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Pharmacokinetic studies of amiloride and its analogs using reversed-phase high-performance liquid chromatography

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Abstract

We have studied the pharmacokinetics of amiloride and its analogs. A high-performance liquid chromatographic method has been adapted for the measurement of amiloride, 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 5-(N,N-hexamethylene)amiloride (HMA) in mouse plasma, kidney, liver and tumor tissues. The method uses a C_8 preparative solid-phase column, followed by separation using a reversed-phase C_{18} column (250×4 mm I.D., 5 μm particle size) with detection by ultraviolet absorption at 365 nm. Reversed-phase separations were performed at ambient temperature using a non-linear gradient method with two different mobile phases: mobile phase A was 100% acetonitrile while mobile phase B was 0.15 *M* perchloric acid at pH 2.20 (flow-rate was 1.2 ml/min). The retention times for amiloride, benzamil (used as an internal standard), EIPA and HMA are 13.4, 19.5, 21.8 and 23.5 min, respectively. The calibration curves are linear over the range of 0.1–50 μM in plasma and in tissues. The half-lives of amiloride, EIPA and HMA (and their confidence intervals) in plasma after intraperitoneal injection of drugs into mice were 68.8±0.2, 31.2±2.5 and 39.3±7.9 min, respectively. Amiloride was detected as a metabolite of EIPA but not of HMA. When EIPA was injected at a dose of 10 μg/g body weight, it was cleared rapidly from liver, but concentrations >1 μM were sustained for at least 2 h in murine kidney and in a transplantable tumor.

Keywords: Amiloride; 5-(N-Ethyl-N-isopropyl)amiloride; 5-(N,N-Hexamethylene)amiloride

1. Introduction

Acidic microenvironments are found in solid tumors and the mean extracellular pH (pHe) tends to be lower than in normal tissues [1,2]. In contrast, measurement of intracellular pH (pHi) in solid tumors has shown no significant difference as compared to that in normal tissues [1] suggesting that cells in solid tumors are actively regulating their pHi to maintain it at physiological levels. The viability of tumor cells under acidic conditions is likely to depend therefore on membrane-based mechanisms which regulate pHi. The major mechanisms for regulation of pHi under acidic conditions are the Na^+/H^+ exchanger and the Na^+-dependent HCO_3^-/Cl^- exchanger [3–5]. The Na^+/H^+ exchanger allows the exchange of intracellular H^+ for extracellular Na^+ and is inhibited by amiloride and its analogs such as 5-(N,N-hexamethylene)amiloride (HMA) and 5-(N-ethyl-N-isopropyl)amiloride (EIPA) [6,7]. An approach to acid-dependent tumor-selective therapy involves the use of agents that are able to inhibit mechanisms which regulate pHi, (such as analogs of amiloride) thereby leading to intracellular

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acidification within the acidic environment of solid tumors. The synthesis of analogs of amiloride was described by Cragoe et al. [6]. The analogs EIPA and HMA are more specific and more potent (~200 fold) agents for inhibition of the Na^+/H^+ exchanger [7–9]. In vitro studies have shown marked pHe-dependent cytotoxicity when ionophores such as nigericin or carbonylcyanide-3-chlorophenylhydrazone (CCCP) (which collapse the pH gradient across the cell membrane) are used with analogs of amiloride [8,9]. Combined use of these agents can also lead to anti-tumor effects in mice [9–11].

Assays for amiloride and a number of its analogs have been reported [9,12,13] but we have found no reports of quantitation of EIPA in plasma or tissues. We have adapted a high-performance liquid chromatographic (HPLC) method that relies on ultraviolet (UV) absorbance at 365 nm for the measurement of amiloride and its analogs in plasma and tissues and have studied their pharmacokinetics in mice.

2. Experimental

2.1. Materials

Benzamil, amiloride and HMA were purchased from Research Biochemicals International (RBI) (Natick, MA, USA). EIPA was synthesized by Aldrich (Milwaukee, WI, USA), as described previously [6]. All chromatographic solvents were HPLC grade and all other chemicals were analytical-reagent grade.

