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Simultaneous Analysis of Codeine, Morphine, and Heroin After B-Glucuronidase Hydrolysis

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ABSTRACT: Analysis of the opiates, morphine and codeine, often proceeds by way of acid hydrolysis for release of the parent morphine from its glucuronide formed during metabolism. Following use, heroin is rapidly deacetylated to 6-monoacetylmorphine (6-MAM), which can be detected in the urine for a short time following injection of heroin. Only a small amount of 6-MAM may be further metabolized to morphine glucuronide. Thus, in general, the urine specimen has not been hydrolyzed prior to analysis for heroin, using a separate procedure from morphine and codeine.

Simultaneous analysis of morphine, codeine, 6-MAM and heroin would be complicated by loss of identity between morphine and heroin when heroin converts to morphine following acid hydrolysis for removal of the glucuronide moiety from morphine glucuronide. Another significant problem in simultaneous analysis is the relative disparity in concentration between morphine/codeine and 6-MAM/heroin (which might be present in the urine specimen).

In the proposed method of analysis, free morphine resulting from B-glucuronidase rather than acid hydrolysis of morphine glucuronide is derivatized with propionic anhydride to form dipropionylmorphine. Heroin that does not react with B-glucuronidase remains unhydrolyzed as the diacetylmorphine derivative. Some of the more exacting steps for the acid procedure are eliminated altogether making overall costs for the enzyme procedure comparable to those of the acid hydrolysis method. The enzyme reaction mixture is purified through a solid phase column system.

The optimal conditions for concentration of enzyme, temperature of hydrolysis and pH are individually characterized for B-glucuronidase hydrolysis and the ions which identify the propionyl derivatives are characterized for the simultaneous analysis of morphine, codeine, 6-MAM and heroin.

KEYWORDS: toxicology, opiate glucuronides, B-glucuronidase, dipropionylmorphine, heroin

Metabolism of codeine or morphine, leads to the formation of their glucuronides in urine [*1*]. Analytical methods based on acid hydrolysis [*2–6*] to free the parent drug from their glucuronides require several tedious and time-consuming steps. Heroin is rapidly metabolized to 6-monoacetylmorphine (6-MAM) by a single deacetylation. This

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requires a separate procedure for its analysis. Further deacetylation would yield free morphine. Attempts at simultaneous analysis for morphine/codeine and for 6-MAM and heroin, if present, would be complicated by the appearance of free morphine from two sources, morphine glucuronide and heroin [*6*], when the analytical procedure involves acid hydrolysis.

Though heroin, as such, is not often encountered in the urine of drug abusers because of its relatively short biological half-life, the current increasing use of heroin suggests occasions when quantitation of morphine, as well as traces of heroin which could be present might be of forensic significance [*13,14*].

The use of B-glucuronidase (E.C.3.2.1.31) [*7–9*] to release the parent drugs from the glucuronides, followed by reaction with propionic anhydride to label the glucuronide-derived morphine, obviates this difficulty because heroin is unreactive to the enzyme. Methods for the simultaneous analysis of codeine, morphine and heroin have been reported [*3,10–12*].

The present communication suggests a procedure for enhancing differentiation between morphine (MOR) and heroin (HER) during simultaneous analysis. B-Glucuronidase is used to release codeine and morphine from their glucuronides [*8,15,16*]. Purification of the enzyme reaction mixture by means of a solid phase extraction system, followed by reaction with propionic anhydride, converts the released morphine to its dipropionyl derivative (DPM). Heroin is not hydrolyzed and remains as the diacetylmorphine. Ions (m/z) unique to each drug specie facilitates quantitation of each drug in spite of differences in concentration between drugs in the urine specimen.

Methods

General

Codeine and morphine used in preparing the controls and standards were obtained as the dry material from AllTech-Applied Sciences Inc., State College, PA, and morphine glucuronide was obtained from Sigma Chemical Co., St. Louis, MO. Heroin was purchased as a 100 μg/mL solution in methanol from Sigma Chemical Co. B-Glucuronidase (E.C.3.2.1.31) (E. coli) was purchased also from Sigma as the dry crystalline material containing 1,900,000 Fishman units/gram (Catalog No. 105-8; Lot No. 51F-3831) (17). For some experiments, Lot No. 110H containing 1,560,000 Fishman units/gram was used. For the analysis, the enzyme was weighed out each day on an analytical balance and was reconstituted with deionized water or with 0.075 M phosphate buffer, pH 6.8, to a concentration such that 0.5 mL of enzyme solution added to the hydrolysis tubes gave a final enzyme concentration of about 1200 Fishman units/mL in the reaction mixture.

