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Comparison of the Rates of Hydrolysis of Lorazepam-Glucuronide, Oxazepam-Glucuronide and Temazepam-Glucuronide Catalyzed by *E. Coli.* β-D-Glucuronidase Using the On-line Benzodiazepine Screening Immunoassay on the Roche/Hitachi 917 Analyzer

REFERENCE: Dou C, Bournique JS, Zinda MK, Gnezda M, McNally AJ, Salamone SJ. Comparison of the rates of hydrolysis of lorazepam-glucuronide, oxazepam-glucuronide and temazepam-glucuronide catalyzed by *E. Coli.* β-D-glucuronidase using the on-line benzodiazepine screening immunoassay on the Roche/Hitachi 917 analyzer. J Forensic Sci 2001;46(2):335–340.

ABSTRACT: The catalytic rates of hydrolysis of lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide when catalyzed by *E. Coli.* β-glucuronidase both in phosphate buffer and buffered drug-free urine were compared as well as the pH dependence of enzyme activity. In 50 mM phosphate buffer pH 6.4, lorazepam-glucuronide has the highest turnover rate of 3.7 s^{-1} with an associated K_m of about 100 μM, followed by oxazepam-glucuronide, which has a turnover rate of 2.4 s^{-1} with an associated K_m of 60 μM. Temazepam-glucuronide has the lowest rate of 0.94 s^{-1} with an associated K_m of 34 μM. In buffered drug-free urine, a similar trend was observed. In addition, an optimal pH for β-glucuronidase was determined to be between 6 and 7 when the enzyme hydrolyzes the benzodiazepine conjugates in buffered drug-free urine. Effects of temperature and incubation time were also examined. It can be concluded that the electron donating or withdrawing of the individual benzodiazepine structure may play an important role in the reactivity of the lorazepam-glucuronide, oxazepam-glucuronide and temazepam-glucuronide catalyzed by β-glucuronidase. This is consistent with other observations made for monosubstituted phenyl-β-glucuronides by Wang et al. (1).

KEYWORDS: forensic science, enzymatic hydrolysis, lorazepam-glucuronide, oxazepam-glucuronide, temazepam-glucuronide, β-D-glucuronidase

The benzodiazepines are among the most widely prescribed drugs in clinical medicine because of their extensive use as anxiolytics, sedative-hypnotics, anticonvulsants, and muscle relaxants (2). Three major metabolic pathways are involved in the metabolism of benzodiazepines: dealkylation, hydroxylation, and glucuronide conjugation (3). Dealkylation occurs more rapidly than hydroxylation in most cases and, therefore, usually occurs at the beginning of the metabolic pathway, followed by hydroxylation at the C3 position. Once formed, the hydroxyl metabolites and compounds such as α-hydroxyalprazolam, lorazepam, oxazepam, and temazepam rapidly undergo glucuronidation and are subsequently excreted in the urine (3). It has been reported that at least 75% of the dose of lorazepam, oxazepam, and temazepam is excreted in the urine as the conjugated form in human beings (4) and almost 100% is so excreted in horses (5). The fact that antibodies in most of the current commercially available immunoassays fail to recognize glucuronide conjugates creates a significant number of false negative measurements in using drugs of abuse screening methods. Hydrolysis is therefore required to ensure adequate detection of these conjugated benzodiazepines (6–10).

β-D-glucuronidase is a lysosomal enzyme catalyzing hydrolysis of natural and artificial glucuronides into glucuronic acids. The catalytically active enzyme exists as a tetramer composed of identical subunits of 60 to 80 kDa each depending on the source of species. For instance, the molecular weight of the *E. Coli.* enzyme is about 290 kDa. By using a series of mono-substituted phenyl-β-D-glucuronides, Wang and Touster (1) showed that both electron-withdrawing and electron-donating substituents facilitate the hydrolysis catalyzed by the enzyme. In the same paper, they also observed a solvent deuterium isotope effect on the enzyme activity. Based on these observations, they concluded that a concerted general acid-base catalysis occurs when the enzyme hydrolyzes the glucuronides, and that a rate-limiting proton transfer is involved in the hydrolysis of both electron-withdrawing and electron-donating substituted phenyl glucuronides. The crystal structure of human β-D-glucuronidase at 2.6 Å resolution (11) supports this hypothesis.

