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Note

High-performance liquid chromatographic determination of amiloride and its analogues in rat plasma

Q.C. MENG*, Y.F. CHEN and S. OPARIL

Hypertension Program, Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294 (U.S.A.)

and

E.J. CRAGOE

211 Oak Terrace Drive, Lansdale, PA 19446 (U.S.A.)

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Amiloride is a guanidine derivative which has been used as a potassium-sparing agent in conjunction with diuretic therapy in the treatment of a variety of disorders, including ascites secondary to hepatic cirrhosis [1–3], congestive heart failure [4] and systemic hypertension [5,6]. Amiloride and related compounds are also useful to the cell physiologists concerned with elucidating the molecular mechanisms of Na^+ and H^+ translocation across membranes [7]. A number of analogues of amiloride which are highly selective for various ion channels and transporters, particularly Na^+ channels and the Na^+/H^+ exchanger, have been synthesized for use in research [7,8] (Fig. 1). Amiloride and its analogues have been used experimentally as probes to examine the role of Na^+ channels and the Na^+/H^+ exchanger, in the control of volume and blood pressure in the whole animal [9,10] and of intracellular pH and Na^+ concentration in cultured cells [11,12]. The unavailability of a convenient, sensitive and specific method for quantitating amiloride and its analogues in biological fluids limits the usefulness of these studies. Assay techniques currently available for amiloride include fluorescence methods [13], radioactive-tracer techniques [14] and high-performance liquid chromatography (HPLC)

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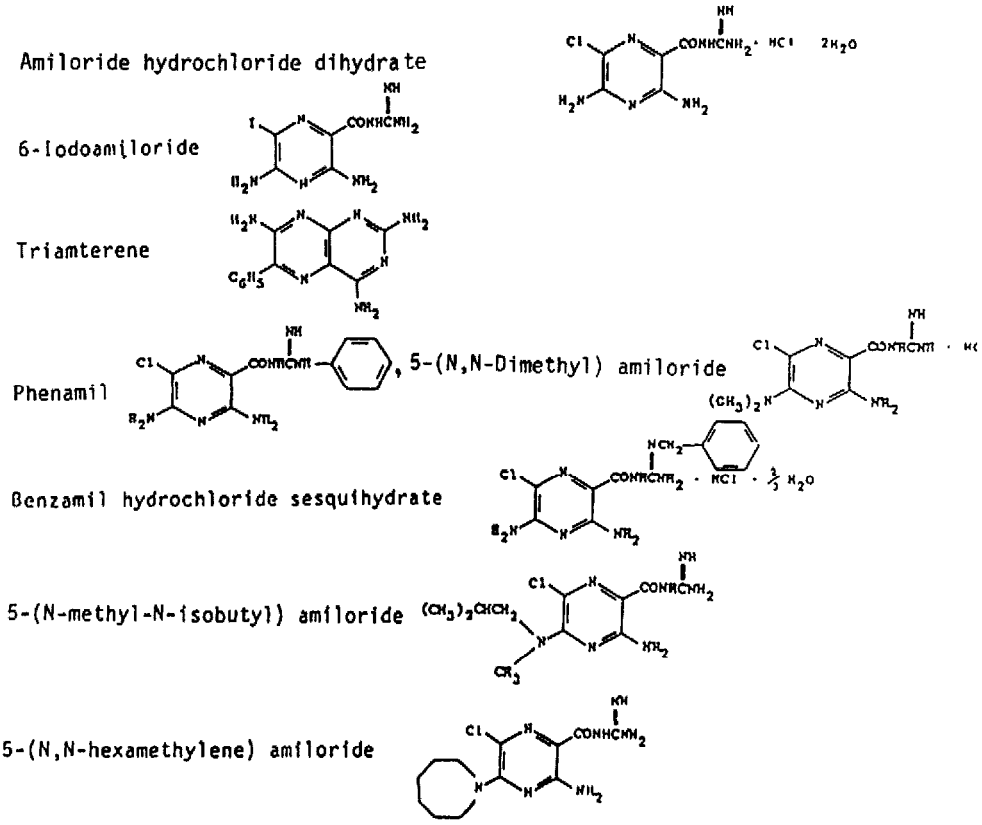

Fig. 1. Structures of amiloride analogues.

with fluorescence detection [15]. Most of these methods are complex and cumbersome or require expensive and specialized equipment and are therefore not suitable for measuring amiloride and its analogues in biological fluids.