Hydrolysis was performed in a water bath maintained at the desired temperature ±1°C. Varian-Analytichem Bond Elut SPE columns were prepared for use according to the Analytichem procedure for basic drugs [*18*].

The columns were eluted through a Baker Extraction System vacuum manifold and then the eluant was evaporated to dryness under nitrogen prior to derivatization by the method of Chen, Taylor and Pappas [*19*] but using 100 μL 1:1 pyridine/propionic anhydride. Pyridine was dried over KOH pellets before use.

All derivatization reactions were performed in Pierce Reacti-Vial reaction vials using a Pierce Reacti-Therm III Heating/Stirring Module pre-set to the desired constant temperature conditions (Pierce Chemical Co., Rockford, IL). At the end of the heating period, the system was cooled and the excess reagents evaporated from the tubes under a stream of nitrogen gas. The desired temperatures were maintained at ±1°C during all reactions.

Quantitation of extracted and derivatized specimen was with the Hewlett-Packard 5890 Gas Chromatograph/5970B Mass Selective Detector equipped with the HP 7673B Auto-

sampler [5]. The GC/MS was fitted with a Hewlett-Packard Ultra 1 capillary column, 12.5 meters × 0.25 mm i.d.; 0.33 um film thickness 100% dimethyl-polysiloxane (gum). The system was operated in the electron impact mode. Split flow was maintained at a ratio of 15:1.

Determination of Optimal Experimental Conditions

Optimal Concentration of Enzyme

Aliquots of a solution of B-glucuronidase (approximate enzyme concentration: 5000 units/mL) were added to a series of tubes containing morphine glucuronide (10 000 ng/mL stock solution) or distilled water as the substrate to give a final enzyme concentration nominally varying between 0 and 5000 units/mL in 0.075 M phosphate buffer, pH 6.8, final volume, 2.0 mL. Incubation was for one hour at 37°C and extraction was through the solid phase extraction (SPE) columns. D3-Morphine and D6-Codeine (0.20 mL of a 40 000 ng/mL stock solution each) was added to each tube as an internal standard. Quantitation of the resulting free morphine was as stated earlier.

Concentration Range of Substrate

Aliquots of stock morphine glucuronide solution containing 0 to 8000 ng/mL were incubated under the conditions given above with 1250 units/mL of B-glucuronidase in 0.075 M phosphate buffer, pH 6.8. The free morphine was recovered in the solid phase column eluate.

