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Robotic Method for the Analysis of Morphine and Codeine in Urine

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ABSTRACT: A totally automated procedure has been developed for the detection and quantitation of morphine and codeine in urine case samples. The samples were initially screened for these drugs by a Syva^R EMIT Toxicology System (ETS™). A Zymate™ laboratory robotic system confirms positive samples from Syva^R ETS by performing the hydrolysis, extraction, and derivatization of morphine and codeine. The derivatized morphine and codeine were detected using gas chromatography/mass spectrometry (GC/MS). Enzymatic hydrolysis conditions were experimentally optimized during method development. The automation of these procedures has proven to be reliable and efficient.

KEYWORDS: toxicology, robotics, codeine, morphine, enzymatic hydrolysis

Opiate abuse, notably heroin, remains a major drug-abuse problem. Only cocaine is seen more often at this laboratory. Testing for opiate abuse generally relies upon detection of "total morphine" [*1*], the sum of free and conjugated morphine.

In order to produce accurate, rapid, and cost-effective results, an automated analytical scheme was developed incorporating an automated screening protocol with a robotic extraction and derivatization procedure for confirmatory testing [*5*]. A preliminary screening test using a Syva ETS™ System was performed in our laboratory. Confirmatory testing of the preliminary positive cases was accomplished using an hydrolysis, extraction, and derivatization procedure performed with a Zymark robotic system [*4*]. An existing solid phase extraction method was modified extensively for this procedure [*2*]. The codeine and morphine were identified using electron impact (EI) GC/MS operating in the selective ion monitoring (SIM) mode.

Experimental

Reagents and Materials

Reagents and disposables for use on the ETS were purchased from the Syva Company. The calibrators, controls, and reagents were prepared according to the manufacturer's

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recommendations [3]. Morphine sulfate, codeine sulfate, morphine-3-glucuronide, deuterated morphine and deuterated codeine were purchased from the Sigma Chemical Company. The working deuterated internal standard was prepared in deionized water to a concentration of 10 μg/mL of each analyte. Analytical grade methanol, isopropanol, chloroform, and dibasic potassium phosphate were obtained from the Baxter Healthcare Corporation. Bis-trimethylsilyl trifluoroacetamide (BSTFA) + 1% trimethlychlorosilane (TMCS) was supplied by Supelco, Inc. Detectabuse™ GC/MS grade extraction columns (Biochemical Diagnostics, Inc.) were used for the solid phase extraction. Glucuronidase enzyme (136 000 unit/mL) was supplied by the Sigma Company.

Instrumentation

A Syva ETS System, an automated analyzer for drug of abuse urine screening was used for the preliminary testing. A Zymate Laboratory Automation System (Zymark Corporation) was used for the hydrolysis, solid phase extraction and derivatization of samples prior to GC/MS analysis (Fig. 1). The robotics system was programmed through the use of Easylab software. The system consisted of various stations secured to sections of wedge shaped aluminum platforms. These mounted stations or pysections are located in a circular pattern around the Zymate II robot. The pysections used are, a temperature controllable sample rack, a test-tube dispenser, a general purpose hand, two pipetting hands and pipet tip racks, a liquid dispensing and mixing station, a Mettler AE 200 analytical balance (Mettler Instrument Corporation), a GC vial crimp capping station, an evaporation station, a hydrolysis station, a disposable chute, two racks for GC vials, four Master Laboratory Stations (MLS), two Power and Event Controllers (PEC), and an Okidata Microline 182 personal printer (Oki America, Inc.). A custom solid phase extraction station was used for holding the Detectabuse SPE GC/MS columns [6].

The chromatographic analysis of the extracted samples was performed on a Hewlett-Packard 5890 Series gas chromatograph equipped with a 5970 mass selective detector (MSD), a 7673A automatic liquid sampler and a HP 59970 MS ChemStation. The fused-silica capillary column was a Hewlett-Packard HP-1 column, 100% dimethylpolysiloxane, 12 m by 0.20 mm id. with a 0.33 μm film thickness. The injection port was a capillary split injector with a split silanized glass insert. The carrier gas, helium, was at a flow rate of 1 mL/min at 200°C oven temperature with a split ratio of 20:1. The septum purge was 2 mL/min. The injector and interface temperatures were 250°C and 280°C, respectively. The temperature program was 180°C to 265°C at 8°C/min. The MSD was used in EI, SIM mode programmed to detect the following ions: silylated codeine m/z 371, 234, and 229; deuterated codeine as internal standard (IS) 374, 237, 232; silylated morphine 236, 196, and 429; and silylated deuterated morphine (IS) 239, 199, and 432. A dwell time of 100 ms was used for each ion. The data acquisition, reduction, and archiving were performed using the Thruput target compound analysis program and in-house created macros. Reports were printed on a Hewlett-Packard Thinkjet dot matrix printer.

