THERAPY FOR UROLITHIASIS BY HYDROXAMIC ACIDS. II.* UREASE INHIBITORY POTENCY AND URINARY EXCRETION RATE OF HIPPUROHYDROXAMIC ACID DERIVATIVES

KYOICHI KOBASHI, KEI-ICHI MUNAKATA,** SACHIKO TAKEBE AND JUN-ICH HASE

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani,
Toyama-shi, 930-01, Japan

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The apparent I_{50} values of various hippurohydroxamic acids against urease activity of sword bean were mostly 0.5 to 2.0 μM regardless of hydrophobicity of their substituents. However, the marked increase of hydrophilicity caused by substitution of trimethoxy groups conspicuously decreased the inhibitory potency. Methylation at α -position of the hydroxamic acid group in these compounds remarkably decreased the inhibitory potency, probably owing to steric hindrance by the α -methyl group. Thenoyl-, furoyl- and nicotino-glycinohydroxamic acids which are bioisostereomers of hippurohydroxamic acid had I_{50} values of 0.64, 1.3 and 5.3 μM , respectively. Furthermore, the inhibitory potency of some substituted hippurohydroxamic acids against the ureolytic activity of intact *Proteus mirabilis* isolated from patients with urinary tract infection, were half to one-tenth of those against urease activity of sword bean. On the other hand, *m*- and *p*-nitro-, *m*- and *p*-methoxy-, *m*- and *p*-acetylamino-hippurohydroxamic acid and furoylglycinohydroxamic acid showed high urinary excretion rates of 14 to 16% of the doses administered orally to rats, while most of the others had excretion rates of about 3 to 5%.

Keywords—urolithiasis; struvite; apatite; urease inhibitor; sword bean; *Proteus mirabilis*; hippurohydroxamic acid; urinary excretion rate

INTRODUCTION

Urinary tract infection by urea-splitting bacteria is regarded as an etiological factor in the pathogenesis of urinary phosphate stones which is one of the incurable disease in the urological fields.¹⁾ Bacterial ureases hydrolyze urea to produce ammonia, and cause alkalization of urine, resulting in crystallization of struvite and apatite stone. Griffith and Musher,²⁾ and Andersen³⁾ reported that acetohydroxamic acid and *p*-chlorhippurohydroxamic acid were useful for the prevention of the stone formation in rats. However, both the hydroxamic acids were reported to be teratogenic⁴⁾ and having low excretion.³⁾ To find a potent urease inhibitor with a high urinary excretion rate and less toxicity, various substituted

hippurohydroxamic acids were synthesized based on drug-design as reported previously.⁵⁾ In the present report, the inhibitory potency of the substituted hippurohydroxamic acids against the ureolytic activities of sword bean and *Proteus mirabilis* isolated from patients with urinary tract infection, and their urinary excretion rates in rats were examined in order to evaluate therapeutic usefulness of these compounds in the treatment of urolithiasis.

MATERIALS AND METHODS

Cultivation of *Proteus mirabilis*—Two strains (A and B) of *P. mirabilis* (P-1950) isolated from patients with urinary tract infection were kindly provided by Dr. Takeuchi, Shiga Medical Univer-

* Part I: See Reference 5).

** Present address: Research Laboratories, Eisai Co., Ltd. Bunkyo-ku, Tokyo, 112, Japan.

sity, Ootsu. Peptone and beef extracts were the products of Eiken Chem. Co., Ltd., Tokyo and BBL, U.S.A., respectively. Strain A was inoculated into broth (pH 7.0) containing 1% bouillon, 1% peptone and 0.5% NaCl and cultivated at 28° for 20 hr with shaking. Cells were harvested by centrifugation of the culture fluid at 7000 *g* for 10 min, washed twice with saline and suspended in an isotonic buffer (pH 7.1) containing 0.93% KH_2PO_4 and 0.86% NaHCO_3 in the final concentration to make 3×10^7 viable cells/ml. The ureolytic activity of the cell suspensions was 984 mIU/ 10^9 viable cells. Strain A with a high ureolytic activity was used for the measurement of the inhibitory potency of hydroxamic acids in the isotonic buffer, but could not be used for further examinations because of morphological changes on successive cultivations. Strain B manifested a low ureolytic activity and was used after induction in an urea-containing culture medium for the measurements of the inhibitory potency of hydroxamic acids in human urine. The strain was cultivated at 37° for 20 hr with shaking in a medium (pH 7.0) containing 0.1% beef extracts, 1% D-glucose, 0.5% NaCl and 1% urea. Cells were harvested by centrifugation, washed twice with saline and suspended in an isotonic 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl to make 1.6×10^9 viable cells/ml. The ureolytic activities of these cell suspensions were 653 ± 249 mIU/ 10^9 viable cells.

