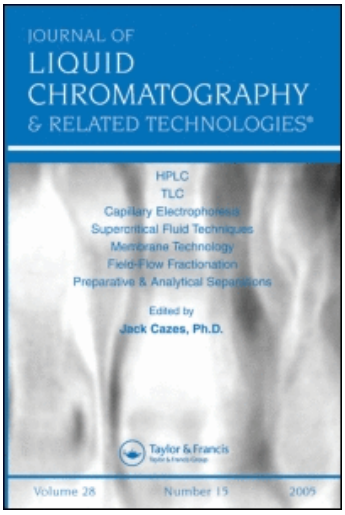

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 31 May 2001

To cite this Article Cheng, Yung-Fong , Neue, Uwe D. , Bonin, Robert , Block, Eric and Bean, Laura(2001) 'SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF CODEINE AND ITS METABOLITE IN URINE AND PLASMA BY LC/UV AND LC/MS USING MIXED-MODE CATION EXCHANGE FOR SAMPLE PREPARATION', Journal of Liquid Chromatography & Related Technologies, 24: 9, 1353 — 1364
To link to this Article: DOI: 10.1081/JLC-100103452
URL: http://dx.doi.org/10.1081/JLC-100103452

J. LIQ. CHROM. & REL. TECHNOL., 24(9), 1353–1364 (2001)

SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF CODEINE AND ITS METABOLITE IN URINE AND PLASMA BY LC/UV AND LC/MS USING MIXED-MODE CATION EXCHANGE FOR SAMPLE PREPARATION

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ABSTRACT

Novel mixed-mode cation exchange (MCX) and HPLC methods were developed to determine codeine and its metabolite from human urine and porcine plasma. For sample cleanup, spiked sample solutions were acidified followed by a well-thought-out MCX method using 3 cc, 60 mg Oasis® MCX cartridges. In this MCX method, there is no need to condition and to equilibrate the sorbent; the sample is loaded directly onto the sorbent. Recoveries from human urine and porcine plasma matrices were greater than 87% with RSDs less than 5.4%.

For the HPLC analysis, the separation was obtained using a Symmetry® C_{18} column with a simple mobile phase of 0.05% TFA/acetonitrile/methanol at 90:5:5. Good peak shapes were

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obtained without the need of addition of any competing reagent to the mobile phase.

INTRODUCTION

Codeine has been used widely as an analgesic and antitussive agent.[1-2] It is used for the relief of mild to moderate pain. The main metabolic pathway of the codeine in humans is via conjugation with glucuronic acid to form codeine-6 glucuronide. Due to the differences in the pharmacokinetics of codeine among different individuals, it is necessary to have a sensitive and reliable method to simultaneously quantitate the codeine and its metabolite in human urine and plasma. Many analytical methods have been applied to the quantitation of the codeine and its metabolite. These include RIA,[3] GC,[4] GC-MS,[5-7] and HPLC.[8-10] For the HPLC assays, many authors[8-10] used ion-pairing chromatography with UV detection to achieve good separation, as well as good peak shapes.

The purpose of the present study is to develop a simple and straightforward mixed-mode cation exchange (MCX) method, as well as a simplified HPLC method for the determination of codeine and its main metabolite from urine and plasma matrices.

EXPERIMENTAL

Reagents and Materials

Codeine, codeine-6-glucuronide, and ranitidine were purchased from Alltech-Applied Science (State College, PA). Methanol, TFA, and phosphoric acid were HPLC-grade quality from J. T. Baker (Phillipsburg, New Jersey). Three different types of human urine were obtained from laboratory personnel, including two Caucasians (male and female) and one Asian (male). Porcine plasma (with EDTA) was purchased from Equitech-Bio (West Ingram, TX). Oasis® MCX cartridges (3cc, 60 mg, 30 μm) were procured from Waters Corporation (Milford, MA). Calibration curves were generated over a concentration range from 0.02 to 0.4 μg/mL for both codeine and codeine-6-glucuronide. Each standard solution contained 0.09 μg/mL ranitidine as the internal standard.

