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Report

Quantification of Three Lidocaine Metabolites and Their Conjugates

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A method has been developed to quantify three lidocaine metabolites, *N*-ethylglycyl-2,6-xylidide (MEGX), glycyl-2,6-xylidide (GX), and 4-hydroxy-2,6-xylidine (4-OH-XY), and their conjugates in pooled human urine using enzymic hydrolysis. The commonly used enzymes, pure β-glucuronidase, sulfatase, and a mixture of the two, were tested for their efficiencies in hydrolyzing the conjugates. Initially, it was found that 4-OH-XY was highly unstable after it was released from conjugates by β-glucuronidase and the enzyme mixture. This problem was corrected by purging the sample with nitrogen prior to incubation. It has been determined that 4-OH-XY is present in human urine exclusively as its glucuronide. The percentage of MEGX in free and in conjugated forms (glucuronide, sulfate, and others) are 44.9 ± 6.8, 16.6 ± 4.5, 6.6 ± 1.8, and 31.9 ± 4.4, respectively. GX was present mostly in the free form (90.6 ± 10.5%).

KEY WORDS: quantification; lidocaine metabolites; conjugates; *N*-ethylglycyl-2,6-xylidide; glycyl-2,6-xylidide; 4-hydroxy-2,6-xylidine.

INTRODUCTION

Lidocaine metabolism has been a subject of interest for a number of years (1–3). The major primary pathway of lidocaine biotransformation in human is apparently *N*-dealkylation, followed by secondary oxidations, conjugations, and hydrolysis (1,4). Recently, we found that in addition to *N*-dealkylation, *p*-hydroxylation could also be an important primary pathway in humans (5). Consistent with literature results (1,4), we have also found that *N*-ethylglycyl-2,6-xylidide (MEGX), glycyl-2,6-xylidide (GX), and 4-hydroxy-2,6-xylidine (4-OH-XY) (Scheme I) are the major metabolites in patients with suspected myocardial infarction (5).The Phase II metabolism of lidocaine is not well characterized because most studies used acid to hydrolyse the conjugates (4,6). It is well known that this method is not discriminatory. Enzyme hydrolysis was used by Nelson *et al.* (2) to study the conjugates of lidocaine metabolites. These investigators reported that in healthy human volunteers MEGX was not conjugated, 60 to 68% of an oral dose of lidocaine was excreted in the form of glucuronide and/or sulfate conjugates of 4-OH-XY, and the enzymes used to hydrolyze conjugates somehow interfered with the extraction of GX.

We have found that 4-OH-XY, in its free form, is highly unstable in neutral and alkaline solutions. More than 50% decomposition occurs within 24 hr at room temperature. It is questionable, therefore, whether the recovery of 4-OH-XY after the traditional 24-hr incubation with enzymes is quantitative.

Based on the available information, it is apparent that the traditional enzyme hydrolysis method for quantifying conjugates of lidocaine metabolites is not of an adequate accuracy. The method reported by Eichelbaum *et al.* (7) was found by us to be unsuitable for the maintenance of 4-OH-XY stability. Therefore, we decided to develop an analytical procedure which would permit quantitative measurements of these lidocaine metabolites without significant decomposition.

MATERIALS AND METHODS

Chemicals. The HCl salts of MEGX, GX, and 4-OH-XY were kindly supplied by Astra Pharmaceuticals (Mississauga, Ontario, Canada). β-Glucuronidases (*E. coli* type VII, lyophilized; and *H. pomatia* type H-1) and sulfatase (limpets type V, lyophilized) were purchased from Sigma (St. Louis, Mo.). All other chemicals were reagent grade.

Preparation of Enzyme Solutions. The β-glucuronidase solutions, type VII [400 Sigma Units (U)/ml] and type H-1 (500 U/ml), and the sulfatase solution (type V, 9 U/ml) were prepared by dissolving appropriate amounts of the enzymes in 0.075 *M* potassium phosphate (pH = 6.8), 0.1 *M* sodium acetate (pH = 5.0), and 0.2 *M* sodium acetate buffer (pH = 5.0), respectively.

