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Enzymatic Hydrolysis of Zenarestat 1-*O*-Acylglucuronide

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Abstract—Zenarestat, (3-(4-bromo-2-fluorobenzyl)-7-chloro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-1-yl) acetic acid, an aldose reductase inhibitor is metabolized mainly to the glucuronide in rat and man. The glucuronide was purified from urine of volunteers after ingestion of zenarestat. The structure of the glucuronide was confirmed by LC-MS and NMR as 1-*O*-acyl-*β*-glucuronide. This compound was unstable at physiological pH, being converted to its structural isomers and the aglycone with half-life of 25 min at pH 7·4 and 37°C in aqueous solution. Enzymatic hydrolysis of the glucuronide was studied in urine, blood and tissues. *β*-Glucuronidase in human urine contributed little to the hydrolysis of the glucuronide, while in rat urine at pH 6, it was degraded by *β*-glucuronidase and the formation of zenarestat was clearly faster than its formation in buffer at pH 6. In both rat and human blood, these reactions were accelerated by albumin, although rat red blood cells may also contribute. The rate of degradation was not affected by red blood cell membrane, haemoglobin, globulin, esterases or *β*-glucuronidase. Arylesterase in rat liver, arylesterase and acetylcholinesterase in the kidney, and *β*-glucuronidase in both tissues may contribute. Thus, enzymatic degradation of zenarestat 1-*O*-acyl-*β*-glucuronide is dependent not only on pH and temperature but also on species and the type of tissue or body fluid.

Zenarestat (3-(4-bromo-2-fluorobenzyl)-7-chloro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-1-yl)acetic acid (Fig. 1), is an aldose reductase inhibitor which is undergoing clinical trials in the treatment of diabetic neuropathy (Kanamaru et al 1989). Its main metabolite in man is the glucuronide conjugate. The instability of acylglucuronides, which depends on pH, temperature (Hasegawa et al 1982), matrix (Smith et al 1990) and substrate (Rachmel et al 1985) is well known for non-steroidal anti-inflammatory drugs.

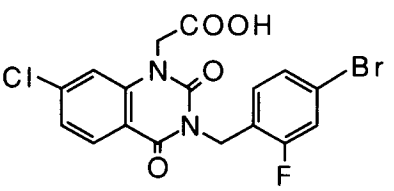

FIG. 1. Zenarestat.

This paper reports on instability in different matrices and enzymatic degradation of zenarestat glucuronide.

Materials and Methods

Chemicals

Zenarestat was synthesized in house and was 99·9% pure by titration. D-Saccharic acid 1,4-lactone, albumin (rat; fraction V), albumin (human; essentially fatty acid free), globulin (human; Cohn fraction II, III), acetazolamide, diethyl *p*-nitrophenyl phosphate and *p*-hydroxy-mercuribenzoic acid were obtained from Sigma Chemical Co (St. Louis, MO). Sodium D-glucuronic acid, neostigmine bromide, phenylmethylsulphonyl fluoride and polyoxyethylene ether W1 were obtained from Nacalai Tesque (Kyoto, Japan).

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Acetonitrile was HPLC grade and all other chemicals used were reagent grade.

HPLC conditions

Determination of zenarestat was as described by Tanaka et al (1991). The column was reversed-phase (TSK-gel ODS 120A, 5 μm, 150 mm × 4 mm i.d., Tosoh, Tokyo, Japan). The mobile phase consisted of 0·02 M phosphate buffer (pH 6·5)/acetonitrile, 50:29 (v/v) for the assay of zenarestat, and 0·1 M ammonium acetate buffer (pH 4·5)/acetonitrile, 5:2 (v/v) for the purification of the glucuronide and the assay of the glucuronide and its degradation products. The flow rate was 1·0 mL min^{-1} in all cases.

