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Short Communication

Determination of active 5-(N,N-hexamethylene)amiloride in pig plasma

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ABSTRACT

A simple, cheap and specific quantitative method for the determination of the selective Na^+/H^+ exchange inhibitor, 5-(N,N-hexamethylene)amiloride, in plasma and aqueous solutions has been developed. The method involves extraction with ethyl acetate, thin-layer chromatography and spectrofluorodensitometry. The compound was separated from several unidentified metabolites in plasma. The detection limit was $6 \cdot 10^{-7}$ *M*. The calculated metabolic extraction by the liver was 29%, and the plasma half-life was 12.8 min. The free, active concentration of 5-(N,N-hexamethylene)amiloride was 19.4% of the total concentration, as determined by equilibrium dialysis.

INTRODUCTION

Amiloride analogues are used as experimental tools to investigate the functional importance of several ion transport mechanisms across membranes [1]. The Na^+/H^+ exchanger is important in the control of intracellular pH, cell growth and differentiation, cell volume regulation and transepithelial transport of Na^+ and HCO_3^- [2]. The maintenance of intracellular pH during pancreatic and bile HCO_3^- secretion requires either acid extrusion or base uptake on the basolateral side.

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The mechanism for this pH control is under investigation. Basolateral H^+ extrusion, mediated by the Na^+/H^+ exchanger, has been suggested as a possible mechanism [3]. Another possible mechanism is basolateral HCO_3^- uptake [4]. Clarification of the mechanism could be important in the treatment of hepatobiliar and pancreatic diseases with affection of HCO_3^- secretion, as well as states with alteration of cellular pH. 5-(N,N-Hexamethylene)amiloride (HMA), which is a more selective inhibitor of the Na^+/H^+ exchanger than amiloride [5], can be used to test if H^+ extrusion *in vivo* is mediated by the Na^+/H^+ exchanger. *In vitro* experiments confirm the presence of Na^+/H^+ exchange in microdissected pancreatic ducts [6].

The aim of the present investigation was to develop a simple analytical method for the free (unbound) and thus pharmacologically active plasma concentrations of HMA in *in vivo* experiments to ascertain that sufficiently high concentrations were obtained to inhibit the Na^+/H^+ exchanger but lower than concentrations affecting other ion transport systems. A high-performance liquid chromatographic (HPLC) method has previously been described [7], but requires expensive equipment and considerable experience and training. We have developed a method for total HMA, which includes extraction from plasma, thin-layer chromatography (TLC) and spectrofluorodensitometry, and we used protein-binding in plasma to estimate free HMA.

EXPERIMENTAL

Chemicals and solutions

HMA was synthesized by one of us (E. J. C., Jr.). The other chemicals used were from Merck (Darmstadt, Germany) and were of analytical grade.

In vivo experiments: source of plasma samples

Plasma samples were obtained from experiments with pigs weighing *ca.* 22 (20–25) kg, anaesthetized as previously described [8]. HMA was synthesized for this study by the method of Cragoe *et al.* [9]. HMA was administered by infusion through a catheter in the thoracic aorta. HMA was dissolved in dimethyl sulphoxide (DMSO) ($1.6 \cdot 10^{-3}$ mol HMA in 80 ml of DMSO and 40 ml of distilled water). The HMA infusion rate was $80 \cdot 10^{-6}$ mol/min for 20 min. Blood samples for the determination of HMA were drawn through catheters in the femoral artery, liver vein and portal vein.

Measurement of HMA in plasma and buffer

Standard preparation. HMA was dissolved in DMSO–distilled water (3:7, v/v) to a concentration of $1 \cdot 10^{-3}$ *M*. Standards in normal pig plasma or in buffer were prepared by spiking with HMA in the range $5 \cdot 10^{-7}$–$5 \cdot 10^{-5}$ *M*. Plasma and buffer standards were always analysed together with samples for preparation of standard curves.

