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Hydrolysis of Morphine Glucuronide

In procedures requiring the quantitative analysis for total morphine in body fluids there is a need to liberate the bound drug, and for the hydrolysis of conjugates in urine there are alternative methods of acid or enzyme hydrolysis. However, with the former method questions are raised as to optimum acid concentration and time of hydrolysis, as well as to the relative efficiencies of autoclaving and refluxing; with the latter there are questions of preferred source of enzyme, pH value of the reaction mixture, and incubation temperature. Before the advent of automated analysis for the rapid determination of morphine, a considerable number of tedious and repetitive analyses would have been required to produce acceptable answers, but the use of an AutoAnalyser® manifold has made it a relatively simple matter.

Most workers would agree with Payte et al [*1*] that the sensitivity for detecting morphine in urine by both thin-layer and gas-liquid chromatography can be increased by acid hydrolysis of the glucuronide metabolite, but the literature on the hydrolysis reveals a wide variation in the conditions used. Oberst [*2*] acidified human urine with one fifth its volume of concentrated hydrochloric acid and refluxed for 3 h. Thompson and Gross [*3*] hydrolyzed samples of urine (from dogs dosed with morphine) with 5 percent volume/volume (v/v) of concentrated hydrochloric acid for 30 min in an autoclave at a pressure of 15 psi; similar conditions were used by Fujimoto et al [*4*], Paerregaard [*5*], and Elliott et al [*6*]. Despite establishing that a mixture containing a concentration greater than 5 percent of concentrated hydrochloric acid resulted in an appreciable loss of morphine, Christopoulos and Kirch [*7*] used 10 percent by volume of concentrated acid for their autoclaving procedure and they found that 15 percent of added morphine was lost when so treated at 121°C for 15 min. Wallace et al [*8*] claimed 95 percent (93–97.5 percent) recovery using the same conditions and then later changed the concentration of acid used to 6 *N*, without comment [*9*]. Other workers favored different procedures without studying yields; Robinson and Williams [*10*] hydrolyzed urine samples with 15 percent weight/volume (w/v) hydrochloric acid on a boiling water bath for 30 min while Bastos et al [*11*] similarly employed 25 percent v/v of 6 *N* hydrochloric acid for 1 h.

Acid hydrolysis has been generally preferred, although the reasons for this preference over the use of enzymes were not usually stated. One can assume economic or time-saving reasons.

Enzymes were employed by Truhaut et al [*12*] who used 100,000 Fishman Units of β-glucuronidase from *Helix pomatia* (together with 800,000 Units of sulphatase) for the

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hydrolysis of morphine glucuronide conjugate. The hydrolysis in 50 ml of urine (pH 5.2) required 24 h at 37°C for completion. No studies on enzymatic hydrolysis of morphine conjugate at higher temperatures have been reported, but Vela et al [*13*], using a one-hour hydrolysis of conjugated pregnane metabolites, have shown that an increase in concentration of β-glucuronidase to 4000 units per ml of urine (pH 4.5) at 60°C gives results comparable with those obtained from a 24-h hydrolysis employing an enzyme concentration of 300 units per ml of urine at 37°C.

Our present interest centered on the possible inclusion of a standardized hydrolysis step in an automated method for the analysis of morphine in body fluids, as advocated by Finkle [*14*], and in view of the uncertainty regarding the best hydrolysis conditions the work reported below was undertaken.

Method

Preliminary studies were made to ascertain the best enzyme source by comparing the hydrolysis potential of several available commercial enzyme products (samples from the Sigma Chemical Company Ltd., London, and C. F. Boehringer & Soehne GmbH, Mannheim). The tests were made on pooled urine obtained from heroin addicts (free morphine concentration in urine approximately 5 mg/l).

A comparison of various hydrolytic methods was carried out with a standard morphine glucuronide solution (see below). Morphine was determined by use of an AutoAnalyser® manifold similar to that reported by Blackmore et al [*15*] but with the following design changes:

(1) A "straight-through" extraction procedure (Hayes, Ref *16*) designed to obviate the continual recirculation of the extracted drug and so improve precision.

(2) The use of sodium acetate buffer (0.5 *M*, pH 6.0), in place of 0.001 *N* sodium hydroxide, to reduce possible "interferences" from coextractives.

(3) The determination of a "blank" reading by splitting the acetate buffer into two streams after re-extraction and adding potassium ferricyanide to one only; each stream was passed through a separate fluorimeter. The blank reading was particularly desirable when urine was acid hydrolyzed and this modification avoided the extra time spent on a blank analysis of each sample.