Solid-Phase Extraction Procedure

Figure 1 outlines the general mixed-mode cation (MCX) extraction procedure developed on the 3cc Oasis® MCX extraction cartridges. The cartridges

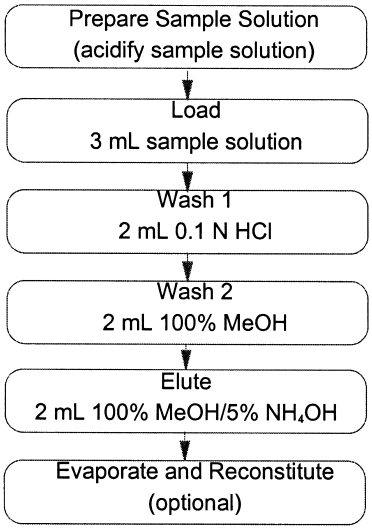


Figure 1. General mixed-cation exchange (MCX) pprotocol for the extraction of basic drugs from human urine and plasma matrices.

were placed on a 20-position Waters Extraction Manifold attached to a Waters vacuum pump.

Two levels of sample concentrations were prepared. The concentrations for codeine and codeine-6-glucuronide were 0.60 μg/mL and 0.12 μg/mL for high and low levels, respectively. Each sample solution contained 0.24 μg/mL of ranitidine as the internal standard. Spiked urine samples were then acidified with hydrochloric acid to bring the final acid concentration to 0.05N of HCl. Spiked plasma samples were acidified with phosphoric acid to a final concentration of 2% phosphoric acid. The acidified sample solutions were loaded onto the extraction cartridges (there is no need for conditioning and equilibration prior to the sample loading). After loading 3.0 mL of acidified sample solution onto each cartridge, it was first washed with 2 mL of 0.1 N HCl, followed by 2 mL of 100% methanol.

Finally, the analytes were eluted with 2 mL of 95% methanol containing 5% ammonium hydroxide. No evaporation and reconstitution was performed. The final eluates were diluted with water in a ratio of 1:3. The final diluted sample solutions were then analyzed via HPLC and also confirmed by LC/MS as described in the following section.

Instruments and Operating Conditions

Isocratic elution was used throughout the entire study. The HPLC system consisted of a Waters Alliance system equipped with a 2690 module and a column heater unit. Detection was carried out using a Waters 996 photodiode detector at 220 nm. The Millennium32 Chromatography Manager was used to control the HPLC system and to perform data acquisition and manipulation. The column used was a Waters Symmetry® C_{18} column (2.1 mm x 150 mm, 3.5 μm particle size) proceeded by a Sentry™ guard column (2.1 mm x 10 mm, 3.5 μm particle size). The elution was carried out at 30°C.

The mobile phase consisted of 0.05% TFA:acetonitrile:methanol at 90:5:5. The flow rate was set at 0.3 mL/min. For the concentration determination of the analytes, 80 μL each of the sample and the standard solution were injected.

Mass Spectrometry was performed on a Waters/Micromass Platform LCZ equipped with a gas-assisted nebulization electrospray interface. Full-scan continuum spectra were obtained in the positive-ion mode by scanning the quadrupole from 50 to 250 Da at a scan rate of 1 second/scan. A sampling cone voltage of 30 V was used for these experiments. The injection volume was 5 μL.

RESULTS AND DISCUSSION

HPLC Separation Using a Simple Mobile Phase

The structures of codeine, codeine-6-glucuronide (the metabolite of codeine), and ranitidine (the internal standard) are shown in Figure 2. Codeine is a basic analyte containing a tertiary amine. The metabolite, codeine-6-glucuronide, is an amphoteric compound containing a tertiary amine and a carboxylic acid group. These analytes interact with the residual silanol sites present on silica-based sorbents, which causes peak tailing in the HPLC separation. To minimize the peak tailing, it has been shown that the addition of sodium dodecyl sulfate is needed in order to obtain good peak shapes and good separation.[8-10] In this study, we were able to obtain good peak shapes using a simple mobile phase, 0.05%TFA-actonitrile-methanol (90:5:5, v/v/v).