Extraction. The determination of HMA in buffer from equilibrium dialysis experiments was similar to that in plasma, except that standards in buffer instead of in plasma were used. Heparinized plasma (1 ml) was adjusted to pH 8.3 ± 0.1 (pK_a value of HMA), giving optimal extraction (Fig. 1), using 0.1 *M* NaOH–0.1 *M* HCl, extracted with ethyl acetate (10 ml) [10], mixed on a whirl-mixer for 30 s and centrifuged for 15 min at 535 *g*. An 8-ml volume of the ethyl acetate phase was evaporated to dryness under a nitrogen stream at 56°C.

Thin-layer chromatography

TLC was performed on 20 × 20 cm plates precoated with 0.25 mm silica gel 60 (Merck). The dry residue was dissolved in 0.1 ml of ethyl acetate and applied to the plate 2 cm from the bottom and 1.5 cm from each side; at most twelve applications were used. The application was performed with a 100-μl Hamilton syringe up to 100 μl per spot, evaporated with a constant stream of warm air. The amount applied was $2 \cdot 10^{-10}$–$5 \cdot 10^{-9}$ mol HMA.

The plate was developed with tetrahydrofuran–3 *M* NH_4OH (9:1, v/v) at saturated conditions up to 2 cm from the top, air-dried, and read in a Zeiss densitometer KM III; excitation 380 nm, filter FL39 (emission above

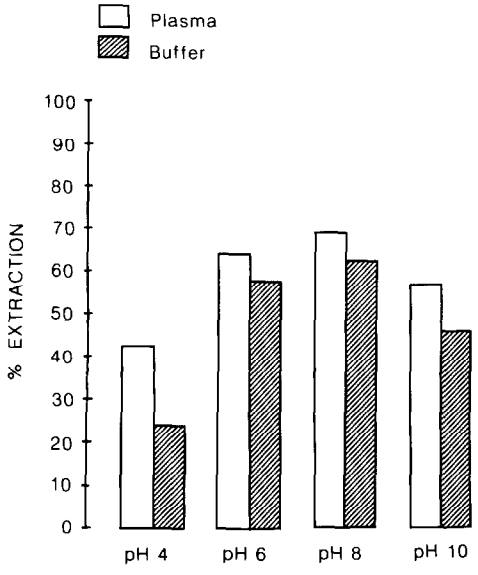

Fig. 1. Influence of pH on recovery of HMA during extraction.

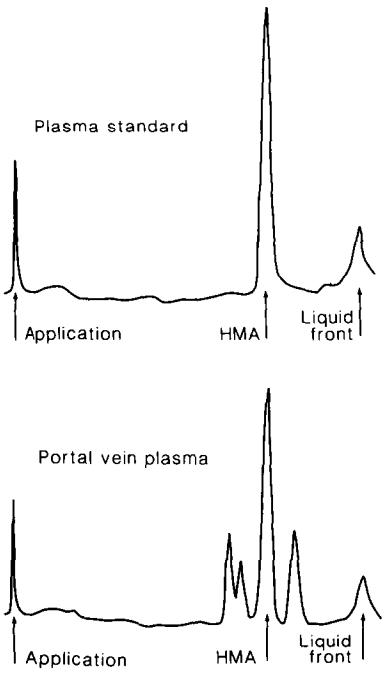

Fig. 2. Densitometer scans of extracted plasma from liver vein and from an extracted plasma standard spiked with HMA to the concentration $1 \cdot 10^{-6}$ *M*.

390 nm). The R_F value was 0.61. A typical densitometer scan is shown in Fig. 2. A log-linear relationship was obtained between the amount of HMA per spot and the fluorescence intensity. The samples were protected from light during the entire analytical procedure because HMA undergoes photodegradation. The limit of detection was determined as described by Kaiser [11].