2.2. Apparatus

Studies using HPLC were performed with a Waters system, which consisted of a Waters Model 6000A solvent delivery system (Waters Assoc., Milford, MA, USA), two Waters Model 510 HPLC pumps, a Shimadzu SIL-9A automatic sample injector (Shimadzu, Kyoto, Japan), a Waters Series 440 UV absorbance detector, a Shimadzu CR501 integrator and a Bio-Sil ODS-5S, 250×4 mm I.D. (5 μm particle size) C_{18} reversed-phase column (Bio-Rad, Richmond, CA, USA).

2.3. In vivo experiments

Amiloride, EIPA and HMA were prepared in 1% DMSO. Mice were injected intraperitoneally (i.p.) with 10 mg/g body weight of EIPA, amiloride or HMA. The maximum tolerated dose is about 20–30 μg/g for each of these agents. At 1, 5, 15, 30, 60 and 120 min after injection, blood samples were obtained by cutting the neck and collected into heparinized Eppendorf tubes. Plasma was separated by centrifugation and stored at −20°C until analyzed. In some experiments, kidneys, livers and tumors were also removed and homogenized in phosphate buffered saline (PBS).

Tumors were generated by intramuscular injection of 1×10^6 KHT cells into hind legs of syngeneic C3H/HeJ mice. Experiments began when the tumor-bearing leg had reached a diameter of 8.5–9.5 mm (equivalent to a tumor weight of 0.3–0.5 g) usually 7–8 days after injection.

2.4. Sample preparation

The same amount of internal standard, 2 μM benzamil was added to plasma or tissue samples to compensate for any loss during the extraction procedure. Samples were applied to C_8 preparative solid-phase columns (Bond Elut, Varian, Harbor City, CA, USA), which had been pretreated with two column volumes of methanol and rinsed with three column volumes of distilled water. After sample application, the columns were washed with two column volumes of distilled water and eluted with 500–750 μl of elution buffer (35% acetonitrile, 45% methanol, 4% glacial acetic acid and 16% water buffered to pH 4.5 with triethylamine). The eluates were then dried in a speed vacuum, stored at −20°C or processed immediately. Samples were reconstituted into 130 μl of 50% methanol–water and centrifuged at 14 000 g prior to HPLC analysis (100 μl of each sample was injected onto the column). The ratios of the areas under the peaks of the compounds of interest and of benzamil were calculated. The concentration of the drug was determined by interpolation following linear regression of the standard curve.

Three methods were used to verify the compound of interest. First, the relative retention time was

compared to that of the standard. Secondly, samples were spiked with standard, resulting in increased amplitude. Thirdly, blank samples did not give rise to peaks with the same retention time as the standard.

2.5. Preparation of a standard curve

Stock solutions of EIPA, HMA or amiloride were prepared separately. Calibration curves were prepared by adding different known concentrations of standard solutions and the same amount of internal standard (2 μM benzamil). These were extracted in a similar manner to that for samples.

2.6. Chromatographic conditions

Reversed-phase separations were performed at ambient temperature using a non-linear gradient method with two different mobile phases A and B. The gradient started with 0% mobile phase A and 100% mobile phase B. Mobile phase A was 100% acetonitrile while mobile phase B was 0.15 M perchloric acid at pH 2.20. Both were degassed and filtered before use. The flow-rate was 1.2 ml/min and the UV detector was set at 365 nm.

3. Results

Typical chromatograms for blank plasma show no interfering peaks, while those for plasma with individual compounds added show narrow peaks with retention times of 13.4 min (amiloride), 19.5 min (benzamil) and 21.8 min (EIPA) as shown in Fig. 1. These retention times correspond to different gra-