The representative chromatograms of human urine blanks, porcine plasma blank, and spiked samples from four different matrices, are shown in Figure 3. The respective blank is always compared to the chromatogram from the same spiked sample solution. All three peaks/components are well separated, and the elution sequence is ranitidine (peak 1, internal standard, spiked concentration at 0.24 μg/mL), codeine-6-glucuronide (peak 2, metabolite, spiked concentration at 0.60 μg/mL), and codeine (peak 3, spiked concentration at 0.60 μg/mL). Good

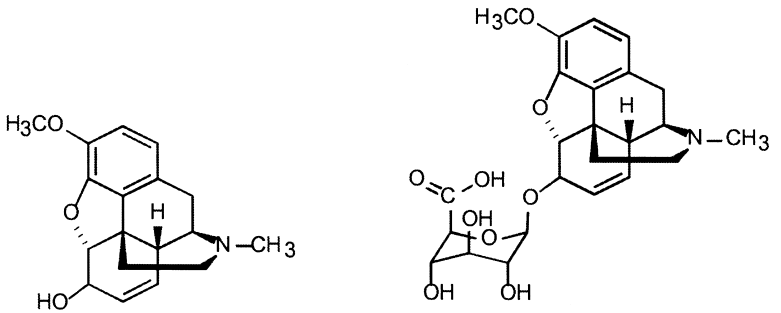


Figure 2. The structures of codeine, codein-6-glucuronide (the metabolite of codein), and ranitidine (used as internal standard). Codeine is a basic analyte containing a tertiary amine. The metabolite is an amphoteric compound containing a tertiary amine and a carboxylic acid group.

peak shapes were obtained, and the USP tailing factors were all between 1.14 and 1.25.

The HPLC calibration curves were based on peak-area ratio to the internal standard, ranitidine, and were performed according to a least-squares fit of the data to a straight line. Within the concentration range described in the experimental section, linear plots were achieved for codeine and its metabolite. The correlation coefficients were 0.999911 and 0.999980 for codeine and its metabolite, respectively.

Recovery of Codeine and Its Metabolite

Sample preparation is required to maximize the quality of the analytical results and to minimize the downtime of expensive, high-overhead, analytical tools such as LC/MS/MS systems. Historically, liquid-liquid extraction and protein precipitation have been the bottleneck in laboratory productivity since they are not easy to automate and the protocol is rather time-consuming. Presently, solid-phase extraction (SPE) has proven to be more readily automated for high sample throughput.[11-12] It was our objective to develop a simple protocol to isolate the basic codeine analyte and its amphoteric metabolite (codein-6-glucuronide) from biofluids. This simple MCX protocol is outlined in Figure 1.

In this protocol, we first acidify the sample solution. The acidified sample solution is directly loaded onto the sorbent without the need to condition and equilibrate the MCX sorbent. The acidification of the sample solution not only facilitates releasing bound drugs from proteins present in the sample matrices,