Binding of HMA in plasma

Equilibrium dialysis was performed using 4 ml of plasma with HMA and 4 ml of Krebs–Ringer bicarbonate buffer, both gassed with CO_2 to pH 7.40 (PHM 75 clinical pH meter, stomach pH electrode GK 2801C Radiometer, Denmark). Dialysis took place under constant shaking in a water-bath at 37°C for 22 h, utilizing a dialysis membrane 20/32 (Union Carbide, Chicago, IL, USA) between two polymethylmethacrylate (Perspex, ICI, Cheshire, UK) cells with a total volume of 5 ml each. The cells were sealed with tape. At the end of dialysis the pH was 7.17 (S.D. = 0.22) on both sides of the membrane. The volume shift across the dialysis membrane due to the colloid-osmotic pressure of serum proteins was determined by weighing the content of the plasma and buffer compartments at the end of dialysis, and was 5.2% (S.D. = 2.9%) in the ten chambers examined.

Estimation of free HMA concentration in plasma samples

The free concentration of HMA in plasma samples from *in vivo* experiments was calculated using the measured total concentration and the free fraction determined by equilibrium dialysis.

RESULTS

Measurement of HMA in plasma

The recovery of HMA from spiked plasma samples was 72.4% (S.D. = 2.8%, $n = 6$). The detection limit was $0.6 \cdot 10^{-6}$ *M*, based on the extraction of 1-ml plasma or buffer samples. The accuracy at $1 \cdot 10^{-6}$ *M* HMA was 1.1% ($n = 10$). The within-assay coefficient of variation (C.V.) at 10^{-6} *M* HMA was 6.4% ($n = 10$), and the day-to-day C.V. over 6 months was 19% ($n = 13$).

Thin-layer chromatograms of extracts from

spiked normal pig plasma standards showed only a single fluorescent spot, with R_F value identical with that of HMA applied from stock solutions. Chromatograms of extracts from pig plasma after administration of HMA showed several fluorescent spots in addition to that identified as HMA (Fig. 2). The increased fluorescence intensity of these additional spots during the course of the experiments, and the higher values in the liver vein than in the portal vein of the femoral artery, indicated that these spots represented metabolites of HMA formed by the liver *in vivo* (Fig. 3). Repeated analysis of stored samples excluded the possibility that HMA was degraded *in vitro* during storage and analysis.

The plasma concentration of HMA in samples from the femoral artery, portal vein and liver vein apparently reached a plateau during the infusion. The values are shown in Table I. The concentrations were lower in the liver vein than in the femoral artery and the portal vein. The calculated metabolic extraction by the liver was 29%. After infusion was stopped, the concentration of HMA apparently decreased monoexponentially from 2 to 15 min. Fig. 4 shows the concentrations during and after infusion in one of the experiments. The half-life of HMA in plasma in that experiment was 12.8 min in the exponential phase (mean value from decay curves in femoral artery, portal vein and liver vein).

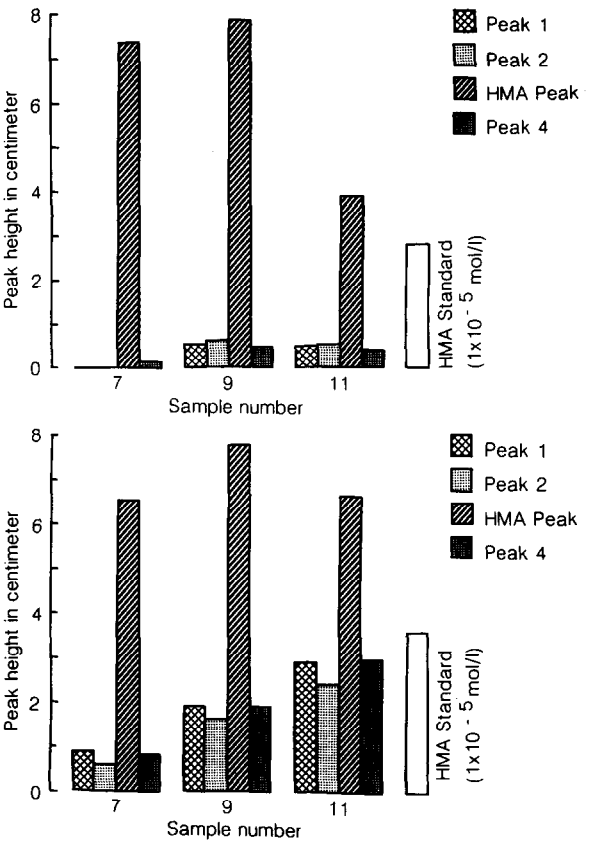

Fig. 3. Peak heights of fluorescent substances (peaks 1, 2 and 4) compared with those of HMA in three pairs of plasma samples from femoral artery (upper part) and liver vein (lower part) from one experiment. Samples 7, 9 and 11 were obtained 5, 13 and 21 min, respectively, after start of infusion.

