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Disposition of the hypolipidaemic agent, 6-amino-2-mercapto-5-methylpyrimidine-4-carboxylic acid, in Sprague-Dawley rats

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Abstract—The disposition of [2-^{14}C] 6-amino-2-mercapto-5-methylpyrimidine-4-carboxylic acid has been determined in rats following intravenous and oral administration. A two-compartment pharmacokinetic model fitted to the blood and urinary data predicted its maximum terminal (β) half-life to be 38 h. Urinary and faecal excretion accounted for approximately 30 and 8% of the administered radioactivity, respectively. The parent compound accounted for 88% of the urine radioactivity after oral administration. In a tissue distribution study, the largest percentages of radioactivity were found in the skin and carcass; by 24 h, all other organs contained less than 1% of the administered radioactivity. The drug was highly water soluble, not extensively bound to plasma proteins, nor taken up by red blood cells. The drug uptake by human fibroblasts or rat aorta cells appeared to be by passive diffusion.

6-Amino-2-mercapto-5-methylpyrimidine-4-carboxylic acid (AMMCA) was shown to be an effective hypolipidaemic agent in rodents at 20 mg kg^{-1} day^{-1}, lowering both serum cholesterol and triglyceride levels (Hall et al 1984, 1985). The agent significantly lowered very low density lipoprotein and low density lipoprotein-cholesterol levels and elevated high density lipoprotein-cholesterol content after 14 days administration in rats (Hall et al 1985). The mode of action of this agent is to interfere with the synthesis of hepatic lipids at HMG-CoA-reductase and phosphatidylate phosphohydrolase steps. Since the compound did not demonstrate any acute toxicity in rats at five times the therapeutic dose, a pharmacokinetic and tissue disposition study was undertaken.

Materials and methods

Synthesis of [^{14}C]AMMCA. A solution of potassium α-methyl-ethoxalylacetonitrile (36·4 mg, 0·189 mmol) in 2·0 mL of 95% ethanol was added to 10·0 mCi of [^{14}C]thiourea (14·3 mg, 0·189 mmol) in 3·0 mL of 95% ethanol containing 8·0 mg (0·143 mmol) of KOH. The reaction was stirred for 35 h at room temperature (20°C) and then acidified to pH 3–4 with dilute HCl. The volatiles were removed under vacuum, and the residue was chromatographed on silica gel with CH_2Cl_2:MeOH:NH_2OH (60:40:1) to yield 27·0 mg (7·8 mCi) of pure, amber solid. Specific activity was 53 mCi mmol^{-1}, 0·29 mCi mg^{-1}.

Animals. Sprague-Dawley male rats were used and dosed with 20 mg kg^{-1} (5·5 μCi) [^{14}C]AMMCA unless otherwise noted.

Disposition studies in rats. (i) Intravenous administration. [^{14}C]AMMCA, dissolved in Minimum Essential Medium (MEM, pH 7·4), was administered directly into the femoral vein of rats (280 ± 8 g; n = 6) housed in Nalge metabolism cages. At various times, blood was collected from the tail vein, 50 μL was used for scintillation counting, and the percent radioactivity per total blood volume calculated. The total blood volume was taken to be 58 mL kg^{-1} (Mitruka & Rawnsley 1977). Urine and faeces were collected at specified times. Faecal samples were homogenized in 10 vol of water. Samples (100 μL) of urine or faecal homogenates were counted and the cumulative percent radioactivity excreted determined.

(ii) Oral administration. Rats (320 ± 6 g; n = 6) received an oral dose of [^{14}C]AMMCA dissolved in 1% carboxymethylcellulose. The rats were placed in metabolism cages and blood, urine, and faeces were collected. Samples were analysed as detailed in the intravenous administration study.

(iii) Absorption of [^{14}C]AMMCA from the stomach and small intestine. Rats (340 ± 8 g; n = 3) were anaesthetized with pentobarbitone (22 mg kg^{-1}) and chlorpromazine (25 mg kg^{-1}). In one group, a ligature was placed around the pylorus, and [^{14}C]AMMCA (in 1% carboxymethylcellulose) was administered by gavage. In the second group, a ligature was placed around the small intestine approximately at the ileum, and another ligature was placed around the pylorus. [^{14}C]AMMCA was introduced below the pylorus. In both groups, blood was collected from the tail vein for 6 h, and plasma radioactivity was determined as described in the tissue distribution study. Results were compared with orally dosed animals without any alimentary tract restriction (see tissue distribution study).

