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High-performance liquid chromatographic method for quantitating plasma levels of amiloride and its analogues

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ABSTRACT

An assay for amiloride was devised for efficient use with the wide variety of analogues available. Amiloride was extracted from 1-ml plasma samples by elution from a C_8 preparative column with 6% acetonitrile–45% methanol–5.4% acetic acid, adjusted to pH 4.0 with trimethylamine. Samples were lyophilized, resuspended in 50% methanol, filtered through 0.22-μm Spin-X cartridges, applied to a reversed-phase C_{18} column, and eluted in a 0–50% acetonitrile gradient in 0.4% acetic acid, pH 4.5 (1.2 ml/min). Detection by ultraviolet absorbance at 360 nm was linear from 1 to 1000 ng. Versatility of the method was demonstrated with the analogues benzamil, 6-hydro-, 6-iodo-, 5-hexamethylene-, and 5-chlorobenzyl-2′,4′-dimethylbenzylamiloride.

INTRODUCTION

It has recently been observed in our laboratory and by others that the ion channel blocker, amiloride, inhibits neovascularization (NV) *in vivo* and *in vitro* [1–5]. However, amiloride has a variety of biological activities in addition to its effects on sodium ion transport [6]. Given the importance of new vessel formation in normal developmental processes, as well as pathogenesis, we are currently engaged in a structure–activity analysis using an array of amiloride analogues both *in vitro* and *in vivo*. Although assays for amiloride and a number of its analogues have been reported [7–11], we have not found any one that is satisfactory for the range of analogues required for our *in vivo* biological experiments. Furthermore, as discussed by Forrest *et al.* [7], these assays rely on radiolabeling or fluorescence detection, and multi-step extractions or other time-consuming steps. We have therefore devised an assay for amiloride and its analogues that is relatively simple, but improved in aspects of resolution, recovery, and versatility.

In this protocol, solid-phase extraction of plasma samples is followed by an exchange of solvents, and then chromatography on a C_{18} high-performance liquid chromatographic (HPLC) column using a gradient elution. The method is compatible in its present form for use with a number of amiloride analogues, and is adapted to others with minor modification. In addition to data on the resolution and recovery of six pyrazinecarbonylguanidines, biological data regarding the uptake and metabolism in rabbits is presented for amiloride and its 5-(N,N-hexamethylene) derivative, HMA.

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EXPERIMENTAL

Materials

Lithium heparin tubes for blood collection were purchased from Becton Dickinson (Rutherford, NJ, USA), Spin-X filter cartridges from Costar (Cambridge, MA, USA), and HPLC solvents from J. T. Baker (Phillipsburg, NJ, USA). Benzamil · HCl was purchased from Research Biochemicals (Natick, MA, USA). All other amiloride analogues were prepared as previously described [12,13]. Amiloride · HCl · $2H_2O$ and all other reagents were obtained from Sigma (St. Louis, MO, USA).

Preparation of standards

Amiloride and benzamil were prepared as stock solutions in 50% methanol at 0.1 mg/ml and stored at −20°C. Working solutions were prepared from the frozen stocks daily and kept on ice. Other amiloride analogues were prepared fresh as stocks of 10 mg/ml in 100% dimethyl sulfoxide and subsequently diluted in 50% methanol.

Animal dosing and collection and storage of blood samples

Rabbits were dosed orally with drugs measured into gelatin capsules at the levels indicated in Results for each experiment. Blood was collected from the central artery of the ear through a sterile 20-g hypodermic needle into 100 × 13 mm vacutainer tubes containing 100 USP units of lithium heparin per tube. The blood samples were centrifuged at 1430 *g* for 10 min at room temperature. Plasma was removed from the tube and aliquots were stored at −20°C until further processing.

