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Note

High-performance liquid chromatographic assay of basic amine drugs in plasma and urine using a silica gel column and an aqueous mobile phase

I. Amiloride

ROBERT J.-Y. SHI, LESLIE Z. BENET and EMIL T. LIN*

Department of Pharmacy, University of California, San Francisco, CA 94143 (U.S.A.)

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Amiloride hydrochloride, a pyrazinecarbonylguanidine, is a potassium sparing diuretic used alone or in combination with hydrochlorothiazide as an antihypertensive agent. Pharmacokinetic and bioavailability studies of amiloride in plasma and/or urine have been done using fluorometry [1], radioactivity measurement [2] and densitofluorometry [3]. A high-performance liquid chromatographic (HPLC) method using ethyl acetate extraction and fluorescence detection has recently been reported [4].

For this study, an increasingly popular technique using a bare silica gel column run with an aqueous mobile phase to separate the drug and biological fluid interference peaks, was employed. The use of an aqueous mobile phase on an unmodified silica gel column has been utilized to determine procainamide [5, 6], quinidine [7, 8], prifinum [9] and lidocaine [10] in plasma and to separate some basic amine drugs in water solutions [11—15]. However, since there are several potential risks to this approach, it has often been avoided. This is because the aqueous mobile phase, particularly at a pH above 8, dissolves the silica gel and consequently distorts column efficiency [16]. This occurs even with the use of a precolumn to saturate the mobile phase. For the assay of basic amines in biological fluids, however, this technique has been very useful. Since, according to the proposed mechanism, only basic amines are retained on the silica gel column [14], very clean chromatograms and a much improved signal-to-noise ratio can be obtained. The drug can also be detected at low nanogram levels simply from deproteinized plasma. This paper demonstrates these advantages.

A simple, selective and sensitive HPLC method for the quantitation of amiloride in plasma and urine is presented here. The sample preparation method for plasma requires only a protein precipitation step. Using fluorescence detection, the sensitivity was 0.5 ng/ml for 0.2 ml of plasma.

EXPERIMENTAL

Materials

Amiloride hydrochloride (USPC, Rockville, MD, U.S.A.) and quinidine hydrochloride (Sigma, St. Louis, MO, U.S.A.) were commercially obtained. Acetonitrile was HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Water was purified through a nanopure apparatus (Barnstead, Boston, MA, U.S.A.). All other reagents were analytical or reagent grade.

Apparatus

The HPLC system consisted of a pump (Model 110, Beckman, San Jose, CA, U.S.A.), an automatic sample processor (WISP 710B, Waters Assoc., Milford, MA, U.S.A.), a 5-μm Ultrasphere Si column, 25 cm × 4.6 mm (Beckman), a fluorescence detector (Model RF-530, Shimadzu, Columbia, MD, U.S.A.) and an integrator (Model 3390A, Hewlett-Packard, Santa Clara, CA, U.S.A.).

Chromatographic conditions

The mobile phase was prepared by mixing 3.96 g of dibasic ammonium phosphate in 1600 ml of water and adding 2400 ml of acetonitrile, then adjusting the pH to 7.0 by adding concentrated phosphoric acid. The solvent was degassed and filtered before use. The flow-rate was 1.0 ml/min and the fluorescence detector was set at 355 nm for excitation and at 410 nm for emission.

Sample preparation

Aliquots of acetonitrile containing the internal standard quinidine sulfate (0.5 ml) were added to 0.2-ml plasma samples. The mixtures were each vortexed for 1 min and centrifuged at 1000 *g* for 10 min. The clear supernatant was evaporated to about 200 μl under nitrogen. Aliquots (75 μl) were injected onto the column, and urine samples (0.2 ml) were diluted with 0.2 ml of mobile phase containing quinidine sulfate. The mixtures were then vortexed for 1 min, and aliquots (5 μl) were injected onto the column.

