Technical Note

Hydrolysis of Conjugated Metabolites of Buprenorphine II. The Quantitative Enzymatic Hydrolysis of Norbuprenorphine-3-β-D-Glucuronide in Human Urine

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Abstract

In gas chromatographic–mass spectroscopic analysis of buprenorphine metabolites, the urine specimen must be first hydrolyzed to release buprenorphine and norbuprenorphine from their glucuronide conjugates. For evaluation of existing hydrolysis methods and to find out the optimal hydrolysis conditions, buprenorphine-3-β-D-glucuronide (B3G) and norbuprenorphine-3-β-D-glucuronide (NB3G) were synthesized. In a previous communication we reported on the optimum conditions for hydrolysis of B3G. Because we have previously shown that no hydrolysis was achieved under basic conditions and that acid hydrolysis resulted in extensive degradation, only enzymatic hydrolysis was examined for NB3G. This study therefore reports on the optimum enzymatic conditions for the hydrolysis of NB3G. Urine fortified with synthetic NB3G was hydrolyzed with β-glucuronidases from different source species, including *Helix pomatia*, *Escherichia coli*, and *Patella vulgata*. Glusulase, a preparation containing both the β-glucuronidase (*H. pomatia*) and sulfatase, was also tested. It was found that marked differences exist in the reactivity of these enzymes. Incubation with glucuronidases from *H. pomatia* or *E. coli* at 37°C for 16 h resulted in quantitative hydrolysis of NB3G. At 60°C, complete hydrolysis was achieved with 1000 units of *H. pomatia* in 4 h and after only 1 h with glusulase. On the other hand, incubation at 60°C for 4 h with *Patella vulgata* resulted in only 14.7% hydrolysis.

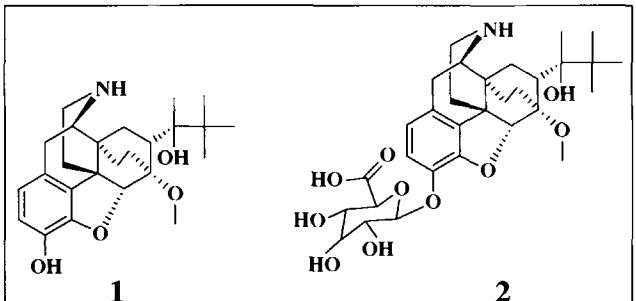

Figure 1. Chemical structures of norbuprenorphine (1) and norbuprenorphine-3-β-D-glucuronide (2).

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Introduction

Buprenorphine (Bup), a semi-synthetic opioid analgesic, is largely used in France for the treatment of about 60,000–80,000 heroin addicts (1). Bup is an oripavine analgesic that is beneficial in the maintenance treatment of opiate-dependent individuals. Although Bup has been studied extensively, relatively little is known about norbuprenorphine (norBup), a major dealkylated metabolite of Bup. NorBup (Figure 1) is a major metabolite of Bup in humans and dogs (2,3). NorBup concentration in blood in 61 fatal cases was < 0.1–65.0 ng/mL (mean: 10.6 ng/mL) (4). NorBup was measurable in plasma between 2 to 3 h after a sublingual dose in human volunteer (4). Bup and its dealkylated metabolite are excreted in urine, almost exclusively as glucuronides (2). Cone et al. (2) reported that no free buprenorphine was detected in urine after administration of the drug to human subjects in various dosages and by different routes. Debrabandere et al. (5) reported that the concentration of Bup and norBup in urine can be less than 1 ng/mL after therapeutic administration, but in abuse situations, it can range up to 20 ng/mL.

In gas chromatographic–mass spectrometric (GC–MS) analysis, Bup and norBup are cleaved from their glucuronide conjugates by hydrolysis before extraction. In our previous work, we reported the hydrolysis of Bup-glucuronide using *E. coli*, *H. pomatia*, and Glusulase, the latter being a common alternative to *H. pomatia* (6).

This investigation was designed to study the effectiveness of commonly used hydrolysis conditions for opiates in the hydrolysis of norBup-glucuronide and to determine the optimal conditions.

Experimental

Materials

β-Glucuronidases from *Helix pomatia* (type H-1, catalog # G-0751, Lot # 119H3380), *Escherichia coli* (type IX-A, catalog # G-7396, Lot # 30K, 8612), and *Patella vulgata* (type L-1, cat-

alog # G-8132, Lot # 60K3780) were obtained from Sigma Chemical Company (St. Louis, MO) as dry powders, and enzyme solutions were prepared at 10,000 or 25,000 Fishman units/mL by dissolving each in appropriate buffer (Table I). Glusulase was purchased from DuPont Chemical Company as a solution that contains approximately 10,000 units/mL of sulfatase and 90,000 units/mL of β-glucuronidase. All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were high-performance liquid chromatography (HPLC) grade. Reference standard solutions of norBup and norBup-d_3 were obtained from Cerilliant (Austin, TX) at 100 µg/mL in methanol and were diluted to working solutions of 5 µg/mL. Bis (trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Sigma Chemical Company (St. Louis, MO) and was used for derivatization.