The quantity of morphine in a solution was found by referring the intensity of fluorescence to a calibration graph on which the morphine concentrations of standard solutions, 2–10 mg/ml, were plotted against respective fluorescence intensities (in arbitrary units).

Comparison of Hydrolysis Efficiency of β-glucuronidase from Various Sources

The quantitative activities of the enzyme samples were initially checked with phenolphthalein glucuronide before use.

To 5-ml aliquots of the addicts' pooled urine were added 2 ml of the appropriate buffer solution followed by a measured quantity of the enzyme (see Fig. 1). Sodium acetate buffer solution (1.0 *M*) was used for pH values 3.0–6.0 and triethanolamine hydrochloride (1.0 *M*) for pH values 6.5–7.5. Succinic acid (0.5 *M*, pH 5.5–6.5) was used with the Sigma Type II enzyme. After overnight incubation at 37°C the samples were made just alkaline with 10 *N* sodium hydroxide solution and diluted to exactly 10 ml with glycine buffer (0.5 *M*, pH 10.2) to facilitate subsequent analysis. Ratios of the quantities of free morphine measured in each urine sample, with and without hydrolysis by the enzyme, were plotted against the pH of the reaction mixture (Fig. 1).

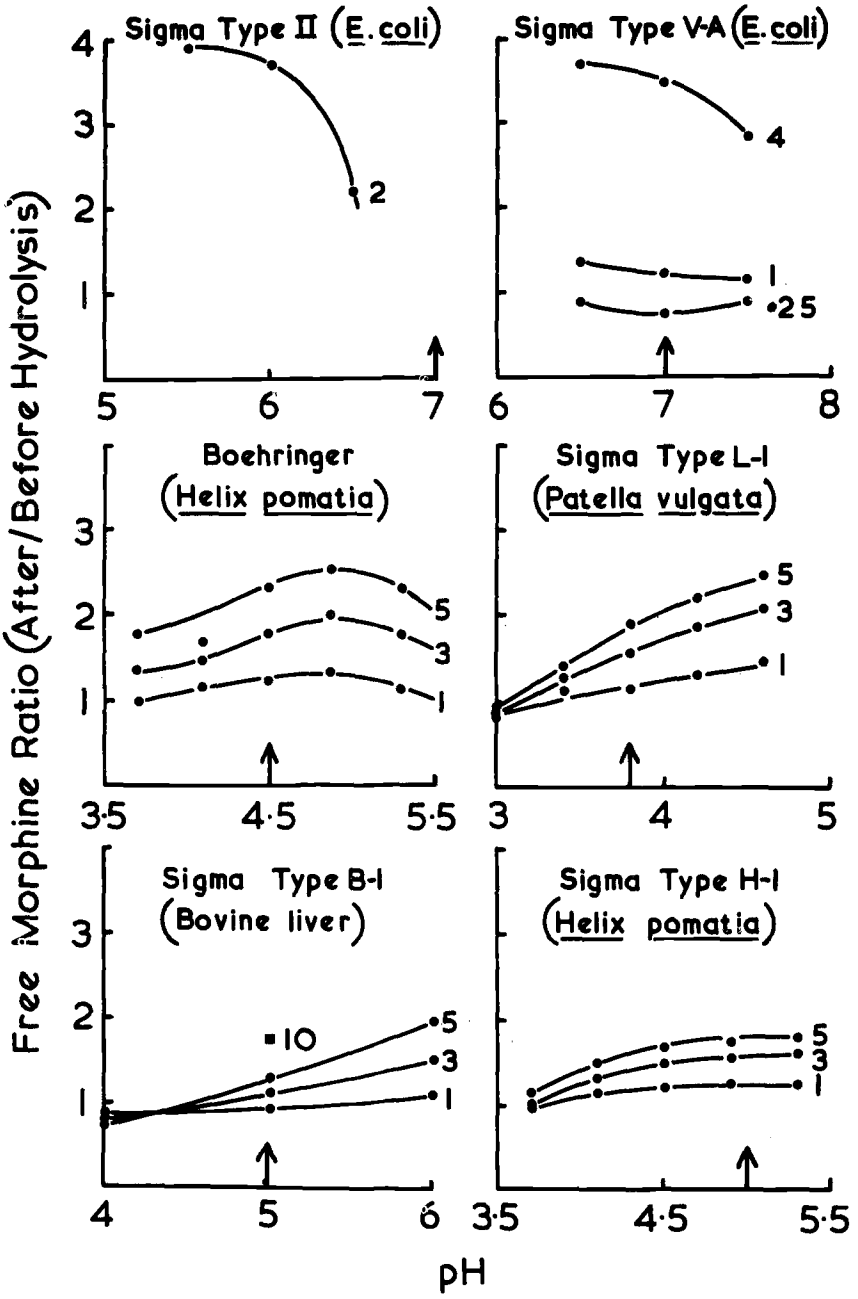

FIG. 1—*Comparison of morphine glucuronide hydrolyses in urine effected by β-glucuronidase derived from various sources. (Numbers beside curves indicate enzyme concentrations in thousands of Fishman Units. Arrows indicate manufacturers' recommended buffer pH values.)*

Preparation of Morphine Glucuronide

Morphine sulphate (2.1 g) was dissolved in saline (0.9 percent w/v, 20 ml) to give a morphine base concentration of 80 mg/ml and the solution was autoclaved at 15 psi for 15 min. It was administered subcutaneously to two male albino rabbits so that each received 600 mg of morphine over 8 h. Urine was collected for 72 h and the morphine glucuronide was extracted by the procedure of Fujimoto and Haarstad [*17*]. The filtered urine was divided and each 100-ml portion passed through a separate Amberlite XAD-2 resin column. The columns were washed with distilled water (100 ml) and the morphine and its metabolites were eluted with methanol. The glucuronide (207 mg) was recovered from the eluate on initial concentration, with an additional 209 mg after further concentration and overnight cooling at 4°C. The glucuronide was recrystallized from water and a high degree of purity was shown by infrared analysis, thin-layer chromatography, and a sharp melting point (244.9–245.6°C).

