

Morphine Glucuronide Hydrolysis: Superiority of β -Glucuronidase from *Patella vulgata*

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β -Glucuronidase from *Patella vulgata*, *Helix aspersa*, *Helix pomatia*, and bovine liver were evaluated for usefulness in routine hydrolysis of drug-glucuronic acid conjugates from equine urine samples. Factors affecting the reaction rate (enzyme concentration, ligand concentration, temperature, and pH) were optimized. A 3-h incubation at 65 °C with 5000 U of β -glucuronidase from *P. vulgata* per milliliter of urine resulted in complete hydrolysis of all morphine glucuronide in the urine samples. Not only was the enzyme preparation from *P. vulgata* the most cost-effective β -glucuronidase source studied, but also its thermal stability is such that it can be used at a temperature high enough to substantially shorten the incubation interval. Preliminary work on other drugs that form glucuronide conjugates indicates that this same procedure is similarly superior for use in their hydrolysis.

Additional Keyphrases: drug assay • drug-glucuronide conjugates • enzymic methods • urine • veterinary chemistry • horses • comparison of enzyme sources for hydrolysis of samples • mollusc enzymes • cost-effectiveness • abused drugs

Many drugs, including the narcotic analgesics, are lipophilic, necessitating metabolism to a water-soluble form for efficient elimination by the kidneys. This occurs most commonly with conjugation to glucuronic acid (1). In humans, 80–95% of morphine recovered from urine is in the conjugated form (2, 3), a form not detected in most assays. Thus, if the analytical procedure includes hydrolysis, the concentration of detectable morphine will be enhanced (4).

Despite this obvious advantage, many laboratories do not routinely hydrolyze samples. Hydrolysis may be performed either with acid or the enzyme β -glucuronidase (EC 3.2.1.31). If any hydrolytic procedure is used, it usually is acid hydrolysis, acids being less costly and more time saving: acid hydrolysis requires only 15–60 min (5); enzymic hydrolysis usually requires 16–24 h (2, 5–8). However, many drugs are destroyed by the extreme conditions of acid hydrolysis. If the concentration of acid is too high, even morphine may begin to break down (5).

Materials and Methods

Apparatus

For all analyses we used a Model 3700 gas chromatograph equipped with a ⁶³Ni electron-capture detector (Varian Instruments, Sunnyvale, CA 94086). The detector was operated at 290 °C. Separations were done on a 1.8 m × 2 mm siliconized glass column packed with SP 2250-DB on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823). Column oven temperature was maintained at 235 °C and injection port

temperature at 250 °C. The flow rate of the carrier gas, prepurified nitrogen, was 30 mL/min.

Materials

Animals. Thoroughbred and standardbred mares, eight to 22 years old, were kept at pasture except on the day of an experiment, when they were housed in box stalls. Drugs were administered intravenously into the left jugular vein. Urine samples were collected by bladder catheterization.

Reagents. We obtained β -glucuronidase from five sources [*Patella vulgata* (Type L-II), *Helix aspersa* (Type HA-4), *Helix pomatia* (Type H-1), bovine liver (Type B-3), and Glucurase®] from Sigma Chemical Co., St. Louis, MO 63178. All except the Glucurase were lyophilized powders. The enzyme from *H. pomatia* and *H. aspersa* was partly purified, while that from *P. vulgata* was a crude powder. Glucurase, a bovine-liver β -glucuronidase solution in acetate buffer, pH 5, was stored at 4 °C. The four lyophilized preparations were stored in a desiccator at –20 °C.

Dichloromethane and ethyl acetate were of "OmniSolve" purity (MCB Manufacturing Chemists, Inc., Cincinnati, OH 45246). Pentafluoropropionic anhydride (Pierce Chemical Co., Rockford, IL 61105) was stored at 4 °C. Morphine sulfate injectable was obtained from Eli Lilly & Co., Indianapolis, IN 45206. The 1.5 mol/L carbonate buffer was prepared by adding a 186 g/L solution of NaCO₃ · H₂O to 500 mL of water containing 63 g of NaHCO₃ until the pH was 8.9.

Procedure

Horses were dosed with 0.1 mg of morphine per kilogram body weight. Urine samples were collected 6 or 12 h later except in the temperature-effect work, for which samples were collected 50 min after dosing. The samples were stored in polystyrene containers at –20 °C until analyzed.

Immediately before an experiment, the lyophilized β -glucuronidase samples were weighed and dissolved in distilled, de-ionized water, with care to protect both the powdered and reconstituted forms from direct light. For the stability study, the reconstituted *P. vulgata* enzyme was stored at 4 °C for the duration of the experiment. The Glucurase solution was used as obtained from the manufacturer.

