Journal of Food and Drug Analysis
1996. 4(1) : 25-34

藥物食品分析
第四卷　第一册

Determination of Codeine, Morphine and 6-Acetylmorphine in Urine

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ABSTRACT

A simple yet effective GC/MS protocol was developed and evaluated for the determination of free codeine, free morphine, 6-acetylmorphine, total codeine and total morphine in urine. Optimal recovery was achieved with extraction conducted at pH 9.0. Trimethylsilyl-derivatives of these analytes and the internal standard (nalorphine) were found adequately stable for a 72-hour period. Quantitative determination of the analytes was performed by selective ion monitoring. Excellent linearity was observed over the 50-1000 ng/mL concentration range studied. The intraday and interday precisions range from 0.47 to 7.72% for codeine, 0.31 to 8.09% for morphine and 0.49 to 6.15% for 6-acetylmorphine. The overall recoveries for codeine, morphine and 6-acetylmorphine were found to be 98.3%, 76.7% and 85.6% respectively.

Key words : GC/MS Quantitation, morphine, codeine, 6-acetylmorphine, urine.

INTRODUCTION

The analyses of codeine and morphine have been traditionally important in clinical laboratories. Recently, it has been advocated that careful evaluations of urinary morphine and codeine concentrations and their ratio may provide valuable information for the differentiation of samples of heroin abusers from those resulting from the ingestion of other opiates-containing items [1, 2]. Since gas chromatography / mass spectrometry (GC / MS) methodologies can best unequivocally identify codeine, morphine and 6-acetylmorphine in biological fluids and tissues, there have been several reports [3, 8] on this topic. Factors concerned in developing a GC/MS procedure for the analysis of opiates include hydrolysis and extraction procedures, derivatization techniques, ionization methods, and chromatography / mass spectrometry conditions [7].

With the unique situation in Taiwan where opium powder-containing Brown Mixture is sold as an over-the-counter drug, we are particularly interested in developing a reliable yet simple (and

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Accepted for Publication: Oct. 24, 1995

economical) procedure to characterize the opiate compositions in Brown Mixture and heroin samples and hopefully to differentiate urine samples collected from Brown Mixture users and heroin abusers [9]. We wish to report a procedure, that has been developed and routinely used in our laboratories, along with several performance characteristics.

MATERIALS AND METHODS

I. *Reagents*

All solvents and reagents were of analytical grade, purchased from Merck (Darmstadt, Germany). One mg/mL methanol solution of codeine, morphine, and nalorphine were purchased from Sigma (St. Louis, MO). 6-Acetylmorphine was purchased from Radian (Austin, TX).

II. *Sample Pretreatment and GC/MS Analysis*

(I) *Sample Pretreatment for the Analysis of Free Codeine, Free Morphine and 6-Acetylmorphine*

Each sample (5 mL) was spiked with 100 mL of nalorphine internal standard (15 µg/mL in methanol), and 1 mL ammonium chloride / ammonium hydroxide buffer (pH 9.5), followed by extraction with 5 mL 4 : 1 (v/v) chloroform : isopropanol mixture on a horizontal shaker for 5 min. The mixture was then centrifuged and the aqueous layer aspirated. The organic extract was transferred to a Reacti-Vial™ (Pierce: Rockford, IL) and evaporated to dryness under a stream of nitrogen at 70 °C.

(II) *Sample Pretreatment for the Analysis of Total Morphine and Codeine.*

Each sample (5 mL) was also spiked with the internal standard, followed by the addition of 1 mL concentrated HCl . The acidified urine was hydrolyzed in a boiling water-bath for one hour. The mixture was cooled to room temperature and extracted with 5 mL 4 : 1 (v/v) chloroform / isopropanol mixture. The aqueous layer was transferred and made basic with 0.95 mL 13 N sodium hydroxide and 1 mL ammonium chloride / ammonium hydroxide buffer (pH 9.5). The same procedure used for the assay of free codeine and morphine was then followed.

(III) *Preparation of Trimethylsilyl Derivatives*

For derivatization, 50 µL pyridine and 50 µL hexamethyldisilazane (HMDS) were added to the residue in the Reacti-Vial™ (room temperature), followed by vortexing for 20 seconds and then heated at 100 °C for 20 minutes. The reaction mixture was allowed to cool to room temperature prior to GC/MS analysis.

(IV) *GC/MS Analysis.*

Selected ion monitoring (SIM) mass spectrometric data were obtained using a Hewlett-Packard (Palo Alto, CA) HP 5890 gas chromatograph interfaced to a Hewlett-Packard HP 5971A mass selective detector (MSD) equipped with HP-G1034C Chemstation software. The gas chromatograph was equipped with a 12-m Hewlett-Packard (Andover, MAA) Ultra-1 (100% dimethyl polysiloxane phase) fused silica capillary column (0.20 mm ID; 0.33 µm film thickness). Helium was used as the carrier gas with a flow rate of 0.5 mL/min with the column head pressure maintained at 4 psi. The injector and interface temperatures were maintained at 260 and 280 °C, respectively. Oven temperature was held at 120 °C for 1 minute, then programmed to 250 °C at 20 °C/min, and held at the final temperature of 250 °C for 5 minutes. The following parameters are used for injecting samples into the GC/MS system: sample size, 1 µL; injection mode, splitless; injector purge-off duration, 1.5 minutes.

