Amiloride: Biological Fluid Analysis by Reverse-Phase HPLC

William C. Vincek[2], Gene A. Hessey II[1,3], Marvin L. Constanzer[1], and William F. Bayne[1]

Received: November 17, 1984; accepted: January 23, 1985.

Abstract: Amiloride is an effective antikaliuretic-diuretic which has been used clinically since 1965. Because of a lack of sensitivity in reported analytical methods, useful pharmacokinetic data have been sparse. This publication describes a sensitive (1 ng/ml), rapid, and reproducible reverse-phase HPLC technique for the quantitative measurement of amiloride levels in both serum and urine specimens. Sample clean-up is based on the adsorption of amiloride to commercially available silica cartridges and its selective elution with perchloric acid. The eluted drug is further resolved from endogenous interferences on a Waters' C-18 μBondapak analytical column with a mobile phase consisting of methanol:sodium perchlorate (0.1 M, pH 4.0) 40:60 v/v and fluorescence detection (λ_{ex} = 368 nm, λ_{em} = 417 nm). This method has been applied to the analysis of human serum and urine samples.

Amiloride is a pyrazinecarbonylguanidine (Fig. 1) possessing both potassium-sparing and diuretic activity. It has been used clinically since 1965 (1), and

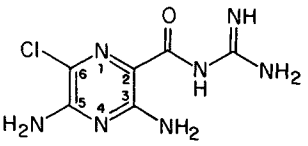

Fig. 1 Amiloride

its metabolism has been studied both in animals (2) and in man (3). Typically, biological fluid analysis was performed either by liquid-liquid extraction and fluorescence detection (2) or by scintillation counting (3–6). Because of the low levels of amiloride present in blood after therapeutic doses (5 to 10 mg amiloride hydrochloride, single oral dose), the only techniques with sufficient sensitivity for serum analysis are scintillation counting and thin-layer chromatography with fluorescent detection (7). The purpose of this report is to describe a new, sensitive (1 ng/ml of serum), rapid, and reproducible reverse-phase HPLC technique for the determination of amiloride levels in serum and urine specimens.

Materials and Methods

Chemicals and Reagents

The water was purified by passing it through a Milli-Q filtration system (18 MΩcm resistivity: cartridge sequence: Super-C, Ion-Ex, Ion-Ex, Organex-Q). Methanol and acetonitrile (HPLC grade) were purchased from Burdick and Jackson. The hydrochloric acid (concentrated), perchloric acid (70–72 %) and sodium perchlorate (HPLC grade) were purchased from Fisher Scientific. The control human sera were purchased from Sera-Tec Biologicals. The drug-free human urine was obtained from MSDRL personnel. The 'Baker-10' extraction system was purchased from J. T. Baker, and the silica extraction cartridges (3 ml) were purchased from either Analytichem International or J. T. Baker. The amiloride analytical standard, 3,5-diamino-*N*-(aminoiminomethyl)-6-chloro-2-pyrazinecarboxamide hydrochloride dihydrate, and internal standard (IS), 3,5-diamino-*N*-(aminoiminomethyl)-6-fluoro-2-pyrazinecarboxamide sesquihydrate, were obtained from the sample repository, MSDRL, Rahway, New Jersey. All standards were prepared in 1:1 v/v methanol: hydrochloric acid (0.01 N) weekly. Stock standards (1.0 mg/ml) of amiloride and IS were stable at 5 °C for at least one month. Working standards of amiloride were prepared at concentrations of 50, 125, 250, 500, and 1250 ng/ml. A working standard solution of IS was prepared at 0.2 μg/ml. All standards were maintained at 5°C when not in use.

Pipets, Instrumentation, and Chromatographic Conditions

Adjustable Pipetman P1000 and P20, in addition to an Eppendorf multipette with 0.5, 5.0, 12.5, and 50.0 ml combitips, were used for critical and/or repetitive solution transfers. The HPLC system consisted of a Waters Model 720 system controller, a Waters Model 6000A pump, and a Waters Model 710B automatic sampler. Peak quantitation was performed by a Hewlett-Packard 3390A integrator with the following parameters: chart speed: 0.5 cm/min; peak width: 64; threshold: 2; attenuation: 2; area reject: 1000; report-peak height ratio (ISTD); and retention times of 4.80 min (IS) and 5.75 min (amiloride) or with an HP 3357-LAS with similar parameters. Peak height was chosen over peak area as the mode of peak quantitation because of the higher precision attained in replicate analyses. The detector was a Perkin-Elmer 650-10S fluorescence spectrophotometer with a Xenon power supply Model 150. The detector settings were as follows: excitation: 368 nm, emission: 417 nm; slit-width: 5 nm; sensitivity-range: 10, fine: 3.5, and response mode: slow. The analytical column was a Waters C-18 μBondapak 10 μm (30 cm x 3.9 mm). The guard column was purchased from Bio-Rad (ODS, 10 μm, 3 cm). The mobile phase consisted of sodium perchlorate (0.1 M, adjusted to pH 4.0 with perchloric acid) and methanol in a volumetric ratio of 60:40, respectively. The mobile phase components were filtered separately, mixed, then degassed by sparging with helium. The flow rate was 1.0 ml/min.