Reports on the β-glucuronidase hydrolysis of conjugated benzodiazepines have accumulated in the last few years (6–10,12); however, there is no quantitative kinetic data on hydrolysis of the different benzodiazepine-glucuronides with β-glucuronidase. Since differences may exist when the enzyme hydrolyzes these structurally different benzodiazepine conjugates, it is important that the rate of hydrolysis of different substrates catalyzed by β-glucuronidase be understood. By taking advantage of four currently available benzodiazepine-glucuronide reference materials: α-hydroxyalprazolam-glucuronide, lorazepam-glucuronide, oxazepam-

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Received 15 Feb. 2000; and in revised form 27 April 2000; accepted 28 April 2000.

glucuronide, and temazepam-glucuronide (all from Alltech), we are able to report the different rates of hydrolysis of these glucuronides when catalyzed with *E. Coli.* β-glucuronidase using the OnLine benzodiazepine screening immunoassay on the Roche/Hitachi 917 analyzer. The optimal pH for the enzymatic hydrolysis was also determined.

Materials and Methods

Reagents—E. Coli. β-glucuronidase was from Roche Molecular Biochemicals. α-hydroxyalprazolam, lorazepam, oxazepam, temazepam, which were in 100% methanol at a concentration of 1 mg/mL, and α-hydroxyalprazolam-glucuronide, lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide, which were in 100% methanol at concentrations of about 100 μg/mL, were purchased from Alltech. The purity of these conjugates is greater than 90%. The solutions of benzodiazepine glucuronide conjugates were prepared by evaporating the methanol under vacuum and reconstituting the residue to the desired concentrations with buffer or buffered drug-free urine. Drug-free urine and the OnLine benzodiazepine assay kit were obtained from Roche Diagnostics Corporation. Sodium acetate, acetic acid, perchloric acid, and potassium carbonate were purchased from Fisher Scientific. EDTA was from Sigma and potassium phosphate monobasic and dibasic were purchased from VWR (Mallinckrodt product). Enzyme concentration determination: protein concentrations were determined by the method of Bradford (13).

Enzyme Hydrolysis—The rate of hydrolysis of benzodiazepine glucuronide was expressed as nmol of product released per minute per mg enzyme. The benzodiazepines released from their conjugates after enzymatic hydrolysis were detected by the Roche OnLine benzodiazepine immunoassay system using the Roche/Hitachi 917 analyzer. To make sure that the initial rate of the hydrolysis is obtained, the enzymatic reaction is quenched with perchloric acid at 2 min after initiating the reaction. The detailed procedure for enzymatic hydrolysis is described as follows: 300 μL of benzodiazepine-glucuronides at various concentrations in 50 mM KP_i buffer, pH 6.4, or buffered drug-free urine containing 2 mM EDTA were added with 10 μL of 10-fold diluted *E. Coli.* β-glucuronidase (with water) and the reaction mixture was incubated for 2.0 min in a 25°C waterbath. The hydrolytic reactions were quenched by adding 30 μL of 14% perchloric acid and incubating in an ice bath for 5 min. After centrifuging for 5 min at 14 000 rpm in an Eppendorf microcentrifuge to precipitate the denatured enzyme, the supernatants were neutralized with 9 μL of 5 M K_2CO_3 and incubated on ice bath for 5 min. The supernatants were then diluted 5-fold with 50 mM KP_i buffer, pH 6.4, and submitted for benzodiazepine analysis.

Control—The control experiment was exactly the same as described above except that 10 μL of distilled water was added instead of the enzyme. The detected amounts of lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide at different concentrations, which were very low without enzymatic hydrolysis because of the low cross reactivity of these conjugates in the assay system, were subtracted from amounts detected after the enzyme hydrolysis.

Calibration—The OnLine benzodiazepine assay was run on the Roche/Hitachi 917 in the semi-quantitative mode using a four-point calibration with nordiazepam as the calibrator at concentrations of 0, 50, 100, and 200 ng/mL.

Standard Curve—Standard curves were generated for lorazepam, oxazepam, and temazepam in 50 mM KP_i buffer and in 50 mM KP_i buffered drug-free urine pH 6.4. 0, 50, 100, 150, 200, 250, 300, 400, 500, and 600 ng/mL of lorazepam, oxazepam, and temazepam were spiked in the buffer and buffered drug-free urine pH 6.4. To 300 μL of the spiked free benzodiazepine solutions, 10 μL of 10-fold diluted *E. Coli.* β-glucuronidase was added. Following perchloric acid addition and K_2CO_3 neutralization as described above, the supernatants were submitted for benzodiazepine measurement. The detected concentrations of benzodiazepines were plotted against the spiked benzodiazepine concentrations to determine a standard curve for each benzodiazepine. The standard curves are used to calculate the amount of products released from benzodiazepine conjugates after enzymatic hydrolysis.