Sample Preparation and Analysis

Preliminary positive samples were prepared for confirmation analysis by the robotic system using the procedure listed in Fig. 2. Two to three mL of each urine sample is poured into 16 by 100 mm disposable test tubes and placed into the temperature controllable sample rack. Further handling of the sample through the incubation, extraction, and derivatization procedures was performed robotically. The sample aliquot and the addition of the internal standard were verified by weight.

Samples that gave an absorbance value of 200 or more units above the low calibrator by EMIT^{R} required a dilution. These samples, which contain a large amount of drug,

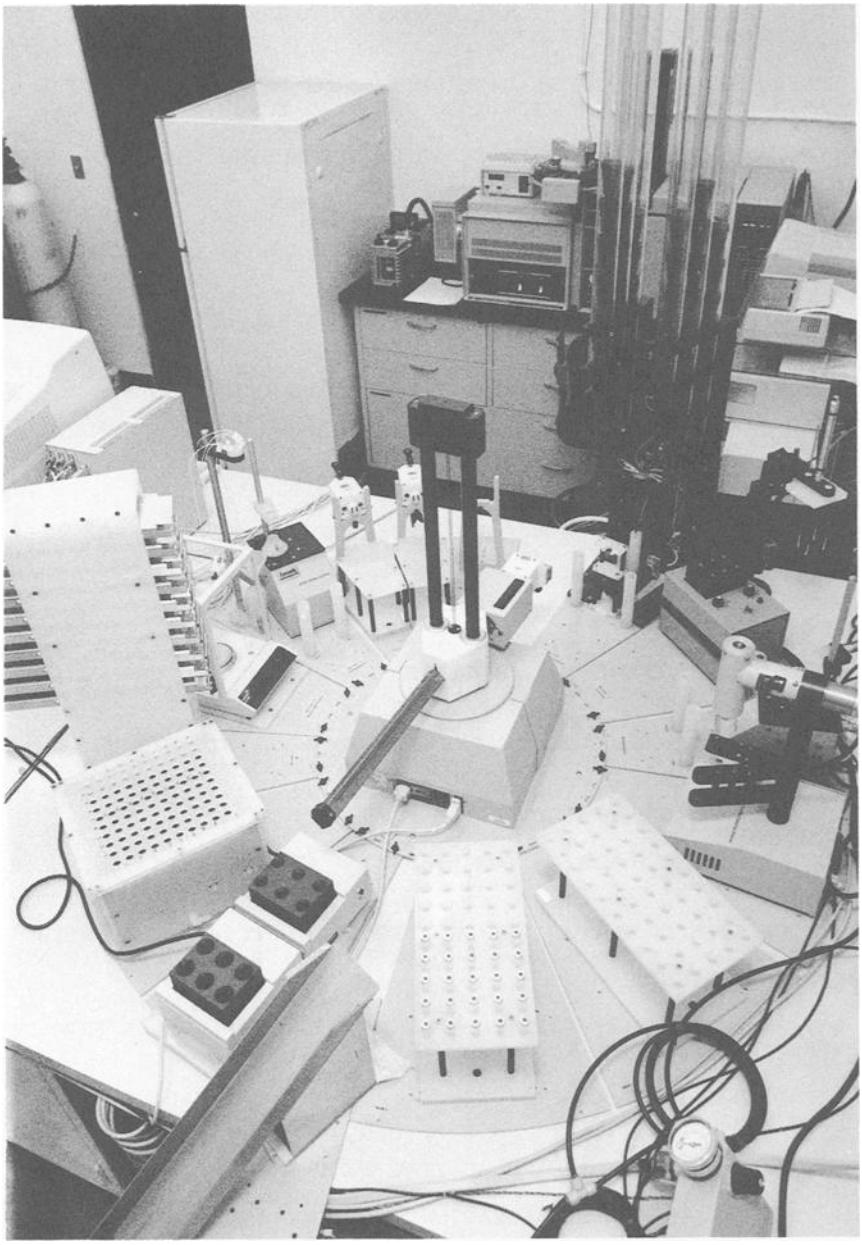

FIG. 1—*Zymark robotics system for hydrolysis, extraction, and derivatization of urine samples.*

are entered into the worklist setup and then automatically diluted by the robot with a chosen amount of deionized water. After vortexing, 1 mL was removed and carried through the procedure. Enzyme buffer was added and the sample incubated for 4 h. The pH was adjusted to 8.8 with 5% NaOH. The reagents for the solid phase extraction were dispensed by a MLS. A uniform flow rate was achieved by air pushes with the MLS syringes. Compressed air was used to dry the column packing before elution. The sample, after evaporation, was reconstituted with BSTFA + 1% TMCS and acetonitrile then

