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Stability and enzymatic hydrolysis of quaternary ammonium-linked glucuronide metabolites of drugs with an aliphatic tertiary amine-implications for analysis☆

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Abstract

Quaternary ammonium-linked glucuronide (N^+-glucuronide) metabolites formed at aliphatic tertiary amine functional groups of xenobiotics have not been previously systematically studied with respect to their stability over a wide pH range and the ease of enzymatic hydrolysis by β-glucuronidase from various sources. Three and four N^+-glucuronide metabolites were respectively studied regarding their non-enzymatic and enzymatic stabilities where the metabolites were quantified by HPLC procedures. The N^+-glucuronide metabolites of clozapine, cyclizine, and doxepin were stored at 18–22°C in buffers at each nominal pH value over the 1–11 pH range. All three metabolites were stable for 3 months over the 4–10 pH range, while two metabolites slowly degraded (k in the range 0.002–0.01 days^{-1}) at each of the other extreme pH values. In the initial enzymatic study the N^+-glucuronide metabolites of chlorpromazine, clozapine, cyclizine, and doxepin were each treated in pH 5.0 and 7.4 buffers at 37°C with β-glucuronidase from three different sources, namely commercial brands from bovine liver, mollusks (*Helix pomatia*), and bacteria (*Escherichia coli*). Clozapine N^+-glucuronide and the standard phenolphthalein O-glucuronide were susceptible to hydrolysis by the enzyme from all three sources. In contrast, the other three N^+-glucuronide metabolites were resistant to hydrolysis, except for the *E. coli* source of β-glucuronidase at pH 7.4. Also when examined at 50-fold increase in concentration of the enzyme sources from bovine liver and *H. pomatia* cyclizine N^+-glucuronide was still resistant to hydrolysis by the former enzyme preparation. The optimum pH for the hydrolysis of each of the four N^+-glucuronide metabolites from the *E. coli* enzyme source was investigated and was found to be in the pH range 6.5–7.4. These data have important implications with respect to the analysis of N^+-glucuronide metabolites formed at an aliphatic tertiary amine: in general, their non-enzymatic stability will not be an important factor in the development of an analytical procedure, and when developing an indirect approach to the analysis of N^+-glucuronide metabolites that involves β-glucuronidase hydrolysis to the aglycone preliminary work should involve determining the appropriate enzyme source, buffer pH, and length of time of incubation.

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1. Introduction

Glucuronidation of either an aliphatic or an aromatic tertiary amine group in a molecule results in a quaternary ammonium-linked glucuronide (i.e. N^+-glucuronide) metabolite. In general, due to their high polarity and low volatility glucuronide metabolites are recognised to require careful selection of the appropriate analytical approaches including extraction, chromatography, and detection. In the case of N^+-glucuronide metabolites, the presence of the permanent positive charge, as well as the ionisable carboxylic acid functional group, imparts physicochemical properties which further impacts the need for careful selection of an appropriate approach in analysis [1]. The techniques most commonly employed in the quantitative analysis of N^+-glucuronide metabolites are HPLC with especially UV detection and and an indirect approach involving analysis of the aglycone released by treatment with either base or β-glucuronidase [1–12]. There has been lack of systematic study of the general characteristics of these metabolites that are useful knowledge when developing a method of analysis and such studies include stability considerations, both with respect to pH and β-glucuronidase. Since there have been marked differences noted between N^+-glucuronide metabolites formed at aliphatic and aromatic tertiary amine atoms with respect to the ease of susceptibility to both non-enzymatic and enzymatic hydrolysis, the present study is limited to the former type of N^+-glucuronide metabolite. The diversity is recognised in the present study, however, in the site of N^+-glucuronidation in that examples are included of metabolites formed at both acyclic and cyclic aliphatic tertiary amine sites. Specifically included in this study regarding substrate stability in aqueous solution and substrate susceptibility to β-glucuronidase are the N^+-glucuronide metabolites of chlorpromazine and doxepin, which are formed at an acyclic *N*,*N*-dimethylaminoalkyl group, and the N^+-glucuronide metabolites of clozapine and cyclizine, which are formed at a cyclic *N*-methylpiperazine moiety.