Results

Cross Reactivity of α-Hydroxyalprazolam, Lorazepam, Oxazepam, Temazepam and their Glucuronide Conjugates

Cross reactivities determined are listed in Table 1. The calculated cross reactivities of α-hydroxyalprazolam, lorazepam, and oxazepam are 86, 30, and 77%, respectively. These values are essentially the same as stated in the OnLine package insert. The cross reactivity of temazepam, which was not reported previously, was estimated to be 62%. The nearly undetectable cross reactivity of lorazepam-glucuronide (1%), oxazepam-glucuronide (0%), and temazepam-glucuronide (0.75%) at the level tested, and the relatively high cross reactivity of α-hydroxyalprazolam-glucuronide (57%) are anticipated because of the structure of the immunogen designed to generate the benzodiazepine antibody (Linkage is out of the N1 position) (14). The cross reactivity of lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide was increased after enzymatic hydrolysis because of release of free

TABLE 1—*Percent cross reactivity of benzodiazepines. To measure the cross reactivity of α-hydroxyalprazolam, lorazepam, oxazepam, and temazepam, these compounds were spiked into 50 mM Kp_i buffer pH 6.4 to the desired concentrations, which are listed in Table 1. The samples were then submitted to the Hitachi 917 for the benzodiazepine measurement. The percent cross reactivities of a-hydroxyalprazolam, lorazepam, oxazepam, and temazepam were calculated according to the following: % Cross Reactivity = (detected drug value (ng/mL)/spiked drug concentration (ng/mL)) × 100%.*

Cross Reactants	Concentration Tested (ng/mL)	Approximate Cross Reactivity (%)
α-Hydroxyalprazolam	100	86%
	150	
Lorazepam	200	30%
	400	
Oxazepam	100	77%
	200	
Temazepam	100	62%
	200	
α-Hydroxyalprazolam-glucuronide	100	57%
	150	
Lorazepam-glucuronide	200	1.0%
	400	
Oxazepam-glucuronide	100	0%
	200	
Temazepam-glucuronide	100	0.75%
	200	

NOTE: N/D: not determined.

benzodiazepines. This makes them suitable for kinetic analysis by using the OnLine benzodiazepine assay system. Insignificant changes were observed in the cross reactivity of α-hydroxyalprazolam-glucuronide with or without enzymatic hydrolysis. Therefore kinetic analysis of β-glucuronidase hydrolyzing α-hydroxyalprazolam-glucuronide has to be carried out by other means and will not be included in this study.

Standard Curve of Lorazepam, Oxazepam, and Temazepam in 50 mM KP_i Buffer and in 50 mM KP_i Buffered Drug-Free Urine pH 6.4

Figure 1 shows the standard curve of lorazepam, oxazepam, and temazepam spiked in 50 mM KP_i buffer. As described in Materials and Methods, calibration standard curves were obtained by spiking 50 mM KP_i buffer pH 6.4 with various concentrations of lorazepam, oxazepam, and temazepam ranging from 0 to 600 ng/mL. To have an appropriate control, these standards were also treated with the $HClO_4$ quenching and K_2CO_3 neutralization, the same procedure as for the enzymatic hydrolysis of the benzodiazepine conjugates. It was notable that the quenching and neutralization procedure had little effect on the cross reactivity of lorazepam, oxazepam, and temazepam. In a similar experiment carried out in 50 mM KP_i buffered drug-free urine pH 6.4, the response curve was almost superimposable with the response curve in 50 mM KP_i buffer pH 6.4. From curve A, B, and C in Fig. 1, it is clear that the detected concentrations of lorazepam, oxazepam, and temazepam when plotted against actual spiked concentration values were not linear throughout the testing range of 0 to 600 ng/mL. The standard curves were optimally fitted with 2nd order regression with the following equation: $y = aX^2 + bX + c$ using SigmaPlot software. This indicates that the cross reactivities of these benzodiazepines vary with different concentrations present in the solution.