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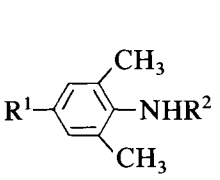

	R^1	R^2
Lidocaine	H	$COCH_2N(Et)_2$
MEGX	H	$COCH_2NHEt$
GX	H	$COCH_2NH_2$
4-OH-XY	OH	H

Scheme I

Hydrolysis of Urine Samples. Aliquots of urine samples from seven patients with suspected myocardial infarction who were admitted to the lidocaine trial (8) were pooled. These patients were given two 100-mg iv bolus injections of lidocaine HCl 30 min apart, followed by infusion (3 mg/min) immediately after the first bolus dose. The duration of infusion ranged from 16 to 48 hr. Individual urine samples, including a predose specimen, were collected until 72 hr postinfusion. Unless otherwise specified, all of the following studies relating to hydrolysis were performed in tightly capped (Teflon-lined) glass tubes.

Enzyme Hydrolysis. Buffered aqueous solutions and buffered enzyme solutions (0.5 ml) were individually added to 0.1 ml of patient urine samples. Two types of control samples were prepared by adding 0.5 ml of buffer solution, with or without the prescribed amount of enzyme, to 0.1 ml of spiked urine sample containing a mixture of MEGX, GX, and 4-OH-XY (concentration ranged from 4 to 600 μg/ml). All samples were diluted to 1.5 ml with deionized water and incubated at 37°C for a maximum of 36 hr. For quantitative measurements, these samples were purged with nitrogen for 1 min prior to incubation.

Acid Hydrolysis. To 0.1 ml of patient or spiked urine sample was added an equal volume of 6 *N* HCl. All samples were incubated at 100°C for a maximum of 2 hr.

Analytical Methods. The treated samples were quantified using the method of Tam *et al.* (5). The concentration of the three metabolites used in this study ranged from 10 to 1200 μg/ml for 4-OH-XY, 10 to 25 μg/ml for MEGX, and 4 to 8 μg/ml for GX.

Data Analysis. All samples were prepared in triplicate and the results were recorded as mean ± SD. It was assumed that acid hydrolysis released free MEGX, GX, and 4-OH-XY from all forms of conjugates of these three lidocaine metabolites. A two-tailed *t* test ($P = 0.05$) was used to evaluate differences between groups.

RESULTS

4-OH-XY. Type VII β-glucuronidase, which contained no sulfatase, is the most efficient enzyme in hydrolyzing 4-OH-XY glucuronide (Fig. 1A). The maximum yield of aglycone was achieved within 30 min, whereas it required 24 hr for the type H-1 enzyme, which is a mixture of β-glucuronidase and sulfatase, to hydrolyze the conjugate. The yield was significantly lower with type H-1 than with type VII enzyme (346 ± 20 vs 484 ± 23 μg/ml, $P < 0.05$). Sulfate-conjugated and free (unconjugated) 4-OH-XY were not detected in urine when the samples were treated with type V sulfatase or buffer containing no enzyme (Fig. 1A).

The decrease in free 4-OH-XY levels in the enzyme hydrolysis–time curve (Fig. 1) is most likely due to the instability of 4-OH-XY. This hypothesis is supported by the continuous decomposition of 4-OH-XY when it was incubated with enzymes (Fig. 1B). However, the enzyme preparations apparently made little contribution to the rate of 4-OH-XY decomposition since the concentration profiles derived from the use of enzyme preparations and buffer controls are superimposable. It is shown in Fig. 1B that the rate of 4-OH-XY decomposition is pH dependent, higher at pH 6.8 than at pH 5.0.