TABLE I

CONCENTRATION OF TOTAL AND FREE HMA IN PLASMA FROM FEMORAL ARTERY, PORTAL VEIN AND LIVER VEIN

Data are based on triplicate analysis of plasma concentrations during infusion of HMA in six different experiments.

	Total concentration (10^{-5} *M*)			Free concentration (mean) (10^{-6} *M*)
	Mean	Standard deviation	Range	
Femoral artery	2.8	1.0	1.4–4.2	5.4
Liver vein	2.0	0.6	0.8–3.2	3.9
Portal vein	2.8	1.1	1.0–5.0	5.4

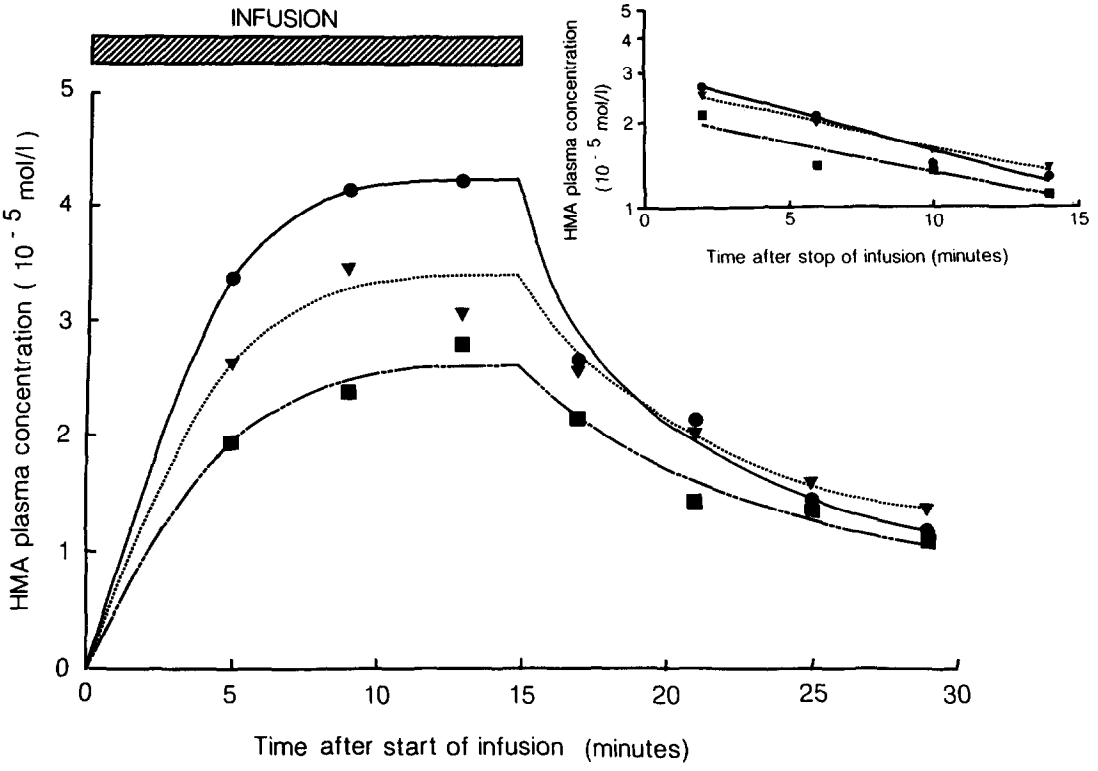

Fig. 4. Plasma concentrations of HMA in (●) femoral artery, (▼) portal vein and (■) liver vein during and after infusion in one experiment (curves drawn by hand). Inset shows the plasma concentration decay curves as a semilogarithmic plot and least-squares regression lines of concentrations from 2 to 14 min after infusion.

