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Note

Simultaneous determination of amiloride and hydrochlorothiazide in plasma by reversed-phase high-performance liquid chromatography

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Amiloride (N-amidino-3,5-diamino-6-chloropyrazine-2-carboxyamide hydrochloride), a potassium-sparing diuretic, is often used in combination with hydrochlorothiazide (6-chloro-3,4-dihydro-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide; HCTZ) to inhibit the loss of potassium associated with the thiazide diuretics (Fig. 1).

Numerous high-performance liquid chromatographic (HPLC) methods for the determination of HCTZ in biological fluids exist [1–9]. Of the methods tested, none were, however, capable of separating HCTZ from amiloride. Because of the low levels of amiloride present in plasma after therapeutic doses (5–10 mg), very sensitive methods are necessary when plasma level determinations are required. Published methods include thin-layer chromatography [10] and HPLC [11–13], all with fluorescence detection.

This report describes a new, sensitive, and reproducible reversed-phase HPLC technique for the simultaneous separation and quantitation of HCTZ and amiloride in plasma. The method was used to determine both compounds in plasma specimens obtained from a clinical trial in which the combination was administered to male volunteers.

EXPERIMENTAL

Apparatus and reagents

Analyses were performed on a modular HPLC system consisting of a loop injector (Model C6UN60, Valco Instruments, Houston, TX, U.S.A.), a Perkin-

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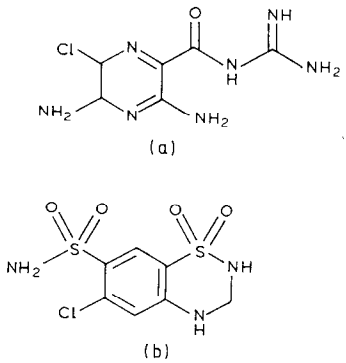

Fig. 1. Structures of amiloride (a) and HCTZ (b).

Elmer solvent delivery system (Model 3B) and fluorescence detector (Model 650-10S) (Perkin-Elmer, Norwalk, CT, U.S.A.), and a variable-wavelength detector (Model 450, Waters Assoc., Milford, MA, U.S.A.). Chromatograms were recorded on two integrators (Model C-R3A Chromatopac, Shimadzu, Kyoto, Japan and Model 3390, Hewlett-Packard, Avondale, PA, U.S.A.).

HCTZ, amiloride and hydroflumethiazide (HFM) were obtained from our departmental reference substance collection. Acetonitrile and ethyl acetate were HPLC grade (Rathburn, Walkerburn, U.K.). All other chemicals were reagent grade (E. Merck, Darmstadt, F.R.G.). Water was purified by passing through a Milli-Q filtration system (18 MΩ cm resistance, Millipore, Bedford, MA, U.S.A.).

Chromatographic conditions

Reversed-phase separations were performed at ambient temperature by means of a 125 × 4.6 mm Spherisorb ODSII (5 μm particle size) column (Bischoff, Leonberg, F.R.G.), using a mobile phase of acetonitrile–methanol–tetraethylammonium phosphate (TEAP) buffer (pH 2.8) (10:9:100). The buffer was prepared by dissolving 15.54 g of tetraethylammonium hydroxide and 2.9 g of orthophosphoric acid (89%) in water and diluting to 500 ml. The mobile phase was filtered (Millipore, Durapore, 0.45 μm) and degassed under reduced pressure before use. The flow-rate was 1.2 ml/min, and the analytical column was protected by a 2-μm in-line frit (Waters Assoc.).

The fluorescence detector wavelengths were 368 nm (excitation) and 415 nm (emission), with 10 nm slit widths for both. The UV detector was set at 271 nm. Both integrators were set at an attenuation of 2 mV full scale.

Standards and extraction procedure

The internal standard solution contained 1 mg HFM in 100 ml water. Calibration curves were prepared by spiking blank plasma with standard aqueous solutions of HCTZ (100 μg/ml) and amiloride (3.2 μg/ml) to cover the range 0–500 ng/ml HCTZ and 0–20 ng/ml amiloride. An aliquot (1 ml) of plasma was transferred to a Quickfit B19 15-ml, glass-stoppered tube (Jobling, Staffordshire, U.K.). After the addition of internal standard solution (25 μl) and 1 ml of buffer (1 *M*

TABLE I

INTER-ASSAY ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMILORIDE AND HCTZ IN PLASMA

Analyte	Theoretical concentrations (ng/ml)	Concentration found (ng/ml)	n	Coefficient of variation (%)
Amiloride	21.2	19.6	9	6.26
	10.8	10.1	11	7.18
	5.23	4.96	11	11.0
	2.09	2.32	9	6.53
	1.05	1.20	9	15.1
HCTZ	572	559	10	4.36
	291	288	10	2.26
	141	133	11	5.56
	56.2	53.1	11	5.85
	28.4	27.2	11	14.6

sodium carbonate–bicarbonate, pH 10), each sample was extracted with 5 ml of ethyl acetate by vortexing for 1 min. The tubes were then centrifuged (1250 g) for 5 min and the ethyl acetate layer transferred to 5-ml glass ampoules prior to evaporation at 45 °C under nitrogen. The residue was redissolved in 100 μl mobile phase, and 50 μl were injected onto the HPLC column.

