Application of the Carbonic Anhydrase Inhibitory Effect of Furosemide to the Study of Furosemide Release from Two of Its Diuretic Derivatives

Gil Navon,*[1a] Jashovam Shani (Mishkinsky),[1b] Ruth Panigel,[1a] and Shlomo Schoenberg[1c]

Department of Chemistry, Tel-Aviv University, Department of Applied Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, and "Teva" Pharmaceutical Industries, Jerusalem, Isreal. *Received December 2, 1974*

Bovine carbonic anhydrase B (CA) inhibitory effect of furosemide was established in two pH values at 26°. FFBu [*N*-furfuryl-4-chloro-5-(butoxymethylsulfamoyl)anthranilic acid] and FFMe [*N*-furfuryl-4-chloro-5-(methoxymethylsulfamoyl)anthranilic acid], two of its alkoxymethyl derivatives, did not exert any CA inhibitory effect at those conditions but were found to inhibit the CA activity after their hydrolysis, which yielded the furosemide molecule. The CA inhibitory effect of furosemide was utilized for determining the kinetic rate constants for the hydrolysis of FFBu and FFMe at various pH and temperature levels. The hydrolysis rate constants of FFBu and FFMe were pH-independent in the pH range tested, and the temperature dependence for FFBu yielded an activation energy of 18 kcal/mol. It is pointed out that the hydrolysis rates of FFBu may be important for the explanation of its possible delayed diuretic effect.

Furosemide, a sulfonamide diuretic, is a potent diuretic and saluretic agent of a debatable mechanism of action; while all authors agree that furosemide inhibits sodium reabsorption both at the proximal convoluted site and in the ascending limb of the loop of Henle,[2] there is a strong controversy about its inhibitory effect of the carbonic anhydrase (CA) activity.[3-7]

In addition to the debate on the mechanism of action of furosemide itself, questions arose concerning the mode of action of two of its active derivatives: *N*-furfuryl-4-chloro-5-(butoxymethylsulfamoyl)anthranilic acid (FFBu) and *N*-furfuryl-4-chloro-5-(methoxymethylsulfamoyl)anthranilic acid (FFMe). Both derivatives are very similar in their diuretic and saluretic activities to furosemide, although their molecules contain only 80–85% of the active furosemide. They are considered chemically as profurosemides, but there is no evidence yet for their in vivo conversion. Preliminary assays on TLC indicated that FFBu and FFMe are substantially stable in acidic aqueous suspensions (*n*/100 HCl) or simulated gastric fluid (pH 1.3) but decompose in vitro at pH 7.4 yielding furosemide.[8] In acid media only small amounts of FFBu are dissolved, which also decompose to furosemide and to 4-chloro-5-sulfamoylanthranilic acid, with an approximate decomposition time of 30 min at 26°.[9] Since FFBu and FFMe, which have their sulfonamide groups blocked, were not expected to exert significant CA inhibition,[10] we decided to follow-up the CA inhibition rate as a parameter for the hydrolysis of FFBu and FFMe at various pH values.

Experimental Section

Bovine (erythrocytes) carbonic anhydrase B, which had been purified according to Lindskog,[11] was purchased from Seravac, England. Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was purchased from Koch-Light, England. Furosemide [*N*-(2-furylmethyl)-4-chloro-5-sulfamoylanthranilic acid], FFBu, FFMe (B.P. 1.364-366, S. Schoenberg, and H. Yellin), 4-chloro-5-sulfamoylanthranilic acid, and 2,4-dichloro-5-sulfamoylbenzoic acid were synthesized in the chemical laboratories of Teva Ltd., Jerusalem, Israel.

Carbonic anhydrase activity was measured spectrophotometrically from the hydrolysis rate of the substrate *p*-nitrophenyl acetate, following the appearance of the *p*-nitrophenolate ion at 400 nm.[12] Enzyme concentrations were estimated spectrophotometrically at 280 nm assuming $E^{280} = 57{,}000\ M^{-1}\ cm^{-1}$.[13]

Carbonic anhydrase inhibition constants were determined by recording the enzymatic activity at various concentrations, thus making use of the following expression

$$[I_0]/(1 - V/V_0) = K_I \cdot V_0/V + [E_0] \quad (1)$$

which follows directly from eq 2 and 3.

$$V/V_0 = [E_{free}]/[E_0] = ([E_0] - [EI])/[E_0] \quad (2)$$

$$K_I = ([E_0] - [EI]) \cdot ([I_0] - [EI])/[EI] \quad (3)$$

$[I_0]$ and $[E_0]$ are the total concentrations of the inhibitor and the enzyme, respectively, [EI] is the concentration of the enzyme–inhibitor complex, while V and V_0 mark the hydrolysis rate with or without the inhibitor, respectively. The dissociation constant of the enzyme–inhibitor complex (K_I) is given by the slope of the linear plot of $[I_0]/(1 - V/V_0)$ vs. V_0/V.