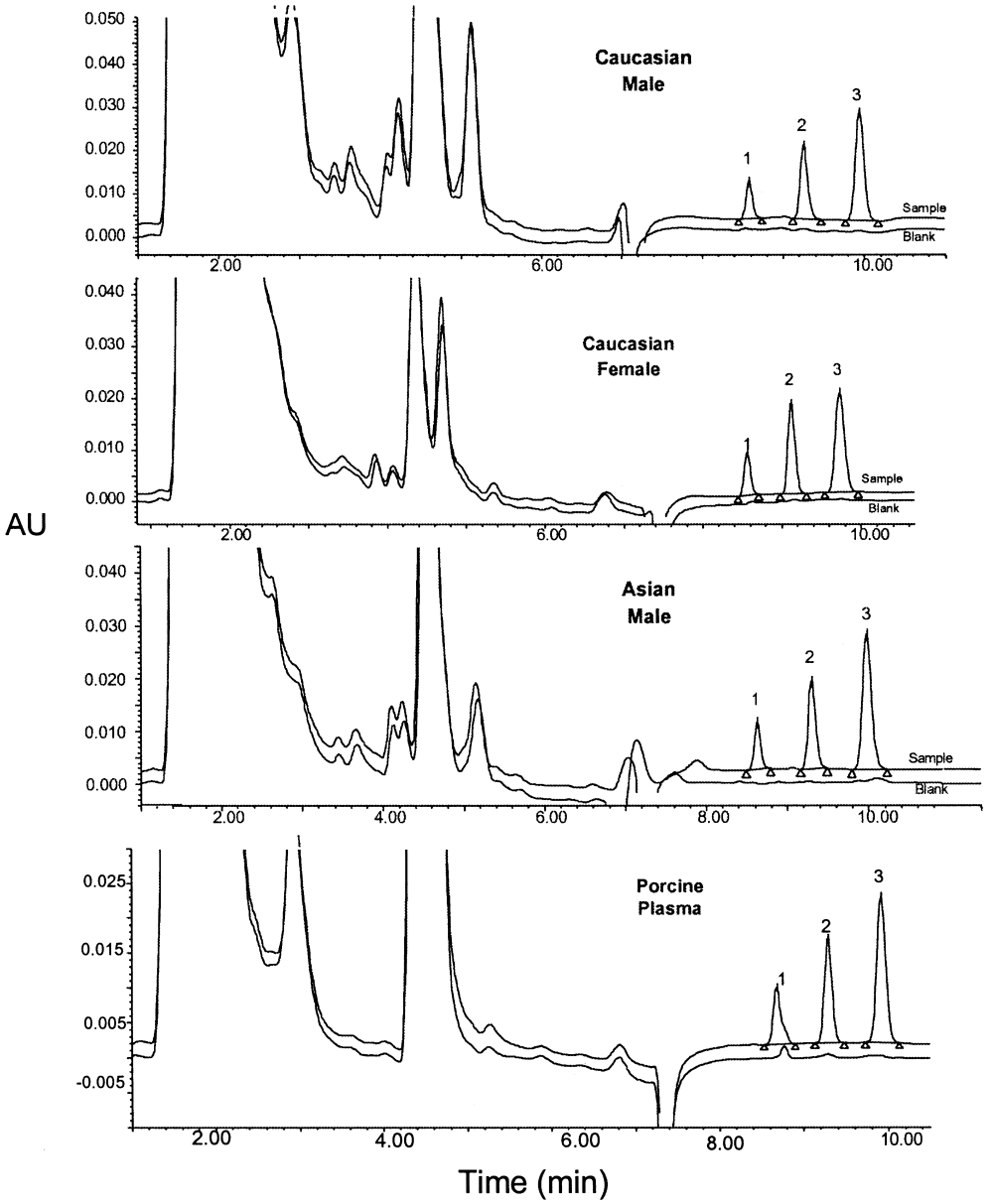


Figure 3. Representative chromatograms of human urine blanks, porcine plasma blank, and spiked samples from four different matrices. Peak 1 is ranitidine (the internal standard); peak 2 is codeine6-glucuronide (the metabolite); peak 3 is codein. Good peak shapes were obtained, and the USP tailing factors are all between 1.14 and 1.25.

but also activates the binding of the analytes onto the cation-exchange sites via the protonated amino groups. The next step (the first wash step) is to wash with 0.1 N HCl; this step is to remove polar non-ionic interferences while maintaining the binding of the basic analytes onto the cation-exchange sites. The third step (the second wash step) is to wash with 100% methanol; the purpose of the methanol wash is to remove hydrophobic (including acidic and neutral analytes) interferences. Once again, the basic analytes are retained on the exchange sites. The fourth step (the elution step) is to elute the analytes of interest with a methanol solution at a high pH value. The change in the pH value interrupts the binding of the basic analytes to the ion-exchanger.

