Biochemical Pharmacology, Vol. 24, pp. 1943–1946. Pergamon Press, 1975. Printed in Great Britain.

FUROSEMIDE ACCUMULATION BY RENAL TISSUE

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(*Received* 16 *January* 1975; *accepted* 4 *April* 1975)

Abstract—Furosemide accumulation by rabbit kidney cortical slices increased with incubation time, reaching a plateau by 60–90 min. Furosemide uptake by kidney slices from rabbits, dogs, mice and rats was inhibited by dinitrophenol, ouabain and probenecid, and was decreased by incubation at 0°, anoxia or the use of sodium-free medium. Furosemide slice/medium ratios decreased with increasing concentration of furosemide in the incubation medium. Addition of furosemide to medium containing rat kidney slices and either *p*-aminohippurate (PAH) or *N*-methylnicotinamide (NMN) produced a dose-related inhibition of PAH, but not NMN, accumulation. It is concluded that furosemide is actively transported by the renal organic anion transport system.

Furosemide, a sulfonamide-anthranilic acid derivative, is a potent diuretic widely used for cardiac and pulmonary edema, hypertension and other indications. Unlike most diuretics, furosemide appears to be effective in the presence of impaired renal function [1, 2] and can produce a diuresis at increasingly high doses when usual doses are ineffective [3, 4]. In addition to water and electrolyte depletion, high doses of furosemide may cause auditory damage, particularly when renal function is impaired [5, 6]. Furosemide has been reported to enhance the nephrotoxicity of several antibiotics given to laboratory animals with transient renal impairment [7, 8]. Earlier experiments have suggested that furosemide is secreted by the renal organic acid transport system [9, 10]. Shelp and Rieselbach [11] indicated that inhibition of the organic anion secretory system by various drugs may result in higher plasma levels of furosemide. The purpose of the present study was to specifically investigate the mechanism and magnitude of furosemide renal tubular secretion.

METHODS

Male New Zealand white rabbits (1–2 kg) were stunned by a blow on the head, while male Labrador dogs (25–30 kg) were anesthetized with pentobarbital (30 mg/kg, i.v.). Male Wistar rats (250–300 g) from Biobreeding Labs and male Swiss–Webster mice (25–30 g; Health Protection Branch strain) were anesthetized with ether. The kidneys, and in some cases the livers, were quickly excised and placed in cold saline. Renal cortical slices or liver slices were prepared free-hand and kept briefly in cold saline until incubated. Approximately 100 mg of slices was incubated in 2·7 ml phosphate buffer medium (pH 7·4) containing 10 mM glucose, 10 mM sodium acetate and either ^{35}S-furosemide (9·2 mCi/m-mole) or unlabeled furosemide (courtesy of Hoechst Pharmaceuticals). In some experiments, either $7{\cdot}4 \times 10^{-5}$M ^{14}C-*p*-aminohippurate (PAH, 12·4 mCi/m-mole) or $6{\cdot}0 \times 10^{-6}$ M ^{14}C-*N*-methylnicotinamide (NMN, 4·6 mCi/m-mole) was added to the medium containing unlabeled furosemide at concentrations ranging from 10^{-6} to 10^{-3} M. Incubations were normally carried out in a Dubnoff metabolic shaker at 25° under a gas phase of 100% oxygen for 60 or 90 min, although the incubation time ranged from 15 to 120 min when studying the rate of uptake. In some cases, slices were incubated under a gas phase of nitrogen or at 0°, while in other studies dinitrophenol (DNP, 10^{-4} M), ouabain (10^{-4} M) or probenecid (10^{-4} M) was present in the incubation medium. When studying the effects of sodium and potassium, an equimolar concentration of sucrose replaced the ion normally present in the medium.