Ions selected for monitoring TMS-derivatives of codeine, morphine, 6-acetylmorphine and nalorphine were m/z 371, 356, 343; 429, 414, 401; 399, 340, 287 and 455, 440, 414, respective-

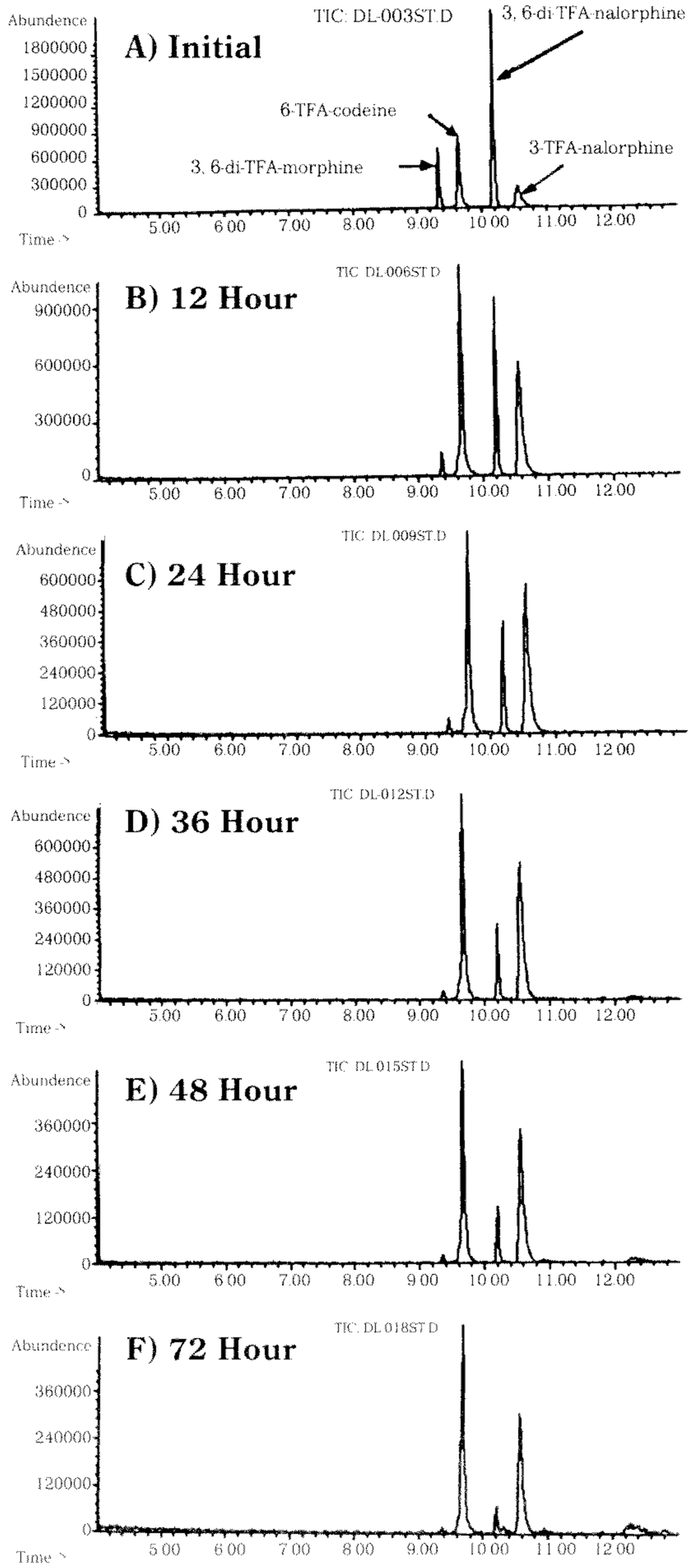

Figure 1. The stability of trifluoroacetylation derivatives of codeine, morphine and nalorphine.