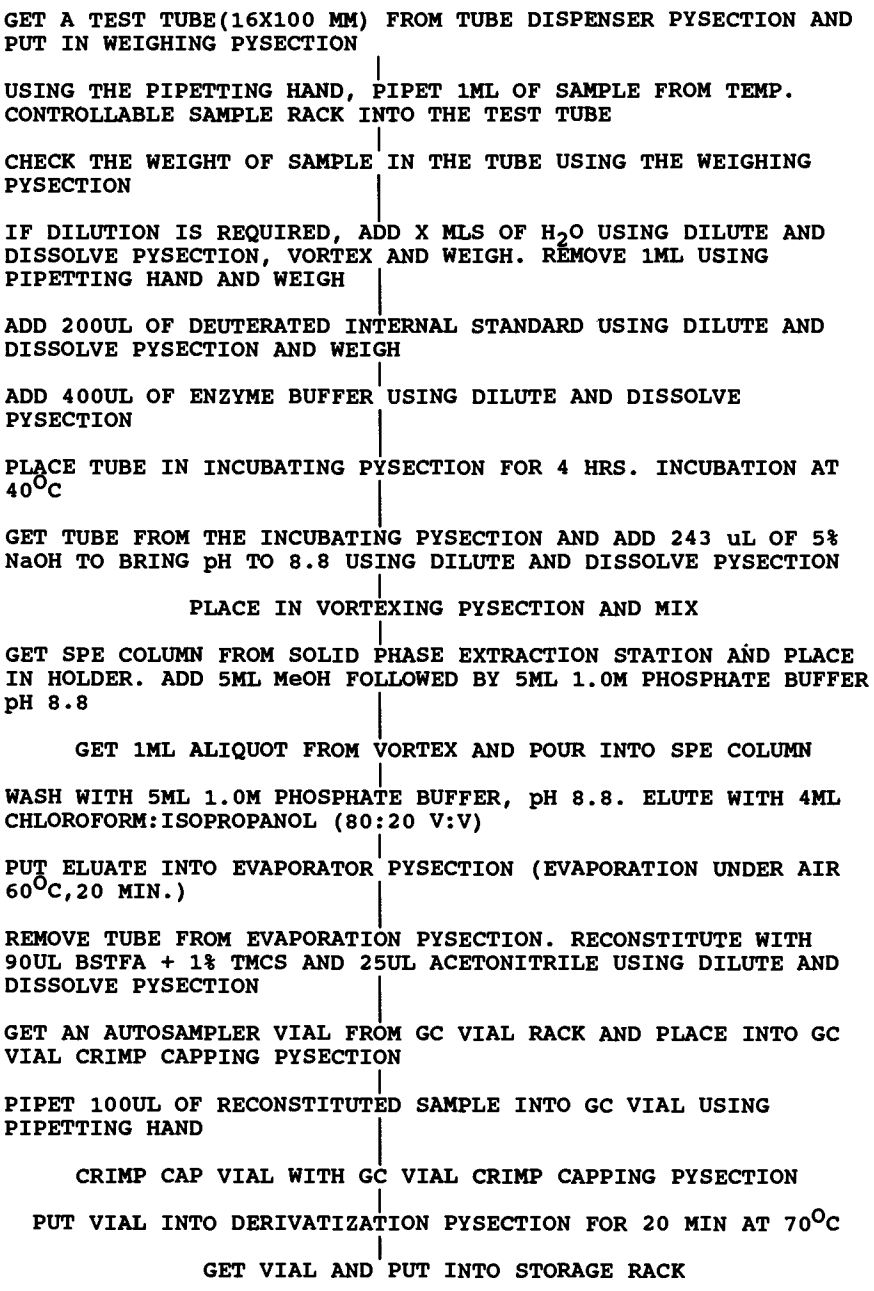

FIG. 2—*Extraction procedure for codeine and morphine.*

pipetted into glass autosampler vials, crimp capped, and placed into the derivatization station for 20 min at 70°C. After derivatization, the sample was stored in the GC vial rack pending placement into the GC/MS autosampler. Three μL of the sample was injected by the autosampler into the GC/MS system. The printout from the Zymate II listed the time the robotic extraction was completed, the sequence number of the sample, and the weights of the sample aliquot, internal standard, diluent, and diluted sample.