Hydrolysis by Enzyme

Morphine glucuronide (4.04 mg) was dissolved in distilled water (100 ml). To 4 ml of this solution in a test tube was added 2 ml of either sodium acetate (1.0 *M*, pH 5.5) or succinic acid (0.5 *M*, pH 5.5) buffer solution, followed by a measured amount of enzyme solution (455 mg Sigma Type II/5 ml H_2O + 0.01 ml $CHCl_3$ = 5000 Fishman Units per ml). The tubes were loosely plugged and incubated for 24 h in a 37°C water bath. The samples were then diluted to 16 ml with glycine buffer solution (0.5 *M*, pH 10.2) before analysis. (For results see Table 1.) Similar experiments were carried out at 60°C with up to 5000 Fishman Units of the Type II enzyme.

TABLE 1—*Hydrolysis of morphine glucuronide in aqueous solution by β-glucuronidase (Sigma Type II) at 37°C.*

Buffer System	pH value	Enzyme, Fishman Units	Liberated Morphine, % of theoretical yield
Acetate	5.5	1000	53
Acetate	5.5	3000	59
Succinic Acid	5.5	1000	64
Succinic Acid	5.5	2000	61
Succinic Acid	5.5	3000	59
Succinic Acid	5.5	4000	59
Succinic Acid	6.0	1000	56
Succinic Acid	6.5	1000	54
Succinic Acid	7.0	1000	51

Hydrolysis by Hydrochloric Acid

Autoclaving at 125°C—To 2-ml volumes of standard glucuronide solution (40.4 mg/l) were added measured volumes of concentrated hydrochloric acid to give different final acid concentrations (see Fig. 2). Sets of three samples were prepared from each of which one was autoclaved for 15 min, one for 30 min, and one for 45 min at 125°C. The samples were cooled and 10 *N* sodium hydroxide solution added by drops until each solution was slightly alkaline. The samples were further diluted to exactly 8 ml with glycine buffer (0.5 *M*, pH 10.2) before analysis. Results are shown in Fig. 2.

Refluxing at 100°C—Measured quantities (1.0, 2.5, and 5.0 ml) of concentrated hydrochloric acid were added to 5-ml samples of standard glucuronide solution and the mix-