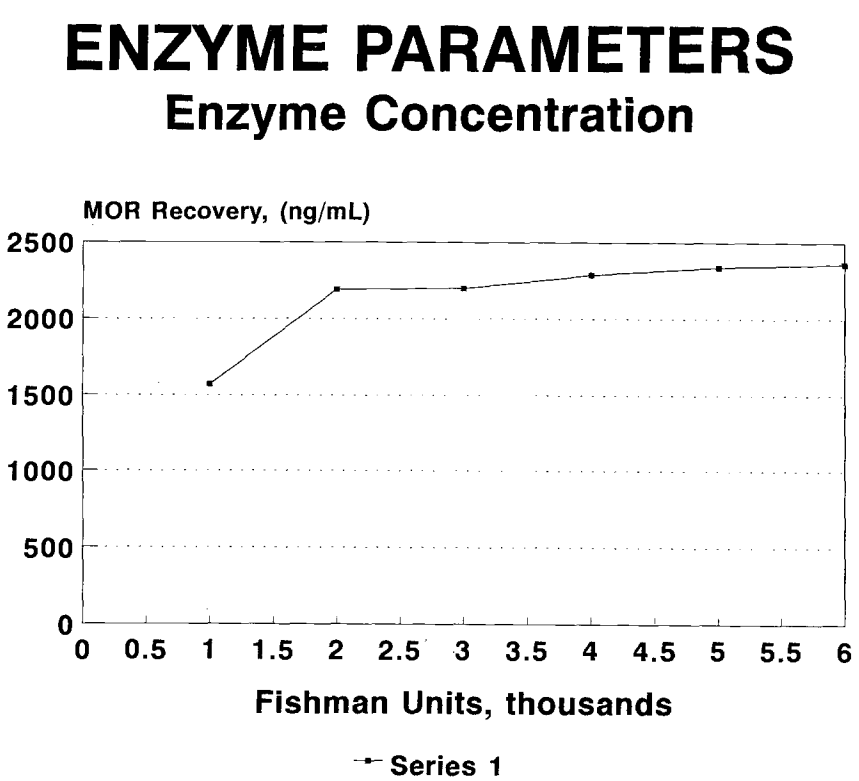

FIG. 1—*Effect of enzyme concentration on hydrolysis rate.*

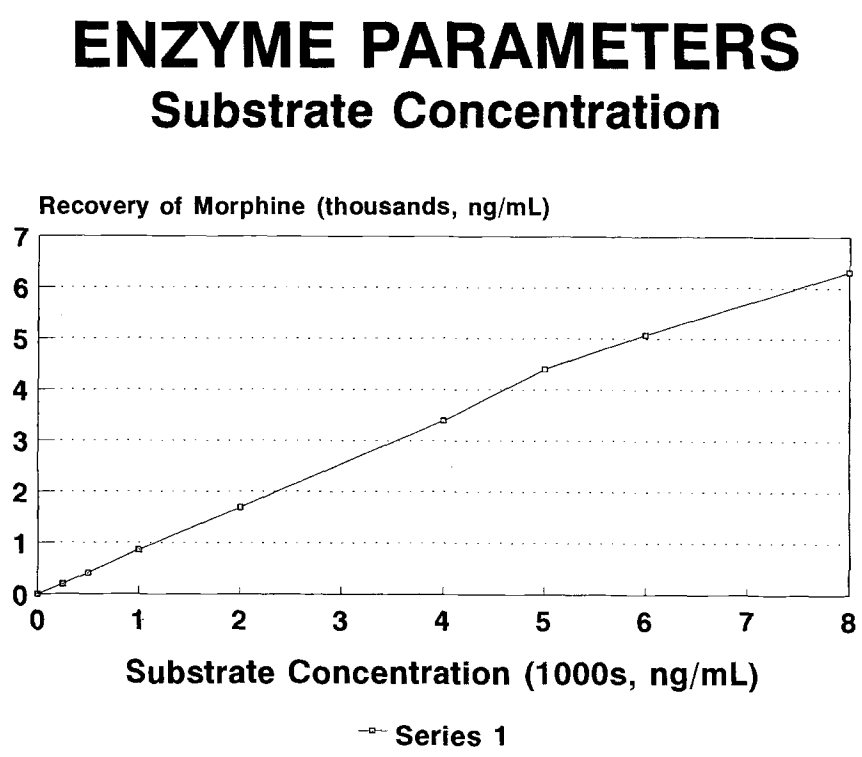

FIG. 2—*Effect of substrate concentration on hydrolysis.*

Optimal Hydrolysis Time

A series of tubes was prepared as above with a constant enzyme (1200 units/mL) and substrate (2500 ng/mL) concentration. These were incubated for 0, 1/2, 1, 2, 3, 4, or 24 hours at 37°C. Recovery was as previously stated.

Optimal pH for Reaction

The optimal pH range was determined over the range of 3.5 to 7.5 under the conditions previously described.

Temperature

The relative rates of enzyme-induced hydrolysis were determined at 25°C, 37°C and 45°C.

Recovery of Heroin

Specimens were prepared containing varying quantities of heroin over the range of 12.5 to 100 ng/mL, 400 ng D6-codeine and 800 ng D3-morphine in 0.075 M phosphate buffer, pH 6.8, in a total volume of 2.0 mL. Three replicate specimens were used at each concentration. After elution through the SPE columns, each eluate was dried and