Results and Discussion

Enzyme Hydrolysis

The amount of enzyme activity needed for hydrolysis, incubation time, and incubation temperature were experimentally optimized during the development of the procedure. The same case samples were used throughout the development of the hydrolysis portion of the procedure.

Required Enzyme Activity

Case samples were analyzed using an in-house acid hydrolysis procedure. The resulting values were designated at 100% for purposes of comparison (Fig. 3). Approximately 12 000 to 14 000 units of enzyme activity in 1 mL of 2 M acetate buffer, pH 4.8, was determined to be appropriate for the procedure.

Incubation Time

Drug-free urine was spiked with 5000, 10 000, and 25 000 ng/mL of morphine-3-glucuronide, then incubated with 0.4 mL enzyme buffer for 3, 4, and 5 h (Fig. 4). Case samples were incubated similarly, with results from an in-house acid hydrolysis procedure designated as 100% for the purposes of comparison (Fig. 5). A 4 h incubation time was determined to be the optimum time, as 5 h incubation did not appreciably change the percent recovery of morphine from morphine-3-glucuronide.

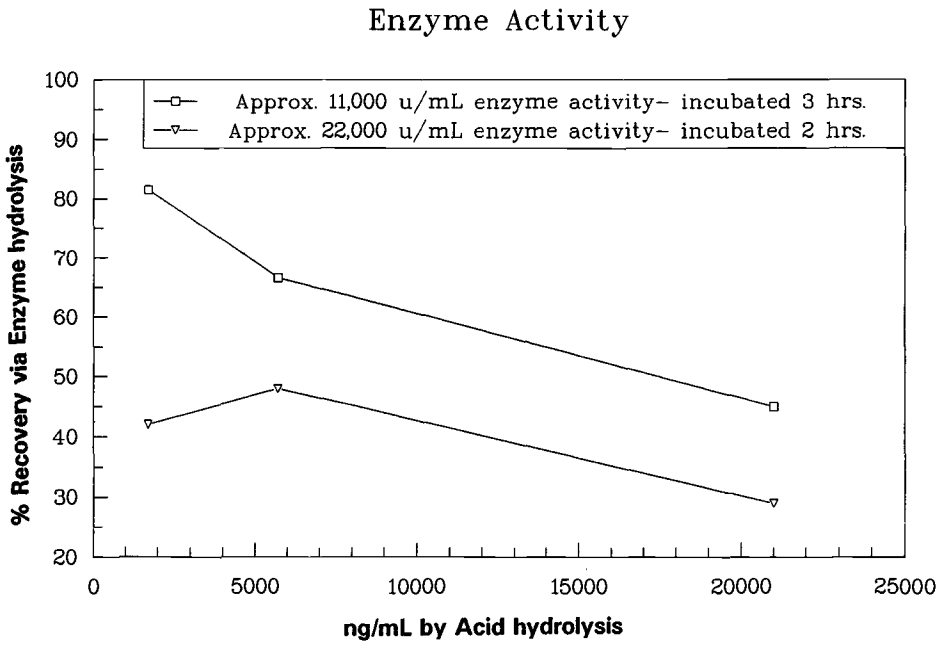

FIG. 3—*Percent recovery in case samples vs. acid hydrolysis (100%). Samples were incubated for 2 h with enzyme activity at 22 000 units/mL and for 3 h with enzyme activity at 11 000 units/mL in 2 M acetate buffer (pH 4.8).*