Hydrolysis rates of FFBu and FFMe were determined by following up the formation of their hydrolysis product, furosemide, using its inhibitory capacity of CA activity. Two alternative procedures were utilized for these measurements. (a) Acetone solutions of FFBu or FFMe were added to a buffered aqueous reaction mixture containing enzyme and *p*-nitrophenyl acetate at a given pH, and the absorbance was recorded as a function of time. The hydrolysis rate at any given time was obtained from the slope of the absorbance recording. Care was taken to ensure that no more than 10% of the *p*-nitrophenyl acetate was consumed during any kinetic run. (b) Buffered aqueous solutions of FFBu or FFMe at various pH values were incubated in a thermostatic bath. Aliquots were taken at various time intervals and added to an enzyme activity reaction mixture, buffered at pH 7.3, and the initial phase of the enzymatic activity was recorded. The same method was utilized for the temperature dependence studies. The enzymatic activity run was performed at one standard condition, but the FFBu solutions were incubated in a separate thermostatic bath, which had been adjusted to the required temperature. For the run which was performed at 37°, where the hydrolysis was too fast to be followed up continuously, aliquots were transferred into an ice bath at 1-min intervals and inhibition of the enzymatic activity was performed at a later time.

In these two methods the liberated furosemide concentration $[I_0]$ was calculated from the following expression

$$[I_0] = K_I(V_0/V - 1) + [E_0](1 - V/V_0) \quad (4)$$

which was obtained by a rearrangement of eq 1.

While method a is a continuous process and requires no manipulations during the kinetic run, it requires an independent determination of the inhibition constant K_I at each particular temperature and pH value. In method b the determination of the liberated inhibitor can be performed in one convenient condition, and therefore the inhibition constant has to be known only at a single specific condition (pH 7.3 in our case). Method b also permits follow-up of the hydrolysis of FFBu and FFMe to completion, while only initial hydrolysis rates are measurable in method a.

Results

All our experiments indicate that while FFBu and FFMe, when added to the reaction mixture, do not exert an immediate inhibitory effect on bovine carbonic anhydrase activity, apparent inhibition develops gradually. It seems that while FFBu and FFMe have very small (if any) carbonic anhydrase inhibitory capacity ($K_I > 1 \times 10^{-4}\ M$), their hydrolysis product, furosemide, has a strong inhibitory effect.

Inhibition constants of furosemide at pH 6.5 and pH 7.3 are given in Table I. It is noteworthy that the binding ca-

Table I. Inhibition Constant of Furosemide and Related Compounds at 26°

Compd tested	K_I, M
Furosemide[a]	3.4×10^{-7}
Furosemide[b]	1.8×10^{-7}
Acetazolamide[b]	2.3×10^{-8}
4-Chloro-5-sulfamoylanthranilic acid[b]	2.5×10^{-6}
2,4-Dichloro-5-sulfamoylbenzoic acid[b]	5.8×10^{-8}

[a]pH 6.5; piperazine N,N^1-bis(2-ethanesulfonate) (Pipes) buffer, 0.025 M. [b]pH 7.3; tris(hydroxymethyl)aminoethane sulfate (Tris) buffer, 0.025 M.

Table II. Rate Constants of the Hydrolysis of Alkoxy Methyl Derivatives of Furosemide at 26°

	FFBu		FFMe	
pH	10^2k, min^{-1}	$t_{1/2}$, min	10^2k, min^{-1}	$t_{1/2}$, min
2.0	5.0	14	5.0	14
4.0	5.3	13	4.6	15
6.1	6.3	12	5.0	14
7.3	6.3	11	6.3	12
8.1	5.3	13	5.0	14

pacity of this inhibitor is weaker only by one order of magnitude than that of acetazolamide. Inhibition constants were also measured for 4-chloro-5-sulfamoylanthranilic acid, which is a minor hydrolysis product, and 2,4-dichloro-5-sulfamoylbenzoic acid, which is a precursor in the furosemide synthesis and may appear as an impurity of furosemide. The results are summarized in Figure 1 and Table I. It is noteworthy that in the plots for the determination of the inhibition constants (Figure 1) the y-axis intercept always coincides with the enzyme concentration as expected according to eq 1.