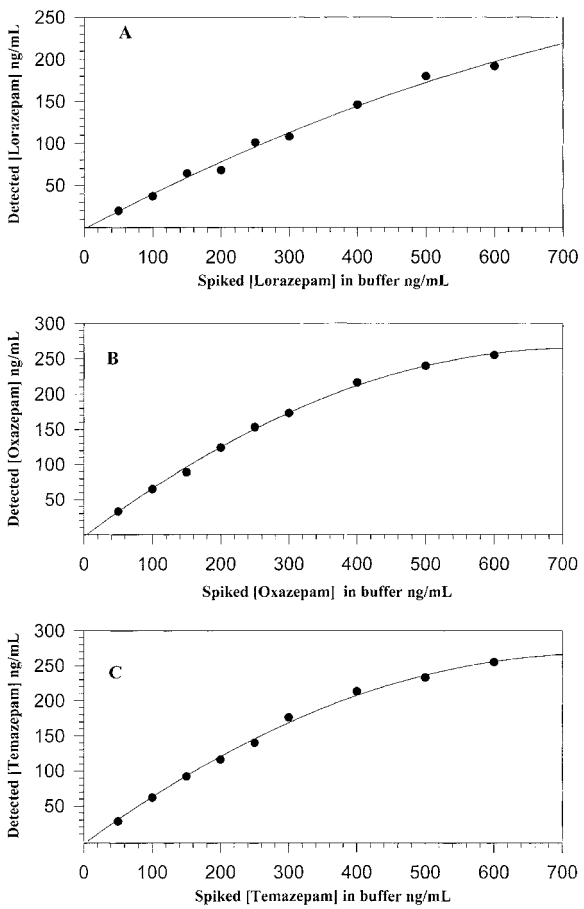

FIG. 1—*Standard response curves of lorazepam, oxazepam, and temazepam: detected ng/mL of benzodiazepines on the Roche/Hitachi 917 analyzer versus actual spiked ng/mL of benzodiazepines in 50 mM KP_i buffer pH 6.4. The curves were fitted with 2nd order regression with an equation:* $y = aX^2 + bX + c$ *in SigmaPlot. Trace A, lorazepam, B, oxazepam, C, temazepam. Detailed procedure is described in Materials and Methods.*

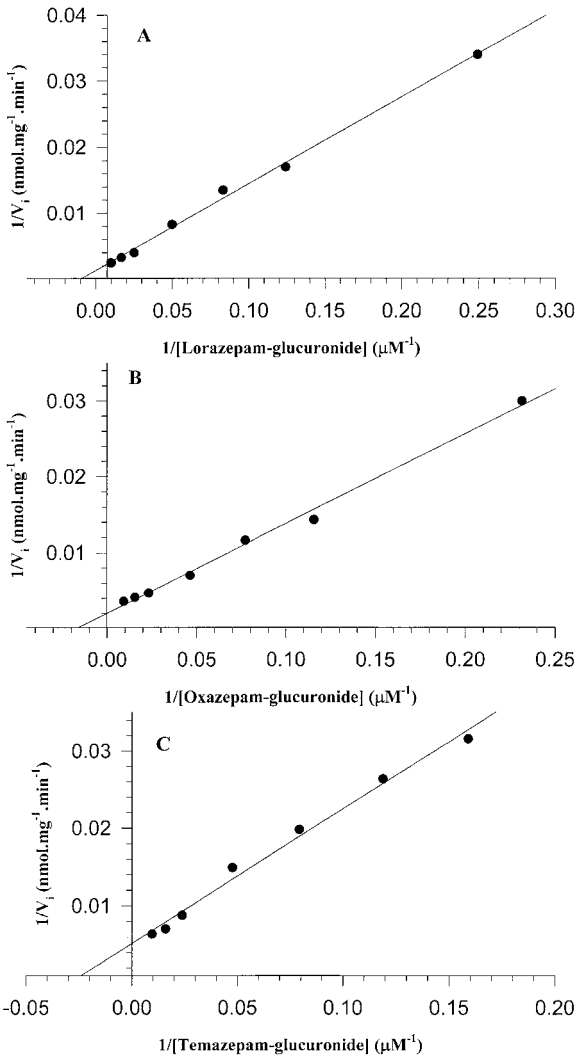

FIG. 2—*Lineweaver-Burk plot ($1/V_i$ versus 1/[benzodiazepine-glucuronide]) of* E. Coli. *β-glucuronidase when hydrolyzing lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide in 50 mM KP_i buffer. Trace A, lorazepam, B, oxazepam, C, temazepam. Detailed procedure is described in the Materials and Methods section.*

Comparison of the Rate of Hydrolysis of Lorazepam-glucuronide, Oxazepam-glucuronide, and Temazepam-glucuronide Catalyzed by E. Coli. *β-D-Glucuronidase*

Figure 2 shows the Lineweaver-Burk plot (I/V_i versus 1/[benzodiazepine-glucuronide]) when *E. Coli.* β-glucuronidase hydrolyzes