2. Experimental

2.1. Chemicals

The following drugs were generously supplied as pure powders by the pharmaceutical company indicated in parenthesis: clozapine (Geneva Pharmaceuticals, Broomfield, CO) and doxepin hydrochloride (Pfizer, Groton, CT). Chlorpromazine hydrochloride, cyclizine hydrochloride, phenolphthalein standard solution (1 mg ml^{-1} ethanol), phenolphthalein *O*-glucuronide, and buffer reagents were purchased from Sigma (St. Louis, MO). The β-glucuronidase enzymes from the following sources (approximate enzyme activity in parenthesis) were also obtained from Sigma: bovine liver (Glucurase®, 5000 U ml^{-1} at pH 5.0), *Escherichia coli* (Type IX-A, 1 560 000 U g^{-1} at pH 6.8), and *Helix pomatia* (Type H-2, 100 000 U ml^{-1} at pH 5.0 and sulfatase 3100 U ml^{-1}). The quaternary ammonium-linked glucuronides of chlorpromazine, clozapine, cyclizine, and doxepin were synthesised according to a previously described procedure [13]. Briefly, this synthetic procedure involved quaternisation of the compound with methyl (2,3,4-tri-*O*-α-D-glucopyranosyl bromide) uronate and sodium bicarbonate in a two-phase system of water and benzene, followed by removal of the protecting groups from the quaternised intermediate in aqueous solution at pH 12.5.

2.2. Instrumentation and chromatographic conditions

The HPLC system used in the analysis of glucuronide metabolites consisted of a model M45 liquid chromatographic pump (Waters Associates, Mississauga, Ont., Canada), a variable wave-

length detector (Waters Associates), and a model C-R3A integrator (Shimadzu Corporation, Kyoto, Japan). Samples were introduced manually through a Rheodyne Model 7125 valve loop sample injector fitted with a 200 μl sample loop (Rheodyne, Terochem Laboratories, Edmonton, AB, Canada).

The isocratic mobile phase consisted of methanol-ammonium acetate (0.1 M, pH 6.8) (70:30 v/v). The mobile phase was filtered through a 0.45 μm Millipore Type HV filter (Millipore Corporation, Bedford, MA). In the case of the four N^+-glucuronide metabolites separations were accomplished on an analytical 100 × 8 mm i.d. C18 Radial Pak column with a particle size of 4 μm (Waters Associates). The flow rate was set at 1.0 ml min^{-1}. The separation of phenolphthalein *O*-glucuronide was achieved on an analytical C18 Ultracarb 5 ODS column (250 × 4.6 mm i.d., 5-μm particle size; Phenomenex, Torrance, CA) in which the mobile phase was operated at a flow rate of 0.5 ml min^{-1}. Detection was made at 254 nm (except in the case of cyclizine N^+-glucuronide where 235 nm was employed) at a sensitivity of 0.02 a.u.f.s.

2.3. Stability of N^+-glucuronide metabolites

Separate solutions of the N^+-glucuronide metabolites of clozapine, cyclizine, and doxepin (approximately 0.050 mM) were prepared in triplicate in citrate–phosphate–borate buffer (0.5 M) over the range pH 1–11 ± 0.05 [14]. Solutions were stored in vacutainer tubes at room temperature (18–22°C) for 3 months. Each solution was assayed for the amount of metabolite remaining after the following time periods: 0, 7, and 21 days, and 1, 2, and 3 months. At the time of analysis, each tube was mixed by vortex and a 10-μl aliquot was directly injected in the chromatographic system. For each analyte quantification was based on peak heights relative to those of freshly prepared solutions prepared in the same manner. Both test and standard solutions were examined in triplicate, and in no case did the coefficient of variation exceed 9%.