Procedure

Serum/Urine – Clinical Samples

To a disposable culture tube (13 x 100 mm) was added serum/urine (1.0 ml), working IS solution (0.050 ml) and methanol:hydrochloric acid (0.01 N), 1:1 (0.020 ml). The solution was mixed (10 sec, SMI multitube vortex). To a silica cartridge (3 ml) attached to a 'Baker-10' extraction system under vacuum was added acetonitrile (1 ml, activates the cartridge) and water (1 ml). The vacuum was released and the serum/urine solution (0.535 ml) was added to the cartridge. To the cartridge placed under vacuum was added water (2 ml), acetonitrile (1 ml), water (1 ml), sodium perchlorate (2.0 M, pH 4,

[1] Merck Sharp and Dohme Research Laboratories, West Point, PA 19486
[2] Smith Kline & French Laboratories, Philadelphia, PA 19101
[3] Correspondence to be addressed to G. A. Hessey II, MSDRL, W26-372

0.250 ml) and the cartridge maintained under vacuum for an additional 1 minute. The vacuum was released, and collection tubes (10 × 75 mm) were positioned beneath the cartridges. To the cartridge placed under vacuum, acetonitrile (for serum – 0.5 ml, for urine – 1.0 ml) was added and the eluate collected. The collected serum eluate was evaporated at 40°C to a volume of ~0.05 ml (~25 min) under a stream of nitrogen. To the residue was added sodium perchlorate (2.0 M, pH 4.0, 0.2 ml). The tube contents were mixed first by sonication (2 min), then by shaking on an SMI multitube vortex. An appropriate volume of the aqueous layer was then injected into the HPLC system. Samples whose expected concentrations were ≤5 ng/ml were injected in a 0.100 ml volume, while those whose expected concentrations were >5 ng/ml were injected in a 0.020 ml volume. The collected urine eluate was mixed (10 sec, vortex) and injected into the HPLC system.

Serum/Urine – Standard Curve

The standard curve range was from 1.0 to 25.0 ng/ml serum or from 0.1 to 5.0 μg/ml urine. To a disposable culture tube (13 x 100 mm) was added serum/urine (1.0 ml), working standard solution (0.020 ml) and working IS solution (0.050 ml). The subsequent steps were identical to the clinical sample procedure.

Serum/Urine – Quality Control Standards

Quality Control (QC) standards were prepared at 2.5 and 10 ng/ml serum or 0.25 and 2.5 μg/ml urine. An appropriate volume of working standard solution was mixed with serum/urine and aliquots (1.25 ml) frozen at −15 ± 5° C.

Results and Discussion

The crux of this assay is in the use of perchlorate to form an ion-pair with amiloride (8). Since amiloride adsorbs strongly to silica, many solvents such as methanol, acetonitrile, dimethylformamide, dioxane, and phosphoric, acetic, or hydrochloric acids, may be used to 'clean-up' the biological sample without causing the release of amiloride from the silica. Once the 'clean-up' has been performed, the addition of sodium perchlorate to the wash creates an amiloride-perchlorate ion-pair which is eluted by the subsequent addition of acetonitrile (0.5 to 1.0 ml).

As an alternative to the extraction method previously described, several deproteinizing schemes were considered for the preparation of serum samples. Precipitating agents ranging from organic solvents to strong inorganic acids were added to provide >99 % protein removal prior to injection. While the chromatographic results were acceptable in most cases, the resultant sample dilution precluded detection of sample concentrations ≤5 ng/ml. Another deproteinizing technique attempted was ultrafiltration with membranes of varying pore size (Amicon Corp.). Once again, chromatographic interference was minimal and sufficient resolution was attained. However, to achieve the desired sensitivity, a concentration step was still needed.