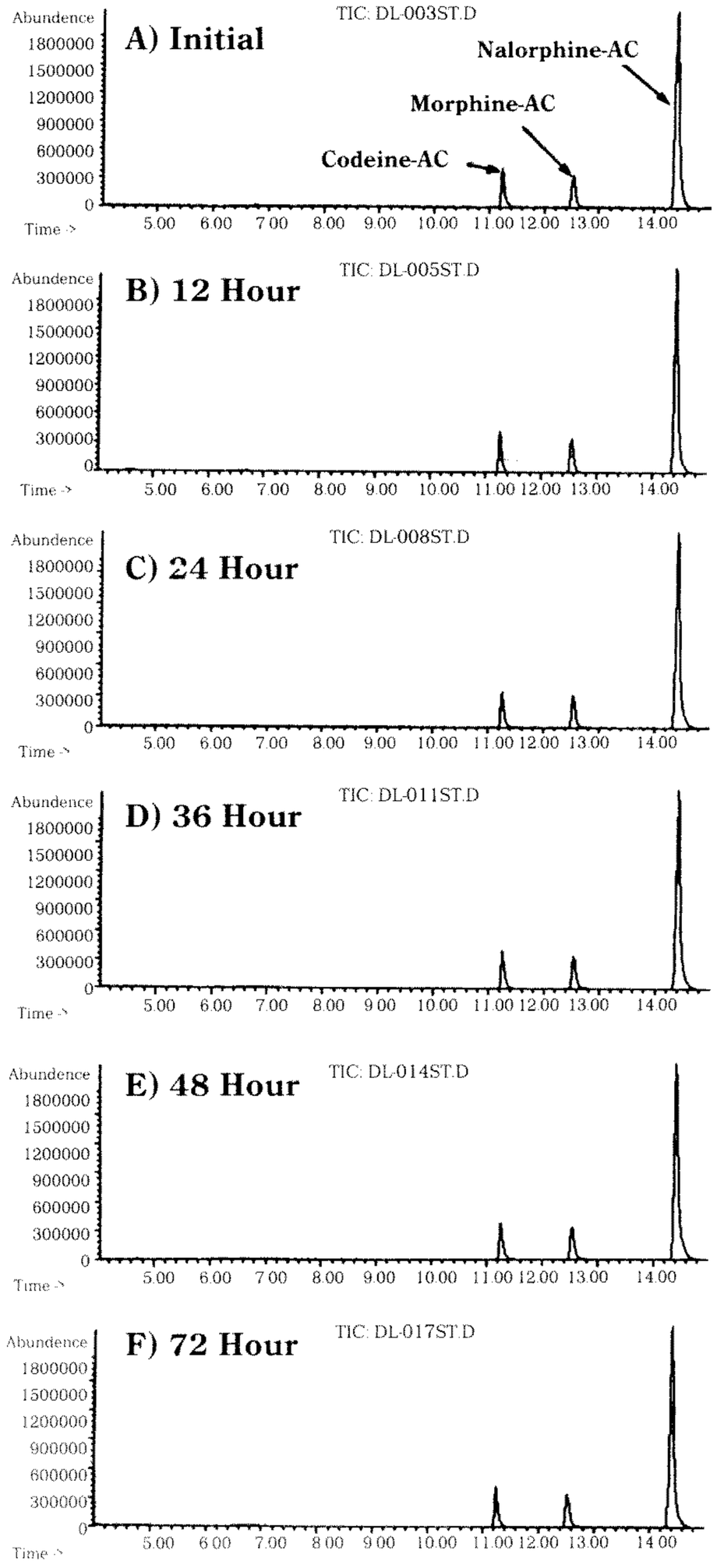

Figure 2. The stability of acetylation derivatives of codeine, morphine and nalorphine.

ly. The first ion (underlined) listed for each compound was used for quantitation using a six-point calibration protocol.

RESULTS AND DISCUSSION

1. *Selection of Derivatization Method and Extraction pH*

Before the over-all procedure is established for the analysis of codeine, morphine and 6-acetylmorphine in urine matrix, two sets of experiments were performed to help select an appropriate derivatization method and the optimal extraction pH condition. Controls containing known amounts of codeine, morphine and 6-acetylmorphine were prepared with distilled water for these experiments.

Trifluoroacetic anhydride, acetic anhydride, and hexamethyldisilazane were evaluated for