The kinetic rates for the hydrolysis of FFBu and FFMe are summarized in Table II, and those obtained in both methods a and b are described in Figures 2 and 3. Since in method a only initial rates were recorded, the rate constants may be calculated directly from the slopes of the absorbance recordings. In method b, where the reaction was followed up to completion, the logarithmic plot was found to be linear, confirming first-order reaction kinetics (Figure 4). In this case the slope is equal to $k/2.303$, where k stands for the hydrolysis first-order rate constant. In both methods a and b, the inhibitor concentrations are calculated from eq 4 and thus are dependent on the specific inhibition constant K_I used for the calculation. The linearity obtained in the kinetic plots confirms that the CA inhibition is caused exclusively by furosemide and not by any other possible minor product.

Discussion

Knowing that sulfanilamide and other sulfamides have varying degrees of CA inhibitory action,[14] furosemide, having a sulfonamide moiety, was expected to have a profound CA inhibition. Therefore, we were attracted by a new furosemide derivative, FFBu, whose preliminary clinical reports disclosed pharmacologic properties which were qualitatively similar to those of furosemide, but with somewhat delayed diuretic and natriuretic effects.[15] It occurred to us that since the delayed effect of FFBu might be attributed

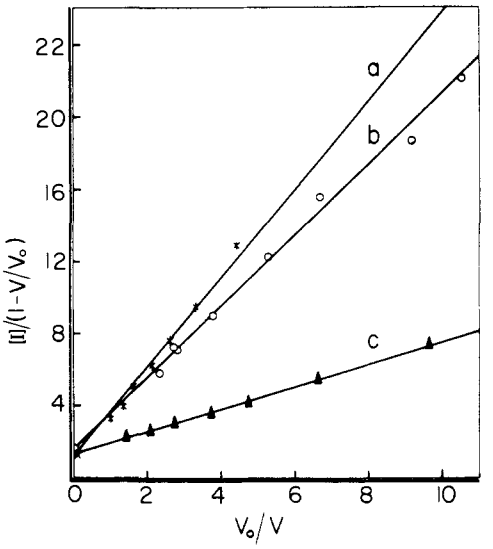

Figure 1. Linear plots for the determination of inhibition constants. Solutions were in 0.025 M Tris–sulfate buffer, pH 7.3, at 26°: (a) 4-chloro-5-sulfamoylanthranilic acid; (b) furosemide; (c) 2,4-dichloro-5-sulfamoylbenzoic acid. The points on the y axis (corresponding to $V_0/V = 0$) are the concentrations of the enzyme.

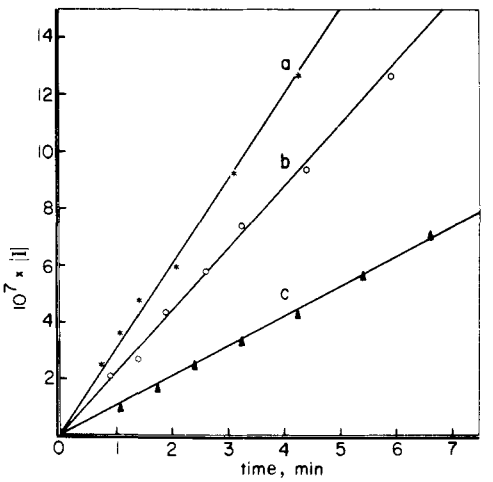

Figure 2. Kinetic plots for the hydrolysis reaction of FFMe according to method a (see Experimental Section). Initial concentrations of FFMe in the reaction mixtures were (a) 1.0×10^{-5} M; (b) 6.7×10^{-6} M; (c) 3.3×10^{-6} M.

to its being a profurosemide, a sensitive method which would accurately determine the rate of furosemide release might assist the elicitation of its mechanism of action and complement recent publication in the pharmacokinetics of oral administration of this drug.[16] CA inhibition was found to be a useful in vitro method for solving both problems: whether furosemide is, or is not, a CA inhibitor and whether FFBu and FFMe exert their (delayed) diuretic effect through release of furosemide, thus being profurosemides or rather delayed acting furosemides. Undoubtedly, metabolic studies with labeled furosemides would be needed to comfirm these findings in vivo.

