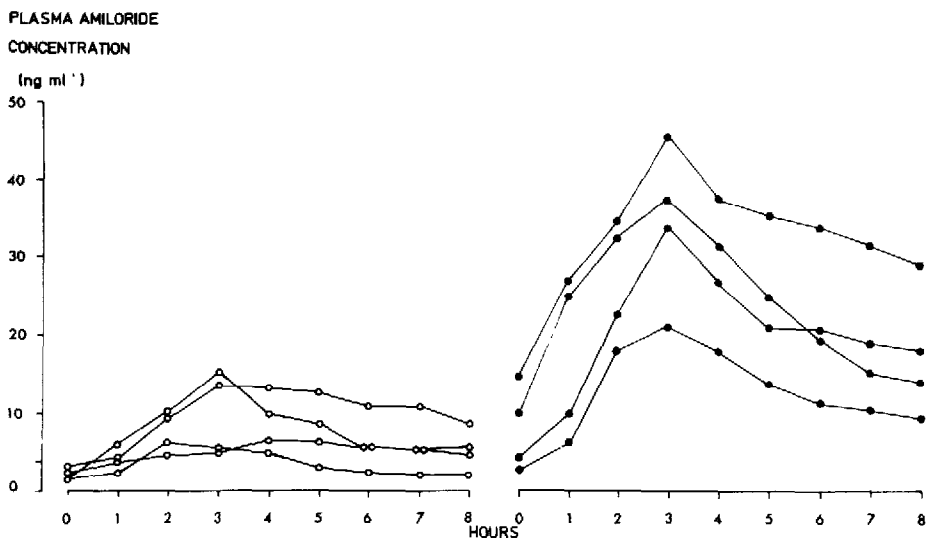

Fig. 3. Plasma concentration–time curves in four representative subjects at each dose of amiloride. (○) 5 mg; (●) 20 mg.

The recoveries of ten urine samples at concentration 1, 2 and 4 $\mu g\ ml^{-1}$ were 1.02, 2.04 and 3.98 $\mu g\ ml^{-1}$ with coefficients of variation of 2.43, 1.79 and 0.88%, respectively.

The assay was used to define plasma amiloride concentration–time profiles in healthy men after repeated administrations at two doses embracing the range usually employed in the clinic. Representative profiles are shown in Fig. 3. Plasma concentrations were directly proportional to amiloride dose and were approximately 500-fold lower than reported by HPLC following intravenous infusions in rabbits [5]. Circulating levels ranged from 0.5 to 47.6 ng ml^{-1}, and of 216 samples

TABLE II

MEDIAN AND RANGES FOR URINE AMILORIDE EXCRETION IN TWELVE HEALTHY MEN DURING REPEATED ADMINISTRATIONS OF 5 and 20 mg AMILORIDE

Subject No.	Amiloride excretion (mg per 24 h)			
	5 mg		20 mg	
	Median	Range	Median	Range
1	0.68	0.46–2.59	7.16	4.29–11.25
2	2.07	1.48–2.48	3.38	1.26– 5.26
3	1.05	0.91–2.09	5.00	3.50– 8.00
4	1.33	0.57–2.14	3.74	1.86–13.00
5	0.72	0.59–1.26	3.94	3.03– 6.04
6	2.22	1.47–3.29	9.65	6.90–12.20
7	0.73	0.52–1.07	5.97	3.79– 7.17
8	1.35	0.73–1.82	4.14	3.67– 7.17
9	0.90	0.73–2.46	9.58	4.95–13.94
10	0.51	0.35–1.60	2.55	0.48– 5.54
11	0.79	0.35–0.97	3.04	0.84– 5.09
12	0.96	0.61–1.87	3.54	1.22– 4.16

analysed only nine (4.2%) contained less than 2 ng ml^{-1}. The precision of the assay at concentrations 2–50 ng ml^{-1} is satisfactory for pharmacokinetic studies.

Results for urine amiloride excretion are shown in Table II. There was considerable inter-individual variability and variability within subjects from day to day but no consistent trend with time was seen. Mean urine recovery in the 24 h after dosing was 23.8% of the prescribed dose following 5 mg and 26.4% following 20 mg amiloride. Previous evidence from radiolabelled drug studies suggested 40–50% recovery in the 24 h after single doses of 20 mg [2,3]. Since urine radioactivity represents parent drug plus any metabolites formed, our findings suggest that amiloride undergoes significant biotransformation in man.

In this study, amiloride was administered with a thiazide diuretic, hydrochlorothiazide, a combination (Moduretic, Merck, Sharpe & Dohme) which is in common clinical use. As hydrochlorothiazide does not appear to interfere with the HPLC assay of amiloride [5], our data may be taken to reflect the pharmacokinetics of amiloride alone.

In conclusion, application of solid-phase extraction to HPLC for amiloride allows rapid sample processing and greatly simplifies an assay which was previously difficult and complicated. Nevertheless, the method retains high precision and reproducibility at the plasma and urine concentrations encountered in man after typical clinical doses, indicating a role in other pharmacokinetic studies.

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