Norbuprenorphine-3-β-D-glucuronide (NB3G), whose structure is shown in Figure 1, was synthesized in-house using norBup and methyl-(2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate as starting materials. NorBup and LiOH (10 eq of norBup) were dissolved in MeOH, and methyl-(2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate was added in four portions (2.5 eq every time) along with second portion of LiOH (10 eq) at room temperature to achieve 25% conversion to NB3G. The structure was characterized by spectral methods and the chemical purity was greater than 99% by HPLC. Stock solution of NB3G at 142.6 µg/mL (equivalent to 100 µg/mL of norBup) and working solution at 7.129 µg/mL (equivalent to 5 µg/mL of norBup) were prepared in methanol.

Urine samples

Negative urine (2 mL) was fortified with NB3G equivalent to 100–200 ng/mL of free norBup. The calibrators at 50, 100, 150, and 200 ng/mL were prepared by fortifying negative urine with norBup standard solutions. NorBup-d_3, the internal standard (40 µL at 5 µg/mL) was added prior to hydrolysis to give 100 ng/mL of norBup-d_3.

Enzymatic hydrolysis

Each urine sample was aliquoted into a 15-mL centrifuge tube, and an appropriate buffer was added. Table I shows the type and volume of the buffer added for each enzyme. Then, 1000 Fishman units of a β-glucuronidase (100 µL of 10,000 Fishman units/mL or 40 µL of 25,000 Fishman units/mL) were added. The samples were capped and incubated in an oven for 1–20 h. The amount of enzyme, temperature, and time varied in the different experiments to determine the effectiveness of the hydrolysis at each condition.

Table I. Optimal pH and Buffers Used for Each β-Glucuronide

Enzyme Origin	pH	Buffer
Helix pomatia	5.0	1.0M acetate buffer (1 mL)
Escherichia coli	6.8	0.1M phosphate buffer (2 mL)
Patella vulgata	3.8	0.25M phosphate buffer (1 mL)
Glusulase	5.0	1.1M acetate buffer (1 mL)

Extraction and derivatization

One milliliter of 40% phosphate buffer (pH 9) was added to each hydrolyzed sample. The samples were gently shaken with 6 mL of $CHCl_3$/isopropanol (9:1). The top aqueous layer was discarded, and the organic layer was washed with 1 mL of deionized water. The organic layer was separated and evaporated to ~ 1 mL under a stream of nitrogen at 50°C. The samples were then transferred to GC vials, evaporated to dryness, and derivatized with BSTFA containing 1% TMSC (50 µL) at 70°C for 30 min. After cooling to room temperature, the samples were transferred to GC vial inserts, and the vials were recapped. The TMS derivatives (1-µL injections) were analyzed by GC–MS.

GC–MS analysis

Analysis was performed on a Hewlett-Packard (Palo Alto, CA) 5890 GC interfaced with a Hewlett-Packard 5970 mass selective detector equipped with a 10-m × 0.18-mm i.d. DB-1 capillary column (0.18-µm film thickness). The initial oven temperature was held at 180°C for 0.5 min, and then increased to 265°C at 30°C/min with a final temperature hold of 9 min. The ions monitored were *m/z* 557, 524, and 500 for norBup and 527 and 503 for norBup-d_3. The retention times for norBup and norBup-d_3 were 6.02 min and 6.00 min, respectively.

Concentrations of norBup cleaved from NB3G by hydrolysis were calculated by plotting drug/internal standard area ratios versus concentration. The curves were linear over the concentration range with r^2 of 1.000.

Results and Discussion

The experiments were carried out in triplicate on urine samples fortified with 100 ng/mL of NB3G. A control group without hydrolysis was included. Incubation with β-glucuronidases

Table II. Effect of Temperature and Incubation Time on the Hydrolysis of NB3G with Four Different β-Glucuronidases

Enzyme	Temperature	Time (h)	% Hydrolysis
E. coli	37°C	1	18.33
		2	31.52
		4	51.26
		16	100.00
H. pomatia	37°C	1	33.22
		2	55.36
		4	79.35
		16	100.00
	60°C	1	71.08
		2	95.99
		4	100.00
P. vulgata	60°C	1	6.75
		2	8.80
		4	14.72
Glusulase	60°C	1	100.00

from *E. coli* and *H. pomatia* at 37°C for 16 h completely hydrolyzed NB3G and gave quantitative recovery of norBup. Earlier studies (7–9) showed that the concentration of the enzyme has greater influence on the recovery of the desired analytes. To determine the amount of enzyme needed to hydrolyze NB3G, varying amounts of *H. pomatia* were added to 2 mL of buffered urine fortified with 100 ng/mL of NB3G and the samples were incubated for 16 h at 37°C. It was found that 1000 units were sufficient to produce quantitative hydrolysis.