2.4. Enzymatic studies

2.4.1. Assays of glucuronide metabolites

Stock solutions (1 mg ml^{-1}) of each metabolite in each of the buffers investigated were prepared and stored in the dark at 4°C. Appropriate dilutions of each stock solution of analyte were made with the appropriate buffer to give a series of solutions at five different concentrations (2.5, 5, 10, 25, and 50 μg ml^{-1}, except for 10, 25, 50, 100, and 250 μg ml^{-1} in the case of doxepin N^+-glucuronide). Each standard was prepared and analyzed in triplicate. These standard solutions were interspersed with test solutions on each day of analysis, and the standard curves were obtained by plotting absolute peak height versus concentration.

In the case of the glucuronides of doxepin and phenolphthalein, because of interfering peaks in the chromatograms from components of the enzymatic mixtures, it was necessary to undertake an extraction procedure. The separation of these two metabolites from the incubation mixture was achieved by solid-phase extraction using disposable octadecyl silane (C18) columns. After loading the incubation mixture (2.5 ml), the column was washed with distilled water (6 ml) and eluted with 70% ethanol (3 ml). A 10-μl portion of the collected fraction or incubation mixture itself (other three N^+-glucuronide metabolites) was injected into the appropriate HPLC system to determine the concentration of the N^+-glucuronide remaining after enzymatic hydrolysis.

The minimum quantifiable level of each analyte was determined at a signal to noise ratio of 3:1. Quality control samples of each metabolite were prepared in duplicate to contain each analyte at the high, medium, and low concentrations of each standard curve range. All the quality control samples were analyzed as unknowns (analyst blind) along with samples for standard curves and test samples.

2.4.2. Incubation of the glucuronide metabolites with β-glucuronidase (40 U enzyme ml^{-1} buffer) from three different sources, examined at pH 5.0 and 7.4

To a solution of the N^+-glucuronide metabolite (approximately 0.035 mM) in 50 ml of 0.1 M

phosphate buffer (pH 5.0 or 7.4) was added a solution containing 2000 U of one of the three enzyme preparations studied. Samples were incubated in the dark over 48 h in vacutainer tubes placed in a shaking water bath (37°C). Samples were withdrawn for analysis at 0, 1, 3, 6, 12, 24, and 48 h after the addition of the enzyme preparation. In order to estimate the contribution (if any) of non-enzymatic hydrolysis, other samples were concurrently treated in the same manner except that no enzyme preparation was added. All samples were prepared in triplicate.

2.4.3. Incubation of cyclizine N^+-glucuronide with β-glucuronidase (2000 U enzyme ml^{-1} buffer) from two different sources, examined at pH 5.0 and 7.4

The enzymatic hydrolysis of one N^+-glucuronide metabolite was additionally examined in a study which differed from Section 2.4.2, in that the enzyme concentration was 50 times greater, and only bovine liver and *H. pomatia* were the enzyme sources studied.

2.4.4. Incubation of the glucuronide metabolites with β-glucuronidase (40 U enzyme ml^{-1} buffer) from E. coli, examined at six pH values

The effect of β-glucuronidase from *E. coli* was examined in more detail than the above study described in Section 2.4.2 in that the incubations were performed at pH values 5.0, 6.0, 6.5, 7.0, 7.4, and 8.0 (0.1 M phosphate buffers) and analyses for the amount of N^+-glucuronide metabolite remaining were performed after incubation for 0, 0.25, 0.5, 1, 2, 3, 6, 12, 24, and 48 h.