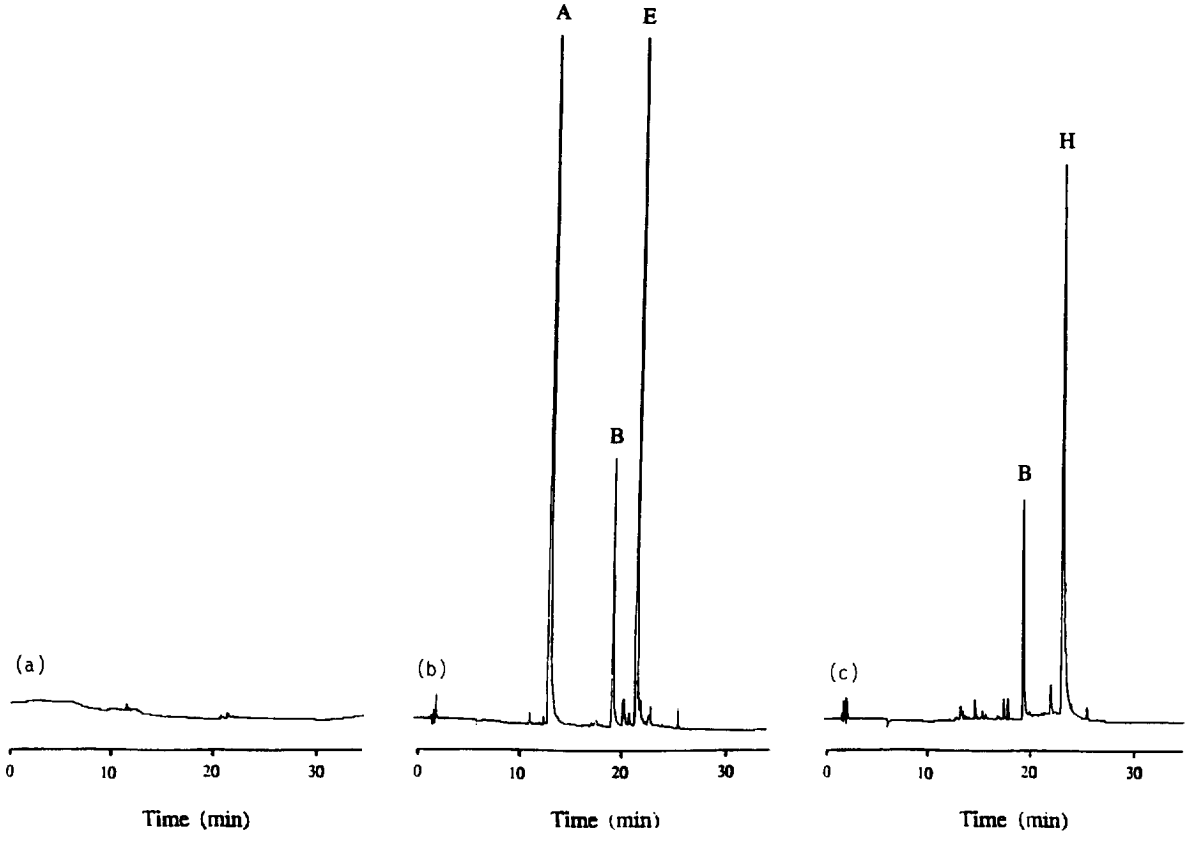

Fig. 1. HPLC profiles of (a) blank mouse plasma, (b) plasma containing (A) 25 μM amiloride, (B) 2 μM benzamil (internal standard) and (E) 25 μM EIPA; (c) plasma containing (H) 30 μM HMA.

dients of the mobile phase: amiloride, benzamil and EIPA (approximately 45, 80 and 86% v/v acetonitrile in 0.15 *M* perchloric acid, respectively). The retention time for HMA was 23.5 min which corresponds to 89% acetonitrile in 0.15*M* perchloric acid (Fig. 1). The calibration curves for amiloride and its analogs in plasma (and extracted tissues) were obtained by plotting the ratio of the area under the peak to that of the internal standard versus the drug concentration. The standard curves were linear over the concentrations used as illustrated in Fig. 2 for EIPA in plasma.

The lowest concentration of drug that could be detected was determined by adding serial dilutions to blank plasma or tissue extract. For each drug this was approximately 0.1 μM in plasma and 1 μM in tissues.

The concentration of amiloride in plasma of Balb/c BYJ mice at intervals up to 2 h following an i.p. injection of 10 μg/g is shown in Fig. 3. The maximum concentration of amiloride was ~70 μM at 30 min and decreased to less ~20 μM at 120 min after injection. The half-life of amiloride in plasma