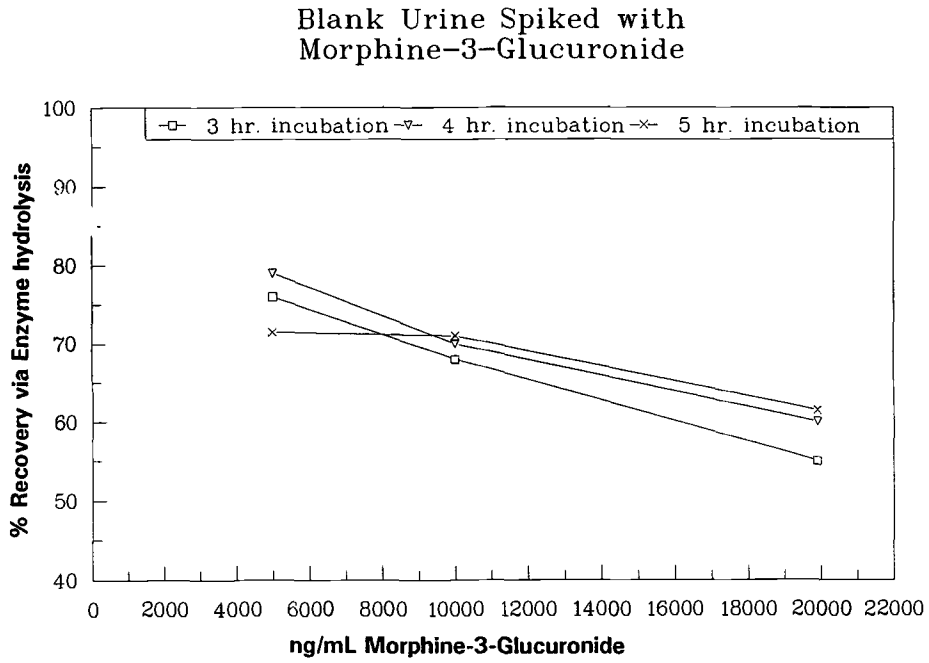

FIG. 4—*Blank urine spiked with Morphine-3-Glucuronide incubated with approximately 5000 Units of enzyme activity in 0.4 mL acetate buffer for 3, 4, and 5 h at 60°C.*

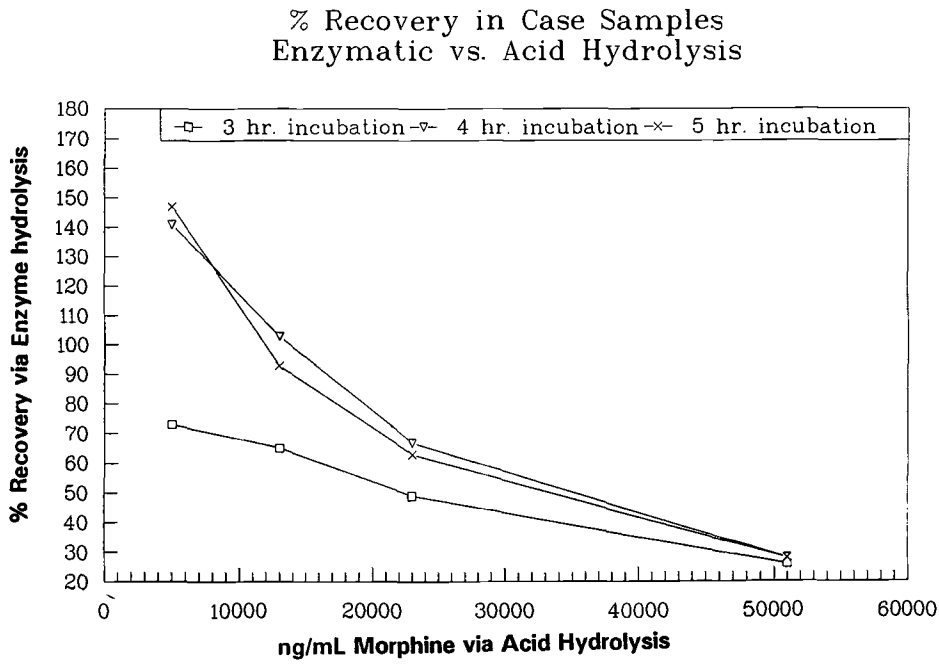

FIG. 5—*Case samples incubated for 3, 4, and 5 h under the same conditions as in Fig. 4. Percent recovery vs. acid hydrolysis (100%).*

Incubation Temperature

Case samples were diluted 1:5 with deionized water and incubated for 4 h with 0.4 mL of enzyme buffer (approximately 5500 units of activity). One run was incubated at 60°C, and another run at 40°C (Fig. 6). The percent recoveries in the diluted case samples were much higher then in the undiluted case samples (Fig. 5). The results indicate more complete hydrolysis in the diluted samples than the undiluted ones. As indicated in Fig. 8, percent recovery drops steadily at morphine glucuronide concentrations greater than 10 000 ng/mL. Therefore, it is recommended that samples with high levels (greater than 10 000 ng/mL) of morphine glucuronide be diluted appropriately. Incubation at 40°C was determined to yield maximum recovery of morphine.

All precision and linearity studies were run with the procedure outlined in Fig. 2.

A typical GC/MS report (Fig. 7) is shown. A sample was reported positive for total morphine or codeine, or both if its retention time and ion ratios were within the limits set by the previously processed standards and if the quantity detected was equal to or greater than 50 ng/mL of either total morphine or codeine, or both.