The current study describes an HPLC method with ultraviolet (UV) detection which separates all of the commonly used amiloride analogues under isocratic conditions with acetonitrile in perchloric acid on a C_{18} reversed-phase column. The procedure is simple, does not involve a large investment in equipment or time and allows for good sample throughput. This method is useful for studying the pharmacokinetics of amiloride and its analogues in vivo.

EXPERIMENTAL

Materials

Amiloride analogues were obtained from Dr. Edward J. Cragoe, Jr. (Oak Terrace Drive, Lansdale, PA, U.S.A.); triamterene, the internal standard for

these studies, was obtained from Sigma (St. Louis, MO, U.S.A.). All chromatographic solvents were HPLC grade and all other chemicals were analytical-reagent grade.

Rat plasma

Two days before the experiment, polyethylene (PE-10 fused with PE-50) catheters were inserted into the femoral vein and artery of 300-g male spontaneously hypertensive rats for drug administration and blood sampling, respectively. On the day of the acute experiment, rats received either a 10-min intravenous infusion of 5-(N,N-hexamethylene)amiloride (HMA) (5 μg/kg) dissolved in 100 μl of 1% (w/v) dimethylsulfoxide (DMSO) or an infusion of an equal volume of 1% (w/v) DMSO vehicle. Prior to (time 0) and 15, 30, 45, 60, 90, 120, 150 and 180 min after HMA administration, 300 μl of blood from the arterial catheter were collected in a heparinized tube for HMA measurement. Blood was replaced volume for volume with donor rat blood. Plasma was separated by centrifugation at 1500 *g* for 20 min at 4°C and stored at −80°C until analyzed.

Sample preparation

After delivering 100 μl of plasma to a clean, dry test tube, 1 ml ethyl acetate, 50 μl sodium hydroxide (5 *M*) and 5 ng triamterene (10 μl) (internal standard) were added. The mixture was mixed on a vortex-mixer for 30 s, then centrifuged at 3000 *g* for 2 min, and 0.8 ml of the upper organic layer was transferred to a tube containing 0.5 ml of 0.1 *M* hydrochloric acid in order to transfer the amiloride analogues and internal standard from the organic phase to the aqueous phase. After vortex-mixing again for 30 s, the solution was dried under nitrogen at 60°C in order to evaporate the residual ethyl acetate and excess hydrochloric acid. The residue was lyophilized overnight to dryness and then reconstituted in 0.25 ml of mobile phase; 200 μl of this were injected into the HPLC system

Standard solutions

Stock solutions of the amiloride analogues (Fig. 1) and triamterene were prepared separately in the optimal mobile phase described below at a concentration of 200 mg/ml and stored in the refrigerator for up to two weeks. On the day of assay, serial dilutions of stock solution were prepared in the mobile phase. Six calibration standards over the range $1.6 \cdot 10^{-9}$ to $3.2 \cdot 10^{-8}$ *M* were used. Calibration curves were linear over the concentration range used. All standard solutions were injected in a volume of 200 μl. In order to test for interference from the DMSO vehicle, 1 ml control rat plasma was mixed with 10 μl DMSO and extracted using the same procedures; 200 μl of this were injected into the HPLC system.

HPLC measurement

HPLC studies were performed with a Waters system, which consisted of a Waters M501 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), a Waters WISP 710 automatic injector and an Alltech Adsorbosphere ODS 250 mm × 4.6 mm I.D. (5 μm particle size) column (Alltech Assoc., Deerfield, IL, U.S.A.) maintained at a constant temperature (35°C) with a BAS-LC-23A column heater (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A tunable absorbance detector (Waters Chromatography Division, Milford, MA, U.S.A.) operated at 361 nm and a Hewlett-Packard HP-3390A integrator (Avondale, PA, USA) were employed. Mobile phases containing 10, 20, 30, 40 and 50% (v/v) acetonitrile in 0.15 *M* perchloric acid, pH 2.2, were prepared to study the effect of the acetonitrile concentration of the mobile phase on the capacity factor (k') of amiloride analogues. From these preliminary studies, a concentration of 48% (v/v) acetonitrile in 0.15 *M* perchloric acid was determined to give optimal resolution of the various amiloride analogues and was used to measure the concentration of HMA in plasma samples. The retention behavior (R) of the amiloride analogues described in Fig. 3 are 0.62, 0.53, 0.42, 0.36, 0.18 and 0.17, respectively, under these conditions.