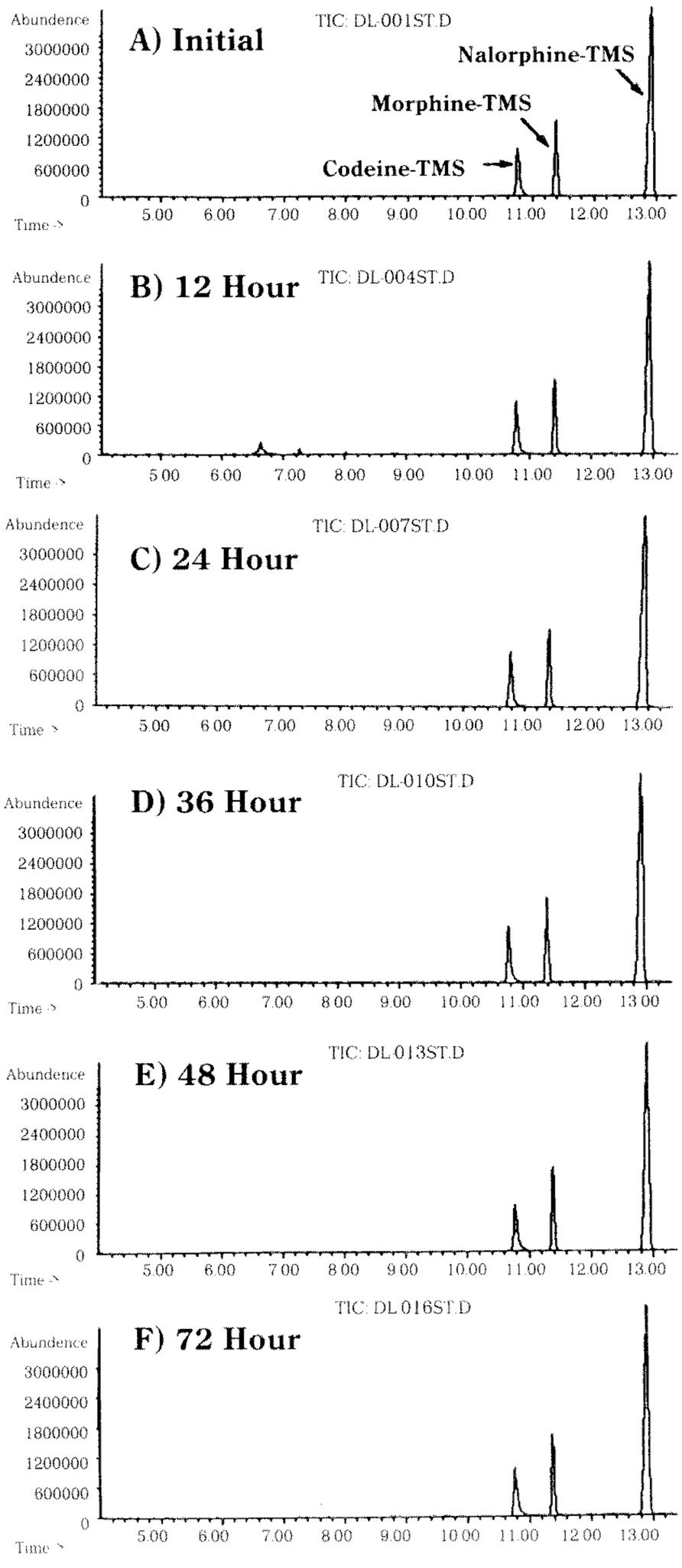

Figure 3. The stability of trimethylsylilation derivatives of codeine, morphine and nalorphine.

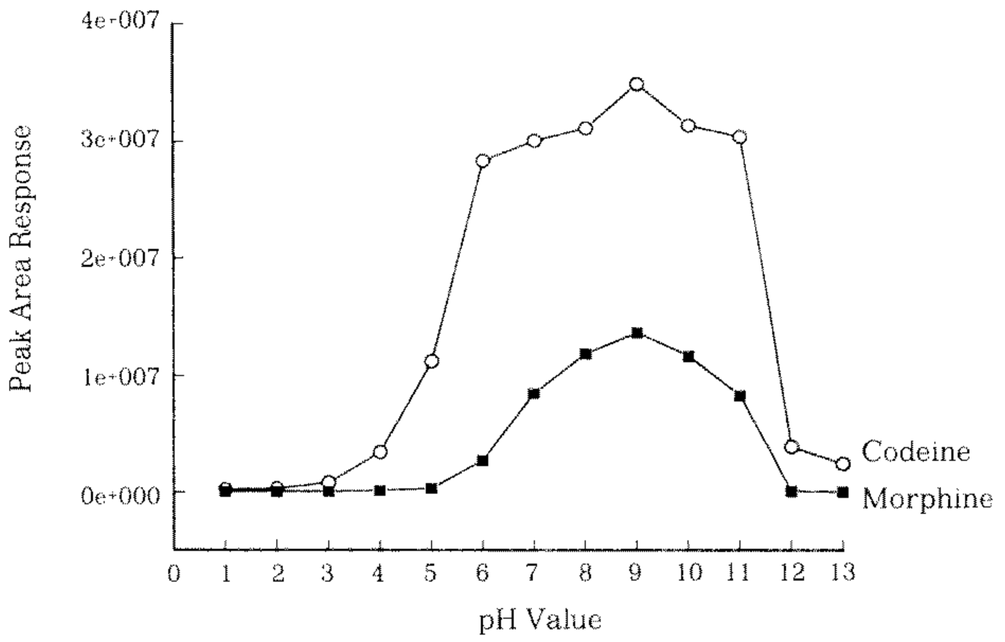

Figure 4. The effect of various pH buffer on recovery rate.

their effectiveness as the derivatization reagent for the determination of codeine and morphine using nalorphine as the internal standard. These derivatization methods were evaluated by examining the full-scan total ion chromatograms resulting from the injection of the derivatization products at time 0, 12, 24, 36, 48 and 72 hours after the completion of the derivatization procedures. Chromatograms in Fig. 1 revealed the formation of two TFA-derivatives for nalorphine, the reduction of some peak intensity, the change of relative peak intensities, and the appearance of additional peaks at retention time 12.3 minutes in chromatograms Fig. 1-D ~ 1-F. Trifluoroacetic anhydride is thus not considered appropriate for this application.