Isolation of amiloride from plasma

Thawed plasma samples were centrifuged in a Beckman microcentrifuge at room temperature for 5 min at 5600 *g* to remove any particulates. After adding benzamil (100 ng/ml of plasma) as an internal standard, samples were applied to 1-ml C_8 preparative solid-phase columns (Bond Elut, Varian, Harbor City, CA, USA), which had been pretreated with 2 × 1 ml of absolute methanol and rinsed three times with distilled water. After sample application, the columns were washed with two additional 1-ml volumes of water, dried by aspiration, and eluted by centrifugation (900 *g*, 10 min) with 500 μl of 6% acetonitrile–45% methanol–5.4% glacial acetic acid, buffered to pH 4.5 with triethylamine (TEA). Samples were filtered through a 0.22-μm low-binding cellulose acetate cartridge (Spin-X), lyophilized, and stored at −20°C or processed immediately. Samples containing more hydrophobic analogues, such as 5-(N-4-chlorobenzyl)-2′,4′-dimethylbenzamil (CBDMB), could be passed through Annotop 10 inorganic filters (Annotec Separations, New York, NY, USA) in lieu of the Spin-X to improve recovery.

HPLC analysis

Lyophilized samples were resuspended in 200 μl of 50% methanol, and microcentrifuged to remove any precipitate. They were then applied to a 15 cm × 4.6 mm I.D. reversed-phase C_{18} column (Beckman Instruments, Fullerton, CA, USA) and equilibrated with 0.4% glacial acetic acid adjusted to pH 4.5 with TEA. Elution was achieved with a 0–50% acetonitrile gradient in 0.4% glacial acetic acid, pH 4.5, over a period of 10 min at a flow-rate of 1.2 ml/min. The column was monitored by UV absorbance at 360 nm. Amiloride levels were derived from the area under peaks using linear regression analysis and known standards. HPLC was performed at 21°C.

RESULTS

Two central features of the protocol described include replacement of acetonitrile with 50% methanol (by lyophilization and resuspension in the latter) and employment of a gradient elution (0–50% acetonitrile) on the reversed-phase column. These two features combined have the effect of shifting the amiloride peak away from the solvent front, as previously observed with solid-phase extractions [7,11], and to the center of the chromatogram. This renders the method immediately compatible with several other amiloride

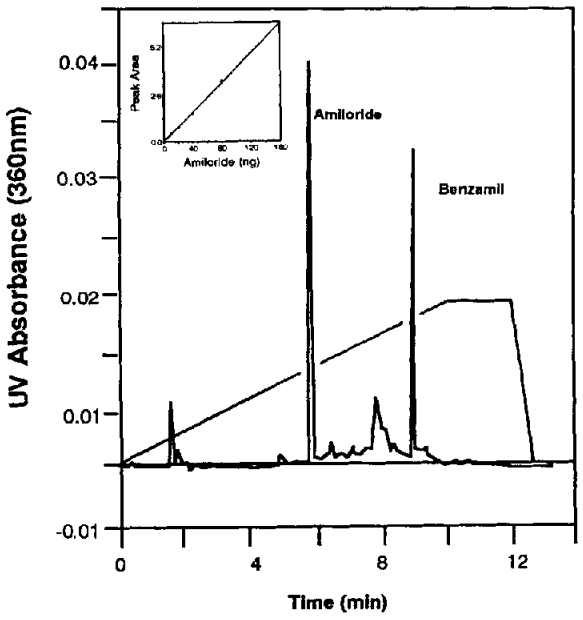


Fig. 1. Chromatogram of amiloride in rabbit plasma. The rabbit was dosed orally with 30 mg amiloride · HCl · $2H_2O$ in a gelatin capsule. Blood was drawn 24 h later. Benzamil was added to the plasma sample as an internal standard. The amiloride peak represents a level of 97 ng/ml. Inset: standard curve for amiloride in rabbit plasma. Values on the abscissa indicate the quantity of amiloride, in ng, added to control plasma samples to generate the standard curve. The correlation coefficient (r^2) associated with this curve is 0.999. The linear range extends from 1 to 1000 ng/ml.