Measurement of Apparent I_{50} Values of Substituted Hippurohydroxamic Acids against the Ureolytic Activity of Sword Bean and *P. mirabilis*—Hydroxamic acids tested were dissolved in 0.1 M phosphate buffer (pH 6.7). Sword bean urease was partially purified according to the method of Uehara and Kobashi⁶⁾ and its specific activity was 70.56 IU/mg of protein. Urease activity was assayed according to the method of van Slyke and Archibald.⁷⁾ The apparent I_{50} value represented the molar concentration of a hydroxamic acid in the preincubation mixture causing 50% inhibition, and was measured by the method described previously.⁸⁾ Apparent I_{50} values against the ureolytic activity of *P. mirabilis* in the

isotonic buffer were measured as follows: a mixture of 0.15 ml of the cell suspension of strain A and 0.55 ml of the isotonic buffer (pH 7.1) containing 0.5 to 10 nmol of the hydroxamic acid to be tested were preincubated at 37° for 30 min. After the addition of 0.05 ml of 5 M urea solution to the preincubation mixtures, which were further incubated at 37° for 30 min. The reaction was stopped by adding 0.2 ml of 1 N sulfuric acid, and the ureolytic activity was assayed by determining the amount of ammonia produced by the indophenol method.⁹⁾ Apparent I_{50} values against the ureolytic activity of the bacterium in human urine were measured as follows: 0.1 ml of the cell suspension of strain B and 0.1 ml of an isotonic buffer (pH 7.1) containing 0.5 to 10 nmol of hydroxamic acid to be tested were added to 0.55 ml of fresh normal human urine. After incubation at 37° for 100 min, the reaction was stopped by adding 0.2 ml of 1 N sulfuric acid to the mixture. The amounts of ammonia produced were estimated by subtracting the amounts of ammonia existing previously in urine used from the total amounts determined by the indophenol method.⁹⁾ Apparent I_{50} values of *m*-methoxyhip-

FIG. 1. Inhibition of the Ureolytic Activity of Sword Bean and Intact *P. mirabilis* by *m*-Methoxyhippurohydroxamic Acid

Against the ureolytic activity of sword bean (—×—), of intact *P. mirabilis* in the isotonic buffer (—○—) and in human urine (—●—).

purohydroxamic acid against the ureolytic activity of sword bean, and of intact cells of *P. mirabilis* in the isotonic buffer and in human urine were measured to be 1.2, 8.8 and 13 μM , respectively, as shown in Fig. 1.

Measurement of Urinary Excretion Rate of Hydroxamic Acids in Rats — Most of hydroxamic acids were dissolved in distilled water to make sample solutions containing 10 to 20 mg of hydroxamic acids/ml, whereas some highly hydrophobic hydroxamic acids were dissolved in 20 to 30% (v/v) ethanol solution. Female Wistar rats weighing 100 to 200 g were fasted (water *ad libitum*) for 24 hr before the experiment. One ml of a hydroxamic acid solution was orally administered to a group of 3 to 5 rats (100 mg/kg) in the metabolic cages. After the oral administration, urine was collected by thoroughly washing the funnels of the metabolic cages with distilled water at 6 and 24 hr, respectively. The amounts of hydroxamic acid excreted in the urine were measured by determining the inhibitory potency of urine against the urease activity of sword bean.¹⁰⁾

RESULTS

Inhibitory Potency of Hippurohydroxamic Acid Derivatives against Ureolytic Activity of Sword Bean and Intact Proteus mirabilis