(iv) Urinary metabolites of [^{14}C]AMMCA. Urine from orally dosed rats (300 ± 6 g; n = 3) was pooled from 0–48 h. Samples of urine were untreated or treated with 1000 units of β-glucuronidase (Sigma Chemical Co.) or for 24 h at 37°C. The samples were then adjusted to pH 5, 7, or 9, and mixed with an equal volume of water-saturated ethyl acetate. The solution was vortexed, allowed to equilibrate for 30 min, centrifuged for 10 min at 3500 *g*, and then samples of the ethyl acetate and aqueous layer were counted. Both the ethyl acetate and aqueous layers were plated on silica gel thin layer chromatography (TLC) plates and eluted with: (a) chloroform–methanol (1:1); (b) chloroform–methanol (9:1); (c) chloroform–ethyl acetate (2:1); (d) chloroform–ethanol (1:1); (e) chloroform–ethyl acetate–acetone (10:60:30); or (f) chloroform–ethyl acetate–methanol (10:50:40). TLC plates were divided into 10 regions, scraped, and counted. Additional plates were also scanned with a Bioscan BID-100 Image Analyzer and the radioactivity quantitated.

(v) Tissue distribution of [^{14}C]AMMCA. Rats (350 g; n = 6) were administered [^{14}C]AMMCA (in 1% carboxymethylcellulose) by gavage. At various times animals were decapitated, blood was collected from the trunk, plasma harvested, and the tissues excised, rinsed, blotted, weighed, and homogenized in 0·025 M sucrose plus 0·001 M EDTA buffer (pH 7·2). The animal carcass, bone, and skin were digested in 30% KOH heated to 55°C for 72 h. Plasma samples (100 μL) were counted, the plasma volume estimated from the percent haematocrit, and the percent radioactivity per plasma volume calculated. Each tissue homogenate (100 μL sample) was counted and the percent radioactivity (g tissue)$^{-1}$ calculated.

(vi) Albumin binding of [^{14}C]AMMCA. An activity of the drug which was equal to the maximum radioactivity found in the plasma after oral administration was incubated with 4·5 mL of

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bovine serum albumin (20%) or rat plasma at 25°C for 72 h. The mixture was placed in a #2 dialysis bag (Spectra/por: mol. wt cutoff 12000–14000) and dialysed at 0°C against phosphate buffered saline (PBS)–EDTA for 3 days. Samples (100 μL) were taken from both the buffer and the dialysis bag contents and counted. Samples of the dialysis bag contents were precipitated with 10% trichloroacetic acid (TCA); the precipitated pellet resuspended and an aliquot counted.

Partitioning studies. (i) Partition coefficient of [^{14}C]AMMCA. The water–octanol (20:80) partition coefficient was determined by the method of Leo et al (1971). The amount of drug in each layer was quantitated by scintillation counting and ultraviolet (UV) detection.

(ii) Red blood cell uptake of [^{14}C]AMMCA. Blood was collected from the abdominal vein of rats and centrifuged at 3500 *g* for 20 min. The red blood cell pellet was resuspended in PBS–EDTA (pH 7·4) and incubated with 3·3 μCi of [^{14}C]AMMCA for 6 h at 37°C. Samples were removed at various times, plated on Whatman #1 discs, washed with boiling 5% trichloroacetic acid (TCA), cold 5% TCA, diethylether–ethanol (1:1), and diethylether. The discs were dried and counted.