RESULTS

Typical chromatograms for blank plasma (urine) and for plasma (urine) spiked with drug and internal standard are presented in Figs. 1 and 2. The retention times for amiloride and internal standard were approx. 7.5 and 10.5 min, respectively. Calibration curves using eight different concentrations (0.5–15 ng/ml) for the plasma assay and seven different concentrations (0.1–2.0 μg/ml) for the urine assay were obtained by plotting the ratio of the peak height of amiloride to that of the internal standard versus amiloride concentra-

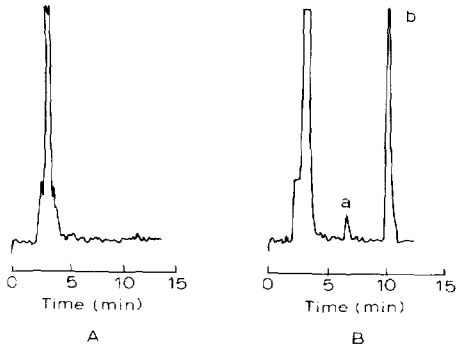

Fig. 1. HPLC profiles of (A) blank plasma and (B) plasma spiked with 1 ng/ml amiloride hydrochloride (a) and quinidine sulfate, internal standard (b).

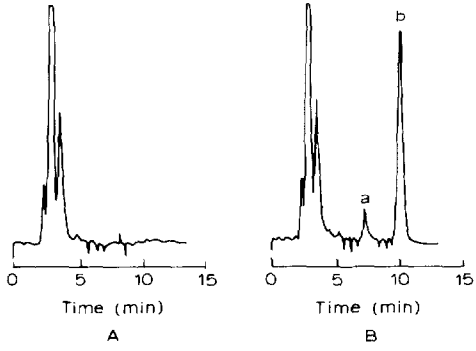

Fig. 2. HPLC profiles of (A) blank urine and (B) urine spiked with 0.1 μg/ml amiloride hydrochloride (a) and quinidine sulfate, internal standard (b).

TABLE I

LINEARITY OF REPRESENTATIVE CALIBRATION CURVE

Plasma			Urine		
Spiked concentration (ng/ml)	Peak-height ratio	Calculated concentration (ng/ml)	Spiked concentration (μg/ml)	Peak-height ratio	Calculated concentration (μg/ml)
0.5	0.065	0.63	0.1	0.053	0.10
1	0.102	1.03	0.2	0.116	0.20
2	0.211	2.21	0.4	0.229	0.40
4	0.387	4.11	0.8	0.454	0.78
6	0.537	5.73	1.2	0.710	1.21
8	0.728	7.80	1.5	0.880	1.50
12	1.082	11.6	2.0	1.180	2.00
15	1.433	15.4			
Intercept	0.0074		−0.0055		
Slope	0.0923		0.5912		
r^2	0.9978		0.9998		

TABLE II

PRECISION OF AMILORIDE HYDROCHLORIDE ASSAY IN PLASMA AND URINE

Spiked concentration (ng/ml)	Inter-day precision			Intra-day precision		
	Calculated concentration (mean ± S.D., $n = 6$) (ng/ml)		Coefficient of variation (%)	Calculated concentration (mean ± S.D.) (ng/ml)		Coefficient of variation (%)
Plasma						
10	10.5	0.350	3.33	10.5	0.514	4.30
5	4.98	0.155	3.11	4.91	0.249	5.07
3	2.90	0.082	2.84	3.02	0.068	2.27
1	1.11	0.068	6.15	1.14	0.070	6.12
Urine						
1.50	1.54	0.050	3.25	1.00	0.062	3.88
1.00	1.02	0.021	2.06	1.06	0.027	2.55
0.50	0.50	0.010	2.00	0.55	0.021	3.81
0.20	0.22	0.008	3.64	0.21	0.008	3.81

tion. Over these concentration ranges the standard curves were linear for both plasma and urine assay with $r^2 > 0.9970$ (Table I).

Precision and accuracy

Method precision over the entire working concentration range was determined by analysis of spiked samples. The inter- and intra-day precision at four different plasma concentrations are presented in Table II. Average coefficients of variation for the method were less than 10% at all concentrations. Recovery was over 90%.

DISCUSSION

The reversed-phase system (alkyl-bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique for the assay of drugs in biological fluids. In this system, the retention mechanism depends mainly on the lipophilic property of the substances to be analyzed. Thus, considerable amounts of various lipid substances in biological fluids (particularly blood, which will be extracted with drug in deproteinized solution) will also be retained on the column and interfere with the drug peaks.