This generic protocol can be used for a range of analytes, which can carry a positive charge. To demonstrate the application of the generic MCX protocol, three different urine and one porcine plasma matrices spiked with codeine and its metabolite were investigated. The chromatograms after the cleanup steps are shown in Figure 3. The recovery results for codeine and its metabolite from these different matrices are summarized in Table 1. For the Asian male urine, the recoveries were all greater than 88.5% with RSDs less than 5.4%. For the Caucasian male urine, the recoveries were all greater than 87.2% with RSDs all less than 5.4%. For the Caucasian female urine, the recoveries were all greater

Table 1. Recoveries of Codeine and Its Metabolite in Spiked Human Urine and Porcine Plasma Sample Matrices. The Urine Sample Was Acidified with 5 N of HCl to 0.05 N HCl, While the Plasma Sample Was Acidified to Contain 2% Phosphoric Acid. No Conditioning and Equilibration Is Needed

		Asian Male Urine		Caucasian Male Urine		Caucasian Female Urine		Porcine Plasma	
Compound	Conc (μg/mL)	Rec (%)	RSD (n=3)	Rec (%)	RSD (n=3)	Rec (%)	RSD (n=3)	Rec (%)	RSD (n=3)
Codeine	0.6 (H)	99.5	1.2	102.5	3.9	110.3	2.4	103.4	0.9
	0.12 (L)	88.5	2.7	87.2	4.6	99.0	2.1	95.4	5.0
Codeine Metabolite	0.6 (H)	98.7	0.4	109.3	5.4	114.1	2.3	104.6	1.3
	0.12 (L)	99.3	5.4	96.5	5.3	111.4	2.1	106.8	1.9
Ranitidine (IS)	0.24 (H)	89.7	1.5	95.9	4.3	94.3	4.1	98.5	0.8
	0.24 (L)	93.1	2.6	96.6	2.9	91.3	3.5	93.3	1.9

than 91.3% with RSDs all less than 4.1%. Lastly, for the porcine plasma matrix, the recoveries were all greater than 93.3% with RSDs all less than 5.0%. The absolute recovery of the internal standard, ranitidine, was determined by comparing the average peak area from replicate analyses to the average peak area of ten injections of standard. The mean recoveries of the internal standard were all greater than 89.7% with RSDs all less than 4.3%. It is noteworthy, that all the experiments discussed above were performed without conditioning and equilibrating the 3cc Oasis® MCX cartridges; no adverse impact of performance was observed. This further simplifies the SPE procedure.

With this MCX protocol (outlined in Figure 1), we can achieve high sensitivity and high selectivity for basic drugs. Most important is the selective retention of basic drugs by a single type of cation-exchange group with a known and high ion-exchange capacity (1 meq/gram). There are no silanol groups to complicate the retention mechanism between the basic drugs and the sorbent. This novel, water-wettable, polymeric sorbent is stable from pH 0 to 14, making the methods development simple and fast. Excellent results were obtained for several basic drugs using the exact same MCX protocol (outlined in Figure 1). Table 2 shows that recoveries greater than 90 % with RSDs less than 3.4% can be achieved routinely across a broad range of basic drugs (containing amine functional group). Silica-based sorbents with mixed-mode functionalities do not perform the same for all basic drugs.