3. Results

3.1. Stability of N^+-glucuronide metabolites

No degradation was observed for the 3 month period at room temperature for clozapine N^+-glucuronide, cyclizine N^+-glucuronide, and doxepin N^+-glucuronide over the pH ranges 4–11, 4–10, and 1–10, respectively (ANOVA, Duncan new multiple range test, $\alpha = 0.05$). Thus, whereas for all three metabolites no degradation occurred over the 4–10 pH range, significant degradation occurred for two of the metabolites at each of the other pH values examined. The first-order rate constants for degradation were calculated from the slopes of the regression lines obtained by plotting the logarithm of the drug remaining against time. All the regression lines had an r value > 0.9, except $r = 0.78$ for cyclizine N^+-glucuronide at pH 11. These data for the degradation rate constants together with the percentage of metabolite remaining after 3 months of storage are given in Table 1. That in all cases the degradation was slow is indicated by the fact that the approximate half life for the degradations varied from 70 days (clozapine N^+-glucuronide at pH 1, 2, and 3) to 350 days (cyclizine N^+-glucuronide at pH 11).

Table 1
Data for the degradation of three N^+-glucuronide metabolites at room temperature as a function of pH, % remaining undegraded at 3 months, and the first-order rate constant k ($days^{-1}$)

N^+-Glucuronide metabolite	Buffer pH[a]	% Metabolite remaining undegraded at 3 months	k ($days^{-1}$)
Clozapine	1	41	0.010
Clozapine	2	29	0.014
Clozapine	3	47	0.0081
Cyclizine	1	73	0.0028
Cyclizine	2	57	0.0052
Cyclizine	3	68	0.0040
Cyclizine	11	78	0.0019
Doxepin	11	59	0.0049

[a] At all other pH values no degradation was observed after 3 months.

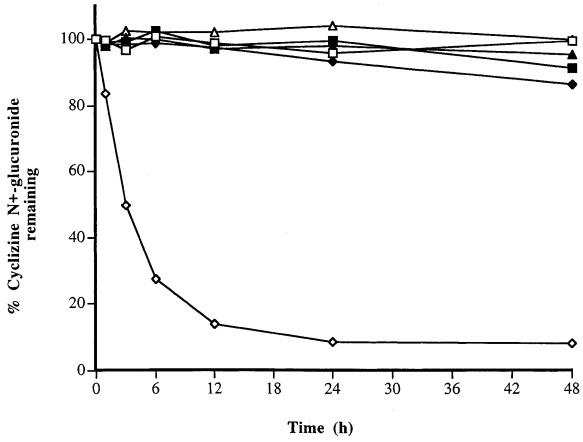

Fig. 1. Effect of β-glucuronidase from three different sources on cyclizine N^+-glucuronide in pH 5.0 and 7.4 buffers (40 U enzyme ml^{-1} buffer). Key: bovine liver, pH 5.0 (—▲—) and 7.4 (—△—); *Escherichia coli*, pH 5.0 (—◆—) and 7.4 (—◇—); and *Helix pomatia*, pH 5.0 (—■—) and 7.4 (—□—).

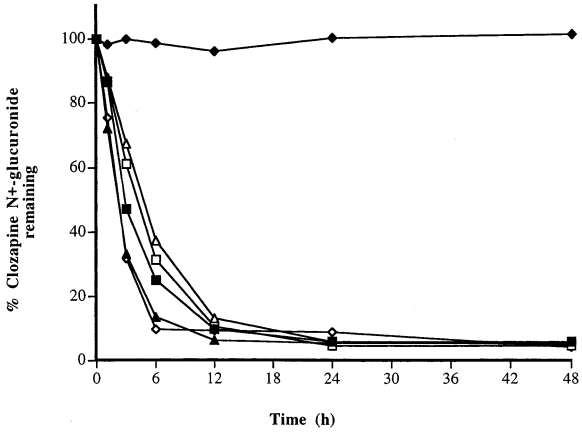

Fig. 2. Effect of β-glucuronidase from three different sources on clozapine N^+-glucuronide in pH 5.0 and 7.4 buffers (40 U enzyme ml^{-1} buffer). Key: bovine liver, pH 5.0 (—▲—) and 7.4 (—△—); *Escherichia coli*, pH 5.0 (—◆—) and 7.4 (—◇—); and *Helix pomatia*, pH 5.0 (—■—) and 7.4 (—□—).