After urine samples were adjusted to the desired pH with glacial acetic acid, 100 μ L of urine, 100 μ L of enzyme solution, and 50 μ L of water were incubated in 1-mL sealed glass ampules in a waterbath. After hydrolysis, 50 μ L of the solution was analyzed as detailed previously (9). The urine, adjusted to pH 8.9 by adding 0.5 mL of the carbonate buffer, was extracted with 4 mL of dichloromethane/isopropanol (9/1 by vol) and centrifuged, and the organic phase was allowed to drip through the silica gel columns. The columns were washed with five 2-mL aliquots of ethyl acetate/methanol/glacial acetic acid (8/1/1 by vol). Morphine was eluted from the columns with 2 mL of ammonia water (2 drops of NH₄OH per milliliter of water) and extracted into 4 mL of dichloromethane/isopropanol. The solvent was transferred into 15-mL conical glass tubes and evaporated under a stream of nitrogen. The morphine was derivatized by adding 25 μ L of pentafluoropropionic anhydride to the residue. The capped tubes were in-

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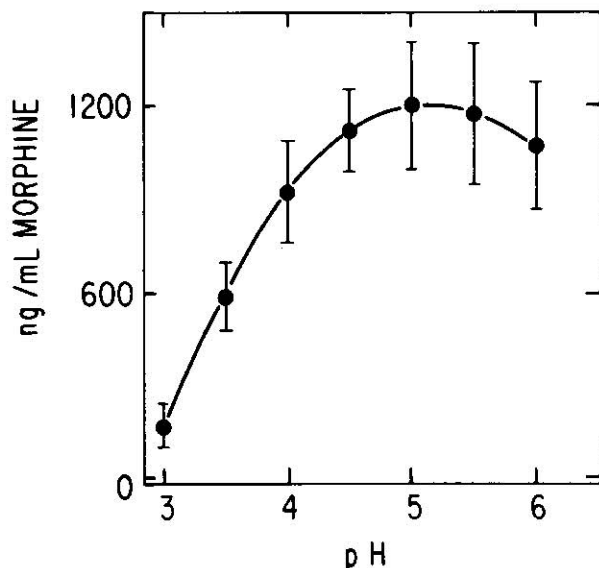


Fig. 1. Effect of pH on β -glucuronidase activity from *P. vulgata*

5000 U of β -glucuronidase from *P. vulgata* per milliliter of urine was used to hydrolyze urine samples from four horses dosed with morphine. Incubation was performed at 0.5 pH intervals from pH 3 to 6 for 24 h at 37 °C. Each data point is the average of four horses \pm SEM

cubated for 25 min at 65 °C. Excess reagent was then evaporated under a stream of nitrogen. The residue was redissolved in 25–200 μ L of ethyl acetate and a 2- μ L aliquot was injected into the gas chromatograph. The retention time for the derivatized morphine was 1.6 min.

Results

β -Glucuronidase from the five sources was examined for optimum conditions for hydrolysis of morphine glucuronide in equine urine.

The first variable studied was pH. Each urine sample collected 12 h after dosing was incubated for 24 h with (per milliliter of sample) 5000 U of β -glucuronidase from each of the five sources at different pH values. Figure 1 shows an example of the series of pH vs activity curves, in terms of morphine released. In each case, variation of pH by 0.5 unit on either side of the optimum made no significant difference in free morphine yield. Table 1 compares the manufacturer's recommended pH, as determined by the activity of the enzyme phenolphthalein glucuronide. The only significant difference is for *P. vulgata*, for which the recommended pH was 3.8, as compared with pH 5, which we found gave the highest yield of free morphine.

We incubated β -glucuronidase from each source with urine samples for 24 h at 37 °C at the optimum pH for each preparation, and found that the β -glucuronidase preparations differed in their ability to free morphine from its glucuronide