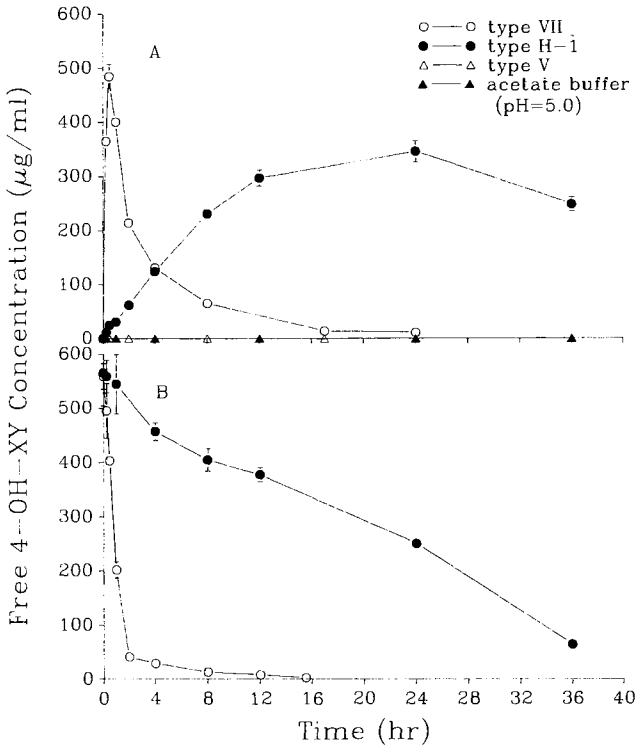

Fig. 1. (A) Profiles of 4-OH-XY release from its conjugate in types VII (pH 6.8) and H-1 (pH 5.0) β-glucuronidase, type V sulfatase (pH 5.0), and acetate buffer (pH 5.0) preparations. (B) Time courses of spiked 4-OH-XY in the two buffered β-glucuronidase solutions studied. All of the samples were not purged with nitrogen before incubation.

Attempts were made to stabilize 4-OH-XY using the method of Eichelbaum *et al.* (7). Ascorbic acid and sodium bisulfite were added to the incubation mixtures, in varying amounts up to 0.43 and 32 mg/ml of each agent, respectively. Both antioxidants failed to prevent decomposition. However, released 4-OH-XY was stabilized when the samples containing its conjugates were purged with nitrogen for 1 min prior to incubation with enzymes (Figs. 2A and B).

Maximal hydrolysis of the conjugate was achieved within 45 min with the type VII enzyme. This result is insignificantly different from that obtained after acid hydrolysis, 1164 ± 27 vs 1204 ± 40 μg/ml, suggesting that 4-OH-XY is present in urine only in the form of glucuronide. This observation is further substantiated by the absence of sulfate conjugate (Fig. 2A). It is interesting to note that the yield of 4-OH-XY after type H-1 treatment is slightly lower (8%) than that obtained using type VII enzyme ($P < 0.05$). This could reflect some decomposition of 4-OH-XY after prolonged reaction (36 hr or more).