On the other hand, in the system consisting of bare silica gel with an aqueous mobile phase, where the retention mechanism results mainly from ion-exchange characteristics and only partially from lipophilic properties [14], endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column. Only the cationic ammonium ions (such as basic amine drugs) will be retained. The interfering substances in biological fluids are eluted at the solvent front, leaving a very clean baseline around the drug's retention time, as shown in Fig. 2. In this method, the mobile phase was first treated by recycling through a short silica gel column overnight and then was kept

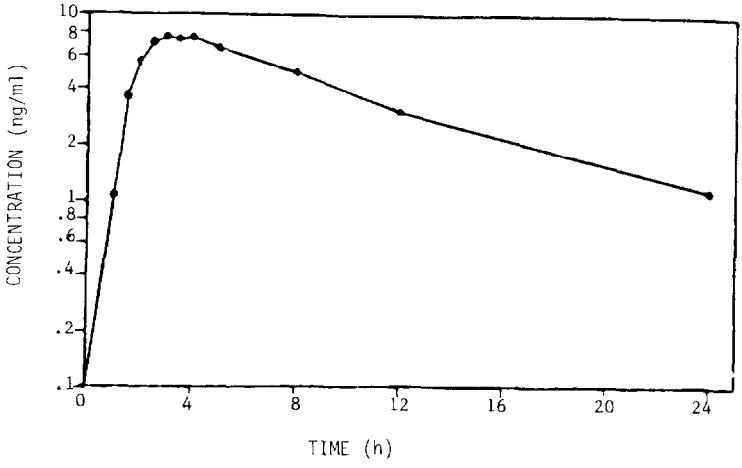

Fig. 3. Amiloride plasma concentration—time profile for one human volunteer given a 5-mg oral dose in fasting state.

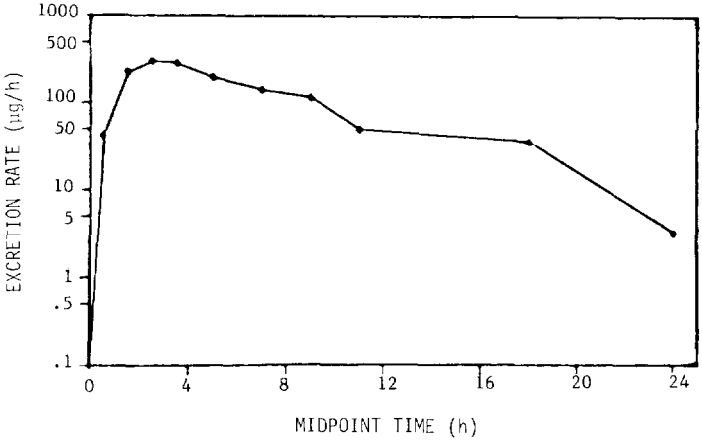

Fig. 4. Amiloride urinary excretion rate versus midpoint time of the collection interval for one human volunteer given a 5-mg oral dose in the fasting state.

recycling while the samples were assayed. No changes in column efficiency were noted.

Recycling the mobile phase may be beneficial to the whole system since the silica gel slowly dissolves in neutral aqueous solution and the water flowing through the silica gel approaches the equilibrium concentration of silica [16]. This recycled saturated mobile phase does not dissolve silica from the column and the degradation of silica will be decreased relative to the single-pass system. During recycling, the pH of mobile phase should be maintained at 7. A higher pH value lengthens the drug's retention time, but quickly dissolves the silica; a lower pH value shortens the drug's retention time and distorts the separation, although it stabilizes the column. Increasing the temperature of the mobile phase rapidly degrades the silica column [16], therefore necessitating that the system be run at room temperature. Under the conditions described above, the column was stable and column efficiency remained

constant throughout 400 injections of deproteinized plasma samples, or alternatively, 800 injections of urine samples simply diluted with mobile phase.

Taking advantage of amiloride's native fluorescence, this HPLC method had a detection limit of 0.5 ng/ml for plasma and 0.1 μg/ml for urine, using 0.2 ml of sample. This method has been utilized in bioavailability studies involving over 800 biological samples. Owing to the method's simplicity, sensitivity, accuracy and precision, reliable measurements of amiloride concentrations were obtained for plasma and urine samples collected for up to 24 h following a 5-mg oral dose. This is exemplified in Figs. 3 and 4, where plasma concentrations and urinary excretion rates of amiloride are depicted for one normal volunteer.

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