The apparent I_{50} value of hippurohydroxamic acid against urease activity of sword bean was 0.77 μM , which is almost the same to that of caprylohydroxamic acid, the most potent inhibitor reported hitherto.⁸⁾ Substitution of halogens such as chlorine, bromine and iodine or of a methyl group onto the benzene ring of the parent compound slightly affected the inhibitory potency, though *o*- and *m*-substituted derivatives were more inhibitory than *p*-substituted ones. 2,4-Dichloro- and 3,4-dichloro derivatives had the I_{50} values of 0.71 and 1.7 μM , respectively. Six alkoxy and 3,4-methylenedioxy derivatives had similar inhibitory potency, showing I_{50} values of 1.2 to 1.7 μM , regardless of the substituent's position or the carbon numbers in the alkoxy group. An increase in the number of methoxy group on the benzene ring remarkably decreased the inhibitory

potency: the I_{50} value of 2,3-dimethoxy derivative was 6.0 μM , 5 times higher than those of monomethoxy derivatives, and those of trimethoxy derivatives were 20 and 100 times higher. Four of five acylamino derivatives tested had a potent inhibitory action similar to one another regardless of the acyl residues, having I_{50} values of 1.0 to 3.2 μM , while *p*-acetylamino derivative had the I_{50} value of 18 μM . Among nitro-compounds, the I_{50} value of the *p*-derivative was 2.9 μM , twice that of the *m*-derivative. Among dimethylamino derivatives, *p*-substitution led to remarkable decrease in the inhibitory potency: the I_{50} value of the *m*-derivative was 2.4 μM , while that of the *p*-derivative was 320 μM . Two sulfamoyl compounds tested showed I_{50} values of about 10.0 μM .

Methylation at α -position of the hydroxamic acid moiety in the substituted hippurohydroxamic acids generally led to a marked decrease in the inhibitory potency: I_{50} values of these α -methyl derivatives were 30 to 300 times higher than those of the corresponding parent compounds, and α, α -dimethyl derivatives were 600 and 2400 times higher.

Three bioisostereomers of hippurohydroxamic acid, containing a thiophene, furan or pyridine ring instead of the benzene ring in the parent compound, had relatively potent inhibitory action, showing I_{50} values of 0.64, 1.3 and 5.3 μM , respectively.

The inhibitory potency of some compounds in this series against the ureolytic activity of intact *P. mirabilis* measured in the isotonic buffer were half to one-tenth of those against the urease activity of sword bean. The inhibitory potency of these compounds measured in human urine was further half of that in the isotonic buffer.

Urinary Excretion Rates of Hippurohydroxamic Acid Derivatives in Rats

Substituted hippurohydroxamic acids excreted into urine in unchanged form were analyzed and more than 80% of the total urinary excretion was determined within 6 hr after the oral administration. The hydroxamic acids tested were not detected in the excreted urine on the second day.

TABLE I. Apparent I_{50} Values against Ureolytic Activities of *Sword Bean* and *P. mirabilis* and Urinary Excretion Rates in Rats of Substituted Hippurohydroxamic Acids