Drug recovery and limit of determination

Recovery was determined by comparing the peak heights from extracted samples without internal standard with those obtained from a direct injection of the same amount of drug in methanol.

The limit of determination was accepted as being twice the concentration at which the coefficient of variation for the determination of spiked samples was equal to 50%.

Precision and stability

The precision of the assay procedure was continuously monitored during the period of assay of the biological samples (26 days) by including quality contol specimens containing known concentrations of the analyte. These in vitro quality control samples were stored under identical conditions (−20 °C) to actual trial samples (Table I). Additional blood was drawn from all volunteers at predetermined times on the first day of the trial, and each sample was divided into seven aliquots. After analysing one aliquot of each sample, four that contained the analyte at concentrations spanning the expected range were selected and reanalysed at different times during the course of the analytical study. These ex vivo quality control samples were stored under identical conditions (−20 °C) to actual trial samples (Table II).

TABLE II

STABILITY OF AMILORIDE AND HCTZ IN PLASMA STORED AT −20°C FOR 26 DAYS

A, B, C and D are ex vivo quality control samples. In all cases $n = 4$.

Analyte	Day	Concentration found (ng/nl)			
		A	B	C	D
Amiloride	1	3.38	7.38	12.5	14.6
	14	4.38	8.36	10.1	14.6
	19	4.17	8.55	13.3	15.6
	26	4.05	8.42	12.7	15.8
Mean		4.00	8.18	12.2	15.2
Coefficient of variation (%)		10.8	6.57	11.6	4.23
HCTZ	1	98.9	163	332	459
	14	109	161	334	446
	19	111	161	386	505
	26	118	163	334	446
Mean		109	162	347	464
Coefficient of variation (%)		7.22	0.71	7.60	6.04

Kinetic study

The kinetic study, approved by the Ethics Committee of the Medical Faculty of the University of the Orange Free State, was conducted on ten healthy non-smoking male volunteers.

Two oral formulations from different manufacturers (MSD and Adcock-Ingram), each consisting of 50 mg HCTZ and 5 mg amiloride, were given to the volunteers after an overnight fast in a cross-over, placebo-controlled design, with

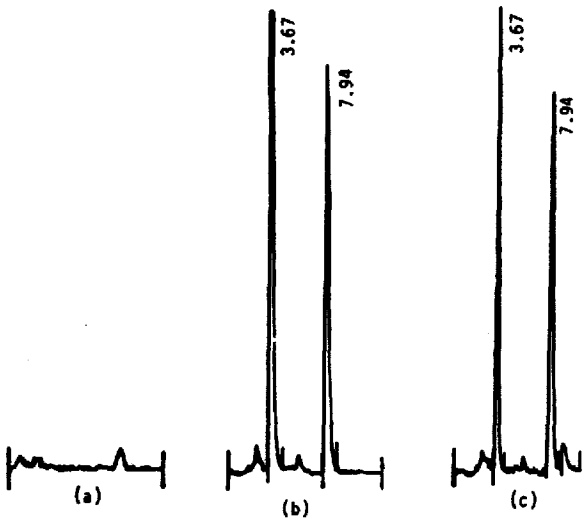

Fig. 2. Chromatograms (fluorescence detection) of extracts from blank plasma (a), plasma spiked with amiloride (20 ng/ml) and hydroflumethiazide (b), and plasma from a trial subject to which HFM has been added (c).

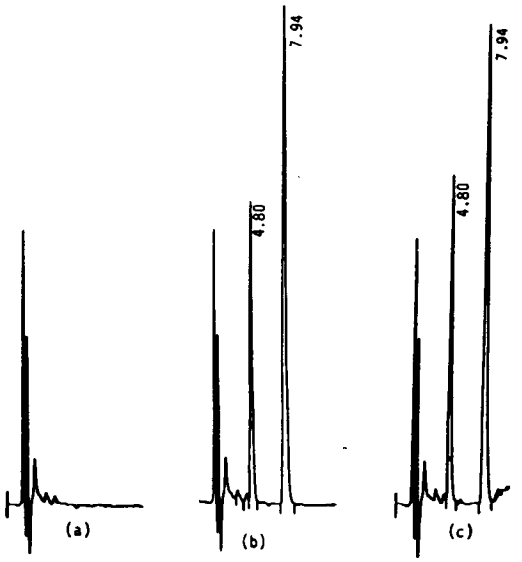

Fig. 3. Chromatograms (UV detection) of extracts from blank plasma (a), plasma spiked with HCTZ (232 ng/ml) and hydroflumethiazide (b), and plasma from a trial subject to which HFM had been added (c).

one week wash-out period between dosage administrations. Plasma samples were collected before drug administration and at 20, 40, 60, 80 and 100 min and 2, 3, 4, 6, 8, 10, 24 and 30 h after drug administration. Blood samples were centrifuged immediately and plasma stored at −20 °C. Plasma amiloride and HCTZ concentrations were determined as described above, and the mean plasma levels for each drug from both formulations are depicted graphically in Fig. 4.