Representative chromatograms of subject serum and urine samples are shown in Figures 2 and 3, respectively. It can be seen in the figures that amiloride and IS are resolved from each other and from endogenous interferences. Other diuretic or diuretic/uricosuric agents, such as hydrochlorothiazide (eluted in the void volume, not detectable) and

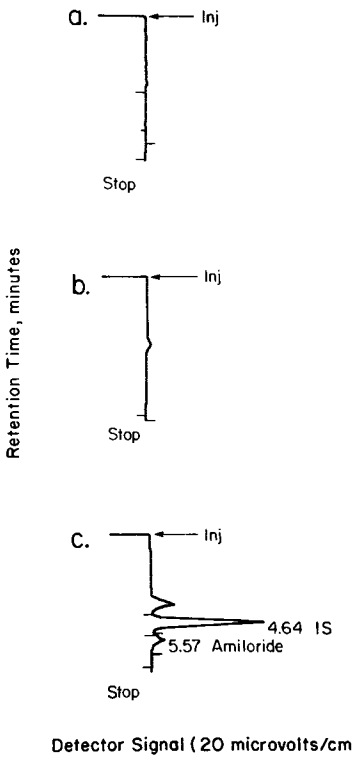

Fig. 3 **a** Control urine blank
b Subject urine blank
c Subject specimen post-10 mg dose (48–72 h, 0.08 μg/ml)
T_r IS = 4.64 minutes

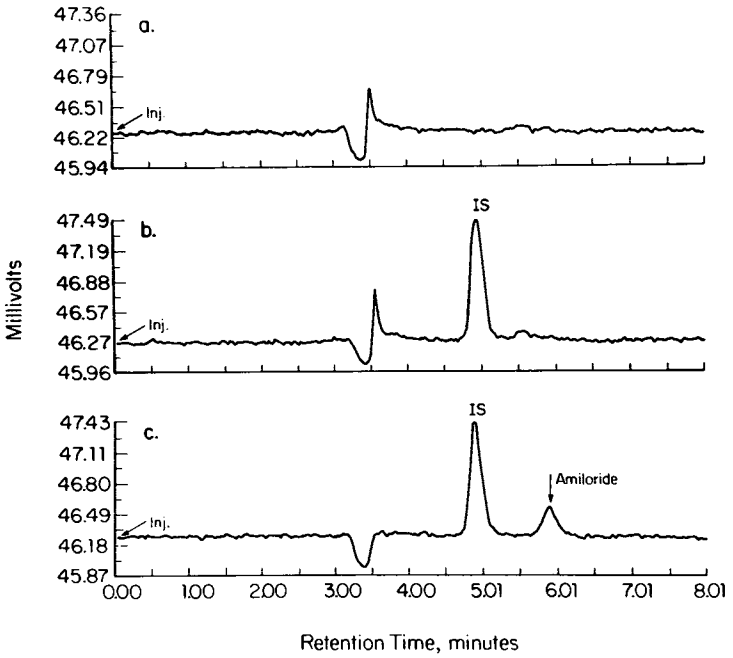

Fig. 2 **a** Control serum blank
b Subject serum blank (IS present)
c Subject specimen post-10 mg dose (1 h, 1.2 ng/ml)

chlorothiazide (void volume, not detectable) do not interfere with the analysis of amiloride, and there are no reported metabolites of amiloride. Standard curves (Fig. 4) were linear (r^2 >0.999) over the range of 1.0 to 25.0 ng/ml serum or 0.1 to 5.0 μg/ml urine. Standards (n = 6) at each point of the standard curve were assayed within 1 day to assess assay reproducibility. The

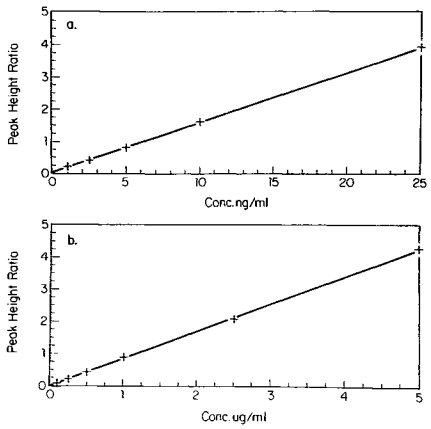

Fig. 4a A representative standard curve of amiloride in serum.
b A representative standard curve of amiloride in urine.

intra-day assay results are shown in Table I. Inter-day assay variability was assessed with the QC standards (Table II). At this time, the lack of long-term analysis of serum samples has precluded inter-day assay variability assessment with serum QC standards. The recovery of amiloride from spiked serum and urine samples was determined by a comparison of a direct injection of the corresponding standard. The drug

Table I. Intra-Day Assay Reproducibility (n = 6).

Serum ng/ml	CV*, %
1.0	3.7
5.0	1.8
25.0	2.7
Urine μg/ml	**CV, %**
0.025	7.7
0.1	1.4
1.0	1.2
5.0	0.4

* CV = coefficient of variation

Table II. Urine Inter-Day Assay Variability (n = 14).