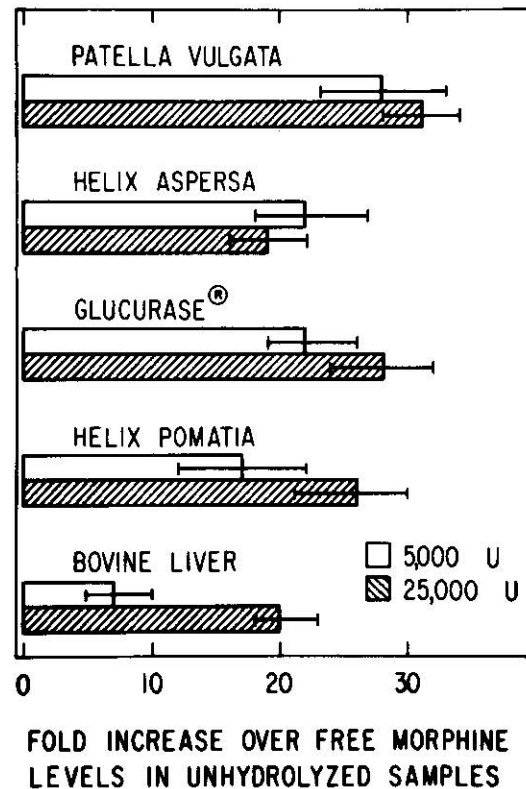


Fig. 2. Effectiveness of 5000 vs 25 000 U of β -glucuronidase

Urine samples from horses injected intravenously with morphine were hydrolyzed with 5000 or 25 000 U of β -glucuronidase from five sources. The increase over free morphine in unhydrolyzed samples is the average from four horses \pm SEM

conjugate (Figure 2). Five thousand units (U) of β -glucuronidase from *P. vulgata* released an average of 1205 ng of morphine per milliliter of urine; the bovine liver extract freed only 320 ng. Figure 2 also illustrates the result of increasing the enzyme concentration from 5000 to 25 000 U of enzyme per milliliter of urine. In general, if 5000 U of an enzyme preparation was effective, fivefold as much did not increase its effectiveness. In contrast, an initial poor activity such as that exhibited by the bovine-liver extract was increased dramatically, seven- to 20-fold more morphine being freed when the enzyme concentration was quintupled.

Figures 3 and 4 show the effect of temperature on reaction rate and yield of free morphine. Urine collected 50 min after dosing with morphine was adjusted to the optimal pH for each enzyme preparation and incubated with 5000 U of β -glucuronidase per milliliter of urine. For all enzyme preparations except *P. vulgata*, 55 °C was the optimum. In general, the rate of reaction had begun to level off by 4 h. If it is assumed that a 120-h incubation at 35 °C would give a 100% yield of free morphine, after a 6-h incubation at 55 °C the reaction of the enzyme preparation from *H. pomatia* was 99% complete, 89% for *H. aspersa*, 61% for Glucurase, and 36% for bovine liver. The enzyme from *P. vulgata* was able to withstand 65 °C, but its activity was attenuated at 75 °C. After a 3-h incubation of urine at 65 °C with the enzyme extract from *P. vulgata*, the urine was found to contain 35-fold more free morphine than without hydrolysis.

Table 2 lists the current cost of β -glucuronidase from each source for the analysis of 0.5 mL of urine, based on 5000 U of enzyme per milliliter of urine and the amount of morphine liberated from 1 mL of urine after a 24-h incubation at 37 °C. From these data we calculated a cost-effect ratio. The enzyme preparation from *P. vulgata* cost 6.7¢ to release 1 μ g of mor-

Table 1. Optimum-pH Studies

β -Glucuronidase source	Optimum pH for phenolphthalein glucuronide hydrolysis	Optimum pH for morphine glucuronide hydrolysis
<i>Patella vulgata</i>	3.8	5.0
<i>Helix aspersa</i>	5.0	4.5
<i>Helix pomatia</i>	5.0	4.5
Glucurase	5.0	5.5
Bovine liver	5.0	5.0

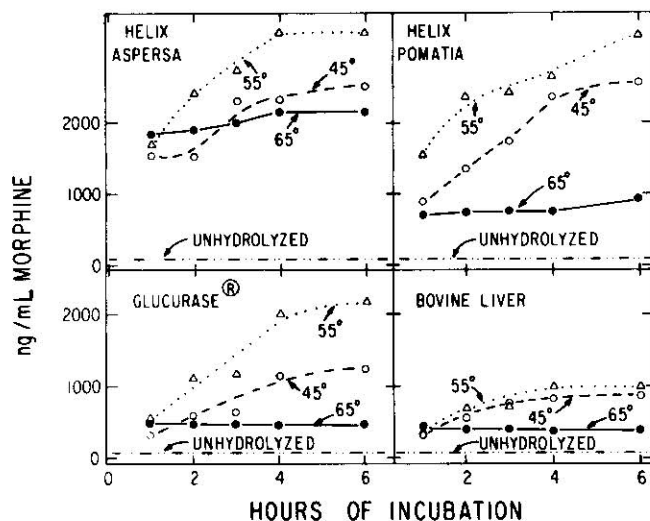


Fig. 3. Temperature effect on enzymic hydrolysis
Four β -glucuronidase preparations were incubated with equine urine containing morphine and its conjugates at 45 °C (○), 55 °C (Δ), and 65 °C (●). The amount of hydrolysis, in terms of free morphine concentration, is compared with free morphine concentrations when the urine samples were not hydrolyzed

phine from its glucuronide; 33.1¢ worth of bovine-liver extract was needed to hydrolyze the same amount of morphine glucuronide.

