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Simultaneous determination of codeine and it seven metabolites in plasma and urine by high-performance liquid chromatography with ultraviolet and electrochemical detection

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Abstract

A sensitive and selective high-performance liquid chromatography method has been developed for the measurement of codeine and its seven metabolites, norcodeine, morphine, normorphine, codeine-6-glucuronide, morphine-6-glucuronide, morphine-3-glucuronide and norcodeine glucuronide, in plasma and urine. The compounds were recovered from plasma and urine using solid-phase extraction with C_{18} cartridges and separated on a reversed-phase C_8 column with a mobile phase consisting of 77% buffer (5 m*M* sodium phosphate monobasic and 0.70 m*M* sodium dodecyl sulfate, pH 2.35) and 23% acetonitrile. Codeine, norcodeine, codeine-6-glucuronide, norcodeine glucuronide and morphine-3-glucuronide were detected by ultraviolet detection at 214 nm, with a detection limit of 0.02 nmol/ml for each compound in plasma. Morphine-6-glucuronide, normorphine and morphine were monitored by electrochemical detection at 350 mV, with a detection limit of 0.003 nmol/ml for each compound in plasma. The assay showed good reproducibility and accuracy using external standardization. The recovery and inter-day variation for all compounds in plasma samples were 63.40–77.90% and 3.49–16.77% (R.S.D.) and while in urine were 64.98–90.13% and 2.93–9.96% (R.S.D.), respectively. © 1998 Elsevier Science B.V.

Keywords: Codeine; Norcodeine; Morphine; Normorphine

1. Introduction

Codeine (COD) is widely used as an analgesic and antitussive agent [1–4]. Codeine is metabolized by *N*-demethylation to norcodeine (NC), by *O*-demethylation to its active metabolite morphine (MOR), and by conjugation to codeine-6-glucuronide (C6G). Subsequently morphine and norcodeine are metabolized by *N*-demethylation and conjugation to normorphine (NM), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and norcodeine glucuronide (NCG). C6G has a similar activity to codeine itself [1]. Following codeine administration, morphine is only present in low concentrations in plasma but contributes substantially to codeine's analgesic effect. M6G has a stronger analgesic effect than morphine [5–10].

In order to define interindividual variability in the

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metabolism of codeine in humans and, particularly, its activation to morphine, a sensitive and reliable method is required to simultaneously measure codeine and its metabolites in human plasma and urine.

Several analytical methods have been reported for the determination of codeine and its metabolites. Findlay et al. first used RIA to measure codeine and morphine [11–13]. Later, gas chromatography–mass spectrometry (GC–MS) was used for the measurement of the two compounds [14–17]. A number of high-performance liquid chromatography (HPLC) methods [18–29] have been described for determination of codeine and/or some of its metabolites. Svensson and co-workers used ion-pair chromatography with ultraviolet (UV) and electrochemical (EC) detection to measure MOR, NM and M6G [18,20]. Codeine and seven metabolites were measured by a modification of Svensson's methods [24]. However the mobile phase pH was not suitable for measuring COD and NC in plasma samples, because there was an interfering peak (UK2) close to COD and NC. Verwey-Van Wissen et al. used HPLC with two electrochemical detectors for determination of codeine and its six metabolites [27]. The use of two EC detectors, however, did not provide much more selectivity than UV and EC, and detection sensitivity for M3G was lost.

In order to achieve complete separation with high sensitivity for simultaneous measurement of codeine and its seven metabolites in human plasma and urine, a modified HPLC method with UV and EC detection coupled to C_{18} solid-phase extraction is described in this paper.

2. Experimental

2.1. Chemicals

Sodium phosphate monobasic and sodium dodecyl sulfate (HPLC grade) were purchased from Fisher (Springfield, NJ, USA), UV grade acetonitrile from EM Science (Gibbstown, NJ, USA), ammonium sulfate, codeine, norcodeine hydrochloride trihydrate, morphine-6-glucuronide dihydrate, morphine-3-glucuronide sulfate from Sigma (St. Louis, MO, USA), normorphine and codeine-6-glucuronide from The National Institute on Drug Abuse (Rockville, MD, USA).

2.2. Stock solutions

All standards of codeine and its metabolites were dissolved in water to yield the following concentrations (nmol/ml): M3G, 407.37; M6G, 82.01; NM, 298.24; MOR, 158.14; C6G, 1286.53; NC, 659.93; and COD, 1670. Quinidine 1541.3 nmol/ml and rifampin 607.0 nmol/ml and erythromycin 340.6 nmol/ml were prepared in methanol–water (50:50).