TABLE 2—*Kinetic parameters for hydrolysis of benzodiazepine-glucuronides catalyzed by* E. Coli. β-*glucuronidase. Michaelis constant* (K_m) *and turnover numbers* (k_{cat}) *and* k_{cat}/K_m *were derived from Lineweaver-Burk plot using Sigma plot software.*

	In 50 mM KP_i buffer pH 6.4			In buffered DFU pH 6.4		
Benzo-glu	k_{cat} s^{-1}	K_m μM	k_{cat}/K_m $s^{-1}\,M^{-1}$	k_{cat} s^{-1}	K_m μM	k_{cat}/K_m $s^{-1}\,M^{-1}$
Lor-glu	3.7	100	3.7×10^4	1.2	150	8.0×10^3
Oxa-glu	2.4	60	4.0×10^4	0.6	122	5.0×10^3
Tem-glu	0.94	34	2.8×10^4	0.23	70	3.3×10^3

lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide in 50 mM KP_i buffer pH 6.4 containing 2 mM EDTA. To ensure that the initial rate is obtained, the enzymatic reaction was quenched with perchloric acid within 2 min after the initiation of the reaction. Kinetic parameters calculated from these plots are listed in Table 2. Obviously, the rates of hydrolysis of the three benzodiazepine-glucuronide conjugates are very different. Lorazepam-glucuronide has the highest rate of 770 nmol hydrolyzed.mg^{-1}.min^{-1} (3.7 s^{-1}), followed by oxazepam-glucuronide, which has a rate of 500 nmol hydrolyzed.mg^{-1}.min^{-1} (2.4 s^{-1}), and finally temazepam-glucuronide, which has a rate of 195 nmol hydrolyzed.mg^{-1}.min^{-1} (0.94 s^{-1}) in 50 mM KP_i buffer pH 6.4. The associated K_m values for these conjugates are 100 μM for lorazepam-glucuronide, 60 μM for oxazepam-glucuronide, and 34 μM for temazepam-glucuronide respectively. However, k_{cat}/K_m values, which are $4.0 \times 10^4\ s^{-1}\ M^{-1}$ for lorazepam-glucuronide, $3.7 \times 10^4 s^{-1}M^{-1}$ for oxazepam-glucuronide and $2.8 \times 10^4\ s^{-1}\ M^{-1}$ for temazepam-glucuronide, did not vary to such a great extent as was seen for k_{cat} or K_m alone. When the enzymatic hydrolysis was carried out in buffered drug-free urine, similar trends were observed, with lorazepam-glucuronide having the highest rates of hydrolysis and temazepam-glucuronide showing the lowest. It was also noticed that the K_m values in buffered drug-free urine are 50 to 100% higher than those observed in 50 mM KP_i buffer.

pH Profile of E. Coli. *β-D-Glucuronidase when Hydrolyzing Lorazepam-glucuronide, Oxazepam-glucuronide, and Temazepam-glucuronide*

It has been reported that β-glucuronidases from different sources have different optimal pH ranges when catalyzing morphine-glucuronide (15,16). To determine the optimal pH of *E. Coli.* β-glucuronidase when hydrolyzing lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide, the enzymatic activities over a pH range from 4.5 to 8.0 in buffered drug-free urine were examined at room temperature. The enzyme was incubated with benzodiazepine conjugates in buffered urine with different pH values for 30 min. The optimal hydrolysis occurred between pH 6 to 7 in buffered drug-free urine (Fig. 3). These results are consistent with those observed for hydrolysis of morphine-3-glucuronide catalyzed by the same enzyme and optimal pH values for hydrolysis of phenolphthalein glucuronide reported by Sigma (16).

Effect of Temperature and Incubation Time on Hydrolysis of 1000 ng/mL of Lorazepam-, Oxazepine-, and Temazepam-glucuronides in Buffered Drug Free Urine pH 6.4.

Figure 4*A* demonstrates the effect of increased temperature on the amount of free benzodiazepines released from their glucuronide conjugates after 2 min hydrolysis in buffered drug free urine, pH 6.4. The release of free benzodiazepines, independent of type, was progressively increased as the incubation temperature raised from 25 to 40°C. Figure 4*B* shows effect of incubation time on the degree of hydrolysis. In the first 15 min incubation period, the degree of hydrolysis was very rapid. Then the rates of hydrolysis slowed and a near plateau was observed at 125 min.