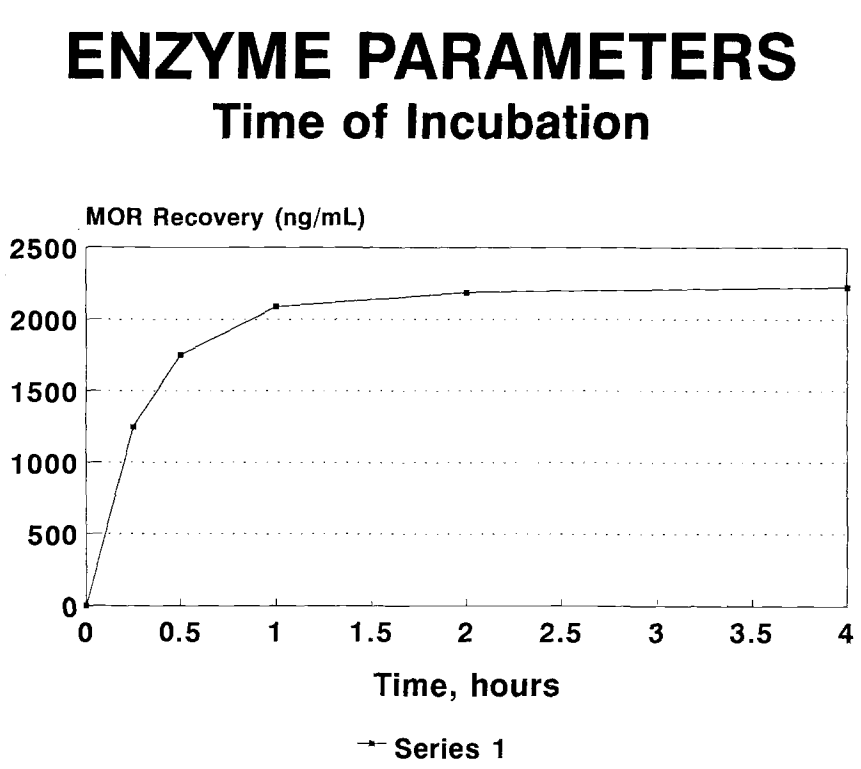

FIG. 3—*Effect of time of incubation on degree of hydrolysis.*

subjected to the derivatization procedure at 40°C for 30 minutes. Quantitation was as previously described using the 369 m/z ion for heroin (see Table 2).

Transacylation Reactions

To determine whether transacylation between acetyl and propionyl ester groups occurred during derivatization, heroin (HER) was treated with pyridine/propionic anhydride and the amount of monopropionyl, monoacetyl morphine (PAM) and the dipropionyl derivative (DPM) were quantitated.

Results

The optimal conditions obtained for evaluation of the six parameters listed above are summarized in Figs. 1 to 5. Between 900 and 1200 units of enzyme/mL in the final hydrolysis mixture appear to give comparable and optimal recovery of free morphine after 1.0 to 1.5 h of incubation. In the range of enzyme used, recovery of free morphine is essentially linear, at least to a substrate concentration of 8000 ng/mL. Under the conditions used in this study with spiked and natural specimens, as much as 93% of the morphine and 89% of the heroin can be accounted for following enzyme hydrolysis and derivatization (Table 1). The fragment ions used for qualifying and quantitating the various morphine and codeine derivatives for opiate GC/MS analysis are summarized in Table 2.