Although the relative peak ratios of the acetyl-derivatives (Fig. 2) appeared more stable than those of the TMS-derivatives (Fig. 3), the latter derivatization procedure was preferred because it can simultaneously determine the concentration of 6-acetylmorphine. The performance characteristics of these derivatization products are consistent with those reported in the literature [4, 7].

The extraction procedure was evaluated by monitoring the codeine and morphine recoveries at different pH conditions (Fig. 4), pH 8.5 ~ 9.5 appeared to be the optimal condition. In this study, NH_4Cl/NH_4OH buffer solution was used. Because NH_4OH is quite volatile, and pH value of the solution would tend to get lower after preparation, so pH 9.5 buffer was used for extraction.

II. *Recovery Efficiencies of the Extraction Procedures*

Controls prepared with known amounts of

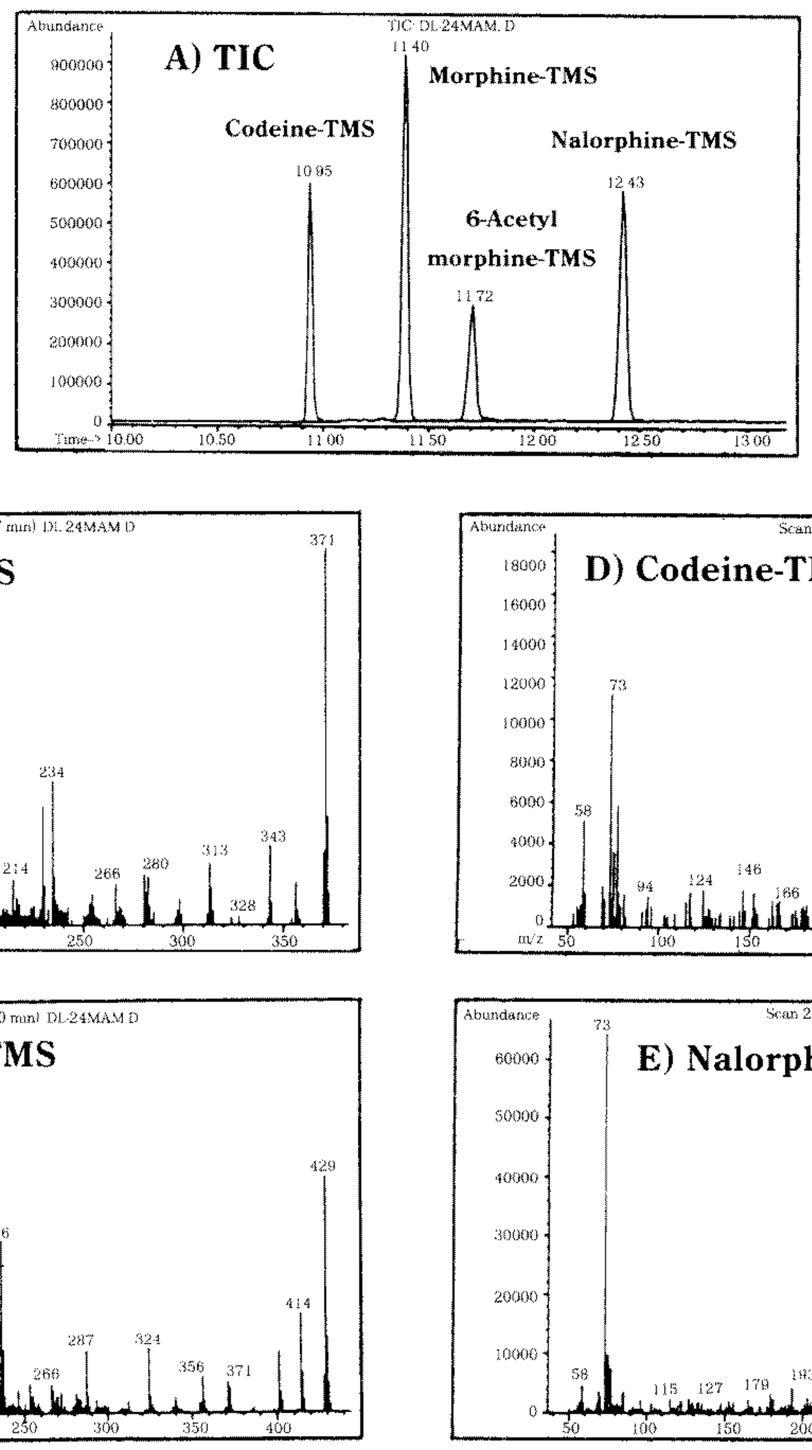

Figure 5. Electron impact mass spectra of total ion chromatogram (A), codeine-TMS (B), morphine-TMS (C), 6-acetylmorphine-TMS (D) and nalorphine-TMS (ISTD) (E).