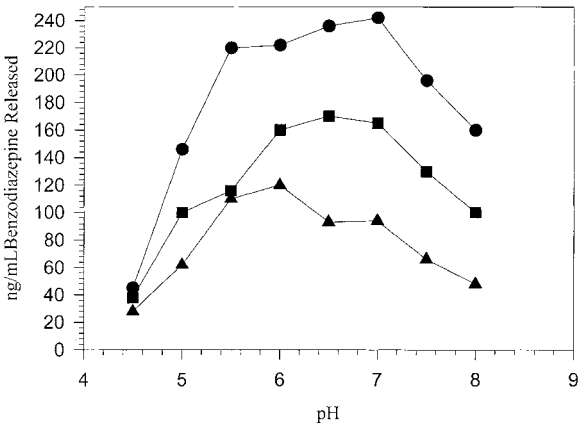

FIG. 3—*Effect of pH on the hydrolysis of lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide catalyzed by* E. coli. *β-glucuronidase in buffered drug-free urine (DFU). 1000 ng/mL of lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide was spiked into 300 μL of 100 mM NaAc-HAc (for pH = 4.5, 5.0, 5.5, 6.0) and 100 mM KP_i (for pH = 6.5, 7.0, 7.5, and 8.0) buffered drug-free urine (DFU) at different pH's containing 2 mM EDTA. Ten μL of 10 fold diluted* E. Coli. *β-glucuronidase was added to each benzodiazepine conjugate solution prepared above. After 30 min incubation in buffered DFU, the reactions were quenched with perchloric acid and neutralized with K_2CO_3 as described in the Materials and Methods. The samples were submitted for free benzodiazepine determination. The amount of conjugate hydrolyzed was derived from the standard response curve. (•) lorazepam-glucuronide, (■) oxazepam-glucuronide, (▲) temazepam-glucuronide.*

Discussion

As predicted, structurally different benzodiazepine-glucuronide conjugates have very different rates of hydrolysis when catalyzed by *E. Coli.* β-glucuronidase in phosphate buffer or buffered drug-free urine. Interestingly, the electron effect of the structure on the reactivity observed for lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide is consistent with that observed by Wang and Touster (10) for monosubstituted phenyl-β-glucuronides. The structures of the four current commercially available benzodiazepine conjugates are shown in Fig. 4. Since the OnLine benzodiazepine assay does not significantly discriminate between α-hydroxyalprazolam and α-hydroxyalprazolam-glucuronide, the hydrolysis study of α-hydroxyalprazolam-glu-