analogues, including ones of significantly higher or lower hydrophobicity. Fig. 1 shows the results obtained with 100 ng of amiloride in 1 ml of rabbit plasma. We found that quantitation was linear over the range 1–1000 ng. The precision and accuracy of the assay is reflected in: (1) coefficients of correlation for standard curves which rarely fell below 0.998 (Fig. 1, inset); (2) variation between identical samples of less than 5%. There are at least two points during sample preparation at which specimens may be stored at −20°C for later use with no loss in recovery: after separation of plasma from whole blood, or after lyophilization.

Fig. 2 is a chromatogram obtained from a sample containing amiloride and five analogues. The closely related compounds 6-hydroamiloride (6-HA) and 6-iodoamiloride (6-IA) are clearly resolved from amiloride, bearing a 6-Cl atom, and analogues with more significant modifications such as HMA or iminohomoamiloride (MK-875) fall easily within the fractionation range. With minor modification of the acetonitrile gradient, the method is useful for analogues of even greater hydrophobicity than HMA, such as the doubly substituted CBDMB (Fig. 3). The recovery of these compounds from rabbit plasma, shown in Table I, ranged from 75% (6-HA) to 99% (6-IA).

The method described above was subsequently used to study plasma levels of amiloride and other potential anti-NV amiloride analogues in rab-

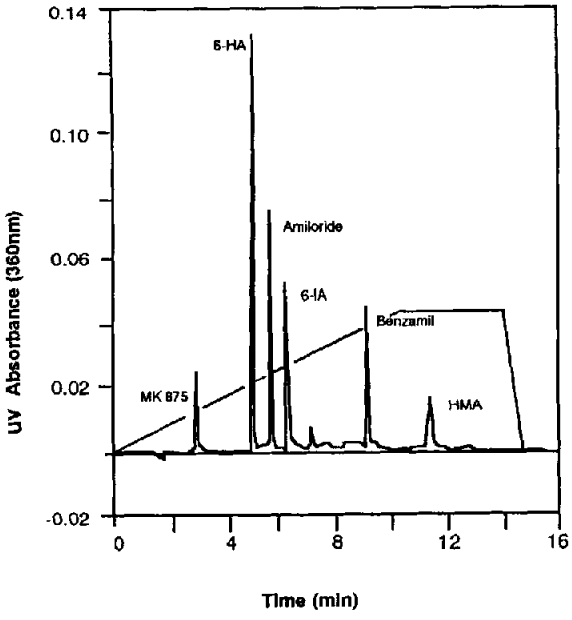
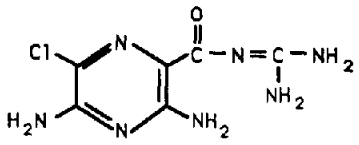
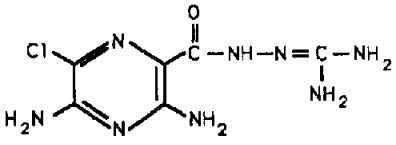
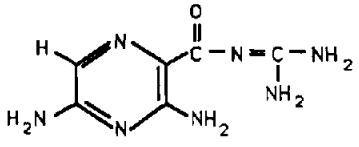
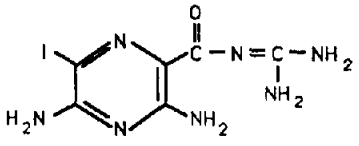
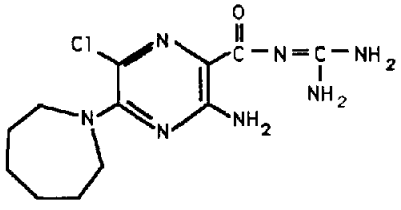
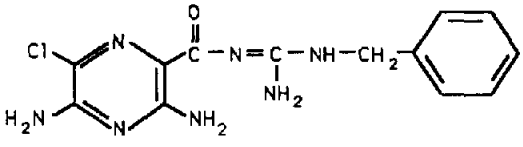