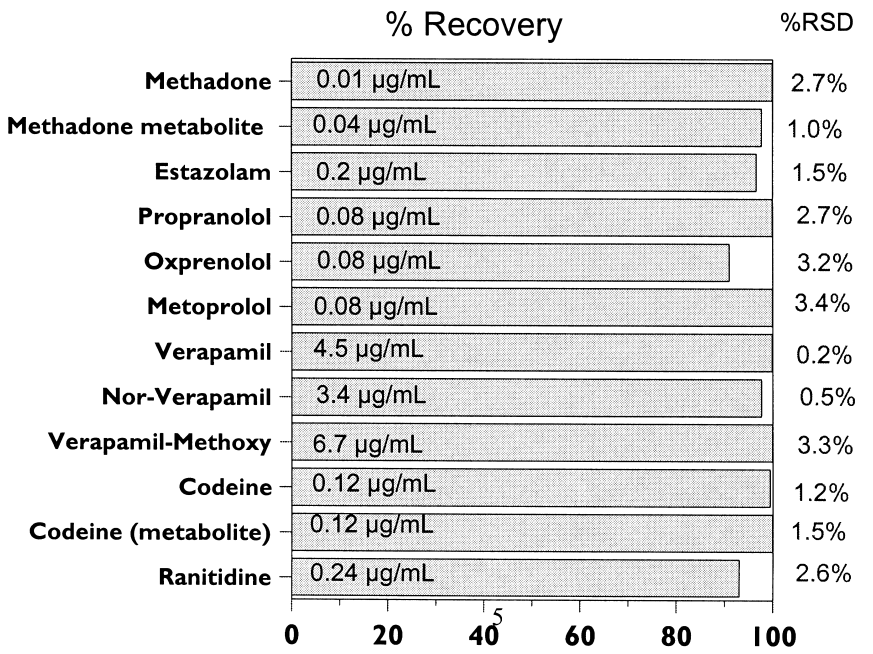
Study of Urine Excretion Profile

The MCX method described above has been applied to obtain urine concentration/time profiles for codeine and codeine 6-glucuronide (the metabolite) from a patient following a single oral dose of 60 mg codeine. Figure 4 depicts the excretion profile of codeine and its metabolite (codeine-6-glucuronide) collected from 0 hour to 21 hours after dosage. This result agrees with the expected urine excretion profile.

The identification of codeine and its metabolite is confirmed by the LC/MS analysis. Figure 5 shows the resulting total ion chromatogram (TIC) obtained from the injection of urine obtained at 10.3 hour. Also, shown in this figure are the mass spectra of the chromatographic peaks observed at 4.4, 5.8, and 7.0 minutes. On the basis of these mass spectra, the peaks are identified as ranitidine, codeine-6-glucuronide, and codeine, respectively.

The mass spectra of codeine and its metabolite are marked by intense protonated molecular ions with little fragmentation, as would be expected due to their highly cyclic structure (see Figure 1). However, under these conditions significant fragmentation was observed for the internal standard ranitidine. The mass spectrum of this compound displayed a strong ion at m/z 270; presumably

Table 2. High Recoveries with Low RSDs (n=3) of Basic Drugs from Human Urine Matrices Following the Same Elution Protocol Outlined in Figure 1



through the loss of dimethyl amine. The ion observed at m/z 224 may arise from the loss of NO_2 from the primary fragment ion at m/z 270, but further confirmatory experiments were not performed.

CONCLUSIONS

We have developed simple HPLC and SPE methods for the determination of codeine and its metabolite in urine and plasma matrices. With a simple HPLC mobile phase, good peak shapes and good separations of the analytes were obtained. With the simple MCX protocol, high and consistent recoveries were obtained using the Oasis® MCX sorbent. With this SPE protocol, there is no need to condition and to equilibrate the sorbent; just load the sample, wash off interferences, and then elute. This further simplifies the SPE method. The method out-