After incubation, the slices were rapidly removed from the beakers, blotted, and weighed. In studies involving ^{35}S-furosemide, the slices were digested in 1 ml soluene. Instagel (Packard) was then added to these vials and to vials containing 0·5 ml incubation medium. Unlabeled furosemide was assayed by a modification of the method described by Hajdu and Haussler [12]. Tissue slices were placed in 2 ml of 1·2 N HCl, while 1 ml incubation medium was acidified with 1 ml of 1·2 N HCl. Samples were heated at 70° for 45 min. After centrifugation, a 1-ml aliquot was diluted with 1 ml of 1·2 N HCl and the Bratton–Marshall reaction was performed using the following reagent concentrations: 0·25 ml of 100 mg/100 ml of sodium nitrite, 0·25 ml of 500 mg/100 ml of ammonium sulfamate and 0·25 ml of 200 mg/100 ml of *N*-(1-napthyl ethylenediamine). In experiments involving ^{14}C-PAH and ^{14}C-NMN, slice and medium samples were prepared as described by Cross and Taggart [13] and Hook and Munro [14] using a modified Bray's solution [15]. Radioactivity in all cases was counted using a Packard TriCarb liquid scintillation spectrometer. Accumulation of furosemide, PAH or NMN was expressed either as μg/g of tissue or as the slice/medium (S/M) ratio, where S equals μg/g or dis./min/g of tissue and M equals μg/ml or dis./min/ml of medium.

Calculations and unpaired Student's *t*-test analyses were performed on a Hewlett–Packard-65 desk calculator. The 0·05 level of probability was used as the criterion of statistical significance.

RESULTS

Rabbit renal cortical slices appeared to actively accumulate furosemide (Fig. 1). The furosemide S/M

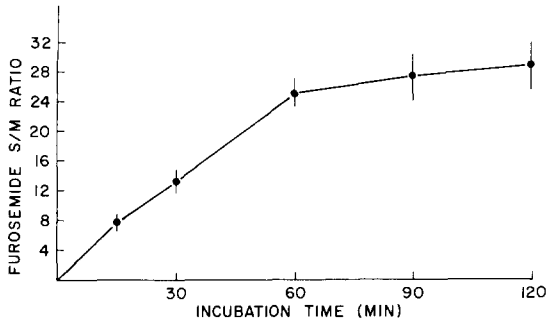

Fig. 1. Accumulation (S/M ratio) of ^{35}S-furosemide by rabbit kidney cortical slices in a medium of 9×10^{-6} M, using increasing incubation times. Points represent means ± standard error from five to eight experiments.

ratio increased with incubation time from 15 to 60 min, reaching a plateau between 60 and 90 min. Furosemide accumulation did not increase significantly when incubation was continued to 90 or 120 min.

Furosemide accumulation by rabbit kidney cortical slices under various incubation conditions further suggested that an energy-requiring process was involved. Dinitrophenol, incubation under 100% N_2 or incubation at 0° reduced the furosemide S/M ratio from a control value of 23 to about 5 (Fig. 2). The presence of 10^{-4} M probenecid also decreased furosemide accumulation (Fig. 2), as did ouabain (Fig. 3). Deletion of sodium from the incubation medium also reduced furosemide uptake, while omission of potassium did not significantly decrease the furosemide S/M ratio.

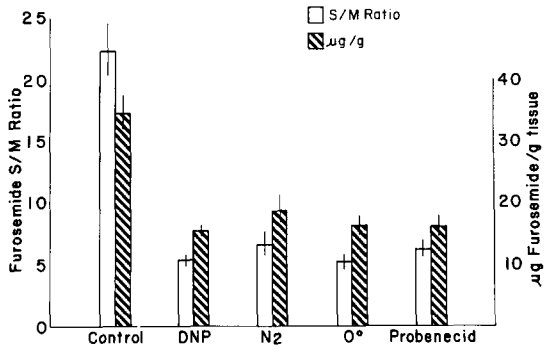

Fig. 2. Effect of metabolic inhibitors and incubation conditions on furosemide accumulation (S/M ratio or µg/g of tissue) by rabbit kidney cortical slices. Values are the means ± standard error from five experiments, with an incubation time of 60 min and furosemide concentration of 9×10^{-6} M.

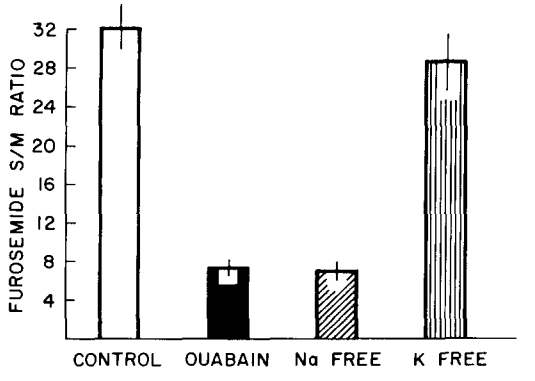

Fig. 3. Effect of metabolic inhibitors and incubation conditions on ^{35}S-furosemide accumulation (S/M ratio) by rabbit kidney cortical slices. Values are the means ± standard error from five experiments, with an incubation time of 90 min and furosemide concentration of 90×10^{-6} M.