Fig. 2. Chromatrogram of amiloride and five analogues: MK-875, 6-HA, 6-IA, benzamil, and HMA. Analogues were added as a mixture to control rabbit plasma (at 100 ng/ml each), and samples were processed as described in Experimental with slight modification. The changes were: 20% acetonitrile in pre-column elution buffer (to maximize recovery of HMA); resuspension of the lyophilized mixture of compounds in distilled water instead of 50% methanol to move MK-875 into the fractionation range. One, both, or neither of these modifications may be used, depending upon the compound(s) of interest. Use of HMA and MK-875 in the same sample was intended only to demonstrate the potential fractionation range of the method and is unlikely to occur in a biological experiment. In fact, the omission of methanol (to accommodate MK-875) had the effect of decreasing HMA recovery and necessitated lengthening the isocratic portion of the acetonitrile gradient.

TABLE I

STRUCTURES AND RECOVERIES OF AMILORIDE AND FIVE ANALOGUES

Compound	Recovery (%)	Structure
Amiloride	97	
MK875	85	
6-HA	75	
6-IA	99	
HMA	96	
Benzamil	98	

bits with PGE_1-induced corneal NV. The kinetics of amiloride uptake and clearance that we observed (Fig. 4) were similar to those reported previously [14,15]. Plasma levels of amiloride peaked at between 2 and 4 h after oral dosing. There was significant variability between individual rabbits, as has also been observed in past studies [7]. Amiloride from a single oral dose of up to 15 mg/kg is largely cleared (>90%) by 48 h. For a 3-kg rabbit given single oral doses of 30 mg per day, the mean plasma level at 24 h was 72 ± 11 ng/ml. Significant inhibition of experimentally induced

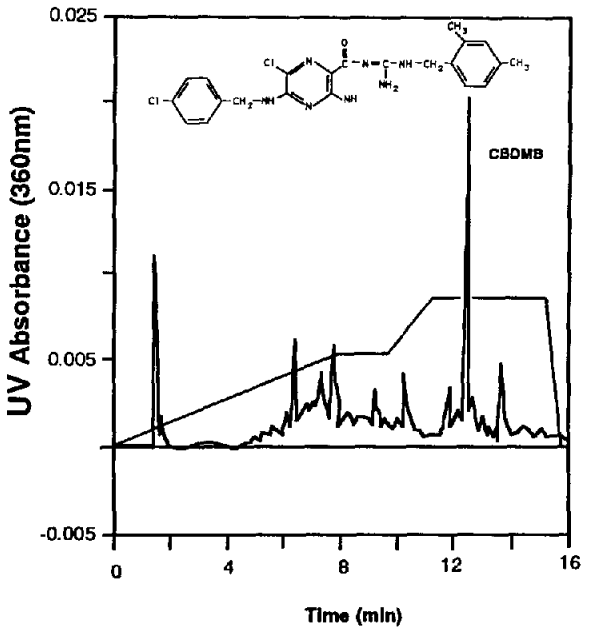


Fig. 3. Chromatogram of CBDMB. Elution from the precolumn was achieved with 40% acetonitrile buffer. The modified gradient (shown) and the use of an Annatop-10 organic filter in lieu of Spin-X are also used to maximize recovery of CBDMB.

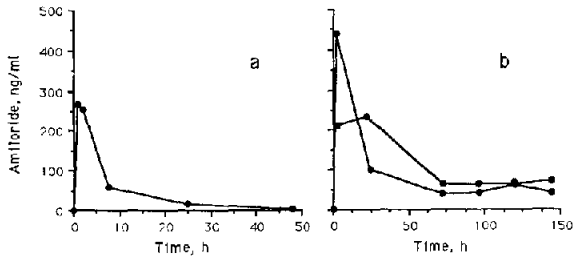


Fig. 4. Uptake and clearance of amiloride from rabbit plasma. (a) The animal was given a single oral dose of amiloride and blood was drawn at the indicated times. (b) Rabbits received daily single oral doses. Blood was drawn at 1 h after the first dose and just prior to each subsequent dose.

corneal NV was observed under these conditions [3].