LC-MS and NMR

A Jasco 880-PU HPLC pump (Jasco, Tokyo, Japan) was used with a TSK gel ODS 120A (5 μm) as above. The mobile phase, 25% acetonitrile combined with 75% aqueous ammonium acetate buffer (pH 5), was eluted at 1 mL min^{-1}. A VG 70-SE plasma-spray LC-MS system (VG Analytical, Manchester, UK) was controlled with a VG 11-250 J data system. The optimum interface temperature was determined by making repeated direct injections of the glucuronide to obtain the maximum intensity for the $(M+NH_4)^+$ ion. The vapourizer was set at 230°C with the probe at 200°C. The system was operated in the positive-ion mode. Proton-NMR spectrometry was performed in DMSO-d_6 in a JNM FX-270 spectrometer (Jeol, Tokyo, Japan). Chemical shifts are reported relative to internal standard, tetramethylsilane.

Purification of zenarestat glucuronide from urine

Volunteers participated in the study after giving written, informed consent. A single zenarestat tablet (600 mg) was ingested with 200 mL water. Urine was collected hourly for 12 h and pH adjusted to below 5 with dilute HCl. Urine was frozen at −80°C until processed. Urine was applied to a Bond Elute C_{18} column (Varian, Tokyo, Japan). The columns were washed with 0·002 M HCl/tetrahydrofuran (THF), 8:2 (v/v) followed by 0·002 M HCl/THF, 7:3 (v/v)

and the glucuronide was eluted with 0·002 M HCl/THF, 6:4 (v/v). The volume was reduced in a rotary evaporator, and the residue was again applied to a Bond Elute C_{18} column, washed with 0·002 M HCl/THF, 8:2 (v/v); the glucuronide was eluted with acetonitrile, and the solvent removed by evaporation to dryness. The residue was analysed by LC-MS and was dissolved in the mobile phase and purified by HPLC. The fractions containing the glucuronide were combined, the volume was reduced in a rotary evaporator, and desalted with a Bond Elute C_{18} column. Purity was determined by HPLC with UV detection at 313 nm, showing a single peak. This fraction was analysed by NMR.

Stability in buffer solution

The glucuronide was dissolved in sodium potassium phosphate buffer at various pH values at 37°C. At the specified times, the solutions were acidified with 1 M HCl and analysed by HPLC. The half-life of the glucuronide was calculated from the decrease of peak height or area of zenarestat glucuronide.

Degradation of zenarestat glucuronide in urine

Acetonitrile solutions were dried under nitrogen and the residues were dissolved in 5·0 mL rat or human urine adjusted to the appropriate pH with dilute HCl or NaOH to a final concentration of about 50 μg mL^{-1}. The urine was filtered through a membrane 0·45 μm and was maintained at 4 or 37°C. Aliquots were taken at specified times and adjusted to about pH 2 with 0·1 M HCl and analysed by HPLC. After 6 h, the residues were applied to a Bond Elute C_{18} column, which was washed with 0·002 M HCl/THF, 8:2 (v/v) before eluting zenarestat-related compounds with acetonitrile. This fraction was subjected to HPLC analysis. Saccharic acid 1,4-lactone, a competitive inhibitor of β-glucuronidase, was used to evaluate the role of β-glucuronidase in the hydrolysis of zenarestat glucuronide in rat urine (final concentration 5 mM).

Degradation of zenarestat glucuronide in blood

Freshly-drawn heparinized blood from male volunteers and male Sprague-Dawley rats was centrifuged at 3000 rev min^{-1} at 4°C for 10 min to separate plasma from the blood cells. The resultant blood cells were washed three times with equal volumes of isotonic saline. Red blood cell (RBC) membrane and cytosol were obtained by adding equal volume 20 mOsm saline to washed RBC and incubating at 4°C for 30 min. The resultant solution was centrifuged at 3000 rev min^{-1} for 10 min to sediment the membranes. When washed RBC and RBC cytosol were incubated with zenarestat glucuronide, each fraction was diluted 1/1 with isotonic phosphate buffer (pH 7·4) or phosphate buffer.

1-*O*-Acylglucuronide (final concentration 50 μg mL^{-1}) was then incubated with 2 mL human or rat blood, plasma, washed RBC, RBC cytosol or purified proteins (4% albumin or 2% globulin in buffer (pH 7·4)) at 37°C. At the indicated times, aliquots were acidified with 20 μL 0·2 M citric acid and the incubation was stopped by addition of 400 μL acetonitrile and the combination was mixed vigorously for 10 s.