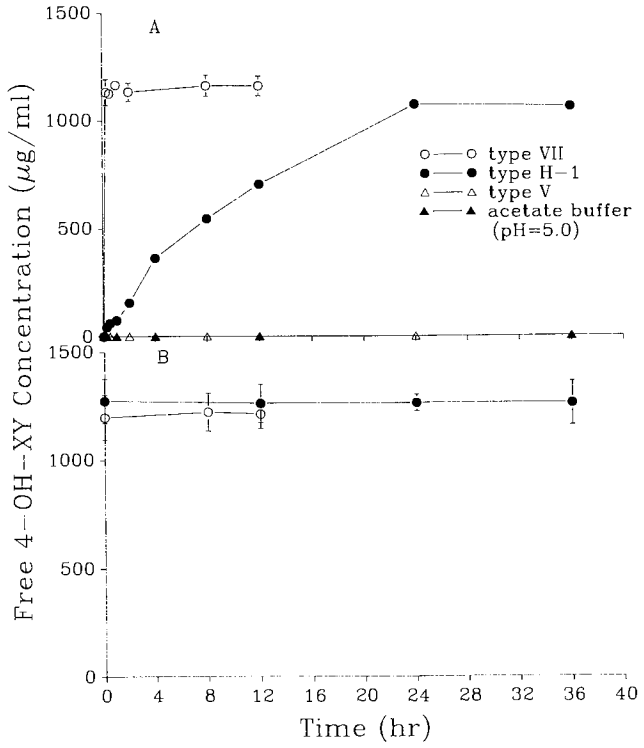

Fig. 2. (A) Profiles of 4-OH-XY release from its conjugate in types VII (pH 6.8) and H-1 (pH 5.0) β-glucuronidase, type V sulfatase (pH 5.0), and acetate buffer (pH 5.0) preparations. (B) Time courses of spiked 4-OH-XY in the two buffered β-glucuronidase preparations. All of the samples were pretreated with nitrogen before incubation.

MEGX. MEGX is present in patient urine in its free form and as sulfate and glucuronide conjugates (Table I). The hydrolysis of the glucuronide and sulfate conjugates was relatively rapid. A maximum yield of MEGX was obtained within an hour with type VII β-glucuronidase and within 2 hr with type V sulfatase treatment. However, it took 12 hr for the glucuronide and sulfate conjugates to hydrolyze when type H-1 enzyme was used. The recovery of free MEGX from type H-1 enzyme hydrolysis was equal to the sum of the free MEGX released from the type VII β-glucuronidase and type V sulfatase, suggesting that these enzymes could be used to measure quantitatively the conjugates of MEGX. Hydrolysis of the urine samples with acid provided a higher MEGX yield than that obtained by hydrolysis with type H-1 enzyme (31.3 ± 3.4 vs 21.5 ± 1.1 μg/ml, $P < 0.05$), indicating the presence of other conjugates of MEGX in urine. MEGX is stable during acid or enzyme treatments. Purging the samples with nitrogen did not change the yield of MEGX.

GX. The treatment of patient urine with type H-1 and VII enzymes showed that no significant amounts of glucuronide and sulfate conjugates of GX were excreted (Table I). Although acid hydrolysis gave a slight increase in the GX response (6.04 ± 0.37 vs 5.09 ± 0.33 μg/ml, $P < 0.05$), this result suggests that GX is present mostly in the free form. GX was stable to both acid and enzyme treatment and nitrogen treatment did not change the yield.

Table I. Mean (±SD) Recovery of Various Forms of 4-OH-XY, MEGX, and GX in Pooled Human Urine

	% recovery		
Form of excretion	4-OH-XY	MEGX	GX
Glucuronide	96.7 (7.01)	16.6 (4.5)	—
Sulfate	—	6.6 (1.8)	—
Other conjugates	—	31.9 (4.4)	9.4 (10.0)
Free	—	44.9 (6.8)	90.6 (10.5)

DISCUSSION

Methods for Stabilizing 4-OH-XY. The mechanism of 4-OH-XY decomposition has been studied (9). It was found that this metabolite is primarily oxidized to the corresponding *p*-quinone via the intermediate imine. This reaction requires oxygen and is pH dependent. The metabolite is most stable under highly acidic conditions and the stability of this compound decreases rapidly as pH increases. Since the enzymes function optimally at pH 5 and 6.8, it is at these pH values that the rates of 4-OH-XY decomposition are high (Fig. 1B). There are two approaches to stabilize this metabolite. One is to use antioxidants. This method was used successfully to stabilize norantipyrine (7), but 4-OH-XY could not be stabilized using this method. The reason could be due to the higher affinity of 4-OH-XY toward the primary reactant, oxygen. The second approach is to remove oxygen entirely from the reaction mixture. Purging the aqueous sample with nitrogen seemed to provide the answer to this stability problem. Whether this simple method is applicable to other unstable metabolites remains to be tested. We have used this method to study the conjugation of the three lidocaine metabolites and the results are listed in Table I.