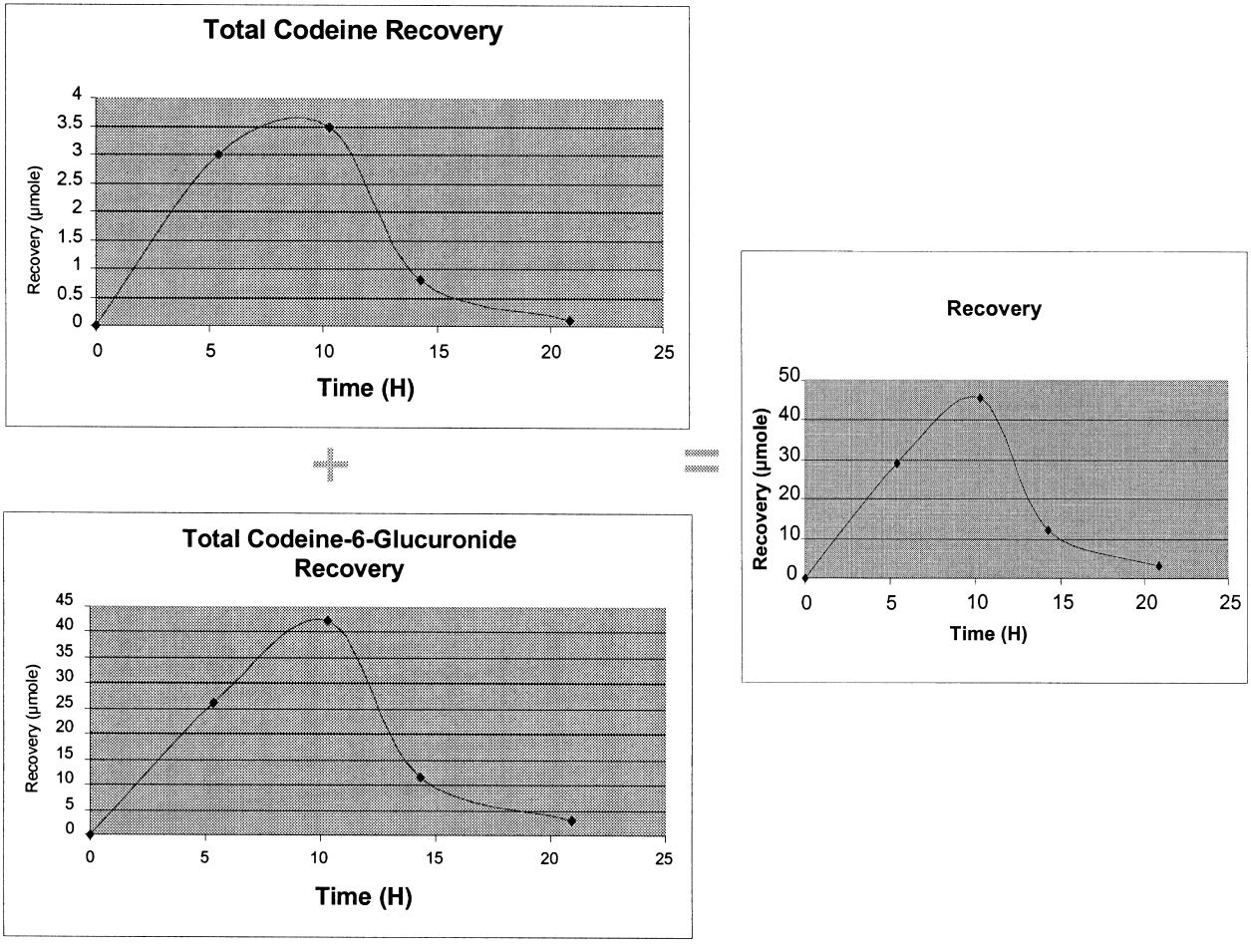


Figure 4. The urine excretion profile from a patient following a single oral dose of 60 mg codeine.