2.3. Chromatography

The chromatographic apparatus consisted of a Model 6000A pump, two 730 data modules, a 740 data module, a Wisp 710A autosampler (Waters Corporation, Milford, MA, USA); the analytical column was a Zorbax C_8 column 150×4.6 mm, 5 μm (MAC-MOD analytical, Inc., Chadds Ford, PA, USA). A spectroflow 773 UV detector (Kratos Analytical Instruments, Ramsey, NJ, USA), set at a wavelength of 214 nm with full scale range 0.002 AU, and a Coulochem 5100A electrochemical detector (ESA, Chelmsford, MA, USA), were used with a 5021 conditioning cell and a 5011 analytical cell, set to an oxidation potential of 200 mV for the conditioning cell and at 250 mV (E1) and 350 mV (E2), using a gain of 66×10. C_{18} cartridges (Waters Corporation, Milford, MA, USA) were used for extraction.

The mobile phase consisted of 77% buffer containing 5 m*M* sodium phosphate monobasic and 0.7O m*M* sodium dodecyl sulfate and 23% acetonitrile. The pH of the aqueous buffer in the mobile phase was adjusted using phosphoric acid to pH 2.35 for plasma samples and 2.90 for urine samples. In order to minimize background noise the solvent mixture was filtered with 0.22-μm Nylon 66 filters (Rainin Instrument, Woburn, MA, USA) and degassed using a magnetic stirrer under a vacuum. A mobile phase flow-rate of 1.0 ml/min was used at room temperature.

2.4. Sample preparation

Plasma and urine were kept frozen (−20°C) until analysis. Following thawing, 1.0 ml plasma or 1.0 ml

diluted urine (urine diluted five times with water) was mixed with 3 ml of 0.5 *M* ammonium sulfate (pH 9.3) and passed through a pretreated Sep-Pak cartridge (pretreatment solvent: 4 ml methanol, 4 ml elution solvent and 4 ml water). The cartridge was then washed with 10 ml of 5 m*M* ammonium sulfate solution (pH 9.3) and 1.0 ml of water. To elute codeine and its metabolites from the C_{18} Sep-Pak cartridge 1.2 ml of 22% acetonitrile in 0.20% phosphoric acid (elution solvent I for plasma) or 28% acetonitrile in 0.20% phosphoric acid (elution solvent II for urine) was used. In order to further purify the sample, the eluate collected from the first cartridge was extracted again using the same technique over another pretreated C_{18} Sep-Pak cartridge. For plasma, 200 μl, and for urine, 50 μl of the eluted solution were injected onto the HPLC.

2.5. Calibration

Standard curves for codeine and its metabolites were constructed by adding standard stock solution to 1 ml blank plasma or 1.0 ml diluted urine. For plasma, the analyte concentrations (n*M*) were: codeine, 33.40–1336.0; NC, 26.39–527.94; C6G, 102.92–20584; MOR, 3.16–158.14; NM, 5.96–715.78; M6G, 3.28–131.22; and M3G, 24.44–814.74 for plasma samples. For 1.0 ml diluted urine (0.2 ml urine added to 0.8 ml water), the concentration of standard solutions (n*M*) were: COD, 167.0–6680.0; NC, 131.99–2639.7; C6G, 462.48–92495; MOR, 15.81–632.56; NM, 29.82–3578.9; M6G, 27.34–1640.2; and M3G, 122.21– 4073.7. External standardization was used to measure the concentrations of all compounds based on peak heights. Peak heights for all peaks were reported in integrator units.

2.6. Reproducibility studies

Five replicate analyses of spiked plasma or urine samples at three concentrations of codeine and its metabolites were performed as described above.

2.7. Drug interference study

Potential interference of quinidine, rifampin and erythromycin with this assay were studied by injecting 10 μl of each stock solution.