In order to validate the HPLC procedure, two approaches were used to establish that the compound extracted from rat plasma was authentic HMA. (1) The retention time of the peak following plasma extraction was compared to that of the HMA standard and was found to be identical. Plasma samples were spiked with HMA standard (2 μg per sample), resulting in increased amplitude of a single peak. (2) Vehicle (DMSO, 1%, w/v) control samples injected into the HPLC system did not give rise to peaks with the same retention time as HMA, indicating that the injection medium did not contain compounds which coelute with HMA.

Data analysis

Chromatography peaks for the amiloride analogues and triamterene were identified by retention times and standard addition protocols [14,16]. All of the detected species were assayed by measuring the chromatographic peak heights. The recovery of HMA from plasma was calculated using the internal standard. Plasma levels of HMA were determined using calibration curves prepared daily. Variability was expressed as standard error of the mean (S.E.M.). Coefficients of variation were determined for inter-assay and intra-assay variation.

RESULTS AND DISCUSSION

Fig. 2 illustrates the effects of altering the acetonitrile concentration of the mobile phase on the chromatographic behavior of amiloride and its analogues. These results indicate that altering the acetonitrile concentration of the mo-

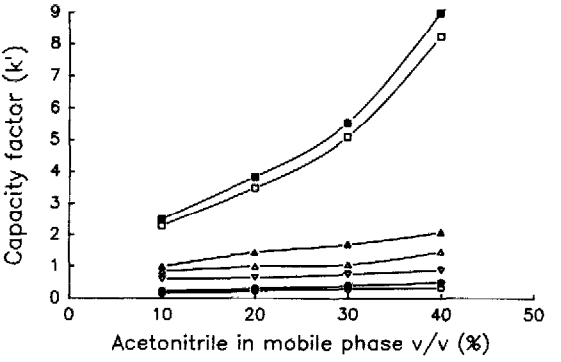

Fig. 2. Effects of acetonitrile content in the mobile phase (in vol. %) on k' values. Column, Alltech Adsorbosphere, 250 mm × 4.6 mm I.D.; flow-rate, 1.1 ml/min; temperature, 35°C; Samples: (○) amiloride hydrochloride dihydrate; (●) 6-iodoamiloride; (▽) triamterene; (△) phenamil; (▲) benzamil hydrochloride sesquihydrate; (□) 5-(N-methyl-N-isobutyl)amiloride; (■) 5-(N,N-hexamethylene)amiloride.

bile phase is effective in changing the solvent selectivity, as shown by the exponential increase in k', the capacity factor, as acetonitrile content is increased. The change in k' with alterations in acetonitrile concentration was abrupt with 5-(N-methyl-N-isobutyl)- and 5-(N,N-hexamethylene)-amiloride, while the other amiloride analogues showed only a gradual increase in k' at low acetonitrile concentrations. This result indicates that the partition ratio of solutes in this chromatographic condition can be drastically affected by the polarity of the mobile phase. The separation of standard preparations of several substituted analogues of amiloride and triamterene under optimal conditions (48%, v/v acetonitrile in 0.15 *M* perchloric acid) is illustrated in Fig. 3a. All of the amiloride analogues studied could be resolved under these conditions except phenamil and 5-(N,N-dimethyl)amiloride, which exhibited identical retention behavior (peak 4). DMSO did not give rise to an interfering peak, nor did it alter the retention behavior of any of the amiloride analogues studied. Plasma to which DMSO alone had been added did not give rise to interfering peaks (Fig. 3b).