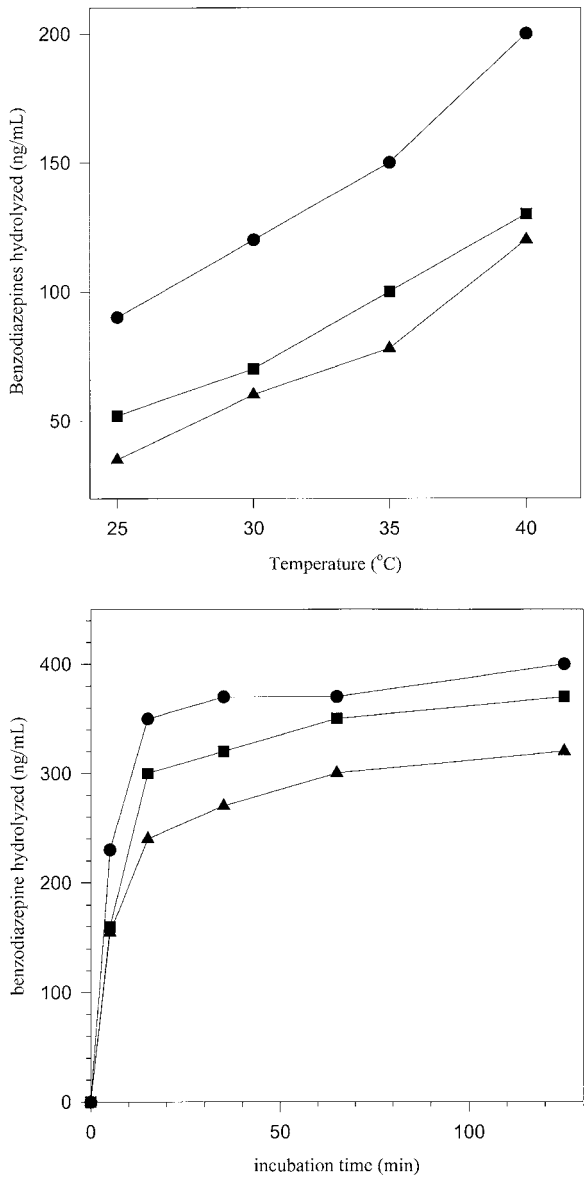

FIG. 4—*Amount of free benzodiazepine released versus incubation temperature and incubation time. (A). Temperature effect on hydrolysis. To 300 μL of 100 mM KP_i buffered drug free urine, pH 6.4 containing 1000 ng/mL benzodiazepine-glucuronides and 2 mM EDTA, 5 μL of* E. Coli. *β-glucuronidase was added to initiate hydrolysis. After 2 min incubation in 25, 30, 35, and 40°C waterbath, the enzymatic reaction was terminated by adding 30 μL of $HClO_4$ and then neutralized with K_2CO_3 as described in Materials and Methods. The resulting solutions were submitted for benzodiazepine analysis. (B). Effect of incubation time on degree of hydrolysis. To 300 μL of 100 mM KP_i buffered drug free urine, pH 6.4 containing 1000 ng/mL benzodiazepine-glucuronides and 2 mM EDTA, 5 μL of* E. Coli. *β-glucuronidase was added to initiate hydrolysis. After 5, 15, 35, 65, 125 min incubation in 37°C waterbath, the solutions were submitted for benzodiazepine analysis. (•) Lorazepam-glucuronide, (■) Oxazepam-glucuronide, and (▲) Temazepam.*

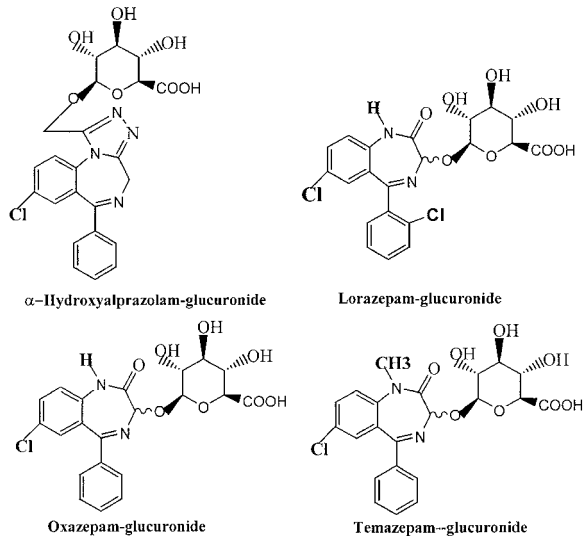

FIG. 5—*Chemical structures of α-hydroxyalprazolam-glucuronide, lorazepam-glucuronide, oxazepam-glucuronide, and temazepam-glucuronide.*

curonide could not be performed in this study. Obviously, the two chlorine atoms at the C7 and C2′ positions in lorazepam-glucuronide have an electron withdrawing effect to the π-conjugate system connected to glucopyranosiduronic acid, which is thereby rendered susceptible to nucleophilic attack. Therefore a higher rate of hydrolysis of lorazepam-glucuronide catalyzed by β-glucuronidase is expected to occur. In fact, among the three benzodiazepine conjugates tested, lorazepam-glucuronide has the highest rate of hydrolysis (3.7 s^{-1}) when the reaction was carried out in 50 mM phosphate pH 6.4. In contrast to lorazepam-glucuronide, oxazepam-glucuronide has only one chlorine at the C7 position. The electron-withdrawing effect is therefore weaker and thus a slower rate is expected (2.4 s^{-1}). In the case of temazepam-glucuronide, there is a chlorine at the C7 position and a methyl group at the N1 position, which is an electron donor. The electron donating effect of the methyl group helps to offset the electron withdrawing effect of the —Cl group. This may explain why temazepam-glucuronide has the slowest rate (0.94 s^{-1}) among the three benzodiazepine conjugates tested.

When the enzymatic hydrolysis was performed in the phosphate buffer, the catalytic efficiency (k_{cat}/K_m) for lorazepam-glucuronide, oxazepam-glucuronide and temazepam-glucuronide does not differ to such a great extent as either k_{cat} or K_m considered alone. The observation that the K_m value becomes smaller in going from lorazepam-glucuronide to temazepam-glucuronide is not consistent with that made by Wang and Touster (1), who demonstrated that unsubstituted phenyl-β-D-glucuronide has the highest K_m, and that both electron-withdrawing and electron-donating substituted phenyl-β-D-glucuronides have lower K_m values. The cause of this discrepancy is not obvious. One possible explanation could be that *E. Coli.* β-glucuronidase, rather than rat liver enzyme is used in this study. If the binding of the substrates to the *E. Coli.* β-glucuronidase is indeed affected by the electron character of the structure of the substrate as shown by Wang and Touster (1), then the prediction would be as follows for benzodiazepine glucuronide conjugates when hydrolyzed by *E. Coli.* β-glucuronidase: the more electron withdrawing substituents associated with the structure, the higher the K_m it will have. Additional benzodiazepine conjugates need to be investigated in order to test this general conclusion.