	I_{50} values (μM) against the ureolytic activity of				Urinary excretion rate				
	<i>P. mirabilis</i> in				0—6 hr		6—24 hr		Total (%)
	Sword bean	The buffer	Human urine	Dose mg/rat	Amount (μg)	(%)	Amount (μg)	(%)	
Hippurohydroxamic acids									
Hydrogen-	0.77	— ^{a)}	6.6	18.3	686	3.7	61.3	0.34	4.0
<i>o</i> -Chloro-	0.50	2.7	—	11.2	413	3.7	18.5	0.17	3.9
<i>m</i> -Chloro-	0.74	7.3	—	15.2	617	4.1	23.4	0.15	4.3
<i>p</i> -Chloro-	1.0	2.1	4.6	7.4	129	1.7	30.8	0.42	2.1
<i>o</i> -Bromo-	0.66	—	—	13.2	691	5.2	6.0	0.05	5.3
<i>o</i> -Iodo-	0.74	—	—	18.0	913	5.1	54.6	0.30	5.4
<i>o</i> -Methyl-	0.86	—	—	9.7	312	3.2	22.7	0.23	3.4
<i>m</i> -Methyl-	0.46	—	—	16.0	413	2.6	49.5	0.31	2.9
<i>p</i> -Methyl-	1.5	—	—	17.5	584	3.3	19.4	0.11	3.4
<i>o</i> -Methoxy-	1.3	3.1	8.3	17.1	506	3.0	18.9	0.11	3.1
<i>m</i> -Methoxy-	1.2	8.8	13	16.0	2443	15.0	94.3	0.59	16.0
<i>p</i> -Methoxy-	1.7	3.2	6.4	18.0	2873	16.0	53.0	0.29	16.3
<i>p</i> -Ethoxy-	1.5	—	—	12.5	700	5.6	187.0	1.50	7.1
<i>p-n</i> -Propoxy-	1.5	—	—	10.0	209	2.1	—		
<i>p-n</i> -Butoxy-	1.3	—	—	10.0	21	0.2	—		
3,4-Methylenedioxy-	1.4	—	—	17.3	1674	9.7	85.7	0.50	10.2
2,3-Dimethoxy-	6.0	—	—	18.0	1334	7.4	139.0	0.77	8.2
2,3,4-Trimethoxy-	27	—	—	10.0	647	6.5	—		
3,4,5-Trimethoxy-	180	—	—	—					
<i>m</i> -Acetyl-amino-	2.0	—	—	10.8	1613	14.9	61.2	0.57	15.5
<i>p</i> -Acetyl-amino-	18	—	—	10.0	1508	15.1	—		
<i>m</i> -Propionyl-amino-	3.2	—	—	15.0	821	5.5	—		
<i>p</i> -Propionyl-amino-	1.0	—	—	10.0	167	1.7	—		
<i>m-n</i> -Butyroyl-amino-	1.4	—	—	14.5	1160	8.0	26.1	0.18	8.2
<i>m</i> -Nitro-	1.2	3.6	9.9	16.5	2405	14.6	54.2	0.33	14.9
<i>p</i> -Nitro-	2.9	—	—	18.3	2569	14.0	70.2	0.38	14.4
<i>m</i> -Dimethyl-amino-	2.4	—	—	16.4	1246	7.6	65.0	0.13	7.7
<i>p</i> -Dimethyl-amino-	320	—	—	—					
<i>p</i> -Methylsulfonyl-	11	—	—	10.0	470	4.7	—		
<i>p</i> -(N,N-Di- <i>n</i> - propylsulfamoyl)-	10	—	—	—					
α -Methyl hippurohydroxamic acids									
<i>o</i> -Chloro-	180	—	—	7.6	544	7.2	—		7.2
<i>m</i> -Chloro-	51	—	—	—					
<i>p</i> -Chloro-	30	—	—	—					
<i>o</i> -Methoxy-	71	—	—	—					

<i>m</i> -Methoxy-	120	—	—	9.8	762	7.8	89.8	0.92	8.7
<i>p</i> -Methoxy-	110	—	—	—					
α , α -Dimethyl hippurohydroxamic acids									
<i>m</i> -Chloro-	1200	—	—	—					
<i>p</i> -Chloro-	620	—	—	—					
Bioisostereomers									
N-(2-Thenoyl)-glycinohydroxamic acid	0.64	0.8	2.6	9.3	549	5.9	31.8	0.34	6.2
N-(2-Thenoyl)- α -methyl glycinohydroxamic acid	47	—	—	—					
N-(2-Furoyl)-glycinohydroxamic acid	1.3	—	—	15.5	2407	15.5	10.6	0.68	16.2
N-(Nicotino)-glycinohydroxamic acid	5.3	—	—	14.6	576	4.6	48.9	0.33	4.3

a) Not determined.

The excretion rate of hippurohydroxamic acid in 24 hr was 4% of the oral dose, which is considerably higher than those of caprylo- and nicotino-hydroxamic acids.¹¹⁾ The excretion rates in 24 hr of halogen and methyl derivatives were about 4% of the dose, while *m*- and *p*-methoxy and *m*- and *p*-nitro derivatives showed higher excretion rates of 14 to 16%. However, *o*-methoxy derivative showed the low excretion rate of only 3% of the dose. An increase in the carbon number of alkoxy groups markedly decreased the excretion rates: the excretion rate of *p*-butoxy derivative was 0.2% within 6 hr. *m*- And *p*-acetylamino derivatives also showed high excretion rates of about 15%, while other acetylamino derivatives showed excretion rates of less than 8% within 6 hr. 3,4-Methylenedioxy, 2,3-dimethoxy and *m*-dimethylamino derivatives showed relatively high excretion rates of 8 to 10%. Among bioisostereomers, furoylglycinohydroxamic acid had a high excretion rate of 16%. On the other hand, *o*-chloro and *m*-methoxy α -methyl derivatives showed relatively high excretion rates of 7 to 9%.