Muschaweck and Hadji found a 50% CA inhibitory concentration of furosemide at 1 mg/ml. This nonphysiological value is higher in over three orders of magnitude than the constant reported by Stein et al.[7] and confirmed by us, a value which has a clinical significance. This finding led the authors to postulate that furosemide in its diuretic concentration would not have CA inhibition, an assumption which was contradicted by Stein's[7] results, not before it was inserted in some textbooks[3] and papers.[5,6] The discrepancy

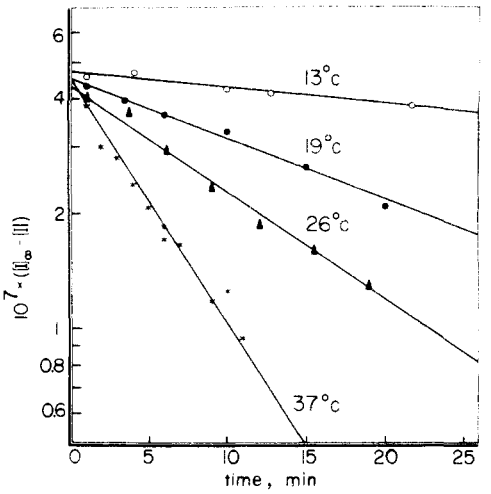

Figure 3. Semilogarithmic kinetic plots for the hydrolysis of FFBu according to method b (see Experimental Section) at various temperatures. Solutions were in 0.025 *M* Tris–sulfate buffer, pH 7.3.

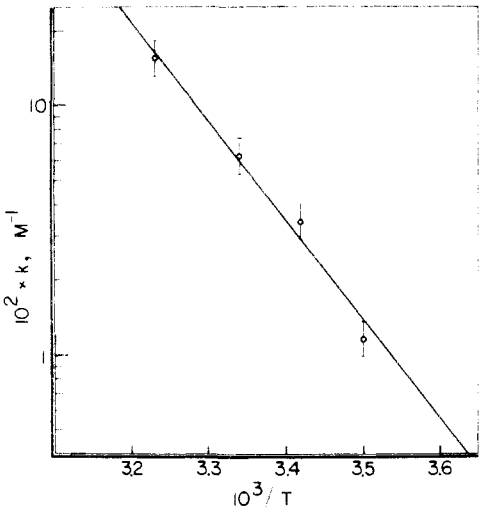

Figure 4. Arrhenius plot for the hydrolysis rate constant of FFBu.

between the two remote inhibitory concentrations may be due to either the source of purity of the carbonic anhydrase used or to aspects of methodology.

We have found that the hydrolysis of both FFMe and FFBu is almost pH independent in the pH range studied (see Table II) and may get hydrolyzed in both acidic and alkaline solutions. These results are in general agreement with values obtained for the decomposition rate of FFBu in blood in vitro, as determined by TLC; FFBu was hydrolyzed by normal full blood (pH 7.4) in vitro to furosemide, at a rate depending on the temperature, ranging from hydrolysis time of 10 min at 37° to 30 min at 15°.[8] However, the low content of furosemide moiety found in acidic pH values is probably due to its low solubility in that medium; at pH 1.3 only 2.1 mg in 100 ml of furosemide is solubilized during 24 hr, as compared to 9.6 or 31.0 mg/100 ml for FFBu and FFMe, respectively. At blood pH (7.4) the solubilities are much higher.[9]

The present method for the assay of the liberated furosemide can be extended and utilized for measuring hydrolysis rates of other sulfamoyl-substituted sulfonamide drugs. It also provides us with a very sensitive method at concentrations down to 10^{-7} *M* for the determination of various other sulfonamides. Our data on the hydrolysis rates of FFBu and FFMe may be used to explain the possible in vivo delayed diuretic effect reported by Licht et al.[15] Moreover, the solubilities of furosemide, FFBu, and FFMe during 2 hr in the simulated gastric fluid (pH 1.3) at 37° are 1.2–3.8 mg/100 ml,[9] and therefore the maximal possible soluble quantities of these drugs in the stomach are small, thus indicating that most of the drug enters the blood stream by a passive diffusion process, where the solubility of FFBu is the lowest of the three compounds, and therefore may account for its proposed delayed onset of action.

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