HMA is a potent NV inhibitor *in vitro* [1,2]. However, unlike amiloride, no effect was observed *in vivo* after oral administration at several doses. We found that this is potentially due to the rapid breakdown and clearance of HMA from the blood. Degradation was apparent within 30 min after dosing, even as uptake from the oral dosage continued (Fig. 5). The HMA peak had increased to maximum level by 2 h post-dosing, but was a minor component of the chromatogram by this time. By 24 h neither the HMA peak nor its putative breakdown products are detectable. Our observations on the stability of HMA incubated at 37°C in either phosphate-buffered saline or 1 *M* HCl indicate that it is stable under these conditions. In a previous study using intravenous injection of rats [11], significant quantities of HMA were recovered in plasma samples 3 h after administration. The difference between these two studies may be due to the route of administration (oral *versus* intravenous) or due to metabolic differences between species. Given the potential of HMA as an anti-NV agent, we are currently analyzing this question in more detail.

DISCUSSION

The occurence of NV in the adult mammal is a relatively rare and closely regulated event. It normally occurs for only limited periods of time, such as during wound healing or ovulation [16]. Unregulated NV is a central feature of neoplasia and other pathologic conditions, such as proliferative diabetic retinopathy. Amiloride has been shown to inhibit NV both *in vitro* and *in vivo*. Although the precise mechanism is not clearly understood, evidence exists suggesting a relationship between the inhibition of NV and the inhibition of urokinase-plasminogen activator and/or cellular Ca^{2+} transport [1–4]. Determining the physiological basis for amiloride's effect on capillary formation can be approached using structural analogues that are enhanced or attenuated in certain of its biological activities.

In vivo, systemic administration of amiloride has been found to be a convenient and effective format for such studies. However, the ability to monitor tissue levels of the compound is essential under these conditions. The assay method chosen should ideally be suited for use with pertinent analogues of amiloride as well. Although several methods for the measurement of plasma amilo-