Table 1. Recovery of codeine, morphine and 6-acetylmorphine (6-MAM) from fortified urine samples

Spiked conc. (ng/ml)		Compound Recovered (%)				
		Free (unhydrolyzed)			Hydrolyzed	
	n	Codeine	Morphine	6-MAM	Codeine	Morphine
50	3	96.2±1.2	77.0±1.7	85.4±2.1	99.5±2.1	87.6±3.8
100	3	103.5±4.1	77.6±4.8	86.7±1.8	97.7±4.2	89.±2.4
200	3	96.8±5.0	76.4±1.6	83.9±2.7	95.5±6.3	90.8±2.2
300	3	97.7±1.1	77.0±3.7	87.1±3.2	90.1±1.7	86.5±0.9
500	3	97.8±3.8	77.4±1.7	84.5±1.5	94.2±4.9	86.8±3.5
1000	3	97.5±2.3	74.9±4.3	85.8±0.9	91.7±3.4	89.6±0.7
Mean±S.D.		98.3±2.6	76.7±1.0	85.6±1.2	94.8±3.5	88.4±1.7

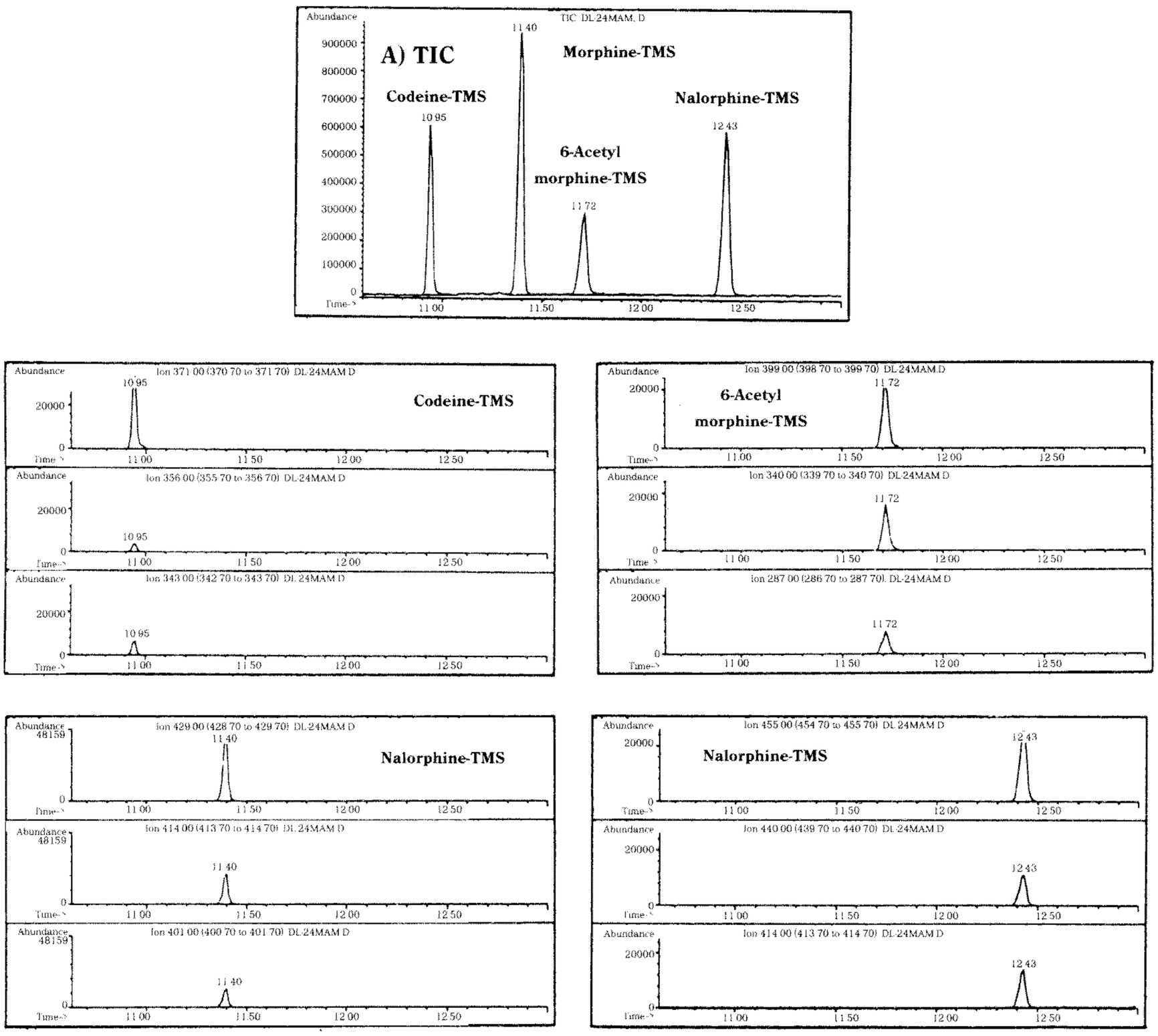

Figure 6. Selected Ion Montoring (SIM) mode of codeine-TMS (m/z 371 and 356, 343); morphine-TMS (429 and 414, 401); 6-acetylmorphine-TMS (399 and 340, 287); nalorphine-TMS (455 and 440, 414).