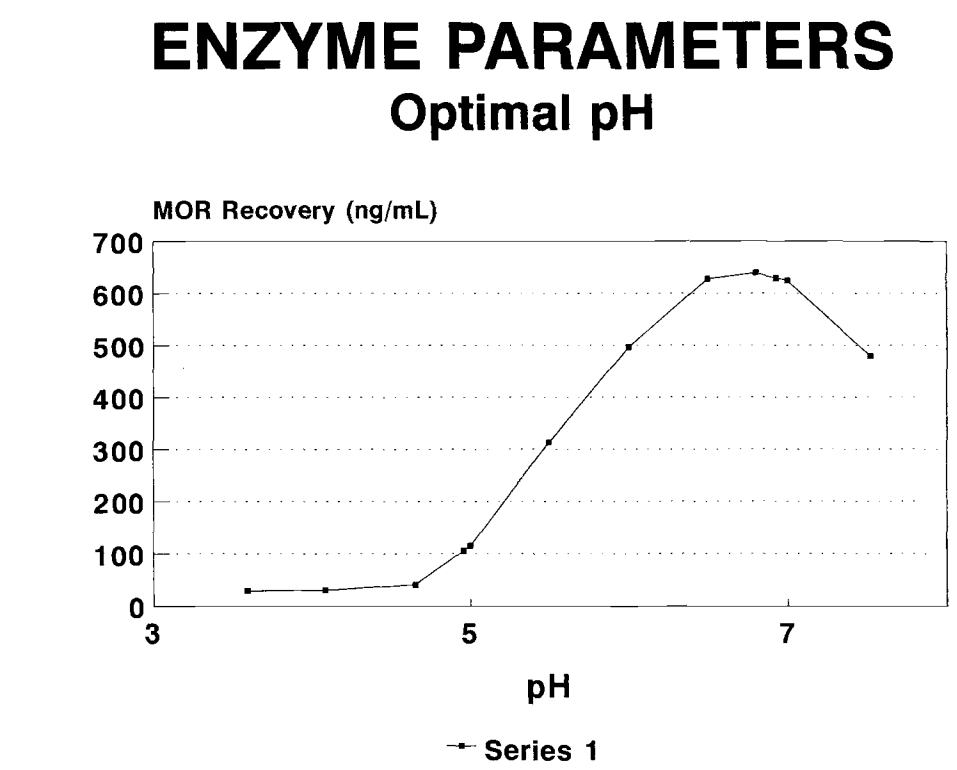

FIG. 4—*Effect of pH on rate of enzyme hydrolysis.*

Conversion of HER to DPM was essentially non-existent at 40°C. Conversion of HER to PAM varied as discussed below.

Typical scans of some opiate derivatives are given in Fig. 6 (MOR, PAM and HER) and Fig. 7 (acetylCOD, propionylCOD and the deuterated internal standards).

Discussion

B-Glucuronidase removes the glucuronide moiety from codeine and morphine with preservation of the acetyl groups on heroin, thereby permitting unique and simultaneous identification of the three opiates. For GC/MS analysis, the polarity of free morphine may result in increased binding at active sites in the analytical system, potentially interfering with proper quantitation. Conversion of the morphine to the diacetyl derivative reduces this polarity, but the unique identification from heroin (diacetylmorphine) is lost. The use of propionic anhydride for derivatization after enzyme hydrolysis also reduces polarity, but enhances differentiation.

Heroin metabolizes to the monoacetylmorphine derivative (6-MAM) within a short time of injection. During the analytical process, 6-MAM appears as the monopropionyl, monoacetyl derivative of morphine (PAM). Quantitation of morphine from morphine glucuronide is as the dipropionyl molecule (DPM) while heroin quantitates as the sum of the diacetyl (DAM) and monopropionyl, monoacetylmorphine compounds.

B-Glucuronidase has been prepared commercially from several species: snail (Helix