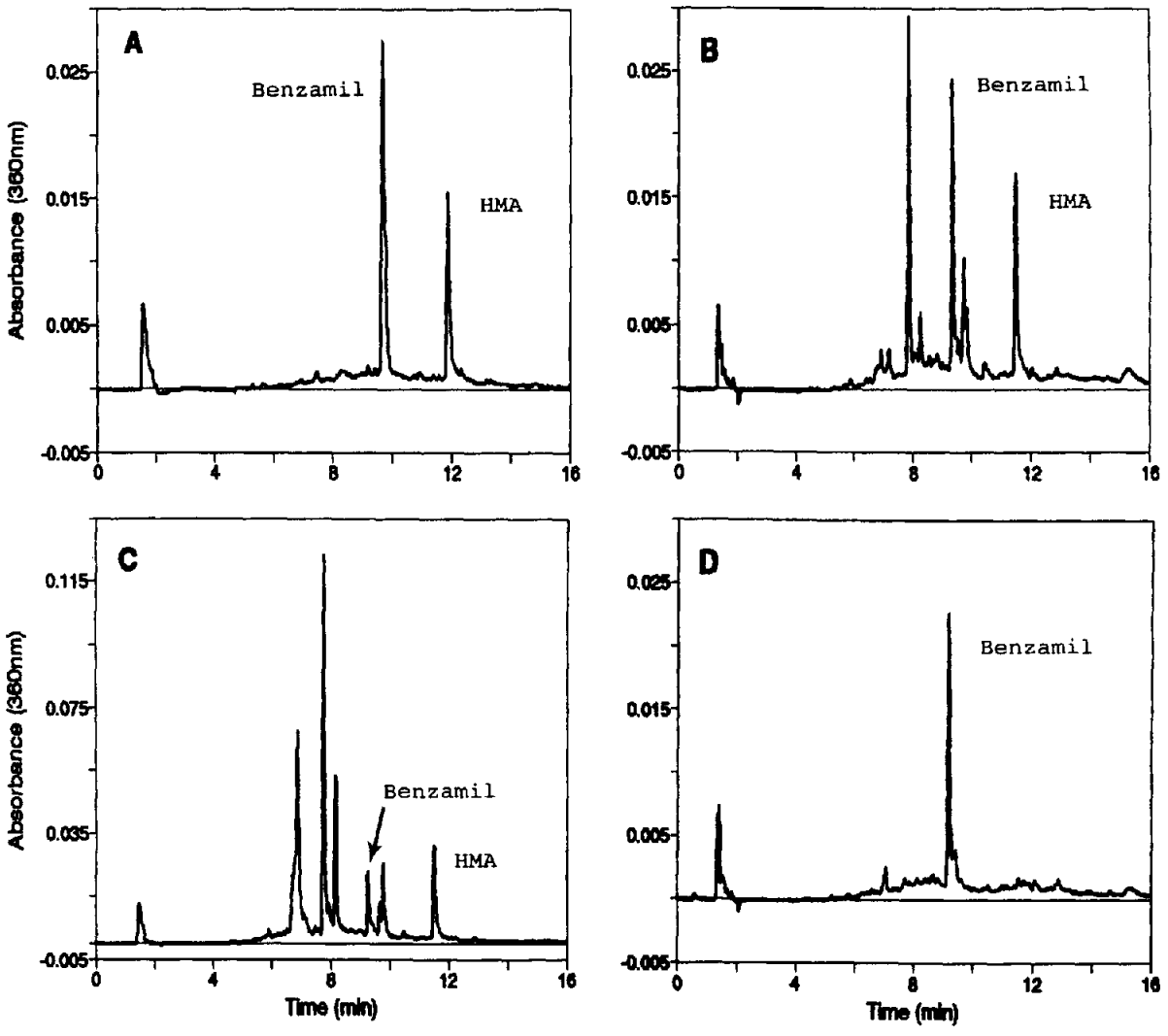


Fig. 5. HMA metabolism in rabbit plasma. HMA was eluted from the precolumn with 20% acetonitrile buffer, as described in the legend to Fig. 3. (A) Control plasma dosed with 80 ng/ml HMA; (B) 30 min after dosing rabbits with 90 mg HMA, only a small proportion is recovered in the HMA peak; several new peaks appear; (C) 2 h post-dosing; the new peaks are enhanced, several additional peaks appear, and the relative quantity of intact HMA diminishes; (D) 24 h post-dosing; neither HMA nor its putative breakdown products are detectable. Note the scale change in panel C, to accomodate increased absorbance in certain peaks.

ride have been reported, we found that none provided the combination of simplicity, sensitivity, and versatility needed for the wide variety of analogues planned for use. The protocol we have evolved appears to fulfill this requirement.

Finally, our observation that HMA is rapidly metabolized and cleared from rabbit blood was somewhat surprising in light of our own previous studies [12] and those by other investigators [11]. The breakdown of 5-substituted analogues is generally slower and results in the stepwise formation of stable, active, amiloride. For example, 5-(N,N-dimethyl)amiloride is converted to 5-(N-methyl)amiloride and subsequently to amiloride. The pathway for HMA degradation, at least in orally dosed rabbits, is apparently different. Given our results with HMA *in vitro* [1,2] and the overall utility of the rabbit corneal pocket as a

model for NV, our results suggest that HMA may require stabilization or an alternative route of administration for *in vivo* experimentation.

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