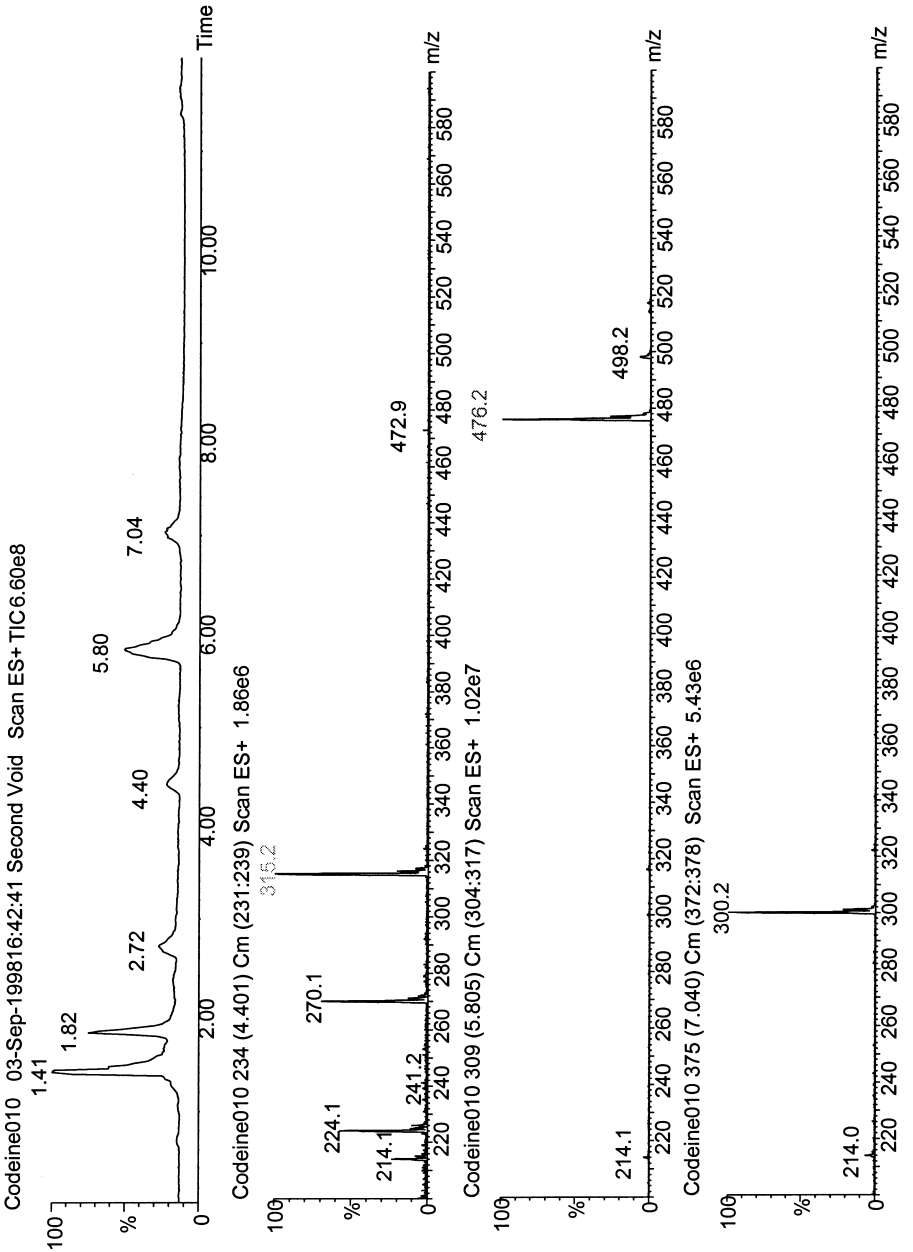


Figure 5. Positive compound confirmation by LC/MS from the injection of sample at 10.3 hours shown in Figure 4.

lined here can be used as a generic method suitable for the extraction and enrichment of analytes with basic functional groups from plasma and urine.

ACKNOWLEDGMENT

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Received October 29, 2000 Manuscript 5429
Accepted November 11, 2000