Denatured proteins were sedimented by centrifugation for 3 min. The supernatant was analysed by HPLC. The effect of esterase inhibitors, acetazolamide, a carbonic anhydrase inhibitor, 0·1 mM; BPNP, a carboxylase inhibitor, 5 mM; neostigmine bromide, a cholinesterase inhibitor, 1 μM; sodium *p*-hydroxymercuribenzoic acid, an arylhydroxylase inhibitor, 10 mM; PMSF, an acetylcholinesterase inhibitor, 1 mM and a β-glucuronidase inhibitor (D-saccharic acid 1,4-lactone) were studied. Plasma samples were preincubated with inhibitors for 10 min at 37°C and incubated for 10 min with the glucuronide.

Degradation of zenarestat glucuronide in tissue homogenate

Male, 240–260 g, and female, 170–190 g, Sprague-Dawley-JCL rats were purchased from Clea Japan, Inc. (Tokyo, Japan). The rats were kept in conditions of constant temperature, humidity, lighting (12 h light/dark cycle) and fed a standard diet (MF Diet, Oriental Yeast Co. Ltd, Japan) with free access to water. Rats were killed by decapitation. The livers were perfused with 1·15% KCl. The liver and kidney were quickly removed and placed in cold isotonic saline on ice. All steps in the preparation of subcellular fractions were carried out at 0–4°C. After the addition of ice-cold 1·15% KCl, 25% (w/v) homogenates were prepared in a glass homogenizer with a Teflon pestle. Homogenates were centrifuged at 1000 *g* for 10 min followed by 9000 *g* for 20 min. The supernatants of the 9000 *g* centrifugation were further centrifuged at 105000 *g* for 90 min to obtain the microsomal fractions. Microsomal pellets were washed once and recentrifuged. Subcellular protein concentrations were determined by the method of Lowry et al (1951). Deglucuronidation activities were assayed in phosphate buffer at 37°C in the 1000 *g* supernatant, 9000 *g* supernatant and microsomal fractions. The assay conditions were checked with respect to protein concentration, substrate concentration, concentration of detergent and linearity of the reaction in time for the microsomal fraction. The incubation mixtures were preincubated for 10 min at 37°C before the addition of the glucuronide. Incubation was terminated after 10 min, and aliquots were added to 1 mL ice-cold 0·1 M HCl. Deconjugation rates were calculated from the amount of parent compound. The effect of esterase and β-glucuronidase

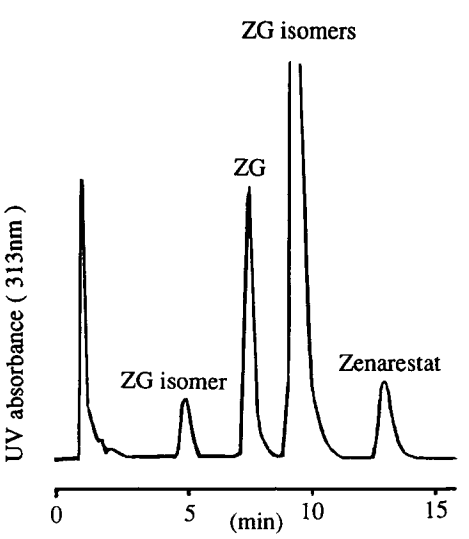

FIG. 2. HPLC of zenarestat glucuronide (ZG) after incubation at pH 7·4, 37°C for 60 min.