There was no free 4-OH-XY detected in the pooled urine samples. Extensive conjugation and/or instability of unconjugated 4-OH-XY could contribute to this observation. It would be extremely difficult to measure accurately free 4-OH-XY in urine, if it were present, because the decomposition of this metabolite is extremely rapid (Fig. 1B). This suggests that significant decomposition can occur in the bladder and/or upon storage.

It is interesting to note that previous investigators (1,4,6) have not identified any problems with the stability of 4-OH-XY. This could be due to the fact that acid hydrolysis was used most of the time to release 4-OH-XY from the conjugate (4,6), and 4-OH-XY is more stable under acidic than basic conditions. We have noticed that, under acidic conditions and at concentrations higher than 20 μg/ml, the decomposition kinetics of 4-OH-XY is apparently zero order (~0.14 μg/min). Since the rate of reaction is slow, the impact on the recovery of 4-OH-XY at high concentrations (>1000 μg/ml) is minimal. Again, the nitrogen pretreatment to remove dissolved oxygen corrects the problem. Therefore, it is advisable to use nitrogen pretreatment for all samples prior to acid hydrolysis.

Based on the results of this study, the quantification method reported by Nelson *et al.* (2) may not be appropriate. These investigators added deuterated 4-OH-XY to fresh urine samples and assumed that the deuterated standard had the same stability as the conjugated metabolite. This as-

sumption is incorrect because the decomposition profile of 4-OH-XY is vastly different from that of the conjugate, which accounts for most, if not all, of the 4-OH-XY in urine. The conjugate, according to our experience, is stable for at least 6 months upon storage (−20°C), whereas the half-life of 4-OH-XY is stable only about 10 days under identical conditions.

MEGX and GX. Although a literature report (2) indicates that MEGX is not excreted in conjugated form, at least three conjugates of MEGX are found in patient urine (Table I). This discrepancy could be related to a number of factors, which include differences in analytical methods, in subject populations, and in the dosages used. The discrepancy cannot be attributed to the different enzymes used to hydrolyse the urine samples. The type H-2 enzyme used by Nelson *et al.* (2) was very similar to the type H-1 enzyme used in this study. In fact, we have confirmed that type H-2 β-glucuronidase (500 U/ml) does hydrolyze our urine samples. The amount of free MEGX released by type H-2 enzyme was virtually identical to that produced by type H-1 enzyme. There is not enough information to evaluate potential difference in the subject populations, therefore, no conclusion can be made. The dosage of lidocaine used in this study is higher than that reported by Nelson *et al.* (2), therefore, it is likely that Phase II metabolism of MEGX could be concentration related. In a lidocaine study, where healthy subjects received single intravenous doses of lidocaine (1 mg/kg), which was considerably less than the 250-mg oral dose used in the study by Nelson *et al.* (2), we found MEGX glucuronide in the urine of these human volunteers. Although other MEGX conjugates could not be found, the reasons for the discrepancy between the two studies remain unclear.

The analytical method used by Nelson *et al.* (2) did not permit the investigators to study GX conjugation because the type H-2 enzyme used to hydrolyze GX conjugates interfered with the extraction of the metabolite. We have circumvented the problem by acetylating free GX in aqueous solution prior to extraction (5).

Effects of Enzyme Concentration. The concentrations of enzyme preparations used in the present study were confirmed to be adequate. When the concentrations of type VII and type H-1 enzymes were increased threefold (from 400 to 1200 and from 500 to 1500 U/ml, respectively), the differences in yields of 4-OH-XY and MEGX were found to be less than 2%.

In conclusion, we have shown that the traditional methods of hydrolyzing conjugates with acid and enzyme preparations have to be used with caution when dealing with an unstable metabolite. A case in point is the quantification of 4-OH-XY conjugates. Additional procedures such as pretreatment of aqueous solutions of conjugates with nitrogen gas may have to be incorporated in order to ensure proper quantification of unstable metabolites. Furthermore, we have revealed that MEGX forms at least three conjugates, and GX exists mainly in its free form in the urine of patients receiving lidocaine.

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