3.2. Enzymatic hydrolysis of N^+-glucuronide metabolites

The concentrations of the glucuronide metabolites in the enzymatic mixtures were determined by separate HPLC assays. The slope of the regression lines for each analyte over the stated standard curve ranges were: chlorpromazine N^+-glucuronide, 2.5–50 μg ml^{-1}, $y = 0.497x - 0.057$ ($n = 5$), $r = 0.9999$; clozapine N^+-glucuronide, 2.5–50 μg ml^{-1}, $y = 0.564x + 0.035$ ($n = 5$), $r = 0.9999$; cyclizine N^+-glucuronide, 2.5–50 μg ml^{-1}, $y = 0.101x - 0.013$ ($n = 5$), $r = 0.9999$; doxepin N^+-glucuronide, 10–250 μg ml^{-1}, $y = 0.295x - 0.047$ ($n = 5$), $r = 0.9999$; and phenolphthalein O-glucuronide, 2.5–50 μg ml^{-1}, $y = 0.321x - 0.082$ ($n = 5$), $r = 0.9999$. The results of the analyses of the quality control samples were such that they met the acceptance criterion of the laboratory, that is for each of the assays four or more of the six samples, including at least one at the lowest concentration examined, showed a bias of $< \pm 15\%$ of the nominal values. Also in all studies control assays were employed concurrently, namely, the glucuronide substrate was incubated in the appropriate buffers without an enzyme preparation. For all control solutions no detected change in substrate concentration occurred over the 48 h of the experiment. Therefore, all observed changes in substrate concentration when the β-glucuronidase is present can be attributed to this enzyme preparation.

3.2.1. Enzymatic hydrolysis of the glucuronide metabolites with β-glucuronidase (40 U enzyme ml^{-1} buffer) from three different sources, examined at pH 5.0 and 7.4

The N^+-glucuronide metabolites of chlorpromazine, cyclizine, and doxepin were similar in that they were resistant to hydrolysis by β-glucuronidase from three different sources at two pH values, except in the case of *E. coli* at pH 7.4. For example, Fig. 1 shows the percentage of substrate remaining-time profiles under six conditions for the effect of β-glucuronidase on cyclizine N^+-glucuronide. In contrast, as likewise shown in Fig. 2, clozapine N^+-glucuronide was relatively rapidly hydrolysed by all three enzyme preparations at both pH values examined, except for *E. coli* at pH 5.0. Table 2 lists the percentage which remains after 24 h for all four N^+-glucuronide metabolites, as well as phenolphthalein O-glucuronide, treated under all the conditions examined. Phenolphthalein O-glucuronide, which served as a

Table 2
The effect of β-glucuronidase from three different sources on four aliphatic type N^+-glucuronide metabolites and phenolphthalein *O*-glucuronide in aqueous buffers (40 U enzyme ml^{-1} buffer) at pH 5.0 and pH 7.4 at 37°C

Enzyme source	pH	% mean ± S.D. remaining after 24 h				
		N^+-glucuronide of				Phenolphthalein *O*-glucuronide
		Chlorpromazine	Cyclizine	Doxepin	Clozapine	
Helix pomatia	5	100 ± 0.9	99.8 ± 1.9	100 ± 1.8	6.3 ± 2.9	3.0 ± 1.4
	7.4	97.9 ± 0.6	96.1 ± 2.5	99.2 ± 2.5	4.8 ± 1.1	6.8 ± 5.8
Bovine liver	5	96.2 ± 0.5	98.0 ± 4.2	97.8 ± 2.5	5.8 ± 4.9	5.4 ± 6.2
	7.4	83.8 ± 0.3	104 ± 2.7	89.5 ± 6.2	6.2 ± 0.4	6.9 ± 2.6
Escherichia coli	5	88.4 ± 1.3	93.3 ± 2.5	95.0 ± 2.8	101 ± 0.6	78.5 ± 2.5
	7.4	11.6 ± 3.2	8.5 ± 5.0	2.9 ± 3.5	9.2 ± 5.6	7.3 ± 3.2

control with respect to the activity of the enzyme preparations was readily hydrolysed under all conditions examined, except for *E. coli* at pH 5.0. In only a few cases was there any appreciable measurable hydrolysis in the 24–48 h time period.