3. Results and discussion

3.1. Chromatography

In order to obtain complete separation of all eight compounds and to resolve them from other peaks in plasma or urine, the influence of pH on column retention was studied. Using a mobile phase pH 2.15 gave good separation of morphine and its metabolites, but it was difficult to simultaneously measure COD and its seven metabolites at this pH, because of an unknown plasma peak (UK2) close to NC and COD. In addition the separation of NC and COD was poor. The retention times of M3G, M6G, NCG, C6G, UK2, NM and MOR were dependent on the pH of the mobile phase. The higher the pH, the shorter the retention time of the first five compounds, and the longer the retention time of NM and MOR. We therefore adjusted the pH of the mobile phase to achieve optimal separation of C6G, NCG, NM, MOR, M3G and UK2 in plasma extracts. The optimal buffer pH of mobile phase for plasma samples was pH 2.35 for separation of M3G, NCG, C6G, NM, MOR, M6G, NC, and COD from each other. This pH also provided good separation of all compounds from the endogenous UK1 and UK2 peaks. For urine samples, pH 2.90 provided optimal separation. Using this mobile phase pH the retention times of M6G and UK2 were very close. However, as UK2 was only detected by UV absorption, it did not interfere with the EC detection of M6G. The concentration of sodium dodecyl sulfate was varied with the concentration of acetonitrile to alter the retention time of all compounds. Generally, the higher the concentration of sodium dodecyl sulfate or the lower the concentration of acetonitrile, the longer the retention time of all compounds. Generally, selectivity was poor at 214 nm UV detection, but its use looks acceptable as compounds are known and sample composition is preliminarily checked. Typical chromatograms showing the separation of codeine and its seven metabolites in plasma and urine can be seen in Figs. 1 and 2. The retention time for each compound is shown in Table 1.

3.2. Extraction

The following solid-phase extraction cartridges were compared: strong cation exchange (SCX),