Table 2. Interday precision for analysis of urine fortified with free codeine

Spiked conc. (ng/ml)	n	Observed Mean conc.	Standard deviation	C.V. (%)
50	9	53.83	5.18	7.72
100	9	101.84	4.53	4.83
200	9	196.02	3.74	2.05
300	9	299.62	9.88	5.76
500	9	492.20	11.93	1.87
1000	9	1003.24	7.83	1.13

Table 3. Interday precision for analysis of urine fortified with free morphine

Spiked conc. (ng/ml)	n	Observed Mean conc.	Standard deviation	C.V. (%)
50	9	57.88	5.70	8.09
100	9	104.59	5.05	4.70
200	9	192.01	5.30	3.61
300	9	297.93	12.58	4.11
500	9	495.88	13.35	1.80
1000	9	1006.46	8.13	0.39

codeine, morphine, 6-acetylmorphine and nalorphine in drug- negative urine were treated by the described procedures. A typical full-scan GC/MS total ion chromatograms is shown in Fig. 5-A. The mass spectra of the TMS-derivatives of these drugs are shown in Fig. 5-B ~ 5-E. Ions selected for monitoring TMS-derivatives of codeine, morphine, 6-acetylmorphine and nalorphine were m/z 371, 356, 343; 429, 414, 401; 399, 340, 287 and 455, 440, 414, as shown in Fig. 6. No noticeable

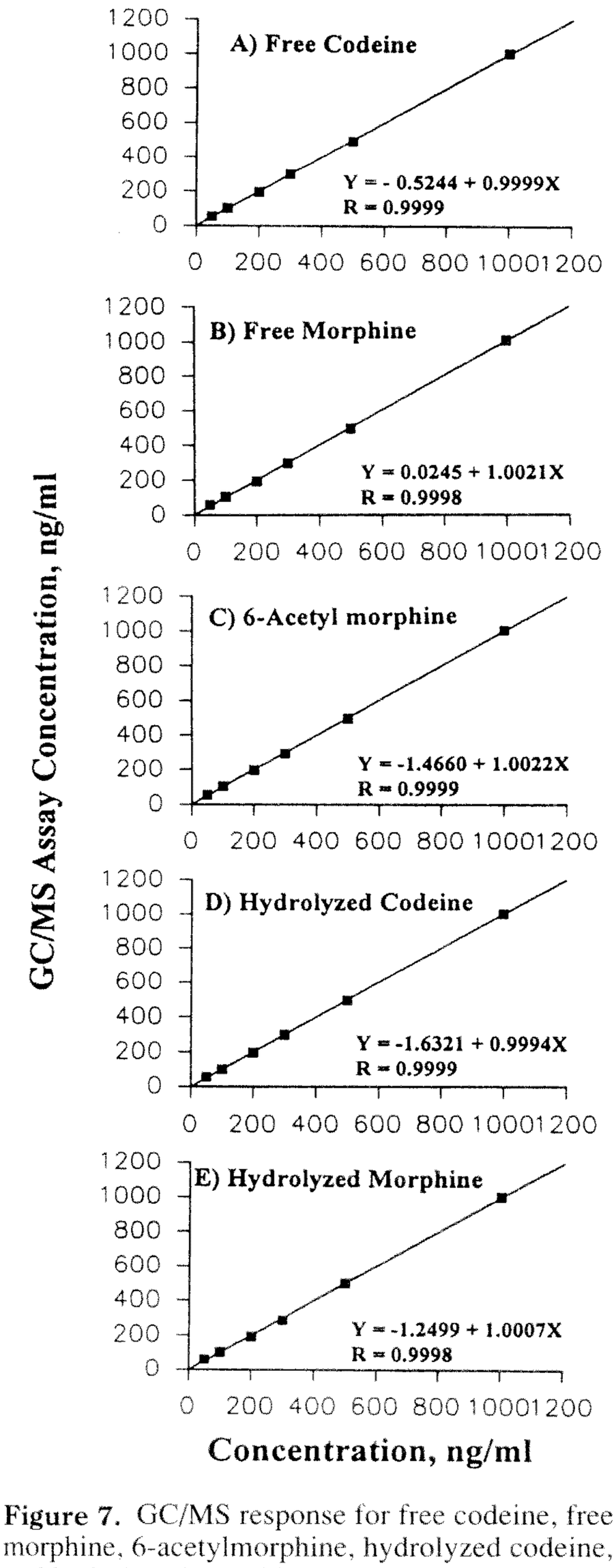

Figure 7. GC/MS response for free codeine, free morphine, 6-acetylmorphine, hydrolyzed codeine, and hydrolyzed morphine versus target concentrations of standard. Each data point represents the mean±SD for nine determinations.