The use of Detectabuse™ SPE columns required a custom made extraction station. These columns were chosen since they had been validated for morphine, codeine, and other drugs by manual methods. They were also inexpensive and a uniform flow rate could be obtained for each column by using the syringe air push.

The reconstitution and derivatization step converted morphine and codeine to their trimethylsilyl ester. This derivatization, which is moisture sensitive, was performed adequately as evidenced by the acceptable peak shape and abundance of the analytes.

Linearity

Linearity was determined by analyzing a series of urines containing codeine with concentrations ranging from 50 to 10 000 ng/mL and morphine-3-glucuronide with concen-

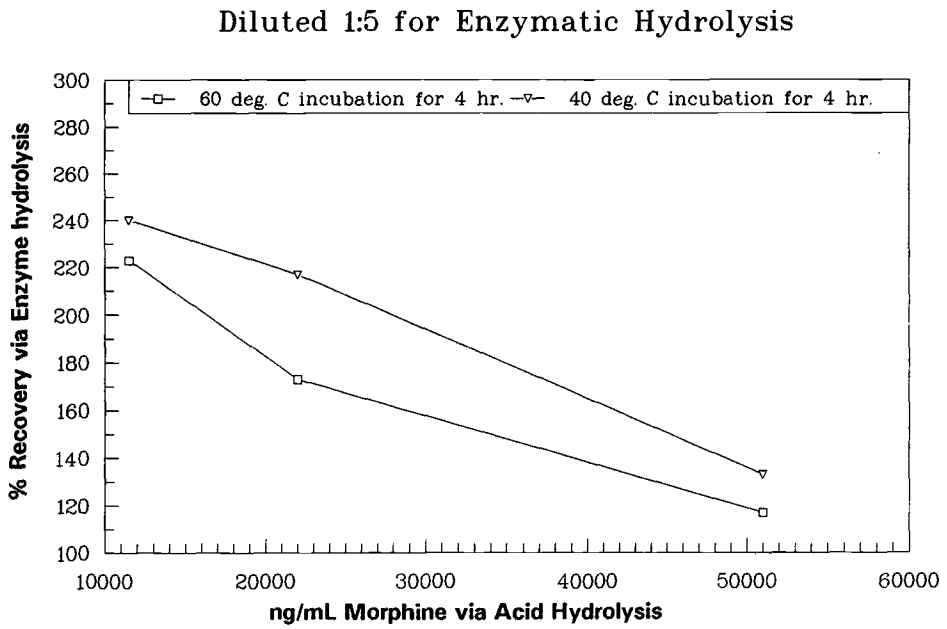

FIG. 6—*The same case samples as in Figs. 3 and 5 were incubated at 40°C and 60°C for 4 h. Samples were diluted 1:5. Percent recovery vs. acid hydrolysis (100%).*