(iii) Human fibroblast and rat aorta cell uptake of [^{14}C]AMMCA. Human BG-9 fibroblasts were plated on tissue culture dishes (5×10^4 cells per plate) suspended in MEM with 10% foetal calf serum and penicillin G (10 int. units) or streptomycin (10 μg). Rat aorta cells were cultured by the method of Bierman et al (1974). Cultures were maintained in MEM with 10% newborn calf serum, non-essential amino acids, and penicillin G or streptomycin. After the cells were confluent, fresh medium containing 50 μL of [^{14}C]AMMCA was added to the dishes. After 10, 15, 30, and 45 min, and 1, 2, 4, 6, and 8 h, the medium was decanted and discarded. The cells were washed (6×) with PBS–EDTA (pH 7·4), lysed with 1 mL 0·1 M NaOH (3×), pooled, and a sample counted. DNA (Chae et al 1970), RNA (Wilson et al 1975), and protein (Sartorelli 1967) were chemically isolated from the respective cells and counted.

Results

After intravenous administration, the averaged radioactivity in the total blood volume declined in a multi-exponential manner (see Table 1). A two compartment phamacokinetic model adequately described the decline in radioactivity versus time, and the terminal (β) half-life ($t_{\frac{1}{2}}$) was estimated to be 37·2 h. Sigma minus and excretion rate plots of the excreted radioactivity yielded $t_{\frac{1}{2}}$ values of 35·4 and 40·4 h, respectively (plots not shown). Since the data represented the total radioactivity in blood, the half-life of [^{14}C]AMMCA would be equal to or less than the $t_{\frac{1}{2}}$ (Gibaldi & Perrier 1975).

Table 1. Averaged percent of radioactivity in total blood volume and cumulative urinary and faecal excretion of [^{14}C]AMMCA.

Time (h)	I.v. administration			Oral administration		
	Blood	Urine	Faeces	Blood	Urine	Faeces
0·12	29·65	2·65	—	2·62	—	—
0·25	10·81	2·92	0·03	4·78	—	0·23
0·50	6·00	—	0·06	6·54	1·99	0·27
0·75	4·83	—	0·08	4·91	—	0·28
1·0	3·80	—	0·08	4·18	2·53	0·44
2·0	2·45	—	0·11	2·79	7·32	0·61
4·0	1·59	9·92	0·35	2·00	13·45	1·13
6·0	1·84	10·92	0·54	1·90	13·82	1·70
8·0	1·24	—	0·66	1·51	14·40	3·14
12·0	1·19	16·36	1·13	1·63	17·26	5·22
24·0	1·05	20·85	4·00	1·16	25·40	7·94
48·0	0·45	24·94	4·86	0·64	28·38	8·38
72·0	0·34	26·75	5·27	0·46	30·03	8·58
96·0	0·38	27·72	5·51	0·28	31·05	8·67
120·0	0·27	28·52	6·45	0·26	31·71	8·75
144·0	—	29·11	—	—	32·41	8·82
168·0	—	—	—	—	33·06	—

Standard deviations were < 5% of the mean d min^{-1} of each time.

Table 2. Averaged percent of radioactivity in plasma volume after [^{14}C]AMMCA administration in various alimentary sites.

Time (h)	Percent radioactivity per plasma volume		
	Oral	Gastric	Duodenum
0·08		0·39	0·98
0·12	1·17		
0·17		0·40	1·14
0·25	1·15		
0·33		0·44	1·21
0·50	1·37	0·42	1·29
0·75	1·14	0·42	1·27
1·0	0·80	0·49	1·24
2·0	0·57	0·56	0·93
4·0	0·74	0·53	0·79
6·0	0·50	0·44	0·46

Standard deviations were < 4% of the mean d min^{-1} of each time.

Oral administration of [^{14}C]AMMCA produced the expected triexponential fit to the averaged radioactivity data and yielded a $t_{\frac{1}{2}}$ of 38·9 h (see Table 1). Sigma minus and excretion rate plots estimated $t_{\frac{1}{2}}$ values of 26·1 and 67·7 h, respectively. The excretion rate plot displayed a great deal of variability at the earlier times thus reducing the confidence in the estimated $t_{\frac{1}{2}}$. [^{14}C]AMMCA was absorbed more rapidly and to a greater extent when placed in the duodenum than the stomach (see Table 2). The levels of drug which resulted from duodenum absorption mimicked the levels after unrestricted oral administration, indicating that the duodenum was the principal absorption site.