To ascertain the stability of the reconstituted enzyme extract from *P. vulgata*, we stored a 5000 kU/L solution in distilled, de-ionized water at 4 °C, using aliquots of this solution for analyses of a split sample (stored at -20 °C) on successive days. The results are plotted in Figure 5. We saw no change in enzyme activity for the first 48 h, but by six days after reconstitution, the enzyme released 9.8% less morphine than on the first day.

There was no difference in enzyme activity among batches of lyophilized β -glucuronidase stored at -20 °C in a desiccator for seven months, three months, or two weeks.

We removed a specific β -glucuronidase inhibitor known to occur in human urine, saccharo-1,4-lactone, and another possible interference, sulfate ions, in an unsuccessful attempt further to reduce the amount of enzyme required. Enzyme activators, including NaCl and bovine serum albumin, were

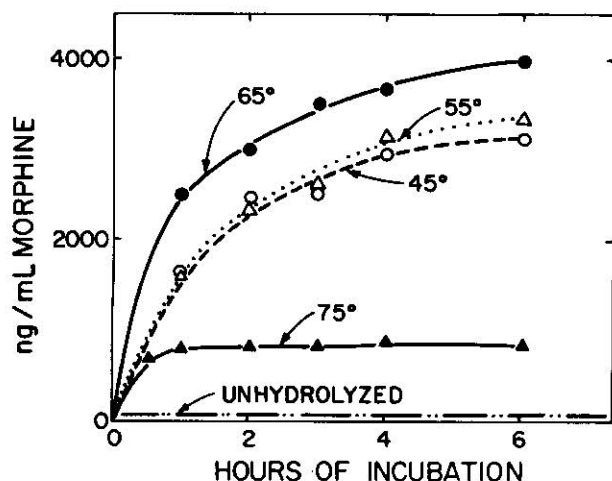


Fig. 4. Temperature effect on hydrolysis by *P. vulgata*
 β -Glucuronidase from *P. vulgata* was used to hydrolyze equine urine containing morphine and its conjugates at 45 °C (○), 55 °C (Δ), 65 °C (●), and 75 °C (▲). The amount of morphine freed is compared with free-morphine concentration when the urine was not hydrolyzed

Table 2. Cost-Effect Ratios of Hydrolyses

β -Glucuronidase source	Cost/tube, ¢	Morphine, mg/L	Cost-effect ratio, ¢/μg
<i>Patella vulgata</i>	8.1	1.205	6.7
<i>Helix aspersa</i>	7.5	0.965	7.8
Glucurase	20.0	0.949	21.1
<i>Helix pomatia</i>	10.6	0.734	14.4
Bovine liver	10.6	0.320	33.1

checked for their ability to increase the free morphine yield, but no significant improvement was noted.

Discussion

For hydrolysis of glucuronide complexes with β -glucuronidase, a wide variety of conditions have been used (2, 5-8). Four of the most important factors affecting enzyme kinetics are enzyme concentration, ligand concentration (substrate, product, inhibitors, activators), pH, and temperature (10). We examined each of these for β -glucuronidase from the five sources and determined optimal conditions.

Earlier work with Glucurase (9) demonstrated the importance of using sufficient enzyme, and 5000 U of Glucurase per milliliter of urine gave a significantly higher yield than 2500 U. For economic reasons, no higher amounts were tested. However, once the cost was brought down by the use of smaller urine volumes and alternative sources of β -glucuronidase, we examined the feasibility of using 25 000 U of enzyme per milliliter of urine. For the *P. vulgata* preparation, increasing the amount of β -glucuronidase from 5000 to 25 000 U increased the yield by only 10%. The cost-effect ratio (30.2¢/μg of morphine released) is too high for routine use of this amount.

There are substances naturally present in urine that either interfere with or inhibit the activity of β -glucuronidase (1, 11, 12). Removal of these compounds should result in less β -glucuronidase being needed. We followed the procedure of Shackleton et al. (12) but saw no significant difference in yield of free morphine by β -glucuronidase in pretreated as compared with untreated urine. Their work was done on human urine; perhaps equine urine has other glucuronides competing for the available enzyme (13).