Notably, both the turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) are attenuated to a great extent (3 to 8.5 fold) when the enzymatic hydrolysis of lorazepam-glucuronide, oxazepam-glu-

curonide, and temazepam-glucuronide was carried out in buffered drug-free urine instead of the KP_i buffer. In contrast, K_m values for the three conjugates are increased by 50 to 100%. This may be caused by some natural compounds being metabolized in the human body and excreted in the urine, which could act as competitive or mixed inhibitors of the enzyme and thus making it less efficient. For instance, saccharo-1, 4-lactone is a very potent competitive inhibitor that has a K_i of 170 nM (10). This observation, along with pH dependence of the reaction rate, indicates that a matrix effect may influence the hydrolysis of urinary benzodiazepine-glucuronide conjugates.

This study demonstrated that one set optimal hydrolysis condition could not assure total hydrolysis of a sample containing mixtures of benzodiazepine glucuronide species, given the fact that structurally different benzodiazepine-glucuronides have very different rates of hydrolysis. In addition, urinary matrix effect should be taken into consideration when trying to optimize the conditions of β-glucuronidase hydrolysis as part of a benzodiazepine assay.

Acknowledgment

We are grateful for Ken Slickers and Irina Baburina for the critical discussion and review of this manuscript.

References

1. Wang C, Touster O. Studies of catalysis by β-glucuronidase: the effect of structure on the rate of hydrolysis of substituted phenyl-β-D-glucopyranosiduronic acids. J Biol Chem 1972;247:2650–6.
2. Klotz U, Kangas L, Kanto J. Clinical pharmacokinetics of benzodiazepines. Progr Pharmacol 1980;3(3), Stuttgart, New York: Fischer.
3. Kaplan S, Jack M. Metabolism of the benzodiazepines: Pharmacokinetics, and pharmacodynamic considerations. The Benzodiazepines: from molecular biology to clinical practice, 1983; In: Costa E, editor, Raven Press, New York.
4. Baselt RC, Cravey RH. Disposition of toxic drugs and chemicals in man, 4th edition, Chemical Toxicology Institute, Forster City, CA, 1995; 432–3, 569–70.
5. Marland A, Sarkar P, Leavitt R. The urinary elimination profiles of diazepam and its metabolites, nordiazepam, temazepam, and oxazepam, in the equine after a 10-mg intramuscular dose. J Anal Toxicol 1999; 23:29–34.
6. Beck O, Lafolie P, Odelius G, Boreus L. Immunological screening of benzodiazepines in urine: improved detection of oxazepam intake. Toxicol. Lett. 1990;52:7–14.
7. Beck O, Lin Z, Bordin K, Borg S, Hjemdahl P. The online screening technique for urinary benzodiazepines: comparison with EMIT, FPIA, and GC-MS. J Anal Toxicol 1997;21:554–7.
8. Salamone S, Honasoge S, Brenner C, McNally A, Passarelli J, Goc-Szkutnicka K, et al. Flunitrazepam excretion patterns using the abuscreen ontrak and online immunoassays: comparison with GC-MS. J Anal Toxicol 1997;21:341–5.
9. Meatherall R. Optimal enzymatic hydrolysis of urinary benzodiazepine conjugates. J Anal Toxicol 1994;18:382–4.
10. Meatherall R. Benzodiazepine screening using EMIT II and TDx urine hydrolysis pretreatment required. J Anal Toxicol 1994;18:385–90.
11. Jain S, Drendel W, Chen Z, Mathews F, Sly W, Grubb J. Structure of human β-glucuronidase reveals candidate lysosomal targeting and active site motifs. Nature Structural Biology 1996;3:375–81.
12. Meatherall R. CEDIA dau benzodiazepine screening assay: a reformulation. J Anal Toxicol 1998;22:270–3.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
14. Antonian E, Courtney JB, Passarelli JJ, Salamone SJ. Eur Pat Appl EP92-103667 920916.
15. Zezulak M, Snyder J, Needleman S. Simultaneous analysis of codeine, morphine, and heroin after β-glucuronidase hydrolysis. J Forensic Sci 1993;38:1275–85.
16. Romberg R, Lee L. Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and β-glucuronidase. J Anal Toxicol 1995;19:157–62.

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