Accurate measurements of plasma levels of amiloride and its analogues are needed in order to interpret studies in which these compounds are introduced into experimental animals and their pharmacologic effects are examined. This information is critical because amiloride and its analogues have multiple mechanisms of action, blocking Na^+ channels, the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger [7]. It has been reported that the native amiloride molecule at low concentrations (0.1–1 μM) blocks the conductive Na^+ channel and at medium (1–1000 μM) and high (0.3–1.1 mM) concentrations blocks the Na^+/H^+ and Na^+/Ca^{2+} exchangers (Table I). Aryl or arylalkyl substitutions on the terminal guanidine nitrogen of the amiloride molecule (e.g.

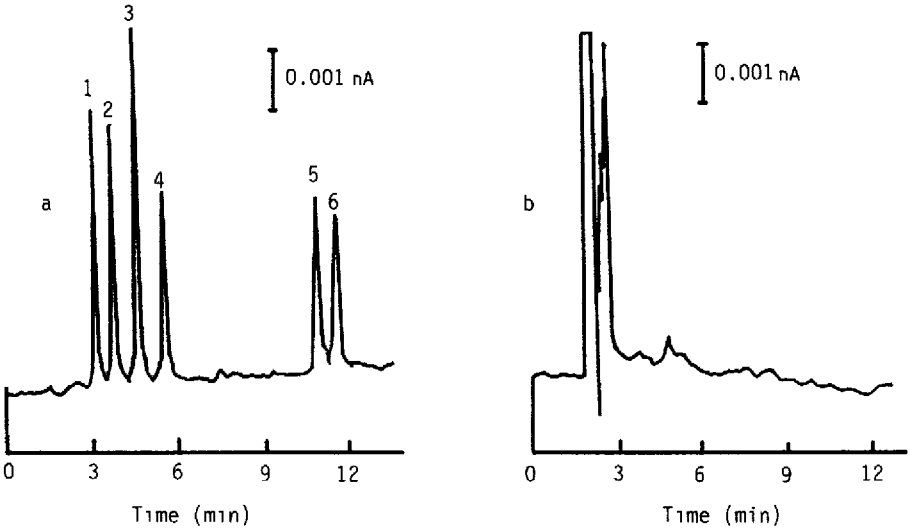

Fig. 3. (a) Separation of amiloride analogues. Column, Alltech Adsorbosphere, 250 mm × 4.6 mm I.D.; eluent, 48% (v/v) acetonitrile in 0.15 *M* perchloric acid (pH 2.2); flow-rate, 1.1 ml/min; temperature, 35°C. Peaks: 1 = dimethylamiloride hydrochloride dihydrate; 2 = 6-iodoamiloride; 3 = triamterene; 4 = phenamil; and 5-(N,N-dimethyl)amiloride; 5 = 5-(N-methyl-N-isobutyl)amiloride; 6 = 5-(N,N-hexamethylene)amiloride; (b) Chromatogram of plasma sample without adding analogues.

TABLE I

K_i VALUES OF AMILORIDE ANALOGUES USING DIFFERENT SYSTEMS

K_i = the concentration required to produce 50% inhibition of either Na^+ transport or other Na^+-dependent transport systems.

Amiloride analogue	K_i (μM of system inhibited)		
	Na^+ channel	Na^+/H^+	Na^+/Ca^{2+}
Amiloride	0.1–1	3–1000	300–1100
6-Iodoamiloride	1.78	18.3	33
Benzamil hydrochloride sesquihydrate	0.008	> 1000	100
Phenamil	0.003	> 500	200
5-(N,N-Hexamethylene)amiloride	> 400	0.16	100
5-(N,N-Dimethyl)amiloride	> 400	6.9	550
Amiloride hydrochloride dihydrate	0.34	84	1100
5-(N-methyl-N-isobutyl)amiloride	> 300	0.44	130

phenamil, Fig. 1, Table I) enhance inhibitory activity toward the Na^+ channel and the Na^+/H^+ and Na^+/Ca^{2+} exchangers by at least one order of magnitude. On the other hand, alkyl substitutions at the ring positions result in a

tremendous diminution of inhibitory activity towards the Na^+ channel, but greatly potentiate the ability of the parent compound to inhibit both the Na^+/H^+ and Na^+/Ca^{2+} exchangers (Fig. 1, Table I) [17]. The current method provides a simple, convenient way to detect and quantitate amiloride and its analogues in plasma after administration to the intact animal. This method will enhance the ability of investigators to examine the pharmacokinetics of amiloride and its analogues in vivo.