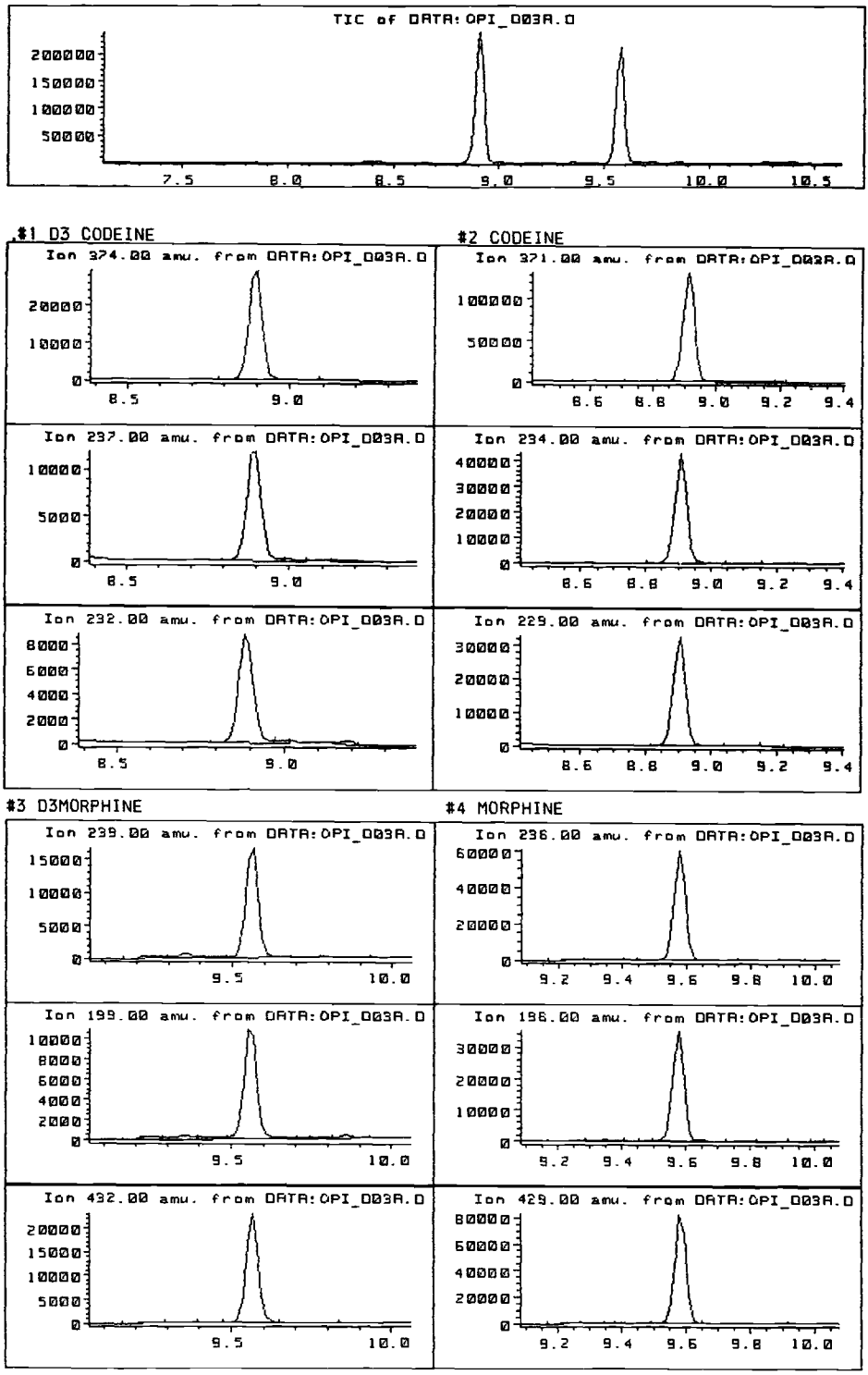

FIG. 7—*GC/MS report of extracted standard containing 5000 ng/mL of codeine and morphine.*

trations ranging from 200 to 25 000 ng/mL (120 to 15 250 ng/mL available morphine). The method was found to be linear for codeine in this range. Linearity for morphine-3-glucuronide was limited by the hydrolysis step. Based on a criteria of 75 to 80% (Fig. 8) recovery of bound morphine, linearity was determined to be 200 to 10 000 ng/mL morphine-3-glucuronide (122 to 6100 ng/mL available morphine) (Fig. 8). For low levels of morphine (50 ng/mL available morphine) the same automated procedure is used with a two mL sample size. Routine runs are calibrated using 150, 1000, and 5000 ng/mL each of morphine and codeine. The correlation coefficients generally range between 0.998 to 1.000 for both analytes.

Precision

The precision of pipetting and dispensing of the sample aliquot, internal standard, diluent, and diluted sample was monitored by weighing (Table 1). The overall precision of each transfer was excellent. The weighing in addition to providing an audit trail of the sample processing, was used to indicate problems such as plugged lines, empty reservoirs, faulty syringes and pumps, worn O rings, and loose connections.

The within-run precision was determined by analyzing six aliquots of morphine-3-glucuronide spiked into drug-free urine. The within-run precision was evaluated at morphine-3-glucuronide concentrations of 500, 1000, and 4000 ng/mL. The data is shown in Table 2. The results show acceptable precision with coefficients of variation (CVs) ranging from 5 to 7.6%. Recovery of available morphine ranged from 80 to 87%.

The between-run precision was obtained by analyzing urines spiked with morphine-3-glucuronide at 500, 1000, 4000, 10 000, and 25 000 ng/mL. The results are shown in Table 3. The between-run precision was acceptable with CVs ranging from 4.7 to 10.9. The