Regardless of the route of administration, urinary excretion accounted for approximately 30% of the administered radioactivity (see Table 1). Most of excreted radioactivity was in the aqueous phase: at pH 5, 71·4%; at pH 7·0, 88·8%; and at pH 9·0, 58·5%. Urine samples pretreated with β-glucuronidase gave the following percentages of radioactivity in the aqueous phase: at pH 5, 80%; at pH 7·0, 71%; and at pH 9·0, 72·6%. These data showed that a glucuronide or sulphate was not a primary metabolite. TLC elution of both the ethyl acetate and aqueous layers with chloroform–methanol (1:1), chloroform–methanol (9:1), chloroform–ethyl acetate (2:1), and chloroform–ethanol (1:1) showed 91, 99, 99·5, and 84·8% of the radioactivity in the chloroform phase, respectively. The parent drug accounted for 88% of the excreted radioactivity and approximately 12% existed as an unidentified metabolite ($R_f = 0·38$). The elution with chloroform–ethylacetate–acetone (10:60:30) or chloroform–ethylacetate–methanol (10:50:40) gave two peaks at $R_f = 0·18$ and $R_f = 0·48$. Blank rat urine (pH 5·0) with added [^{14}C]AMMCA eluted two peaks at $R_f = 0·19$ and $R_f = 0·48$. Therefore, the drug is thought to exist as a zwitter-ion.

The percent distribution of radioactivity in tissues after oral administration of [^{14}C]AMMCA showed that heart, spleen, brain, adrenals and thymus levels were less than 0·6% at all times (data not shown). The lungs, kidneys, and reproductive organs varied in the time of peak radioactivity (0·25–4 h), but the peak radioactivity was only 2–4% (data not shown). The maximal percent in the liver was 7·3%, and occurred at 1 h (see Fig. 1). Peak times (and percent

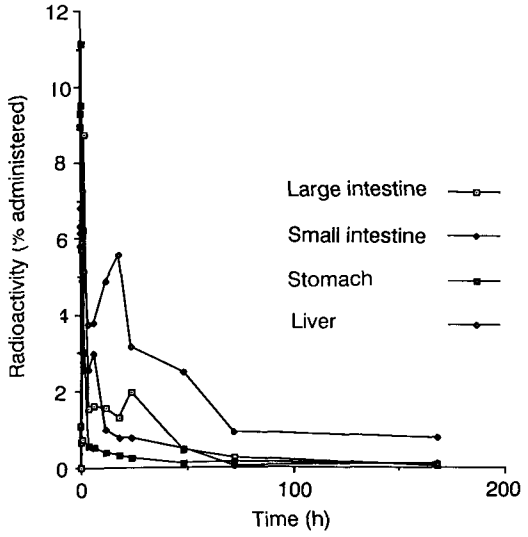

FIG. 1. Mean radioactivity (expressed as percent of total radioactivity administered) found in large intestine, small intestine, stomach, and liver after oral administration of 20 mg kg^{-1} (5·5 μCi) of [^{14}C]-AMMCA (standard deviations were < 5% of the mean d min^{-1} of each time).

radioactivity) in the stomach, small intestine, and large intestine were 0·75 h (11·1%), 0·5 h (5·7%), and 2·0 h (8·7%), respectively. The skin and carcass radioactivity accounted for the largest percentages of [^{14}C]AMMCA at sites external to the alimentary tract (see Fig. 2). The maximum skin percentage was 22·4%, reached at 2 h. The carcass percentage peaked at 6 h (18·4%), and declined to less than 3% by 168 h. The urinary and faecal excretion data shown in Fig. 2 is cumulative data.

In-vitro studies demonstrated that 2·1% of [^{14}C]AMMCA was bound to bovine serum albumin. The TCA treatment of the plasma protein bound drug demonstrated that 1·6% remained firmly bound. The rat plasma protein binding of [^{14}C]AMMCA was 1·8%. This amount appeared securely bound since 1·7% of [^{14}C]AMMCA remained bound to the plasma proteins after TCA treatment.