Binding of HMA in plasma

The mean free fraction of HMA in normal pig plasma was 19.4% (S.D. = 3.55%). The concentration of HMA in plasma at the end of dialysis was $7.1 \cdot 10^{-6}$–$8.2 \cdot 10^{-6}$ *M* (S.D. = 0.9). No concentration dependence of binding was observed. The calculated recovery of HMA based on the measured concentrations in the plasma and buffer compartments at the end of dialysis was 86%, indicating that the extent of degradation of HMA during dialysis was small. The relatively long dialysis time (22 h) was necessary to obtain equilibrium. The volume shift was only *ca.* 5%, thus causing little dilution of the plasma proteins during dialysis. Consequently, no correction for decreased binding owing to protein dilution was performed.

Free HMA concentration in plasma samples

The mean values for the estimated free concentration of HMA in plasma samples from the *in vivo* experiments are shown in Table I.

DISCUSSION

Evaluation of the dose regimen of HMA *in vivo* requires the determination of the free plasma concentration, since it is a generally accepted pharmacological principle that the effect of a drug *in vivo* is more closely related to the free drug concentration in plasma than to the total plasma concentration [12]. For this reason, adequate comparison of the effects of a drug *in vivo* and *in vitro* can be done only when free drug concentrations are known.

In this study we have developed a method for the determination of the total concentration of HMA in plasma and buffer. Combined with data on the extent of binding to plasma protein, the approach enabled us to estimate the free concen-

tration. The quantitative method for the determination of HMA described here was specific for HMA, as other amiloride derivatives have different R_F values in the chromatographic system used [13]. The chromatographic step was necessary, as shown by the appearance of additional fluorescent spots on the chromatograms of plasma extracts from *in vivo* samples. Although direct proof of the identity of peak 3 (HMA) was not obtained, identical R_F values for HMA standards and peak 3 strongly indicate that the latter represented HMA. The method was sensitive enough to determine HMA in plasma samples after administration of HMA to pigs *in vivo*, and in plasma and buffer after equilibrium dialysis *in vitro*.

The data on plasma pH [14] and volume shift [15] during equilibrium dialysis exclude large systematic errorrs in the determination of HMA binding due to these factors. Grayson *et al.* [16] reported that amiloride is not protein-bound, whereas Spahn *et al.* [17] found 40% to be protein-bound in human plasma. The higher binding of HMA observed in the present study than previously observed for amiloride is not unexpected when the higher lipophilicity of HMA is taken into account.

Although a plateau was apparently reached in the plasma concentration during infusion, a steady state might not have been reached in view of the half-life of 12.8 min. The rapid decrease in plasma concentration after infusion was stopped probably due to a distribution phase. The concentration difference between the portal vein and the liver vein was probably due to metabolism of HMA in the liver, in accordance with increasing levels of non-HMA fluorescent substances in plasma.

The estimated free concentrations of HMA in plasma after infusion of $80 \cdot 10^{-6}$ mol/min were high enough to inhibit the Na^+/H^+ exchanger by 89–98%, based on an inhibition constant (K_i) of $1.6 \cdot 10^{-7}$ *M* [5], calculated according to Cheng and Prusoff [18] and assuming that the inhibition obeys the law of mass action. This concentration would inhibit the Na^+/Ca^{2+} exchanger by only 5% based on the K_i value of $1 \cdot 10^{-4}$ *M* [19]. Because no significant inhibition of secretin-stimulated bile canalicular and pancreatic duct HCO_3^- secretion was produced by HMA in *in vivo* experiments using this infusion rate [6], it appears that the Na^+/H^+ exchanger is not important for secretin-stimulated HCO_3^- secretion.

In conclusion, the described method allows the determination of active, unmetabolized HMA in plasma in the biologically interesting concentration range.

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