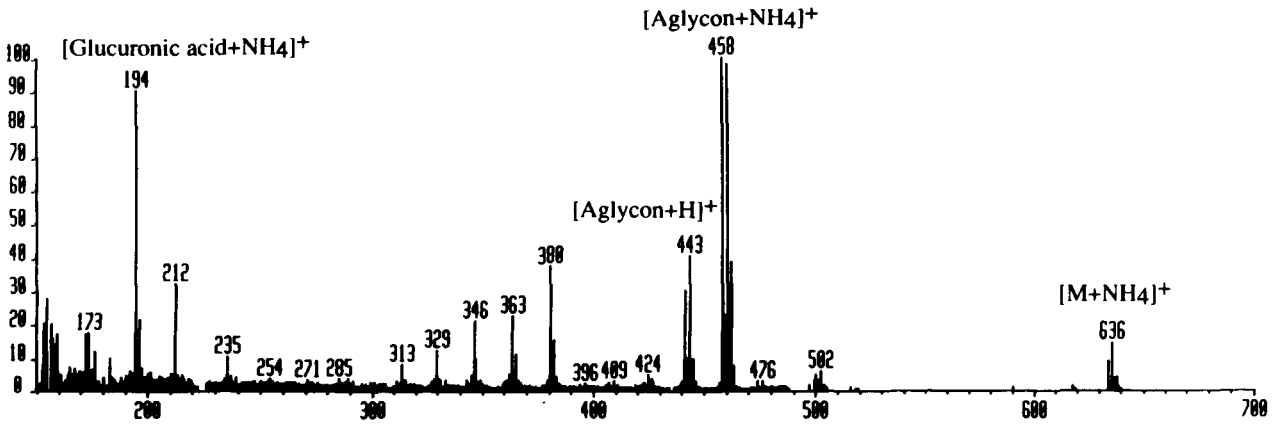

FIG. 3. Positive-ion mass spectrum of zenarestat 1-O-acyl glucuronide obtained by plasma-spray LC-MS.

inhibitors was studied in the 1000 *g* supernatant fraction at pH 6 and pH 7·4. A blank incubation with only the solvents was carried out to determine the non-enzymatic deconjugation.

Results

Fig. 2 shows the HPLC of 1-*O*-acyl glucuronide, isomers and parent compound. Fig. 3 shows the spectrum of the glucuronide obtained from on-column analysis by positive-ion plasma spray LC/MS. In addition to the abundant $(M + NH_4)^+$ ion at mass 636, the aglycone fragment m/z 460, 443, and sugar fragment ion of mass 194 were detected. Glucuronide isomers gave similar spectra (data not shown). Purified glucuronide gave proton NMR signals at 7·06–8·05 ppm; 5·49 ppm (d, J = 7·9); 5·04 ppm (s); 5·06 ppm (m); 3·83 pp (d, J = 9·2). The doublet at 5·49 ppm was determined to be the anomeric proton of glucuronic acid, having the same coupling constants as the 1β proton of glucuronic acid, but 1.1 ppm further downfield. Fig. 4 shows the rate of glucuronide loss at different pH values in water. The decomposition rate increased markedly at pH 7·4 and 7·8 ($t_{\frac{1}{2}}$ (h): pH 5, 15·2; pH 6, 6·3; pH 7·4, 0·42; pH 7·8, 0·20). The rate of loss of glucuronide in human and rat urine at 37°C increased as the pH increased (Table 1). The effect of pH on the appearance of the aglycone was more complex: pH 5 and pH 7·4 > pH 6 in human urine and pH 8·5 and pH 6 > pH 5 > pH 7·4 in rat urine. The addition of saccharic acid 1,4-lactone, a β-glucuronidase inhibitor, in rat urine reduced the appearance of the aglycone and the loss of glucuronide.

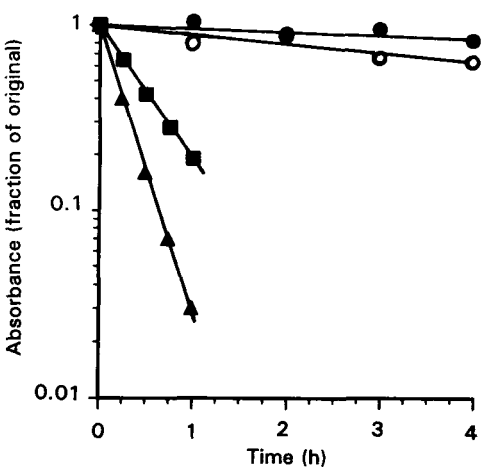

FIG. 4. Apparent first-order plot for the degradation of zenarestat 1-O-acylglucuronide in water at 37°C. ● pH 5, ○ pH 6, ■ pH 7·4, ▲ pH 7·8.