The absolute recoveries of amiloride analogues and triamterene from rat plasma and water were determined by extracting the compounds using the aforementioned procedure and comparing the chromatograms to those obtained from an unextracted aqueous solution. Mean ($\pm$S.E.M.) recoveries of HMA and triamterene were 68.1 ± 6.0 and $91 \pm 5.2\%$, respectively. To determine the intra-assay variability of the plasma HMA measurement, six plasma samples containing 1.5 μg/ml HAM and six samples containing 18.1 μg/ml HMA were prepared and extracted by the method described above on a single day. Six replicates of each of the above solutions were assayed on the same day based on a single calibration curve. The mean ($\pm$S.E.M.) measured concentrations of HMA were 1.55 ± 0.9 and 18.9 ± 0.7 μg/ml. The intra-assay coefficient of variation was 3.7%. To determine the inter-assay variability of the plasma HMA measurement, six plasma samples containing 1.5 μg/ml HMA and six samples containing 18.1 μg/ml HMA were prepared and extracted by the method described above on six different days. The mean ($\pm$S.E.M.) measured concentrations of HMA were 1.6 ± 1.1 and 19.3 ± 0.9 μg/ml. The inter-assay coefficient of variation of the procedure was 5.5%.

In the preparation of plasma samples for assay, the technique of lyophilization described here offers several advantages over published methods. (1) Volatile compounds which otherwise would have interfered with the UV detection of the amiloride analogues are removed during the evaporation procedure. (2) After lyophilization, samples can be reconstituted to precise volumes, eliminating the variability introduced by changing sample volumes in the evaporation step. (3) Lyophilization is a simple one-step procedure suitable for concentrating large numbers of samples of biological fluids, such as plasma, facilitating the accurate quantitation of amiloride and its analogues in biological fluids in which they are present in very low concentrations. With these simple procedures thirty samples can be extracted on the same day by one operator. With an automatic injector, all of these samples can be measured in a single day by HPLC.

HMA is an analogue of amiloride which has a hexamethylene ring at position 5 of the native amiloride molecule (Fig. 1). HMA is highly selective for the Na^+/H^+ exchanger. The K_i of HMA for the Na^+/H^+ exchanger is 0.16 μM, 600–2500 times more selective than its effect on Na^+/Ca^{2+} exchanger ($K_i = 100$ μM) or the conductive Na^+ channel ($K_i = 400$ μM) (Table I). The HMA assay

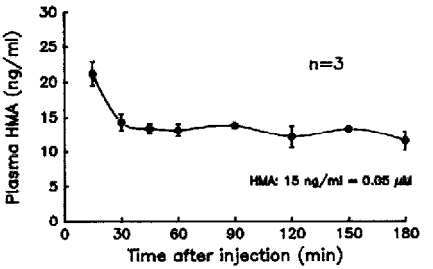

Fig. 4. Plasma (5-N,N.-hexamethylene)amiloride concentrations following intravenous bolus administration to rats (5 μg/kg). Column, Alltech Adsorbosphere, 250 mm × 4.6 mm I.D.; flow-rate, 1.1 ml/min; temperature, 35 °C.

was used to explore the pharmacokinetics of this agent in rats. Rats receiving a 5 μg/kg intravenous bolus dose of HMA exhibited the plasma disposition profile illustrated in Fig. 4. The plasma HMA concentration was 20 ng/ml 15 min after the initiation of the HMA injection. HMA levels then decreased to 15 ng/ml (0.05 μM) in 30 min and remained stable throughout the experiment (180 min). Thus, steady state plasma HMA concentrations achieved with this dose of HMA were within the range of the K_i for the Na^+/H^+ exchanger, but far below the K_i for the Na^+/Ca^{2+} exchanger and the conductive Na^+ channel, suggesting that HMA administered at this dose was highly selective for the Na^+/H^+ exchanger.

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