DISCUSSION

The urease inhibitory potency of hydroxamic acids has been affected by the hydrophobicities of

their acyl residues, and their ionic properties, such as pK_a values only affected the velocity of the urease inhibition.^{12,13)} The apparent I_{50} values of most of substituted hippurohydroxamic acids were 0.5 to 3.0 μM regardless of hydrophobicity of the substituents on the benzene ring, though I_{50} values of the compounds having hydrophobic substituents such as halogen or methyl groups were smaller than those of the compounds with hydrophilic ones such as nitro or acetylamino groups. These observations suggest that the presence of the $-\text{CONHCH}_2-$ moiety between the alkyl residue and the functional hydroxamic acid group might lead to the optimal binding with the active site of the enzyme and so the difference in hydrophobicity of the substituents did not markedly affect the inhibitory potency. However, a marked increase in hydrophilicity of the benzene ring caused by substitution with trimethoxy group led to the remarkable decrease in the inhibitory potency. The remarkably decreased inhibitory potency of *p*-dimethylamino derivative might be due to the formation of the cationic site (δ^+) on the dimethylamino moiety, which was induced by conjugating with the carbonyl group on the benzene ring, while such a conjugative effect is not induced in the corresponding *m*-derivative. The formation of the cationic site is

probably disadvantageous for the interaction with the residues of hydrophobic amino acids of the active site of urease.¹²⁾ In general, the *p*-derivatives were less inhibitory than the corresponding *o*- and *m*-derivatives, for which there is, as yet, no satisfactory explanation. A relationship between hydrophobicity and the inhibitory potency was also observed in the bioisostereomers: their inhibitory potencies were found to be in the ascending order of hydrophobicity of the rings. The remarkable decrease in the inhibitory potency caused by methylation at α -position of the hydroxamic acid group might be attributable to steric hindrance of the α -methyl groups near the functional hydroxamic acid moiety. These observations are similar to the " α -effect" in the phenyl substituted *n*-aliphatic hydroxamic acids and the "*ortho* effect" in the substituted benzohydroxamic acids as for steric hindrance: α -phenyl and *o*-derivatives have markedly less inhibitory potency than the corresponding β - and γ -derivatives and *m*- and *p*-derivatives, respectively.⁸⁾

The lower inhibitory potency of the hydroxamic acids against the ureolytic activity of intact *P. mirabilis* than those against urease activity of sword bean might be due to a difference in enzymatic properties between plant and bacterial ureases and/or to the lower permeability of the hydroxamic acids across the bacterial cell membrane, because the bacterial urease is the intracellular enzyme and the test bacterium showed no capacity to metabolize the hydroxamic acids as verified experimentally. The lower inhibitory potency observed in human urine than in the isotonic buffer might be the result of the influence of about 0.3 M urea in the urine, because hydroxamic acid competes with urea at the active site of urease, and ammonia has no effect on the reaction at the concentrations found in urine.¹⁴⁾

Urinary excretion rates of most of the test compounds in this series were about 3 to 5% of the doses within 24 hr after the oral administration. On the other hand, *m*- and *p*-derivatives of methoxy, nitro and acetylamino hippurohydroxamic acids and furoylglycinohydroxamic acid, which had relatively more hydrophilic

characters, showed high excretion rates of about 15%. The differences in the excretion rates of the test compounds might be attributable to their metabolic rates in liver and their renal clearances, possibly owing to their hydrophilicities. Furthermore, α -methyl derivatives did not show high excretion rates, which suggested α -methyl group could not effectively prevent metabolic degradation of the hydroxamic acid moiety.

As the therapeutic agent for urolithiasis, a potent urease inhibitor with a high urinary excretion rate is required. In order to evaluate various compounds the "Effective Index" was calculated from the equation described below.

$$\text{Effective Index} = \frac{\text{Urinary excretion rate (\%)}}{\text{Apparent } I_{50} (\mu\text{M})}$$

Wherein apparent I_{50} values used were those against urease activity of sword bean. Among the compounds tested, *m*- and *p*-methoxyhippuro-, *m*-nitrohippuro-, thenoylglycino- and furoylglycinohydroxamic acid gave high effective indexes, of 13.3, 9.41, 12.4, 9.75 and 12.5, respectively. On the other hand, aceto-²⁾ and *p*-chlorohippurohydroxamic acid,³⁾ which have been reported to be effective therapeutic agents, had low effective indexes of 2.1 and 2.7, respectively. *m*-Methoxyhippurohydroxamic acid having the highest effective index among these compounds is a hopeful therapeutic agent for the treatment of urolithiasis, because it showed high urinary concentration (about 1 to 2 mM) enough for inhibition of bacterial urease activity in consideration of its I_{50} value (13 μM).

Further pharmacological and toxicological studies are required before it can be used clinically for the treatment of urolithiasis.

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