The partition coefficient (log P) between octanol and water

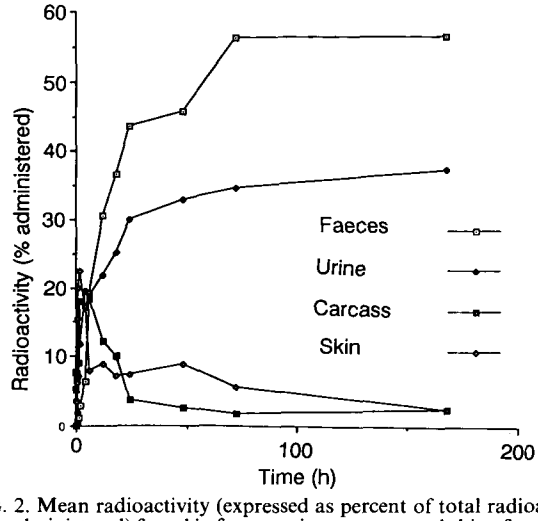

FIG. 2. Mean radioactivity (expressed as percent of total radioactivity administered) found in faeces, urine, carcass, and skin after oral administration of 20 mg kg^{-1} (5·5 μCi) of [^{14}C]AMMCA (standard deviations were < 5% of the mean d min^{-1} of each time).

was 0·078 indicating that the drug was highly water soluble. The maximum UV absorption of the drug was 290 nm. Red blood cell uptake of [^{14}C]AMMCA was small; the peak uptake (3·9%) was reached after 4 h. Tissue culture studies with human fibroblasts (BG-9) and rat aorta cells suggested that the drug was taken up in the cells by passive diffusion. Cell uptake occurred within 10 min with 1·7% in the fibroblasts and 1·4% in the aorta cells. Maximum accumulation was 2·9% at 8 h in fibroblasts and 3·4% at 6 h in aorta cells. After 24 h incubation in human fibroblasts, more [^{14}C]AMMCA was bound to DNA (4·5%) than RNA (3·1%), and protein contained higher radioactivity (3·4%) than RNA. Rat aorta cells demonstrated an entirely different pattern with 1·5% of drug bound to DNA, 1·9% to RNA, and 0·4% to proteins.

Discussion

The intravenous and oral administration studies showed a very similar percentage of excreted radioactivity in urine and faeces (see Table 1). This could indicate that absorption was complete and biliary recycling minimal. However, the studies also showed that approximately 38% of the administered radioactivity was excreted by 168 h, or 4·4 half-lives. By that time, most of the drug that was going to be excreted should have been excreted via these routes; thus, 62% of the drug was unaccounted for at a time when elimination was essentially complete. In the intravenous and oral administration studies, faecal samples were collected from faeces actually excreted from the animal. But in the tissue distribution study, faeces were expressed from the caecum and large intestine, and combined with the excreted faeces (see Fig. 2). In the tissue distribution study, the "faecal excretion" accounted for 56·7% of the total radioactivity, and when combined with the cumulative urinary excretion (37·6%), accounted for approximately 90% of the radioactivity. Since the faecal collection procedure was different in the intravenous study and the tissue distribution study, conclusions about the absorption efficiency or biliary excretion of [^{14}C]AMMCA are not possible. However, its placement directly into the duodenum led to approximately twice the rate and extent of absorption compared with gastric or unrestricted oral administration. It is likely that the gastric pH or constitutents are responsible for the altered absorption.

The blood radioactivity vs time data after intravenous administration of [^{14}C]AMMCA was adequately described by a two-compartment pharmacokinetic model indicating that tissue distribution played a significant role in its disposition. The tissue disposition study showed that it was not sequestered in any of the tissues. [^{14}C]AMMCA showed minor distribution into the primary organs (brain, heart, lung, kidney, spleen, adrenals, reproductive organs). Higher levels were found in the small and large intestine, stomach, and liver. The highest distribution of radioactivity was found in skin and carcass; even at 168 h, 3% of the administered radioactivity remained in these two organs. Such a distribution pattern coupled with the fact that approximately half of the urinary and faecal excretion had occurred by 4 h would be expected of a compound that showed significant water solubility, low protein binding, and minimal red cell uptake.