The apparent half-lives for the loss of glucuronide, and the percentage of the glucuronide, its isomers and the aglycone in the total substrates after 30 min in blood are shown in Table 2. Various esterases and saccharic acid 1,4-lactone had no effect on the degradation of zenarestat glucuronide and the appearance of the aglycone in human or rat plasma.

Deglucuronidation in kidney microsomes was shown to be linear for protein concentrations up to 4·0 mg mL^{-1}, with incubation times up to 15 min, and was maximum with

Table 1. pH-dependent degradation of zenarestat glucuronide and the formation of zenarestat in rat and human urine at 37°C. Values represent percentage of the total substrate.

		Aglycone			Percentage after 6 h		
Species	pH	2 h	4 h	6 h	Glucuronide	Isomers	Aglycone
Rat	5·0	12	22	34	67	4	29
	6·0	19 (4)[a]	37 (8)[a]	48 (13)[a]	22 (54)[a]	26 (33)[a]	51 (13)[a]
	7·4	12	18	23	0	73	27
	8·5	29	47	59	1	35	64
Human	5·0	8	18	25	67	5	28
	6·0	5	12	18	46	33	20
	7·4	9	16	23	0	70	30

[a] In the presence of the β-glucuronidase inhibitor, saccharic acid 1,4-lactone.

Table 2. The degradation of zenarestat glucuronide and the formation of zenarestat in rat and human blood at 37°C.

	Half-life of glucuronide (min)	Percentage after 30-min incubation		
		Zenarestat 1-*O*-acylglucuronide	Isomers	Aglycone (zenarestat)
Buffer				
pH 7·4	25	42·0	52·9	3·7
pH 7·8	11·8	15·8	73·6	7·6
Rat				
Plasma	8·8	7·9	47·9	41·0
Whole blood	17·8	26·9	44·7	26·9
Washed RBC	33·7	52·5	43·7	3·8
RBC cytosol	20·6	43·5	46·4	10·1
Albumin	13·2	22·1	30·3	42·7
Human				
Plasma	4·8	2·3	55·6	39·0
Whole blood	8·3	7·7	54·6	35·8
RB[a]	44·7	63·9	32·9	3·3
Albumin	10·9	16·8	40·4	39·1
Globulin	55·3	67·9	28·2	3·9

[a] RBC membrane plus cytosol.

0·025% of the detergent. The rate of appearance of zenarestat was constant up to a substrate concentration of 0·23 mM. The rate of reaction was $1{\cdot}03 \pm 0{\cdot}05$ nmol min^{-1} (mg protein)$^{-1}$ in male rat kidney and $0{\cdot}98 \pm 0{\cdot}23$ nmol min^{-1} (mg protein)$^{-1}$ in the female (mean $\pm$ s.d., n = 8). At pH 6, saccharic acid 1,4-lactone almost completely inhibited the production of the aglycone both in liver and kidney. At pH 7·4, only *p*-hydroxy-mercuribenzoic acid (2·5–10 mM) inhibited 75–90% of the activity in liver, and *p*-hydroxy-mercuribenzoic acid at 10 mM and PMSF at 1 mM provided 41% and 24% inhibition of activity in the kidney, respectively. Other inhibitors had no effect in either the liver or the kidney.

Discussion

1-*O*-Acylglucuronides are generally considered to be unstable at physiological pH, but the half-life for intramolecular migration at pH 7·4 and 37°C varies by more than 100-fold and the lability of intramolecular rearrangement will depend on the electronic nature of the aglycone moiety and the relative configurations at the adjacent centres involved (Dickinson et al 1985). Zenarestat glucuronide was found to be unstable at physiological pH, leading to the isomers and the aglycone. Enzymatic hydrolysis of the glucuronide in various matrices was studied to understand its true disposition.