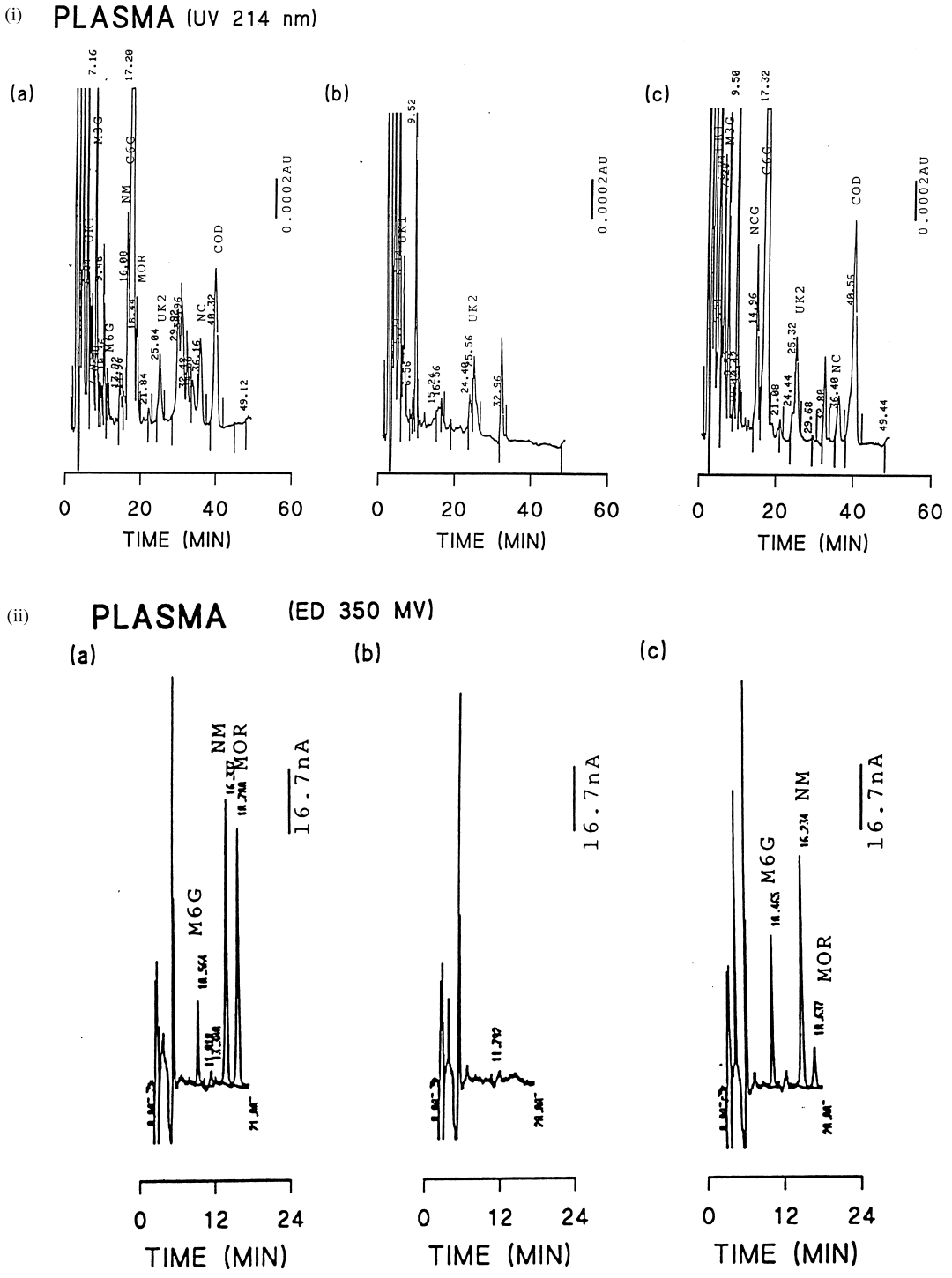

Fig. 1. Chromatograms of (a) human plasma to which has been added standard solution yielding 0.33 nmol/ml of M3G; 0.05 nmol/ml of M6G; 0.24 nmol/ml of NM; 0.06 nmol/ml of MOR; 2.57 nmol/ml of C6G; 0.20 nmol/ml of NC, and 0.50 nmol/ml of COD; (b) human plasma and (c) human plasma collected 90 min after administration of 120 mg of codeine.