Table 1. Effect of medium concentration of ^{35}S-Furosemide on its accumulation (S/M ratio) by renal cortical slices from rabbits and dogs*

Concn (M)	Furosemide S/M ratio	
	Rabbit	Dog
4.5×10^{-7}	30.0 ± 4.6	43.1 ± 4.1
4.5×10^{-6}	27.4 ± 3.4	27.7 ± 2.6
4.5×10^{-5}	25.0 ± 2.3	21.4 ± 3.7

* Values are the mean furosemide S/M ratios (±standard error) from three to six experiments, with an incubation time of 60 min.

The furosemide S/M ratio declined with increasing concentration of furosemide in the incubation medium in both rabbit and dog renal cortical slices (Table 1). Furosemide accumulation under several different incubation conditions was compared using renal cortical slices from dogs, mice, rats and rabbits (Table 2). The results were comparable in these species in that inhibition of cellular metabolism by various procedures such as the use of DNP, probenecid, sodium-free media or the use of N_2 as the gaseous phase reduced furosemide accumulation. Liver slices were unable to accumulate furosemide to a substantial degree in any of the species studied (Table 2).

Addition of furosemide to incubation beakers containing rat kidney cortical slices, media, and either ^{14}C-PAH or ^{14}C-NMN produced a dose-related depression of PAH accumulation (Fig. 4). A concentration of 3×10^{-5} M furosemide reduced PAH uptake by approximately 35 per cent, while 3×10^{-4} M furosemide reduced the PAH S/M ratio by 75 per cent. NMN accumulation, on the other hand, was not affected by furosemide except at the high concentration of 3×10^{-3} M, at which concentration there likely was a general inhibition of renal cellular metabolism.

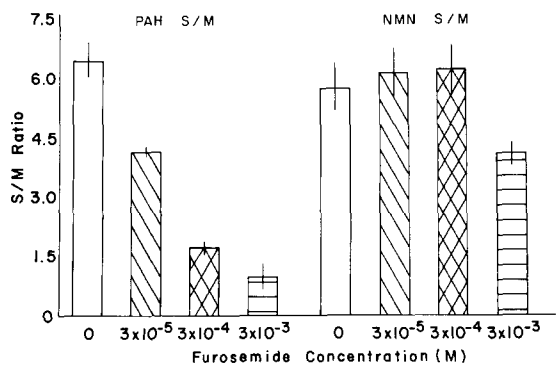

Fig. 4. Effect of varying furosemide medium concentrations on PAH or NMN accumulation (S/M ratio) by rat kidney cortical slices. Values are the means ± standard error from five experiments, with an incubation time of 90 min.

Table 2. Effect of metabolic inhibitors and incubation conditions on ^{35}S-Furosemide accumulation by kidney and liver slices from various species*

	Kidney slices					Liver slices
	Control	DNP (10^{-4} M)	Nitrogen	Probenecid (10^{-4} M)	Na-free	Control
Rabbit	22·5 ± 2·1	5·4 ± 0·6†	6·5 ± 1·2†	6·2 ± 0·8†	6·9 ± 1·0†	2·5 ± 0·3†
Dog	27·7 ± 2·6	5·7 ± 0·3†	8·2 ± 2·0†	9·6 ± 1·4†		3·8 ± 0·5†
Mouse	9·5 ± 0·2	3·7 ± 0·1†	4·5 ± 0·2†	3·6 ± 0·1†	5·2 ± 0·2†	
Rat	14·3 ± 0·7	3·7 ± 0·2†	4·7 ± 0·3†	3·6 ± 0·1†	9·6 ± 0·3†	2·6 ± 0·5†

* Values are the mean furosemide S/M ratios (± standard error) from three to six experiments. The concentration of furosemide in the medium was 9×10^{-6} M; the incubation time was 60 min except in mice where it was 90 min.

† Values significantly different from their respective controls, $P < 0.05$.