In rat urine, at pH 6·0, which is the optimal pH of rat β-glucuronidase (Ho et al 1979), the percentage of the aglycone was more, and that of glucuronide was less than in the buffer. After the addition of β-glucuronidase inhibitor, urine and buffer levels were similar. This suggests that β-glucuronidase contributes to the hydrolysis of the glucuronide in rat urine at pH 6. At pH 5, glucuronide in rat and human urine was slightly less than in the buffer, although at this pH β-glucuronidase activity is about 70% of the maxima in both species; the β-glucuronidase of man is optimum at pH 4·5 (Goldbarg et al 1959). The role of β-glucuronidase in the hydrolysis of the glucuronide is minor, although intrinsic urinary inhibitors such as D-glucano-1,4-lactone (Marsh 1963; Ho & Ho 1981) may be involved. Thus, β-glucuronidase seemed to contribute to the instability of the glucuronide in rat urine only at pH 6 and not to contribute to that in human urine in-situ.

The instability of acylglucuronide in urine was thought to be similar to that in water, so that adjustment of pH below 5 and cooling the samples can alleviate this problem (Hasegawa et al 1982).

Mulder et al (1988) reported that after the administration of radio-labelled ponarestat (structurally similar to zenarestat) to female rats, most of the radioactivity in the urine was in the form of unchanged compound at the collected pH of around 8·5, and only the glucuronide conjugate was observed when rats had been pretreated with ammonium chloride so that their urine became slightly acidic (pH 5·5–6·0). In similar experiments with zenarestat using cannulae inserted into the bladder in female rats, and immediate analysis of the urine collected, we detected only the unchanged drug (Tanaka et al 1991); under these conditions the glucuronide and its isomers would be detectable if present.

The reactions of acylglucuronide in plasma and albumin solution have been previously studied (Wells et al 1987), but the stability in RBC and globulin, and the contribution of esterase in blood to reactions of ester glucuronides have not been studied, although various esterases (Williams 1987) and β-glucuronidase (Plaice 1961) are known to exist in rat and human blood. Rat and human plasma, and albumin catalysed the conversion of the glucuronide and its isomers to the aglycone. This is different to the activity of β-glucuronidase which does not convert the isomers to the aglycone. In rat RBC cytosol, the glucuronide and the isomers were converted to the aglycon for a lesser extent than in plasma. Esterases (Quon & Stampfli 1985) rather than haemoglobin will catalyse these reactions, since human RBC cytosol did not catalyse these reactions. No inhibitor effected the degradation of the glucuronide in human or rat plasma. Rat and human RBC membrane, and human globulin and

esterases in plasma appeared not to catalyse the reaction. Thus, both in rat and human blood, only albumin is a major contributor to the catalysis of zenarestat glucuronide and its isomers to the aglycone, although rat RBC cytosol may also contribute. This suggests more clearly than before that albumin can react with the acylglucuronide and this adduct may contribute to its toxicity (Benet & Spahn 1988).

Smith et al (1990) suggested that acetylcholinesterase activity is responsible for hydrolysis of zomepirac glucuronide in guinea-pig liver in-vivo. β-Glucuronidase and arylesterase in rat liver, and β-glucuronidase, arylesterase and acetylcholinesterase in rat kidney contributed to the degradation of zenarestat glucuronide. These suggested that enzymatic hydrolysis of acylglucuronide in tissues is dependent on species and tissues.

Rats showed a marked sex difference in the urinary excretion of zenarestat (Tanaka et al 1992); only about 1% of the radioactivity was excreted in males, while 45% of the radioactivity was excreted in females. Deglucuronidation of zenarestat glucuronide in rat kidney microsomes showed no sex difference. Although sex differences in glucuronidation of *p*-nitrophenol in rat kidney contributed to the sex differences in *p*-nitrophenylglucuronide excretion (Rush et al 1983), deglucuronidation may not play a role in sex differences in urinary excretion of zenarestat.

Thus, enzymatic degradation of zenarestat glucuronide is dependent not only on pH and temperature, but also on species and the tissue or fluid under investigation.