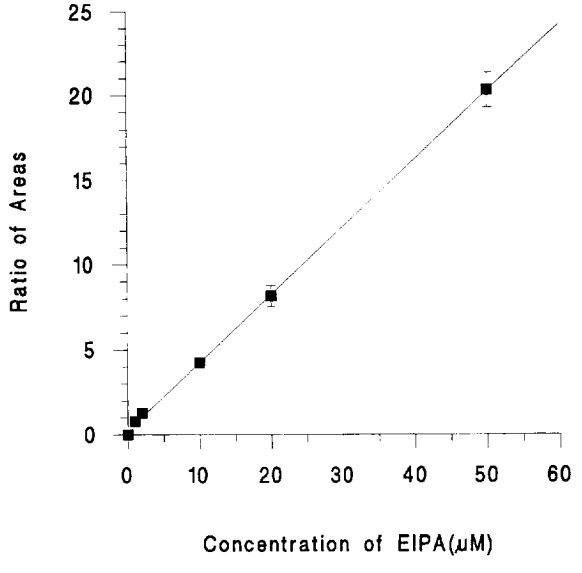

Fig. 2. Standard curve for EIPA in plasma. The ratio of areas under the peaks for EIPA and benzamil are plotted as a function of concentration of EIPA. The relationship is linear (r^2=0.999). Each point represents the mean of three experiments. Bars=SEM (standard errors of the mean unless error bar is less than the height of the symbols).

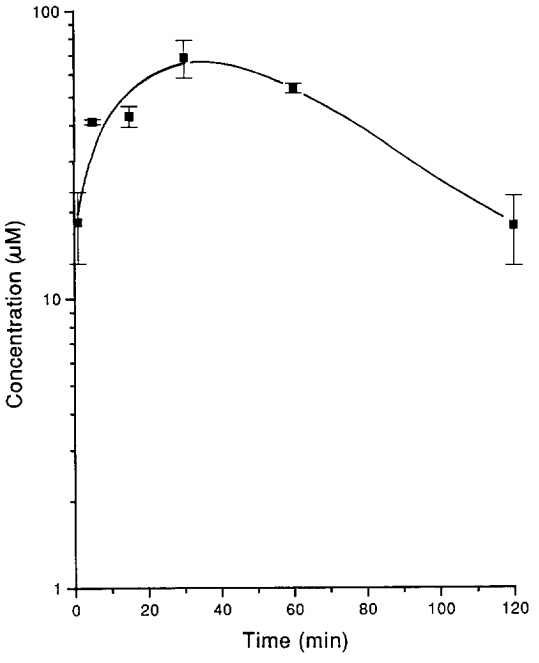

Fig. 3. Plasma concentration–time profile following a single i.p. injection of 10 μg/g of amiloride in Balb/c BYJ mice. Each point represents the mean of three experiments. Bars=SEM (standard errors of the mean).

determined from a regression line fitted to data points at 30, 60 and 120 min is 68.8±0.2 min.

The concentration of EIPA and HMA in plasma of C3H/HeJ mice was measured at varying times after an i.p. injection of 10 μg/g. The concentration of EIPA was ~3.5 μM at 5 min after injection, while that of HMA was ~6 μM (Fig. 4). The half-lives of EIPA and HMA (determined by regression lines fitted to data points from 5 to 60 min and their confidence intervals) are 31.2±2.5 and 39.3±7.9 min respectively in plasma. Similar results were obtained with EIPA (data not shown) and HMA [9] in plasma of Balb/c BYJ mice. In addition, amiloride was detected as a metabolite in plasma following injection of EIPA in C3H/HeJ mice (Fig. 4a), but not following injection of HMA. Approximately 1 μM of amiloride was detected at 15 min after injection of EIPA and the concentration of amiloride increased to ~10 μM at 60 min.

Kidneys, livers and tumor tissues in Balb/c BYJ and C3H/HeJ mice were extracted and analyzed in a similar manner to the plasma samples. The con-