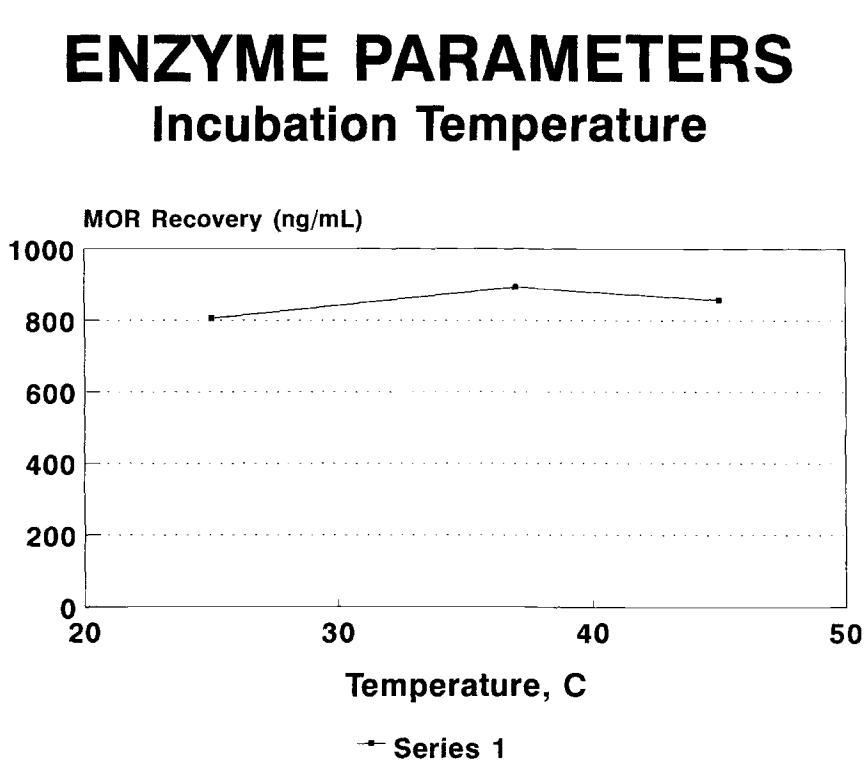

FIG. 5—*Effect of incubation temperature on rate of hydrolysis.*

pomatia), beef liver, abalone, limpets (Patella vulgata), scallops (Chlamys opercularis), and bacteria (E. coli) (8), and non-commercially from a number of other species [*6,8,17*]. Though each performs the same basic reaction, their rates of hydrolysis vary considerably, the preparation from the bacterial source being the most reactive [*17*]. In addition, the pH optimum for the snail (5.0) and mollusk (3.8) preparations differs from that for the beef and bacterial preparations (pH 6.8) [*17*]. Sulfatase, as well as other enzyme activities, contaminate even the best of the snail preparations [*8,17*]. Because one objective of this study was the development of a practical analytical procedure, only the bacterial enzyme was used in the present work.

TABLE 1—*Recovery of heroin.*

Theoretical Concentration[a]	Heroin Recovered	Percent Recovery
12.5	10.41 ± 1.6	83.0
25.0	24.06 ± 3.2	96.2
50.0	46.90 ± 3.1	93.8
75.0	70.51 ± 2.8	94.0
100.0	97.95 ± 7.6	98.0
Mean		93.1 ± 5.7

[a]Concentrations expressed as ng/mL.

TABLE 2—*Identifying parameters for opiate GC/MS analysis.*

Ionized Fragments[a] m/z			Retention Time (Nominal) min.	Chemical Description Parent Compound
400	344	327	6.92	d3-N-methyl-3,6-dipropionyl morphine (d3-DPM)
397	341	324	6.96	3,6-dipropionylmorphine (DPM)
383	327	310	4.80	3-propionyl, 6-acetyl morphine (PAM)
369	327	310	4.33	3,6-diacetylmorphine (HER)
384	**383**	324	5.00	6-acetylmorphine (6-MAM)
361	304	288	3.97	d3-O-methyl, d3-N-methyl, 6-propionylcodeine
355	282	229	4.01	6-propionylcodeine
347	341	282	3.17	6-acetylcodeine

[a]Quantifying ion in bold.
Morphine is identified as 3,6-diacetylmorphine (heroin) in NDSL SOP [*15*].

One point of concern was whether the acetyl groups of heroin might undergo transacylation during derivatization of free morphine with propionic anhydride since this could blur the distinction between morphine and heroin. Partial transacylation of the dipropionyl compound (DPM) may yield propionyl, acetylmorphine (PAM), while full transacylation would yield heroin.

Conversion of HER to DPM was not observed, while the conversion of HER to PAM occurred to a variable extent ranging from 'not detectable' to as much as 20% (once), essentially independent of the temperature of incubation. Estimates of HER conversion to PAM could readily be made from authentic material included in every analytical set.

In no instance was there detectable interference in heroin quantitation from morphine, though codeine, as the propionyl derivative, does elute approximately 0.3 min before heroin under the isothermal chromatographic conditions used. Therefore, there may be a potential interference with mass identity ratios if codeine is present in large quantities.

The single extraction step based on use of a solid phase extraction system is simple. The ability to analyze all common opiates together represents a significant time and cost advantage over separate analyses for each. The ability to simplify the manual operations of opiate extractions proposed in the current procedure compensates for the slight additional cost of the bacterial enzyme compared with total chemical costs. At an incubation time of 1.5 h and 1000 Fishman units/tube, the overall costs for the enzyme-propionic anhydride procedure is quite comparable to that of acid hydrolysis methods, and includes the additional benefit of not requiring chlorinated solvents which are costly to discard. For specimen containing very high concentrations of codeine or morphine glucuronides, more enzyme may be required to complete hydrolysis or, preferably, longer incubation times will be necessary. There is no indication that the 64-fold excess of morphine over heroin used in this study in any way represents the maximum differentiation which can be achieved with this method. The identification of 6-MAM in the presence of codeine/morphine proceeded without difficulty throughout the study. Some of the more exacting steps of the acid hydrolysis method used in this laboratory [*2,5*], such as autoclaving and careful adjustment of pH (to 9.0–9.2) to achieve separation of codeine from morphine, while ensuring proper recovery of both substances, are eliminated altogether. Enzymatic hydrolysis provides a more efficient means for removal of the glucuronide from morphine, 91.49 ± 1.67% recovery, compared with 84.10 ± 5.23% recovery of free morphine from acid hydrolysis.