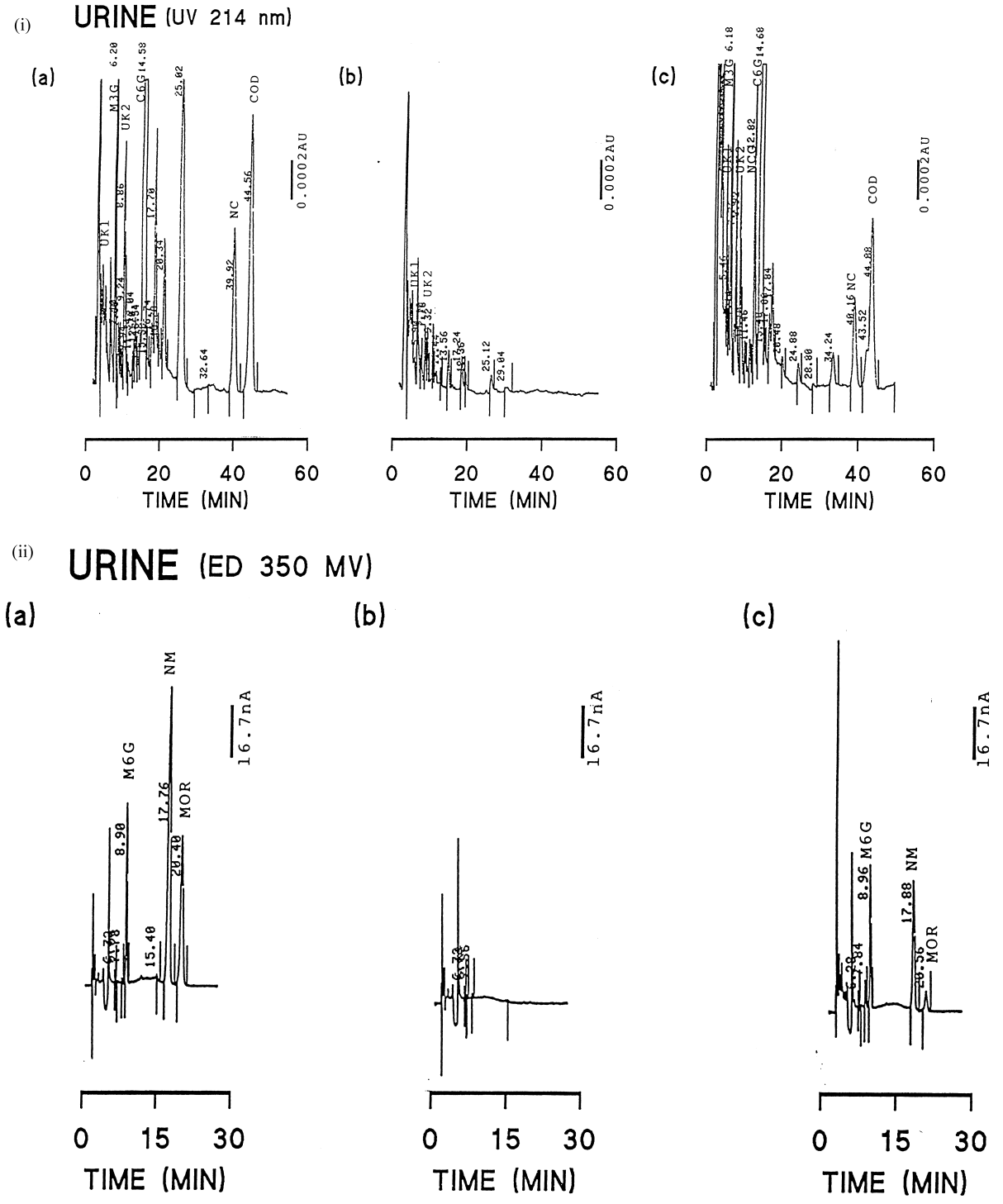

Fig. 2. Chromatograms of (a) human urine to which has been added a standard solution yielding 1.63 nmol/ml of M3G; 0.55 nmol/ml of M6G; 1.19 nmol/ml of NM; 0.24 nmol/ml of MOR; 11.56 nmol/ml of C6G; 1.32 nmol/ml of NC, and 2.50 nmol/ml of COD; (b) human urine and (c) human urine collected 0–11 h after administration of 120 mg of codeine.

Table 1
Retention time of codeine and its metabolites

Compound	Retention time (min) (mean±S.D., $n=5$)	
	Plasma samples[a]	Urine sample[b]
UV (214 nm)		
UK1	6.08±0.10	5.32±0.30
M3G	7.50±0.33	6.04±0.25
UK2	32.11±1.71	8.87±0.10
NCG	15.90±0.49	11.80±0.73
C6G	18.37±0.67	13.61±0.99
NC	44.16±1.77	37.07±2.72
COD	49.30±2.03	41.39±2.97
EC (350 mV)		
M6G	10.87±0.51	8.49±0.45
NM	19.00±0.72	16.91±0.85
MOR	21.88±0.84	19.28±1.13

[a]Mobile phase (pH 2.35).
[b]Mobile phase (pH 2.90).

carboxylic acid cation exchange (CBA), silica (Varian, Harbor City, CA, USA) and C_{18} (Waters). Many interfering peaks were found when using the SCX and CBA cartridges, and low recoveries of M3G and M6G were obtained using the silica cartridges. We therefore optimized the extraction procedure using C_{18} cartridges. We used the previously described buffer (0.5 *M* ammonium sulfate, pH 9.3) [18], along with an elution mixture consisting of 22% acetonitrile in 0.2% phosphoric acid for plasma samples and 28% acetonitrile in 0.2% phosphoric acid for urine samples. These two solvent mixtures eluted all of the compounds from C_{18} cartridges with good recoveries and fewer interfering peaks than other solvent systems. We had previously tried to use the C_{18} cartridges with the buffer system described by Yue et al. [24]. However we found that the recovery of M3C and M6G using the Yue solvent at a high percentage of acetonitrile was low, or conversely at a low percentage of acetonitrile the recovery of COD and NC was low.

3.3. Detection limit and validation

The detection limit of the assay was 0.003 nmol/ml for M6G, NM, and MOR; 0.02 nmol/ml for M3G, C6G, NC and COD in plasma ($S/N>3$). The concentrations of codeine and its metabolites in urine were about 20-fold higher than those in plasma. So that the detection limit for plasma and urine samples were similar. The correlation coefficients of the standard curves for all compounds in plasma and urine were excellent (Table 2). Because a NCG reference standard was not available, the concentrations of NCG in plasma and urine were calculated using standards of C6G at appropriate concentrations.

The precision of the assay was determined from the repeated analysis of three concentrations of all compounds on 1 day and over 5 days ($n=5$), and resulted in satisfactory intra- and inter-day coefficients of variation for plasma and urine samples as shown in Table 3. The extraction recoveries from plasma and urine were also good and are summarized in Table 4.