Table 4. Interday precision for analysis of urine fortified with 6-acetylmorphine

Spiked conc. (ng/ml)	n	Observed Mean conc.	Standard deviation	C.V. (%)
50	9	54.21	4.24	5.73
100	9	103.78	6.73	6.15
200	9	195.34	6.46	3.18
300	9	292.47	8.93	4.26
500	9	495.23	10.29	1.78
1000	9	1004.89	7.63	0.49

Table 5. Interday precision for analysis of urine fortified with hydrolyzed codeine

Spiked conc. (ng/ml)	n	Observed Mean conc.	Standard deviation	C.V. (%)
50	9	53.49	3.23	4.13
100	9	99.23	6.61	5.83
200	9	194.54	6.30	1.49
300	9	296.88	15.02	6.27
500	9	494.42	11.64	1.51
1000	9	1000.41	9.12	0.47

Table 6. Interday precision for analysis of urine fortified with hydrolyzed morphine

Spiked conc. (ng/ml)	n	Observed Mean conc.	Standard deviation	C.V. (%)
50	9	58.90	3.55	5.10
100	9	102.54	6.83	3.56
200	9	192.53	5.61	3.13
300	9	287.84	13.91	4.98
500	9	498.87	7.70	7.59
1000	9	1003.24	4.14	0.31

by-products or impurities were observed. Two sets of controls containing 50, 100, 200, 300, 500, and 1000 ng/mL of morphine and codeine in drug-free urine were prepared to evaluate the efficiencies of the extraction and the hydrolysis/extraction procedures in recovering free morphine and codeine from the urine matrix. These two sets of controls were processed with the respective procedures without the addition of the internal standard until they are ready for the

derivatization step. A third set of controls containing the same corresponding amounts of morphine and codeine in methanol was prepared from the concentrate stock (1mg/mL). Controls in this set were derivatized in parallel with the controls in the other two sets to provide the basis for calculating the recovery efficiencies of the two procedures under evaluation. Results shown in Table I indicate the recovery for codeine and morphine using the extraction and the hydrolysis/extraction procedures are 98.3, 76.7; 94.8, 88.4%, respectively.

III. *Precision and Linearity of the Overall Procedure*

Controls containing 50, 100, 200, 300, 500, and 1000 ng/mL of codeine,morphine and 6-acetylmorphine in drug-free urine were prepared to evaluate the assay precision and the linearity of the over-all procedures. Both extraction and hydrolysis/extraction procedures were carried out. For precision evaluation, triplicates of controls at each concentration levels were analyzed for three times in one day and in three consecutive days. Interday precisions were calculated using one-way ANOVA [10]. Data thus obtained are shown in Table II ~ VI. The interday precisions range from 0.47 to 7.72% for codeine, 0.31 to 8.09% for morphine and 0.49 to 6.15% for 6-acetylmorphine.

Urine specimens containing known concentrations of 6-acetylmorphine, codeine and morphine were analyzed for the contents of 6-acetylmorphine, free codeine, free morphine, hydrolyzed codeine and hydrolyzed morphine by GC/MS assay. All concentrations were prepared and analyzed for nine times. The data are illustrated in Fig. 7 with regression statistics. The accuracies of the assays are reflected in the slope and intercept parameters. The GC/MS response was linear across each of the concentration ranges tested.

In summary, this method is simple and rapid to detect codeine, morphine and 6-acetylmorphine in urine with a single extraction procedure. With this detection system, the peak symmetry and quantitation have been very good and reproducible. The stabilities of trimethylsilyl-derivatives of codeine, morphine, 6-acetylmorphine and nalorphine also make this procedure very useful when storage of the derivatives at room temperature is unavoidable. This method is in routine use in our laboratory for the confirmation of enzyme immunoassay opiates-positive urine samples.

ACKNOWLEDGEMENT

The authors are thankful to Dr. Ray H. Liu of the University of Alabama at Birmingham, Birmingham, AL, U.S.A. for helpful discussion and assistance in the preparation of the manuscript.

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Journal of Food and Drug Analysis. 1996. 4(1)

尿中可待因、嗎啡與單乙醯嗎啡之簡易分析法

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摘　要

本研究開發並評估一簡易的氣相層析／質譜儀方法，供定量尿中free codeine, free morphine, 6-acetylmorphine, total codeine, total morphine。抽取用之緩衝液以 pH 值為 9.0 時，其抽取回收率最高。抽取回收率，free codeine 為98.3%，free morphine 為76.7%，6-acetylmorphine為85.6%，三甲基矽化衍生物在七十二小時內均非常安定，適合其定量分析。nalorphine 為內標準品，以 SIM (Selected Ion Monitoring) 方式定量。濃度在 50 至 1000 ng/mL 範圍內均具有良好的線性關係，日內及日間精密度試驗顯示，codeine 的變異係數為 0.47 ~ 7.72%，morphine 為 0.31 ~ 8.09%，6-acetylmorphine 為 0.49 ~ 6.15%。

關鍵詞：GC/MS定量，嗎啡，可待因，單乙醯嗎啡，尿液。