DISCUSSION

These studies were undertaken to determine the extent to which renal tissue is capable of concentrating or accumulating furosemide from an incubation medium. The approach *in vitro* was utilized in order to reduce problems of rates of metabolism, plasma binding, drug distribution and other factors. The accumulation process *in vitro* reflects the capacity of the kidney *in situ* to accumulate a drug from plasma, thereby raising its effective concentration within kidney cells. Renal cortical slices from all four species studied accumulated furosemide to a significant extent, with S/M ratios in rabbits and dogs being about 25–30 at a concentration of 9×10^{-6} M. Uptake of furosemide increased with incubation time and reached a plateau at about 60 min. The data suggest that furosemide accumulation by renal cortical slices involves the organic acid transport system. The observation that the furosemide S/M ratio decreased as the furosemide medium concentration was increased also indicates the involvement of a saturable carrier-mediated process. Although furosemide is highly bound to plasma proteins [16], Berndt and Mudge [17] have shown that extensive protein binding does not inhibit organic anion secretion.

The relatively specific organic anion inhibitor, probenecid [18, 19], markedly reduced the uptake of furosemide. Hook and Williamson [20] reported that probenecid was able to block the diuretic response to furosemide in dogs. Similarly, low concentrations of furosemide specifically reduced the uptake of PAH while having no effect on the organic base NMN. Hook and Hirsch [21] showed that an actively transported anion added to the incubation medium would inhibit PAH but not the base, whereas a general metabolic inhibitor would inhibit both acid and base transport.

It appears that the major dependence for renal cellular transport or accumulation of furosemide is upon oxidative tissue metabolism. The effects of low temperature, anoxia and metabolic inhibitors all suggested that a metabolically dependent active uptake process for furosemide is involved. Quite clearly from our results, however, a portion of the observed furosemide uptake was not abolished by reduction of the incubation temperature to zero degrees, by substitution of nitrogen for oxygen as the gaseous phase, or by the presence of metabolic inhibitors. This residual fraction presumably is the result of nonspecific uptake or binding of furosemide. Similarly, the magnitude of furosemide accumulation by the liver in rabbits and dogs was very low, possibly reflecting nonspecific binding and indicating organ specificity for furosemide accumulation in the kidney. Dinitrophenol has been shown to block the transport of various organic acids handled by the PAH transport system [22].

The inhibitory action of ouabain on furosemide uptake suggests that the organic acid transport system is sensitive to cardiac glycosides. In this regard, Charnock and Almeida [23] have suggested that the ability of ouabain to reduce ethacrynic acid uptake was related to a dependence of the PAH transport system on the maintenance of adequate sodium ion gradients. The requirement for sodium is also indicated in the present studies by the reduction in furosemide accumulation when sodium-free media were used. Gerencser *et al.* [24] have recently reported that PAH uptake is dependent on the presence of sodium, supporting earlier work by Chung *et al.* [25] showing the dominant role of sodium in organic acid transport. The role of potassium in furosemide accumulation by the organic acid transport system is unclear, since the furosemide S/M ratio, using unleached slices, was not inhibited when potassium was deleted from the incubation medium. Phenolsulfathalein uptake is inhibited less than that of PAH in potassium-free medium [25], suggesting that the potassium requirement for organic acid transport varies somewhat. Additional studies similar to those done with sodium by Gerencser *et al.* [24] will have to be carried out to determine if potassium has any effects on other furosemide transport parameters, including V_{max} and K_m.

The importance of furosemide transport, as demonstrated in these studies *in vitro*, is supported by the work of Burg *et al.* [26], who recognized the importance of a high renal concentration of furosemide, and by the work of Calesnick *et al.* [27], who found that humans excreted 77 per cent of an injected dose in the urine within 4 hr. However, the half-life of furosemide is prolonged in patients with renal disease [28], although nonrenal excretion then increases. Since the renal organic anion transport system plays a predominant role in the excretion of furosemide, it is evident that furosemide should be used with care when renal function is impaired. Patients with renal failure have higher serum levels of unbound furosemide available for metabolism in the liver, where fur-

osemide can be converted to a toxic arylating metabolite [29]. Future studies will be directed toward evaluation of the role that tubular secretion plays in furosemide efficacy and its possible interaction with other drugs.

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