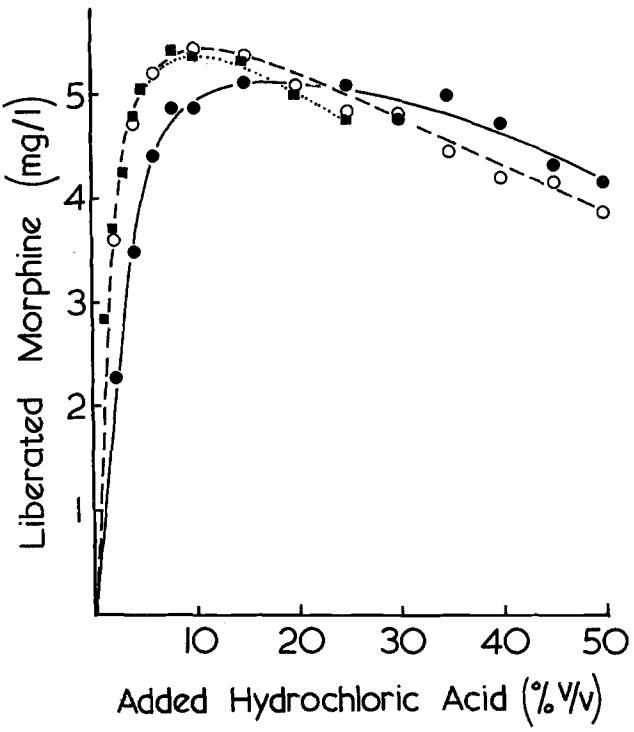

FIG. 2—*Hydrolysis of morphine glucuronide in aqueous solution by varying concentrations of hydrochloric acid—autoclaving at 125°C.* (●——●, 15 min; ○----- ○, 30 min; ■···· ■ 45 min).

tures refluxed vigorously (water-cooled condensers) for up to 40 min, under controlled conditions, by means of heat applied to a brass plate on which the flasks were placed together. The samples were made alkaline and diluted to 20 ml as above. For results see Fig. 3.

Water Bath Heating—Measured volumes (1, 2, and 3 ml) of concentrated hydrochloric acid were added to 5-ml samples of standard glucuronide solution, contained in test tubes. The tubes were placed in boiling water for up to 2 h. The samples were then cooled, made alkaline, and diluted to 20 ml as above. For results see Fig. 4.

Decomposition of Morphine by Hydrochloric Acid—To demonstrate decomposition of morphine by hydrochloric acid, various amounts of the concentrated acid were added to 5 ml of morphine hydrochloride solution (≡5 mg morphine base/100 ml of water). Duplicate samples were autoclaved for 30 min at 125°C, cooled, neutralized with 10 *N* sodium hydroxide solution, and diluted to 25 ml with glycine buffer (0.5 *M*, pH 10.2).

Results and Discussion

The rapid automated method for the determination of morphine based on the fluorescence of pseudomorphine was completely reliable and gave reproducible results (coefficient of variation 0.8 percent).

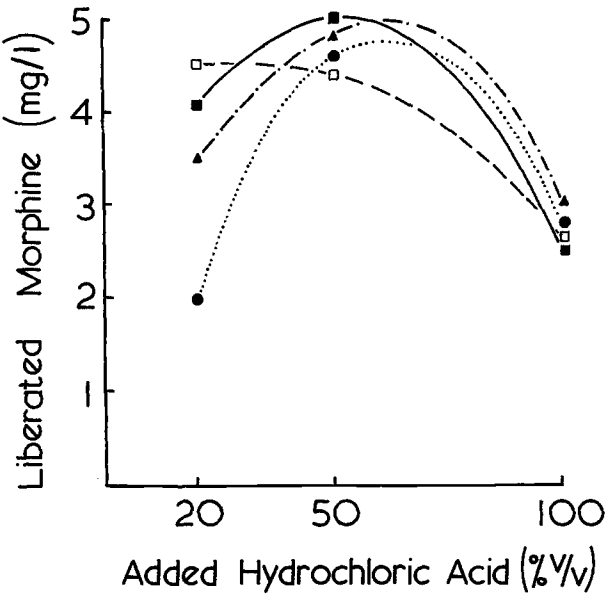

FIG. 3—*Hydrolysis of morphine glucuronide in aqueous solution by varying concentrations of hydrochloric acid—refluxing at 100°C.* (●····●, 10 min; ▲----▲, 20 min; ■——■, 30 min; □----□, 40 min).