3.2.2. Enzymatic hydrolysis of cyclizine N^+-glucuronide with β-glucuronidase (2000 U enzyme ml^{-1} buffer) from two different sources, examined at pH 5.0 and 7.4

In order to investigate whether the enzyme concentration was a major factor for the observed lack of effect by β-glucuronidase from bovine liver and *H. pomatia* enzyme sources on three of the N^+-glucuronide metabolites, a follow up study was performed with one of these substrates. Consequently cyclizine N^+-glucuronide was treated as before with each of these two enzyme sources, except that the enzyme concentration was increased fifty-fold. That hydrolysis was still not extensive at this far higher concentration of enzyme, except in the case of *H. pomatia* at pH 7.4 is illustrated by the observed remaining percentage of substrate after 24 h under the various conditions: 45.9 ± 8.0%, *H. pomatia* at pH 5.0; 10.9 ± 4.3%, *H. pomatia* at pH 7.4; 61.0 ± 9.5%, bovine liver at pH 5.0; and 53.1 ± 6.1%, bovine liver at pH 7.4. Also the hydrolysis rate (4.55×10^{-5} nmol ml^{-1} h^{-1}) by the *H. pomatia* enzyme source at pH 7.4 was relatively slow in comparison to the optimum rates determined for *E. coli* (Section 3.2.3 and Table 3).

3.2.3. Enzymatic hydrolysis of the four N^+-glucuronide metabolites with β-glucuronidase (40 U enzyme ml^{-1} buffer) from E. coli, examined at six pH values

In initial studies *E. coli* was the only one of the three enzyme sources examined which resulted in extensive hydrolysis of all N^+-glucuronide metabolites examined. Therefore, follow-up studies were conducted with this enzyme source in order to determine the approximate optimal pH for hydrolysis of each of the four substrates. The profiles of the four substrates for the percentage of substrate remaining against time were similar in that the most extensive and rapid hydrolysis occurred over the pH range 6.5–8, as shown for doxepin N^+-glucuronide in Fig. 3. The observed optimal pH was found to be in the range 6.5–7.4 (Table 3), although it should be noted that clozapine was the only substrate where the hydrolysis

Table 3
Hydrolysis rates at the observed optimal pH for β-glucuronidase from *Escherichia coli* on four aliphatic type N^+-glucuronide metabolites

N-Glucuronide metabolite	Optimal pH	Substrate hydrolysed (nmol ml^{-1} h^{-1})
Chlorpromazine	7.4	6.51×10^{-4}
Clozapine	6.5	8.04×10^{-4}
Cyclizine	7.0	1.73×10^{-4}
Doxepin	7.4	5.29×10^{-4}

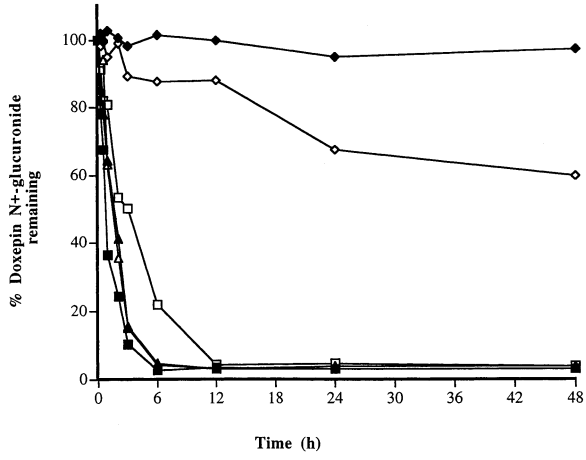

Fig. 3. Effect of β-glucuronidase from *Escherichia coli* on doxepin N^+-glucuronide in buffers over the range pH 5.0–8.0 (40 U enzyme ml^{-1} buffer). Key: pH 5.0 (—◆—), 6.0 (—◇—), 6.5 (—▲—), 7.0 (—△—), 7.4 (—■—), and 8.0 (—□—).