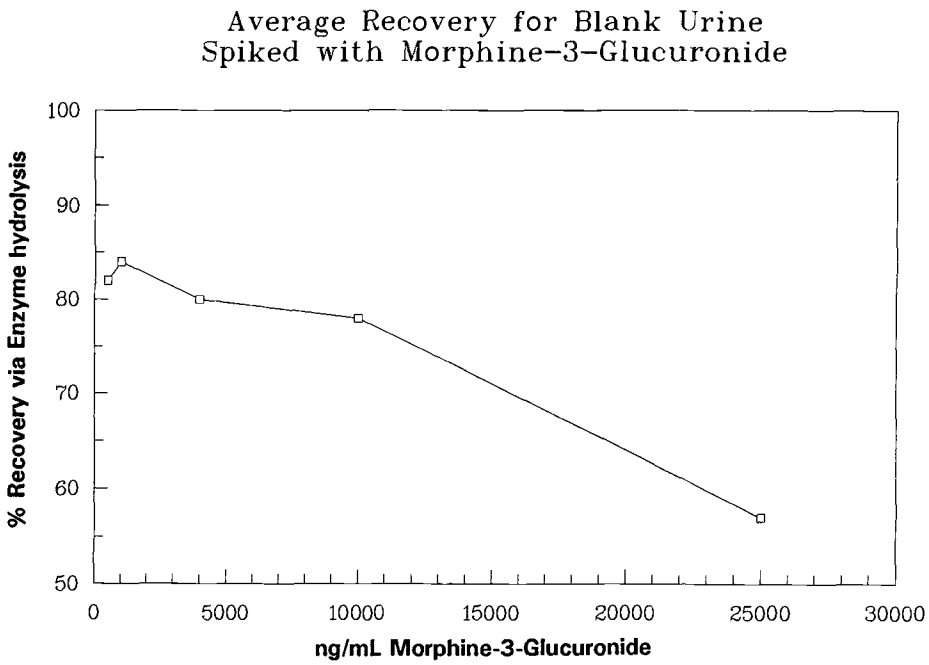

FIG. 8—*Average absolute recovery of morphine from blank urine spiked with 500, 1000, 4000, 10 000, and 25 000 ng/mL morphine-3-glucuronide. Samples were processed by procedure in Fig. 2.*

TABLE 1—*Precision in pipetting and dispensing of samples and reagents.*

Operation	CV (%)
Pipetting of sample (N = 50)	0.8
Dispensing of IS (N = 50)	1.8
Dispensing of diluent (N = 25)	0.4
Dispensing of diluted sample (N = 25)	0.5

TABLE 2—*Within-run precision for morphine-3-glucuronide.*

Conc. (ng/mL)	Avail. Morphine (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)	Recovery (%)
Morphine-3-Glucuronide						
500	305	6	245	12.2	5.0	80
1000	610	6	528	40.2	7.6	87
4000	2440	6	1979	102.7	5.2	81

TABLE 3—*Between-run precision for morphine-3-glucuronide.*

Conc. (ng/mL)	Avail. Morphine (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)	Recovery (%)
Morphine-3-Glucuronide						
500	305	10	250	11.9	4.7	82
1 000	610	14	514	56.0	10.9	84
4 000	2 440	8	1951	101.4	5.2	80
10 000	6 100	10	4738	248.9	5.3	78
25 000	15 250	8	8492	620.7	7.3	56

CV for the 1000 ng/mL morphine-3-glucuronide was slightly high, probably from the variability introduced by the hydrolysis step.

The between-run precision for free codeine and free morphine was obtained by analyzing urines spiked at 150, 300, and 4000 ng/mL. The results are shown in Tables 4 and 5.

The within-run precision was obtained by analyzing urines spiked with free codeine and free morphine at 150, 300, and 4000 ng/mL. The results are described in Tables 6 and 7.

Conclusion

Due to the forensic significance of morphine as an analyte in urine, procedure development and performance evaluation were focused on morphine and the hydrolysis of morphine-3-glucuronide for quantitation by GC/MS.

TABLE 4—*Between-run precision for free codeine.*

Conc. (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)
150	10	154	15	9.5
300	10	293	14	4.8
4000	10	4100	165	4.0

TABLE 5—*Between-run precision for free morphine.*

Conc. (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)
150	10	158	14	8.9
300	10	299	29	9.7
4000	10	4233	333	7.9

TABLE 6—*Within-run precision for free codeine.*

Conc. (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)
150	5	162	22	13.6
300	6	305	21	6.9
4000	7	3283	11	0.4

TABLE 7—*Within-run precision for free morphine.*

Conc. (ng/mL)	N	Mean (ng/mL)	SD (ng/mL)	CV (%)
150	5	166	17	10.0
300	5	267	28	10.4
4000	7	3936	44	1.1

The abuse and legitimate use of codeine can usually be distinguished from heroin or morphine use by the presence of a high ratio of codeine to morphine in urine [7].

The Zymate Laboratory Automation System has enabled the testing for morphine and codeine to be fully automated, including the hydrolysis of urine samples. In addition to saving staff-hour expenditures, it decreases human exposure to hazardous chemicals and specimens. The robotic system has demonstrated that it can be a reliable, accurate, precise, and cost effective alternative to manual techniques.

Acknowledgments

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