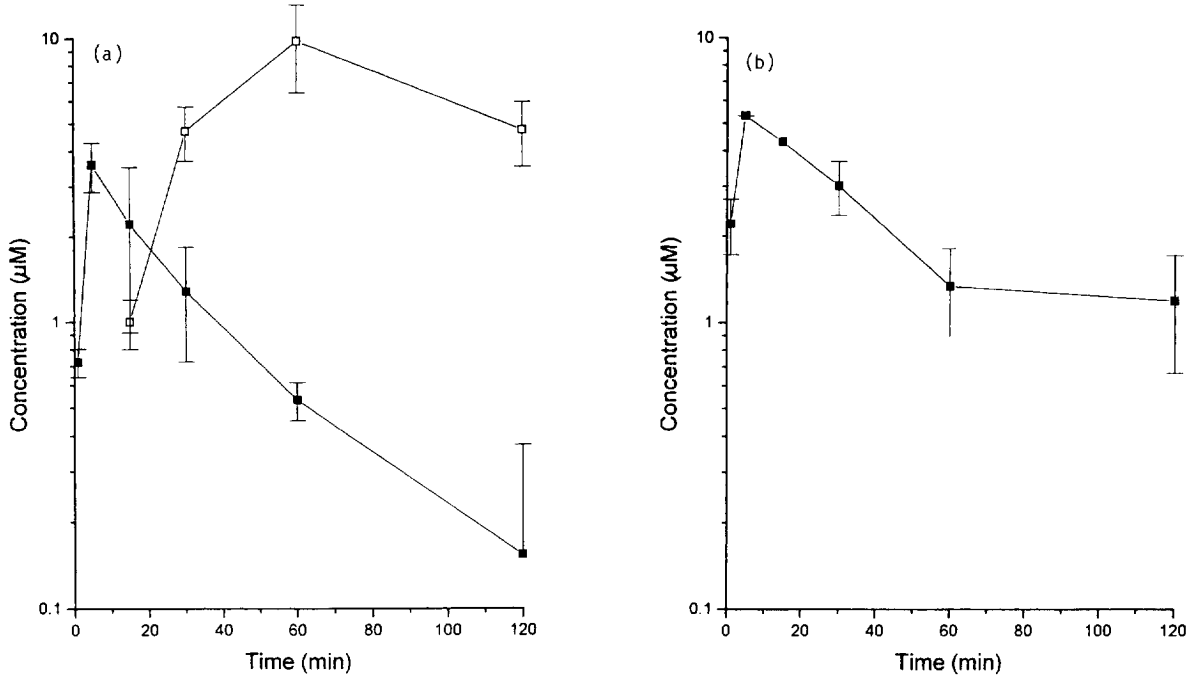

Fig. 4. Relationship between plasma concentration (filled squares) and time following a single i.p. injection of 10 μg/g body weight of (a) EIPA or (b) HMA in C3H/HeJ mice. Amiloride (open squares) was detected as a metabolite following injection of EIPA but not HMA. Each point represents the mean of three experiments. Bars=SEM (standard errors of the mean).