Nominal μg/ml	Found μg/ml*	CV, %
0.25	0.25	5
1.0	0.99	2

* Mean over 7 days of duplicate analysis.

recovery from serum at 1.0, 5.0, and 25.0 ng/ml was 72.1 ± 4.2, 71.4 ± 2.6, and 65.9 ± 3.2%, respectively. The recovery of amiloride from urine samples at 0.1, 1.0, and 5.0 μg/ml was 62.0 ± 0.8, 66.8 ± 1.3, and 67.6 ± 0.6%, respectively. The recovery of the IS was 81.4 ± 2.4% from serum and 56.0 ± 1.2% from urine samples.

The described method is the first reported high performance liquid chromatographic procedure for the analysis of amiloride in serum samples at the 1 ng/ml limit of detection. The profile of amiloride in the serum of a subject dosed with 10 mg of amiloride hydrochloride is shown in Figure 5 (see reference 3 for human plasma profile generated by scintillation counting technique). Because of the sensitivity and reliability of this method, it will be possible to study the pharmacokinetic behavior of amiloride without the use of radiolabeled drug and with the speed and reliability of an HPLC technique.

Yip et al. have recently reported an HPLC method for the measurement of amiloride in rabbit plasma and urine following a 50 mg intravenous bolus dose (9). The method of Yip et al. (9)

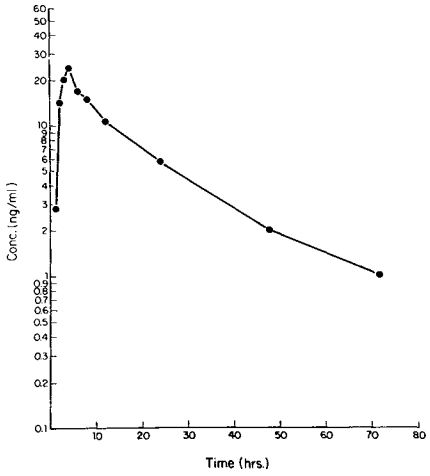

Fig. 5 The amiloride serum profile of a subject who was dosed with amiloride hydrochloride (10 mg).

employs a multi-step liquid-liquid extraction scheme in comparison to the liquid-solid extraction previously described. Since both methods involve an evaporation step, there is presumably no appreciable difference in sample preparation time. The HPLC conditions of Yip et al. (9) provide slightly shorter retention times [T_r amiloride = 2.3 min, T_rIS (triamterene) = 3.8 min] primarily because of an increase in mobile phase flow rate (2 ml/min). Otherwise, both HPLC systems utilize C-18 analytical columns, isocratic conditions, and fluorometric detection. The 50 mg i. v. dose studied by Yip et al. is in excess of therapeutic doses administered to man (5 to 10 mg amiloride hydrochloride, single oral dose). This excess is even more disparate when considering amount per unit body weight. Yip et al. (9) achieved a detection limit of 4 ng/ml, bearing a signal-to-noise ratio of 3.0. However, because of the large doses administered, it was sufficient to validate the plasma assay to a lower limit of approximately 1.0 μg/ml [intra-assay precision (n = 6) at 1.1 μg/ml, C.V. = 2.5%]. In contrast, because of the low levels of amiloride present in human sera after therapeutic doses, it was necessary to validate the assay described herein to a limit of 1 ng/ml [intra-assay precision (n = 6), C.V. = 3.7%]. Thus, the described assay provides a suitable method to ascertain the pharmacokinetics of amiloride following various dosage regimens.

References

(1) Moukhecbir, N. W., Kirkendall, W. M. (1965) Clin. Res. 13, 425.
(2) Baer, J. E., Jones, C. B., Spitzer, S. A., Russo, H. F. (1967) J. Pharmacol. Exp. Ther. 157, 472–485.
(3) Weiss, P., Hersey, R. M., Dujovne, C. A., Bianchine, J. R. (1969) Clin. Pharm. Ther. 10, 401–406.
(4) George, C. F. (1980) Br. J. Clin. Pharmacol. 9, 94–95.
(5) Grayson, M. F., Smith, A. J., Smith, R. N. (1971) Br. J. Pharmacol. 43, 473–474.
(6) Smith, A. J., Smith, R. N. (1973) Br. J. Pharmacol. 48, 646–649.
(7) Reuter, K., Knauf, H., Mutschler, E. (1982) J. Chromatog. 233, 432–436.
(8) Burns, D. T., Hanprasopwattana, P. (1980) Anal. Chim. Acta 118, 185–189.
(9) Yip, M. S., Coates, P. E., Thiessen, J. J. (1984) J. Chromatog. 307, 343–350.