rate was optimal at pH 6.5 and data at pH 7.0 was not obtained due to the lack of availability of an adequate amount of this substrate. Also at pH values in the vicinity of the observed optimal value there was at least one pH value with hydrolysis rates similar to the observed optimal pH, which is readily illustrated by comparing for doxepin N^+-glucuronide in Fig. 3 the decline in substrate values at pH 6.5 and 7.0 values over the first 6 h with that for the observed optimal pH 7.4. The observed optimal hydrolysis rate for cyclizine N^+-glucuronide was 3.1–4.6-fold less than for the other three substrates (Table 3).

4. Discussion

Stability and enzymatic hydrolysis studies were performed with three and four aliphatic type N^+-glucuronide metabolites, respectively. Chlorpromazine N^+-glucuronide was not included in the stability studies since it is well established that phenothiazine drugs readily undergo various chemical reactions in solution, including ring *S*-oxidation [15]. At laboratory temperature the three N^+-glucuronide metabolites examined were stable for 3 months in aqueous solution over a wide range of pH values, and even at extreme pH values the rate of degradation was slow. Also there was no degradation detected for all four metabolites at 37°C over 48 h in the 5–8 pH range when examined in control experiments in the enzymatic hydrolysis studies. Therefore, and since the metabolites studied were structurally diverse in encompassing examples of both acyclic and cyclic N^+-glucuronide metabolites, these data indicate that in general there should be no stability problems in certain aspects of analysis with respect to the N^+-glucuronide C–N linkage, including the various steps of an analytical procedure such as solvent extraction and chromatographic separation. The stability of the metabolites in biological media was not addressed in the present work, consequently such issues as the long term storage of biological samples prior to analysis requires further study. In one reported study the storage stability of diphenhydramine N^+-glucuronide in human urine at −20°C was demonstrated [12].

There was no consistency for the three metabolites examined in the extreme pH values at which instability was detected in that the N^+-glucuronides of clozapine and cyclizine were unstable in the pH 1–3 range and the N^+-glucuronides of cyclizine and doxepin were unstable at pH 11 (Table 1). This instability at high pH is consistent with the fact that treatment with hot alkali has been employed as a means to generate the aglycone in indirect qualitative and quantitative analysis of aliphatic type N^+-glucuronide metabolites [7,16]. However, the observed instability in acidic medium at room temperature, albeit slow, is surprising in that there are reports to the stability of this type of N^+-glucuronide metabolite in such medium, including hot 1–3 N mineral acid [8,17–19]. Nevertheless, despite the absence of a previous systematic study over a wide pH range to the stability of aliphatic type N^+-glucuronide metabolites, all except one literature report is consistent with the present observation that they are stable in solution over wide pH values and except under extreme conditions. The only non-supportive report known to the authors is the observation that about 40% hydrolysis of cyproheptadine N^+-glucuronide in pH 8.5 buffer occurred over 24 h at 37°C [20].

Three sources of β-glucuronidase were employed in the enzymatic studies, one commercial brand from each of the three types of sources commonly utilised in in vitro research: bacteria, bovine liver, and mollusks. That the scope of the work could only encompass one brand of each type of source of β-glucuronidase is a limitation that needs to be realised when interpreting these data. In the initial enzymatic study the pH values of 5.0 and 7.4 were employed since for the three β-glucuronidase preparations investigated pH values similar to either of these values were used in previous studies involving N^+-glucuronide metabolites and activity information (from the supplier) at similar pH values was available.