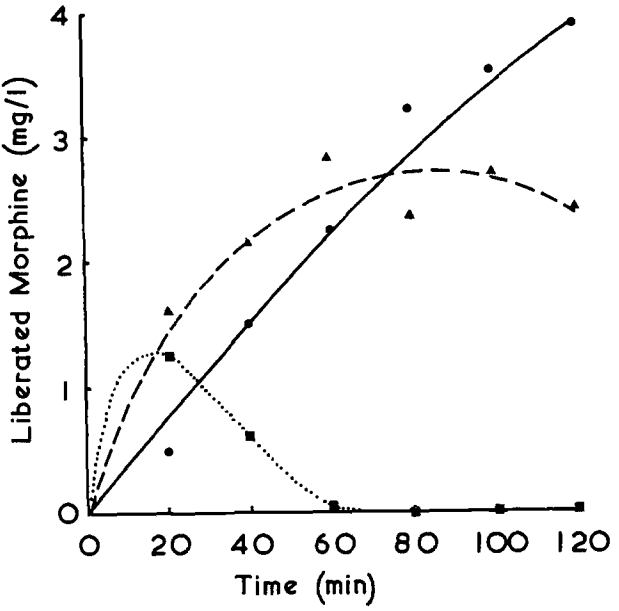

FIG. 4—*Acid hydrolysis of morphine glucuronide in aqueous solution with varying time—water bath heating (v/v of added concentrated hydrochloric acid* ●——●, 20 percent; ▲----▲, 40 percent; ■····■, 60 percent).

From Fig. 1 it can be seen that there was a wide variation in the potency of β-glucuronidase from different sources, that from *Escherichia coli* consistently showing the greatest hydrolysis efficiency. The pH conditions recommended for the use of the enzyme were generally not the optimum for the liberation of free morphine from the glucuronide. Using a diluted solution of prepared morphine glucuronide dihydrate ($C_{23}H_{27}O_9N \cdot 2H_2O$) from which the theoretical maximum yield of morphine would be 57.3 percent of 10.1 mg/l = 5.8 mg/l, and treating with the most efficient enzyme (Sigma Type II) under optimal conditions, 64 percent of the morphine available was liberated (3.7 mg/l). From Table 1 the importance of buffer choice, pH, and enzyme concentration can be seen. Studies with the Sigma Type II enzyme at 60°C, similar to those performed by Vela et al [*13*], not only failed to reduce the lengthy incubation time at 37°C but also failed to liberate more than 5 percent of the conjugated morphine.

The yields of morphine after enzyme hydrolysis were disappointing compared with those obtained by acid hydrolysis. For the latter the maximum yield of free morphine liberated from the glucuronide solution was 5.4 mg/l ≡ 93 percent of available morphine (Fig. 2). This resulted from autoclaving at 125°C for at least 30 min using between 8 and 15 percent v/v of added concentrated hydrochloric acid. These are conditions very similar to those used by Thompson and Gross [*3*].

Refluxing is a satisfactory alternative to autoclaving, although the yield is slightly lower (maximum 5.0 mg/l ≡ 86 percent available). Choice of time and acid concentration are matters of compromise (Fig. 3), 40 min with 20 percent of added concentrated hydrochloric acid being clearly superior to shorter times with this amount of acid but inferior to refluxing for 30 min with 50 percent by volume of added concentrated acid. Using the latter the yield is decreased after 40 min due to decomposition of the morphine base [*7*]. This point was corroborated by studying the breakdown of morphine (as hydrochloride). When autoclaved for 30 min with different amounts of acid (5, 10, 20, 30, 40, and 50 percent by volume of concentrated hydrochloric acid added), solutions showed a decrease from an initial 10 mg/l to the following levels, respectively: 9.9, 9.7, 9.5, 9.3, 8.7, and 8.3 mg/l.

By comparison with autoclaving or refluxing, heating on a water bath is much less effective (Fig. 4) unless a low acid concentration (20 percent added) is used for a prolonged time. However, even after 2 h the yield was only 3.85 mg/l ≡ 66 percent of available morphine.

Although the recommended acid hydrolysis of urine samples usually results in the development of a brown coloration and some black precipitation, when the samples are subsequently made alkaline a granular precipitate settles out. Within about 20 min standing or after 2 min centrifuging (3000 rpm), the supernatant can be aspirated for analysis. Consequently, gummy residues can be avoided without resorting to the use of an enzyme for hydrolysis with its resulting "clean" action but lowered efficiency.

Summary

The quantities of morphine liberated by various acid and enzymatic hydrolyses have been compared using an automated method of analysis. The potency of β-glucuronidases from various sources was evaluated, that from *Escherichia coli* being the most efficient of those studied. With β-glucuronidase the buffer system used and its pH are important, whereas the conditions for high temperature acid hydrolysis by autoclaving or refluxing are not so critical.

The recommended procedure is autoclaving urine samples with between 8 and 15 percent (v/v) of added concentrated hydrochloric acid for 30–45 min at 125°C or, alternatively, refluxing with 50 percent (v/v) of added concentrated hydrochloric acid for 30 min.

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