Pharmacokinetic analysis of both blood and urine radioactivity data showed a $t_{\frac{1}{2}}$ of 38 h. Urinary excretion data showed that 88% of the excreted radioactivity was the parent compound. Since the majority of the radioactivity remained as the unchanged parent compound, there was increased likelihood that the $t_{\frac{1}{2}}$ of [^{14}C]AMMCA was approximately 38 h.

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A comparison of aminopeptidases from excised human buccal epithelium and primary cultures of hamster pouch buccal epithelium

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Abstract—Aminopeptidase activity associated with human buccal tissue and primary cultures of hamster buccal epithelium homogenates was assayed fluorometrically using 4-methoxy-2-naphthylamides of leucine, alanine, and arginine. Kinetic parameters, K_m and V_{max}, for all substrates were estimated. Aminopeptidase parameters for human tissue were similar to those for the in-vitro system and reported literature values for rodent buccal tissue aminopeptidases. Aminopeptidases of both tissues were also found to be similarly sensitive to typical inhibitors, bestatin and puromycin. Overall results suggest that appropriate in-vitro systems derived from animal tissues may be useful in assessing the role and localization of peptidases associated with buccal tissue.

Because of the significant proteolytic activity encountered along the gastrointestinal tract following oral administration of peptides, recent attention has focused on systemic delivery of these agents by intranasal, ocular, rectal, transdermal and buccal routes (Banga & Chien 1988; Audus et al 1990). As an alternative, the application of peptides in certain dosage forms on buccal epithelium has been suggested for the systemic delivery of selected peptide hormones (Ishida et al 1981; Nagai & Machida 1985; Merkle et al 1990) but a major limitation to adequate bioavailability appears to be the absorption process (Gupta et al 1990). Another possible limiting factor in the buccal delivery of peptides, may be degradation due either to interactions with secreted peptidases in the oral cavity or to epithelial peptidases. The possible role of peptidases in buccal delivery has been suggested from rodent studies (Kashi & Lee 1986; Stratford & Lee 1986; Garren & Repta 1988; Tavakoli-Saberi & Audus 1989a; Garren et al 1989) and the enhancement of the permeation of aminopeptidase labile substrates across rodent buccal tissues in-vitro (Garren et al 1989).

In this study, we have examined aminopeptidases in excised human buccal tissue. The results obtained were compared with an in-vitro system comprised of primary cultures derived from buccal epithelium of a representative animal model (hamster) for studying the buccal route of administration (Tavakoli-Saberi & Audus 1989a, b).

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Materials and methods

Materials. Aminopeptidase substrates, 4-methoxy-2-naphthylamides, and inhibitors, bestatin and puromycin, were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest grade commercially available.

Buccal tissue preparation. Superficial human buccal tissues were surgically removed postmortem from tissue donors at the VA Medical Center, Kansas City, MO. Anonymous donors chosen were of any age, either sex, but were non-smokers without oral disease. Tissues from individuals were collected within about 4–5 h of death and transported to the laboratory within 1 h in ice-cold phosphate buffered saline, pH 7·4 (PBS). The tissue probably included some minor components of underlying tissues. Based on the observation of Garren et al (1989) that aminopeptidase activity resides predominantly in the epithelial cell layers, further dissection of the tissues was not made.

Homogenous populations of hamster buccal epithelial cells were isolated and grown in primary culture (Tavakoli-Saberi & Audus 1989a, b). Expression of aminopeptidases has been shown to be retained in such primary cultures when compared with freshly excised hamster tissue (Tavakoli-Saberi & Audus 1989a). The viability of the tissue cultures was 100% as estimated by trypan blue exclusion before collection and homogenization for aminopeptidase assays.

Homogenate preparations. Either excised human buccal tissue or primary cultures of hamster buccal epithelial cells grown in 100 mm culture dishes (Tavakoli-Saberi & Audus 1989a, b) were rinsed three times with phosphate buffered saline, pH 7·4 (PBS). Hamster epithelial cells grown in 100 mm dishes were scraped from the dishes and collected in 2–3 mL of PBS containing 0·32 M sucrose. Excised human buccal tissues were also placed in PBS containing 0·32 M sucrose and cut into small cubes (1 mm).