Three of the N^+-glucuronide metabolites were similarly affected by the three enzyme preparations at the two pH values examined, namely resistance to hydrolysis except in the case of *E. coli* at pH 7.4. This relative resistance to hydrolysis of three of the N^+-glucuronide metabolites to β-glucuronidase from bovine liver and *H. pomatia* was indicated by comparison with the similarly obtained data for not only clozapine N^+-glucuronide but also for the reference standard phenolpthalein *O*-glucuronide. Furthermore, the resistance of cyclizine N^+-glucuronide to the effects of β-glucuronidase from especially bovine liver was demonstrated by a follow up study in which the enzyme concentration was increased 50-fold. This study is noteworthy since indirect assays of glucuronide metabolites are invariably performed with a great excess of β-glucuronidase. The present studies which collectively indicate that aliphatic type N^+-glucuronide metabolites are resistant to the effects of β-glucuronidase from certain sources have implication with respect to conducting an indirect analysis of this type of metabolite involving enzyme hydrolysis. That is, the source of the enzyme is of the utmost importance and of three enzyme sources examined in the present work the *E. coli* source is the most appropriate for the analysis of this type of N^+-glucuronide metabolite. This is consistent with reports where comparison of *E. coli* (at pH 6.8) and *H. pomatia* (at pH 5.0) indicated that there was a relative resistance with various aliphatic type N^+-glucuronide substrates examined to the latter enzyme source [12,19].

These observations regarding the resistance of aliphatic type N^+-glucuronide metabolites to β-glucuronidase are surprising in view of the fact that the most commonly reported conditions to the enzymatic hydrolysis of these metabolites involve bovine liver [2,3,5,11] or mollusks [3,4,8,21], rather than *E. coli*, as an enzyme source. Also, it is noteworthy that preliminary studies to determine appropriate conditions of enzymatic hydrolysis in indirect analysis of these metabolites has been only referred to in a few studies [4,12]. In one of these reported studies [4] it was found that a *H. pomatia* source of β-glucuronidase was optimum at pH 6.5, which contrasts to the use of this enzyme source at pH 5.0–5.2 in reported studies to the hydrolysis of N^+-glucuronide metabolites [3,8], but is consistent with the present observation of a greater sensitivity of cyclizine N^+-glucuronide to such an enzyme source at pH 7.4 than pH 5.0.

All enzymatic studies were carried out for 48 h, yet in no case was complete hydrolysis observed. Even in those cases where the most rapid hydrolysis occurred the amount of N^+-glucuronide metabolite remaining after 48 h was quantitated to be in the 3–12% range (data not shown). Apart from the likelihood that the hydrolysis was in fact incomplete, other possible explanations for this observation include that there were reaction products other than the parent drug and one or more of these had a HPLC retention time similar to the measured substrate. It should be noted that no contaminant(s) with similar HPLC characteristics was present in the in-house synthesised substrates. There have been previous observations to the incomplete hydrolysis of N^+-glucuronide metabolites by β-glucuronidase [6,12,16], which in some cases was far greater than that observed in the present work [6,16].

In the study performed to investigate the optimum conditions at which to utilise *E. coli* as a source of β-glucuronidase to hydrolyse N^+-glucuronide metabolites, all four of the substrates examined exhibited optimal hydrolysis in the 6.5–7.4 pH range. The hydrolysis rates at the observed optimal pH varied appreciably between substrates which was reflected in the fact that the time point at which no further hydrolysis was observed oc-

curred at 24 h for cyclizine N^+-glucuronide but at either 3 or 6 h for the other three metabolites examined. Thus the present work illustrates that to develop for an individual N^+-glucuronide metabolite an indirect analytical method involving β-glucuronidase it is important to perform preliminary studies to determine not only a suitable enzyme source but also the appropriate incubation conditions including buffer pH and incubation time.

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