3.4. Drug interference

The medications, quinidine, rifampin or erythromycin were coadministered to alter codeine's metabolism. After injection of all three compounds, no peaks were found in either detection system. Com-

Table 2
Regression equations for determination of codeine and its metabolites in plasma and urine

Compound	Plasma			Urine		
	Calibration range (n*M*)	Regression equations	R^2	Calibration range (n*M*)	Regression equation	R^2
M3G	24.44–814.74	$y=2.95x-0.02$	0.9905	122.21–4073.7	$y=1.13x+0.03$	0.9967
C6G	102.92–20584	$y=1.16x+0.20$	0.9984	462.48–92495	$y=0.49x+0.38$	0.9941
NCG	102.92–2573.1	$y=1.20x+0.10$	0.9974	462.47–11562	$y=0.54x+0.08$	0.9955
NC	26.39–527.94	$y=0.70x+0.001$	0.9980	131.99–2639.7	$y=0.30x+0.007$	0.9944
COD	33.40–1336.0	$y=0.54x-0.004$	0.9941	167.00–6680.0	$y=0.26x+0.009$	0.9945
M6G	3.28–131.22	$y=1306.3x-2.38$	0.9967	27.34–1640.2	$y=232.85x+7.22$	0.9942
NM	5.96–715.78	$y=1325.0x-20.46$	0.9957	29.82–3578.9	$y=183.77x+9.80$	0.9914
MOR	3.16–158.14	$y=2701.1x-5.19$	0.9970	15.81–632.56	$y=416.44x+8.37$	0.9940

Table 3
Inter- and intra-day variation of codeine and its metabolites in human plasma and urine ($n=5$)

Compound	Plasma			Urine		
	Conc. (n*M*)	Inter-day (R.S.D.%)	Intra-day (R.S.D.%)	Conc. (n*M*)	Inter-day (R.S.D.%)	Intra-day (R.S.D.%)
M3G	48.88	13.57	10.26	407.37	9.35	7.59
	488.84	4.90	6.11	1629.5	4.52	7.19
	814.74	7.57	5.15	3258.9	9.96	5.81
C6G	205.84	13.94	5.25	2312.4	8.34	7.05
	7719.2	6.95	12.90	11 562	9.74	6.10
	20 584	4.39	13.95	57 810	7.15	4.39
NC	39.59	16.77	12.71	659.93	9.59	7.77
	264.00	3.49	13.01	1319.9	9.73	5.18
	527.94	3.58	11.07	1979.8	8.22	7.60
COD	66.80	4.68	6.19	835.00	9.80	6.23
	668.00	6.87	13.81	2505.0	2.53	6.67
	1336.0	5.68	9.13	5010.0	4.22	6.54
M6G	9.84	9.84	8.14	136.69	7.78	5.61
	65.61	9.33	9.13	546.74	9.00	7.59
	131.22	8.89	4.02	1093.5	8.28	7.55
NM	23.86	11.13	5.75	298.24	3.97	7.72
	357.88	9.31	7.49	1192.9	3.85	5.35
	715.78	8.28	7.26	2385.9	5.85	6.69
MOR	12.65	5.41	11.02	79.07	6.58	4.80
	94.88	8.73	8.42	316.28	3.61	5.39
	158.14	8.06	5.98	474.42	4.64	4.52

Table 4
Extraction recoveries of codeine and its metabolites from human plasma and urine ($n=5$)

Compound	Plasma			Urine		
	Added conc. (m*M*)	Measured conc. (m*M*) mean±SD	Recovery (%)	Added conc. (m*M*)	Measured conc. (m*M*) mean±SD	Recovery (%)
M3G	48.88	36.49±4.13	74.65	407.37	367.20±23.23	90.13
	488.84	362.41±17.77	74.14	1629.5	1373.1±89.41	84.26
	814.74	603.70±45.69	74.10	3258.9	2532.4±147.17	77.70
C6G	205.84	139.92±19.50	67.99	2312.4	2098.8±147.36	90.78
	7719.2	5526.4±384.3	71.59	11 562	9427.3±327.20	81.55
	20 580	13 320±584.6	64.72	57 810	42 193±1851.4	73.00
NC	39.59	28.83±4.84	72.81	659.93	511.79±39.76	77.56
	264.00	183.05±6.39	69.34	1319.9	1078.9±55.85	81.74
	527.94	358.70±12.84	67.95	1979.8	1475.9±112.20	74.55
COD	66.80	43.21±2.02	64.69	835.00	603.28±37.60	72.25
	668.00	431.78±29.65	64.64	2505.0	2059.6±137.40	82.22
	1336.0	846.98±48.11	63.40	5010.0	3629.7±237.40	72.45
M6G	9.84	6.36±0.63	64.92	136.69	100.87±5.66	73.79
	65.61	48.96±4.57	74.63	546.74	466.71±33.74	85.37
	131.22	102.21±9.09	77.90	1093.5	832.48±51.90	76.13
NM	23.86	17.59±1.96	73.60	298.24	205.09±18.53	68.78
	357.88	268.90±25.04	75.13	1192.9	816.65±23.30	68.45
	715.80	538.34±44.58	75.21	2385.9	1550.4±148.1	64.98
MOR	12.65	9.04±0.49	71.15	79.07	58.17±2.79	73.54
	94.88	72.31±6.31	76.20	316.28	224.19±12.08	70.88
	158.14	121.35±9.79	76.76	474.42	333.71±28.15	70.34