Pharmacokinetic parameters measured were the maximum concentration (C_{max}), the time to reach C_{max} (T_{max}) and the area under the concentration–time profile (AUC).

RESULTS AND DISCUSSION

To date, no published methods for the simultaneous determination of amiloride and HCTZ in plasma could be found. HCTZ has no native fluorescence and was therefore determined by UV absorption at 271 nm. The low levels of amiloride in plasma after therapeutic doses (5–10 mg) could not be detected by UV absorption with the equipment at our disposal. The strong fluorescence of amiloride, however, permitted quantitation at these low levels.

Using the described assay conditions, baseline resolution between HCTZ, HFM and amiloride was achieved. In Figs. 2 and 3, the chromatograms of blank plasma extracts (a) show that no interfering peaks are presents at the retention times of HCTZ, HFM or amiloride. Typical chromatograms of spiked plasma extracts (b) and plasma samples from a volunteer (c) show that the three components appear as sharp, symmetric peaks with retention times of 3.67 min (amiloride), 4.8 min (HCTZ) and 7.94 (HFM). The calibration curves of HCTZ/HFM and

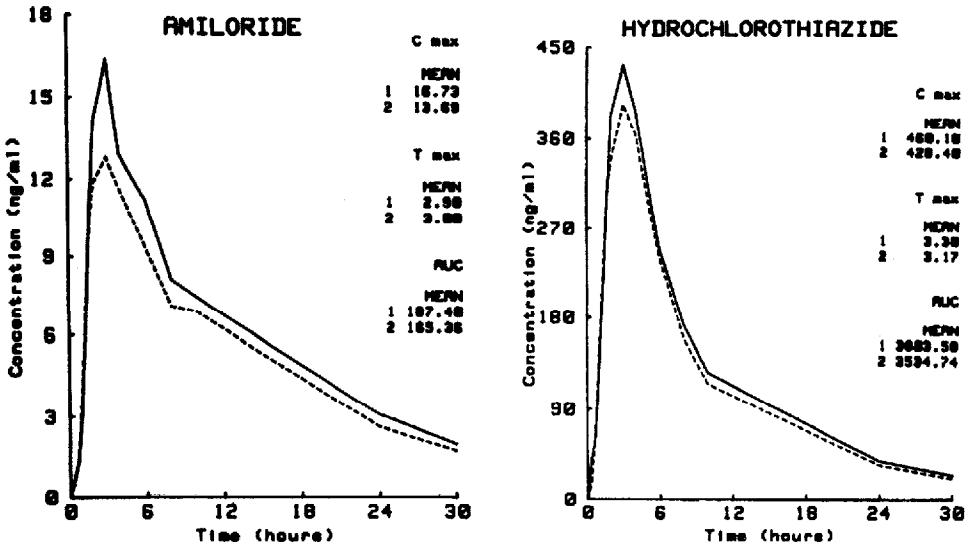

Fig. 4. Mean plasma concentration–time profiles of ten volunteers after simultaneous administration of 10 mg amiloride and 100 mg HCTZ from two formulations; (—) formulation 1; (---) formulation 2.

amiloride/HFM peak-height ratios versus concentration are linear over the ranges 10–550 ng/ml for HCTZ ($r^2=0.9985$, $y=0.004+0.002x$) and 0.5–20 ng/ml for amiloride ($r^2=0.9988$, $y=-0.045+1.01x$).

The detection limits of the proposed method were 10 ng/ml for HCTZ and 0.5 ng/ml for amiloride, which were more than adequate for measuring plasma levels after therapeutic doses of the two compounds.

Table I depicts the accuracy and precision of the method as determined over a period of 25 days.

No evidence of decomposition of either HCTZ or amiloride in plasma stored at −20°C was observed over a period of 26 days, as can be seen from the results in Table II.

Graphical representations of mean plasma concentration–time profiles ($n=10$) are depicted in Fig. 4. The AUC was calculated by the linear trapezoidal rule between the first and last concentration–time data points. C_{max} and T_{max} values were obtained directly from the individual observed concentration versus time profiles. For amiloride, the mean values were: $C_{max}=15.2$ ng/ml; $T_{max}=2.95$ h; AUC = 176 ng/ml h. For HCTZ the mean values were: $C_{max}=444$ ng/ml; $T_{max}=3.24$ h; AUC = 3669 ng/ml h.

The results obtained indicate that this method for the simultaneous determination of amiloride and HCTZ in plasma is accurate and sufficiently sensitive to follow the plasma concentrations for 30 h after a single dosage and rapid enough for routine determination of therapeutic concentrations in plasma.

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