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References

Benet, L. Z., Spahn, H. (1988) Acyl migration and covalent binding of drug glucuronides—potential toxicity mediators. In: Siest, G., Magdalou, J., Burchell, B. (eds) Cellular and Molecular Aspects of Glucuronidation. Colloque Inserm/John Libbey Eurotext Ltd, pp 261–269

Dickinson, R. G., Kluck, R. M., Eadieand, M. J., Hooper, W. D. (1985) Disposition of β-glucuronidase-resistant "glucuronides" of valproic acid after intrabiliary administration in the rat: intact absorption, fecal excretion and intestinal hydrolysis. J. Pharmacol. Exp. Ther. 233: 214–221

Goldbarg, J. A., Pineda, E. P., Banks, B. M., Rutenberg, A. M. (1959) A method for the colorimetric determination of β-glucuronidase in urine, serum, and tissue; assay of enzymatic activity in health and disease. Gastroenterology 36: 193–201

Hasegawa, J., Smith, P. C., Benet, L. Z. (1982) Apparent intramolecular acyl migration of zomepirac glucuronide. Drug Metab. Dispos. 10: 469–473

Ho, K. J. H., Ho, L. H. C. (1981) Inhibitory effect of bile acid on the activity of human β-glucuronidase at its optimal pH. Proc. Soc. Exp. Biol. Med. 167: 304–309

Ho, K. J. H., Ho, L. H. C., Kruger, O. R. (1979) Characterization of the activity of biliary β-glucuronidase in rats. J. Lab. Clin. Med. 93: 916–925

Kanamaru, M., Mizuno, A., Nagashima, S., Uematsu, J., Nakashima, M., Terakawa, M., Sugiyama, A. (1989) Pharmacokinetics of FK366 and its effect on aldose reductase and serum uric acid in healthy volunteers. Eur. J. Clin. Pharmacol. 36 (Suppl.): A315

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Marsh, C. A. (1963) Metabolism of D-glucuronolactone in mammalian systems. Biochem. J. 86: 77–86

Mulder, G. J., Wierckx, F. C. J., Jansen, P. L. M., Warrander, A. (1988) Acyl-glucuronidation of ponarestat in the rat in vivo and its role in the sex difference in urinary excretion of the drug. In: Stest, G., Magdalon, J., Burchell, B. (eds) Cellular and Molecular Aspects of Glucuronidation. Colloque Inserm, John Libbey Eurotext Ltd, pp 271–278

Plaice, C. H. J. (1961) A note on the determination of serum beta-glucuronidase activity. J. Clin. Pathol. 14: 661–665

Quon, C. Y., Stampfli, H. F. (1985) Biochemical properties of blood esmolol esterase. Drug Metab. Dispos. 13: 420–424

Rachmel, A., Hazelton, G. A., Yergey, A. L., Liberato, D. J. (1985) Furosemide 1-O-acyl glucuronide in vitro biosynthesis and pH-dependent isomerization to β-glucuronidase-resistant forms. Drug Metab. Dispos. 13: 705–710

Rush, G. F., Newton, J. F., Hook, J. B. (1983) Sex differences in the excretion of glucuronide conjugation; the role of intrarenal glucuronidation. J. Pharmacol. Exp. Ther. 227: 658–662

Smith, P. C., McDonagh, A. F., Benet, L. Z. (1990) Effect of esterase inhibition on the disposition of zomepirac glucuronide and its covalent binding to plasma proteins in the guinea pig. J. Pharmacol. Exp. Ther. 252: 218–224

Tanaka, Y., Deguchi, Y., Ishii, I., Terai, T. (1991) Sex differences in excretion of zenarestat in rat. Xenobiotica 21: 1119–1125

Tanaka, Y., Fujiwara, T., Esumi, Y. (1992) Sex difference in the excretion of zenarestat in mice, rats, dogs and humans. Xenobiotica 22: 941–947

Wells, D. S., Janssen, F. W., Ruelius, H. W. (1987) Interactions between oxaprozin glucuronide and human serum albumin. Xenobiotica 17: 1437–1449

Williams, F. M. (1987) Serum enzymes of drug metabolism. Pharmacol. Ther. 34: 99–109