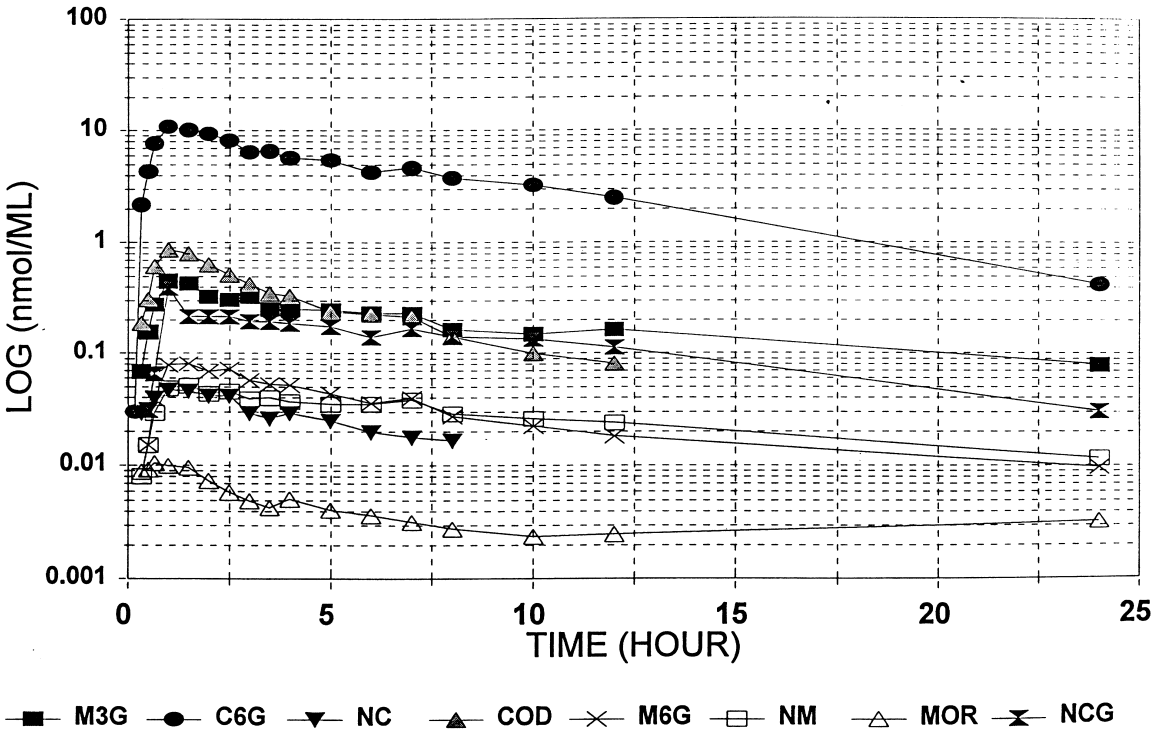

Fig. 3. Plasma concentrations of codeine and its seven metabolites over a 24-h period following administration of 120 mg of codeine.

pared to blank plasma or urine, no interfering peaks were observed in either the plasma or urine samples.

3.5. Application

This method has been used to simultaneously define plasma and urine concentration/time profiles for codeine, and its seven metabolites, in subjects following a single oral dose of 120 mg codeine. Fig. 3 shows an example of the plasma concentrations in one of those subjects.

4. Conclusion

The assay described here is a sensitive, selective and reliable method for determination of codeine and its seven metabolites in plasma and urine. We have used the method to successfully measure thousands of samples to define the effects of variability in cytochrome p450 (CYP) enzyme activity on the pharmacokinetics of codeine and its metabolites. By manipulating that activity with known inhibitors (quinidine, erythromycin) and an inducer (rifampin) of CYP activity, it is possible to determine the effect of enzyme activity on codeine's activation to morphine.

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