In a previous report, apart from *H. pomatia*, we have also studied different other types of β-glucuronidases from other source origins including *E. coli* and *P. vulgata* for hydrolysis of buprenorphine-3-β-D-glucuronide (B3G) under different hydrolysis conditions. These materials and those different incubation conditions were applied to the hydrolysis of NB3G. Table II and Figure 2 show the percent hydrolysis of NB3G under different temperatures and incubation times with enzymes from three different sources using 1000 Fishman units of each. The experiments were carried out at the reported (10) optimal pH for each enzyme (*H. pomatia*, 5.0; *E. coli*, 6.8; and *P. vulgata*, 3.8). The fastest rate was achieved by *H. pomatia*, which hydrolyzed ~ 96% at 60°C and ~79% at 37°C after 4 h incubation. For *E. coli* and *H. pomatia*, hydrolysis was complete after 16 h at 37°C. Because in our previous report we showed that β-glucuronidase from *P. vulgata* hydrolyzed only 26% of B3G after a 16-h incubation at 37°C and 35% at 60°C after 4-h incubation, in this study we only examined the activity of the enzyme from *P. vulgata* at 60°C. The hydrolysis rate was much slower compared with *E. coli* and *H. pomatia* enzymes, where only 15% of NB3G was hydrolyzed after a 4-h incubation at 60°C with the *P. vulgata* enzyme.

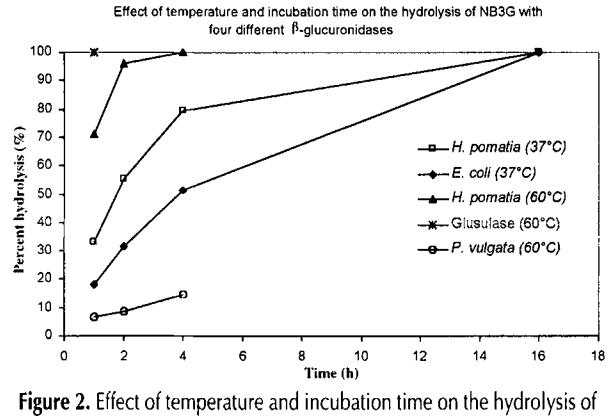

Figure 2. Effect of temperature and incubation time on the hydrolysis of NB3G with four different β-glucuronidases.

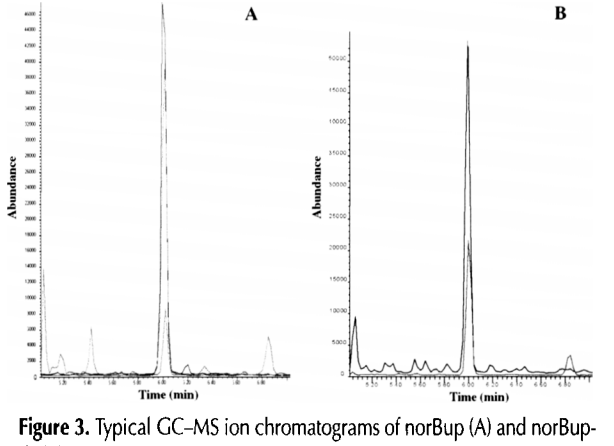

Figure 3. Typical GC–MS ion chromatograms of norBup (A) and norBup-d_3 (B).

Enzyme activity is also influenced by temperature. In our previous publication, we reported that the time required to complete hydrolysis of B3G with *H. pomatia* at 60°C was only 4 h as compared to 16 h at 37°C. Table II and Figure 2 show that the time required for complete hydrolysis of NB3G with *H. pomatia* was also 4 h at 60°C as compared to 16 h at 37°C. With *P. vulgata*, the percent hydrolysis reached 14.72% after incubating for 4 hours, although the rate at 60°C was five times the rate at 37°C. Therefore, the hydrolysis rate for NB3G is much lower than B3G with *P. vulgata*. These results affirm the substrate and source-dependent nature of enzymatic hydrolysis, when β-glucuronidases are used.

One of the objectives of this study was to develop a practical hydrolysis procedure. We compared the effectiveness of hydrolysis of NB3G at higher concentration levels (500, 1000, and 2000 ng/mL) with *H. pomatia*. The amount of enzyme used was 1000 Fishman units and incubation was at 37°C for 16 h. Quantitative hydrolysis of NB3G was achieved up to 1000 ng/mL. Only 75% hydrolysis occurred for the 2000 ng/mL concentration using the 1000 units of enzyme, suggesting that the hydrolysis is also concentration dependent given a limited amount of enzyme.

It is evident that there are measurable differences between the different enzymes effects on B3G and NB3G, most notably the fact that although the *E. coli* glucuronidase resulted in almost quantitative hydrolysis of B3G in 1–2 h at 37°C (6), only 50% hydrolysis of NB3G occured under the same conditions. However, there were no noticeable differences on the effect of any enzyme systems on the cleanliness of the chromatograms. Figure 3 shows a typical ion chromatogram for norBup and its internal standard.

In conclusion, for the analysis of Bup and norBup in urine, quantitative hydrolysis of B3G and NB3G could be achieved using β-glucuronidases from *E. coli* and *H. pomatia* and incubation at 37°C for 16 h, *H. pomatia* at 60°C for 4 h, or Glusulase at 60°C for 1 h.

Acknowledgment

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