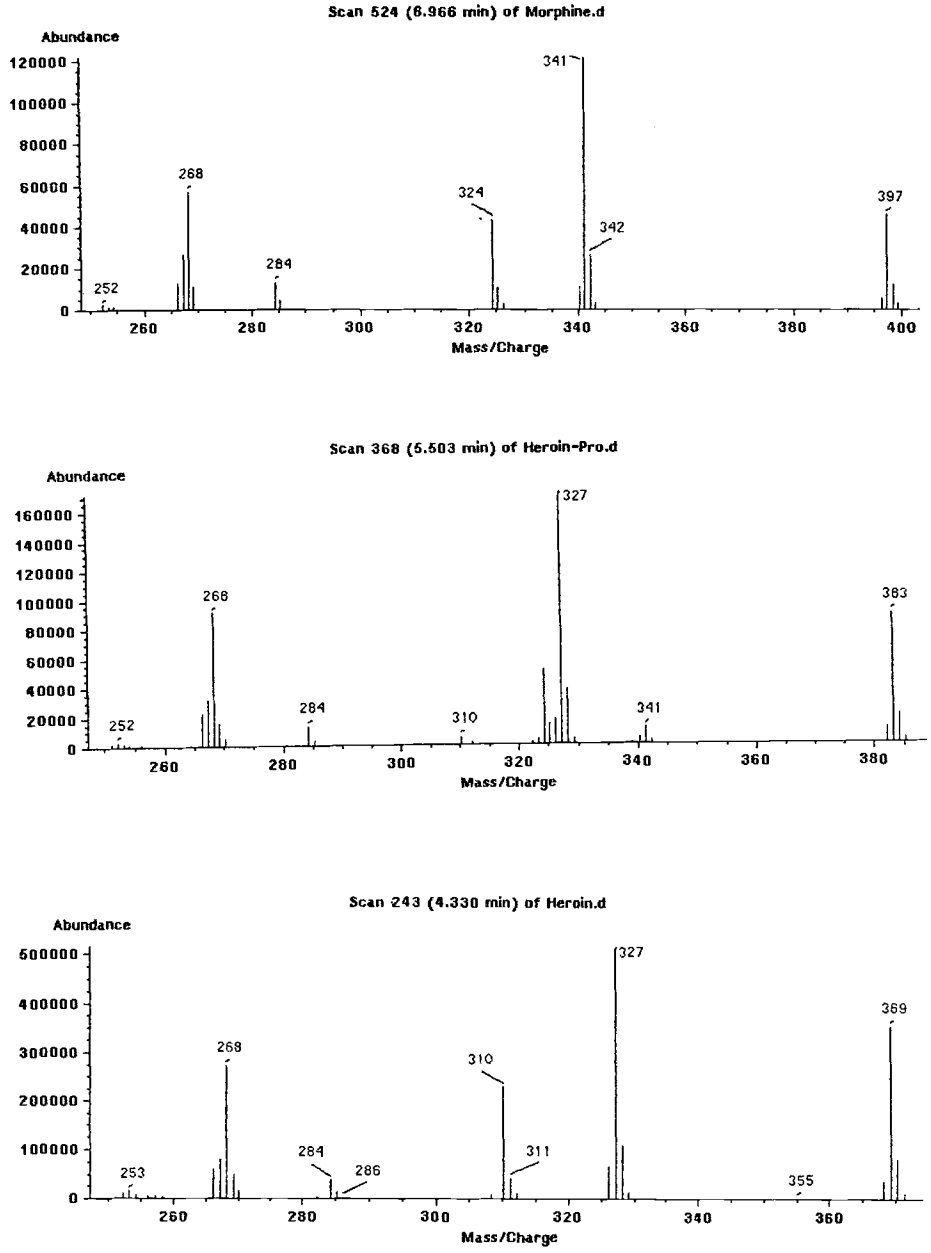

FIG. 6—*GC/MS scans of some morphine derivatives.*

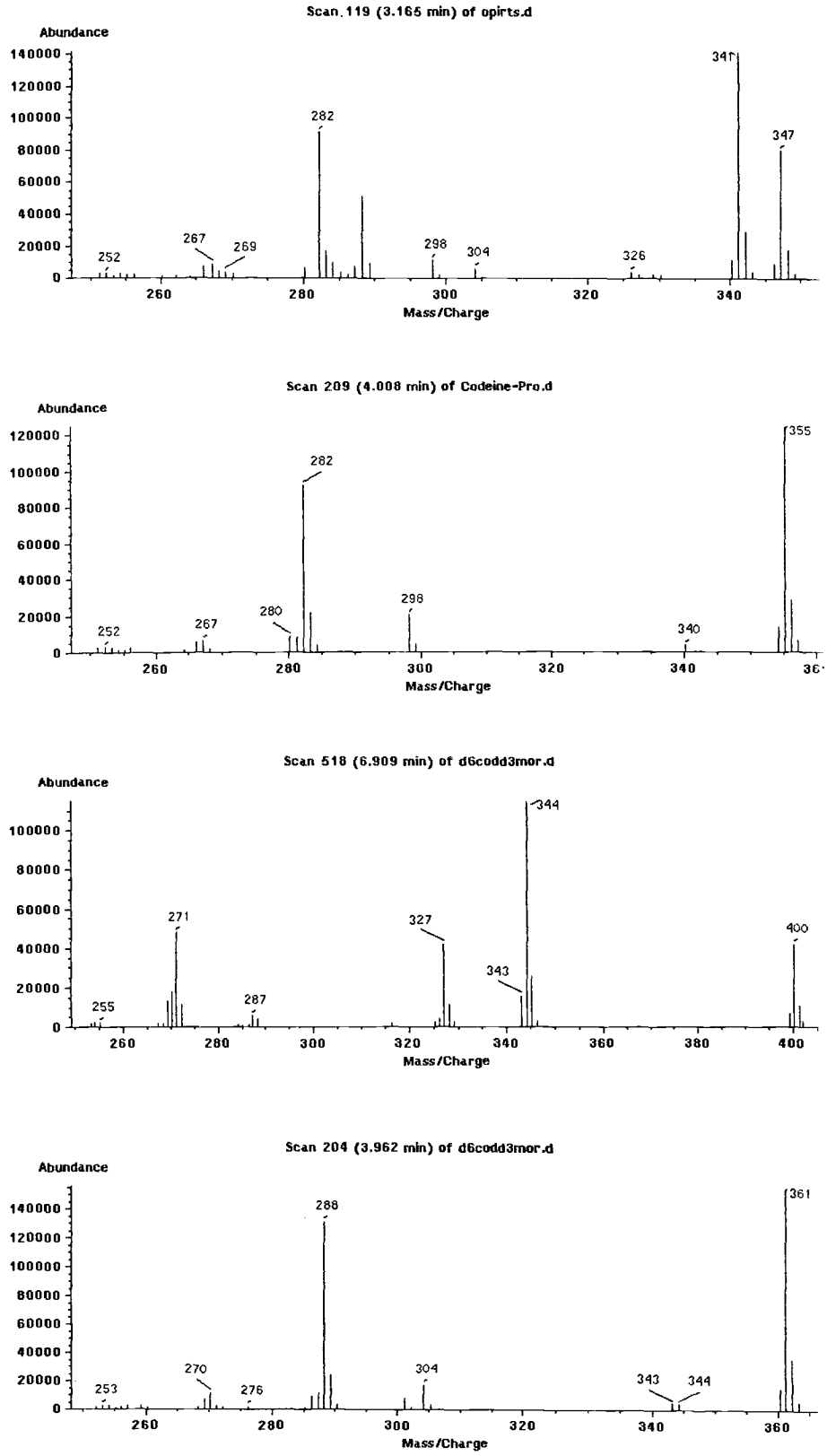

FIG. 7—*GC/MS scans of codeine derivatives and internal standards.*

While only Varian Bond Elut SPE cartridges were used in this procedure, it is expected that columns from other manufacturers could be substituted with only minimal procedural modifications [*10*].

Because the Navy Drug Screening Laboratory is neither authorized nor in a position to administer drugs to volunteer subjects for research studies, and urine specimen from authentic heroin users are not currently available to us, all studies were performed with authentic negative urine spiked with heroin. In every instance in which heroin-spiked urine specimen were used, analyses were in the expected range for heroin.

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