The equilibrium point of the reaction catalyzed by β -glu-

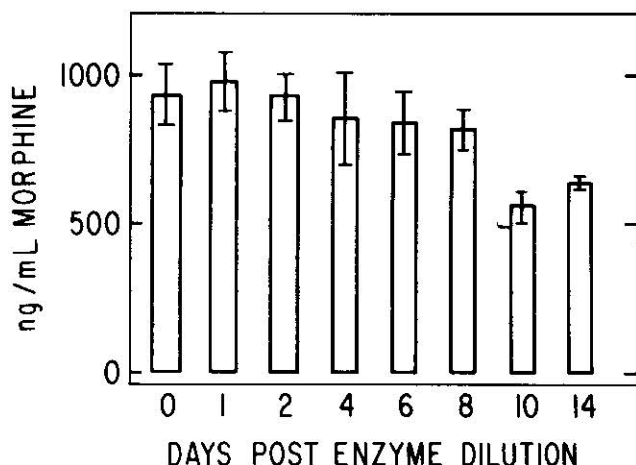


Fig. 5. Stability of β -glucuronidase from *P. vulgata* in water
5000 U of β -glucuronidase was stored in 1 mL of distilled, de-ionized water at 4 °C. This solution was used to hydrolyze the same equine urine sample containing morphine and its conjugates on successive days, to determine the stability of the enzyme preparation in water

curonidase lies far in favor of hydrolysis (1), so we did not check the effect of product concentration. Increasing substrate concentration shifts the optimum of a molluscan preparation toward a higher pH, because only un-ionized substrate is attacked by the enzyme (1). This may be one reason for reported variations in pH optima, as will be discussed next, but otherwise poses no major problem for most assays.

The effect of pH on β -glucuronidase hydrolysis has been reported in the literature (1, 5). In agreement with the fact that pH has little effect on the ionization of a β -glucuronidase, in most cases the nature of the aglycone has little effect on the pH optimum (1). Fish and Hayes (5), however, studied six enzyme preparations and found that the manufacturer's recommended pH did not always match the optimum pH for hydrolysis of morphine glucuronide. Like them, we found that in general the optimum pH for morphine glucuronide hydrolysis was within 0.5 pH unit of that reported by the manufacturer to be optimal for freeing phenolphthalein, the main exception being β -glucuronidase from *P. vulgata*, for which the optimum pH for morphine glucuronide hydrolysis was 5 and for phenolphthalein glucuronide hydrolysis 3.8. This discrepancy may be partly explained by differences in substrate concentration between the two assays. Mammalian preparations of β -glucuronidase are known to have multiple kinetic forms, a low- K_m form predominating at pH 3 and a high- K_m form predominating at pH 5.5 to 6 (14). Differences in incubation time and temperature for hydrolysis of morphine and phenolphthalein glucuronides may be another reason for the discrepancy in optimum pH.

To our knowledge, use of an incubation temperature $>37^\circ\text{C}$ has been reported only once (8), and in that study both the amount of enzyme used and the temperature were increased (8). Because in general the reaction rate will double with each 10°C increase in temperature (15), a reaction will arrive at the same endpoint in half the time, if the temperature is increased by 10°C . Enzyme preparations are denatured by too high temperatures, but those we examined could withstand far warmer conditions than the commonly used 37°C . Surprisingly, the common limpet, whose sea coast habitat would not lead one to expect the presence of a high-temperature-sensitive enzyme, proved to be the source of β -glucuronidase with highest activity at 65°C . We checked the applicability of the above generalization to the *P. vulgata* preparation. Incubation at 35°C for 120 h was assumed to give 100% recovery of free morphine. The proportion of free morphine recovered was essentially the same for incubation at 35°C for 24 h as for 45°C for 12 h, 55°C for 6 h, or 65°C for 3 h.

Repeatedly, under a variety of conditions, the enzyme preparations from *P. vulgata* freed more morphine in less time. Preliminary work on other drugs forming an ether glucuronide indicates that *P. vulgata* β -glucuronidase is also the best enzyme preparation for their hydrolysis.

Several factors probably contribute to the high activity

observed for the *P. vulgata* preparation. Of more than 300 mammalian and nonmammalian tissues assayed, the visceral hump of *P. vulgata* was among the highest in β -glucuronidase activity (1). Moreover, *P. vulgata* extracts hydrolyze α -glucuronides and α -galacturonides (1); *P. vulgata* preparation was only a crude extract, so many other conjugate-hydrolyzing enzymes could be present.

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