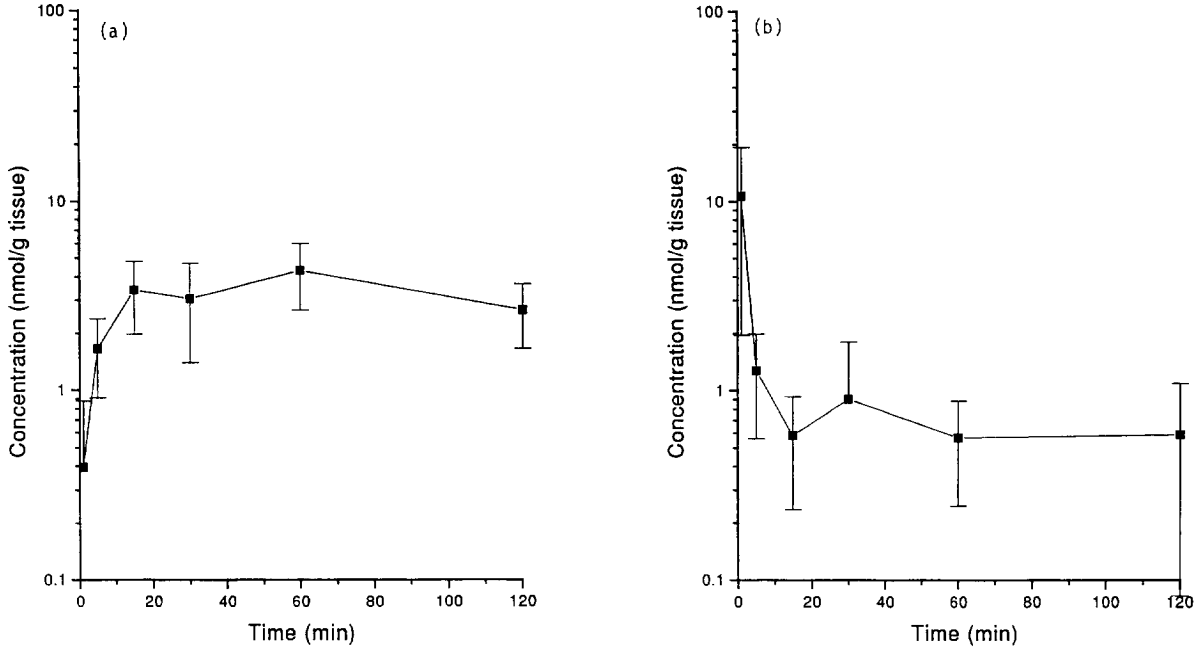

Fig. 5. Concentration of EIPA in (a) kidney and (b) liver following a single i.p. injection of 10 μg/g body weight in Balb/c BYJ mice. Each point represents the mean of two experiments. Bars, range. Values below 1 μM cannot be separated reliably from background.

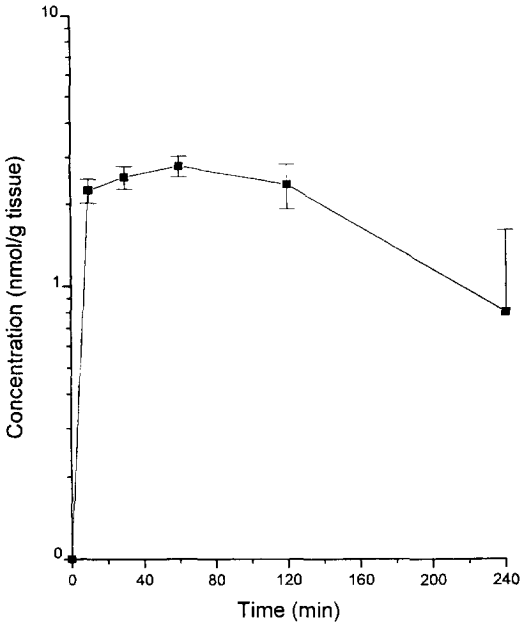

Fig. 6. Concentration of EIPA in the KHT tumor following a single i.p. injection of 10 μg/g body weight. Each point represents the mean of two experiments. Bars, range. Values below 1 μM cannot be separated reliably from background. The metabolite amiloride could not be quantitated since there was an endogenous peak eluting at the same time.

centration of EIPA in kidney increased immediately after an i.p. injection and reached a level of ~3 μM at 15 min and then remained constant for up to 2 h. In liver the concentration of EIPA increased to ~10 μM immediately after i.p. injection, but fell to undetectable levels (<1 μM) by 15 min (Fig. 5).

The concentration of EIPA in the KHT tumor was ~2 μM at 10 min and reached a maximum of 3 μM at 60 min. The concentration then decreased slowly to 1 μM at 4 h. (Fig. 6). Amiloride could not be detected in these experiments since there was an endogenous peak eluting at the same time as amiloride.

4. Discussion

This report describes a simple yet sensitive HPLC method using UV detection for the quantitation of amiloride and two of its analogs in plasma and tissues. It extends the work of others [9,12,13] by assaying the potent analog EIPA and by studying the concentration of these agents in various tissues of mice. Existing methods using ^{14}C-labeled compound [14,15] or fluorometric analysis [16] do not have specificity for the detection of amiloride, EIPA and HMA at the same time in plasma. The method is very sensitive in plasma (limit of detection ~0.1 μM) but the limit of detection in tissues (~1 μM) was higher since there was more background noise in tissue samples.

Pharmacokinetic studies were undertaken to provide a rational basis for delivery and maintenance of an effective concentration of these drugs in vivo. Plasma concentration–time curves indicate that these agents are cleared rapidly from plasma. These results suggest that repeated dosing or constant infusion will be necessary to maintain plasma levels at a constant value, although more constant levels of EIPA are maintained in an experimental tumor and in kidney for at least 2 h. A tissue concentration of >1 μM EIPA is usually sufficient to inhibit Na^+/H^+ exchanger activity [8] so that slow clearance from these tissues might provide anti-tumor effects through inhibition of regulation of pHi in an acidic microenvironment and diuresis through effects in the kidney. Amiloride was detected as a metabolite (but not after injection of HMA), although its lower potency for inhibition of Na^+/H^+ exchanger activity suggests a marginal contribution to the above effects. Results presented in this paper will be used to optimize schedules of administration of EIPA or HMA as a component of pH-dependent approaches to tumor therapy.

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