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SIMULTANEOUS DETERMINATION OF CODEINE, NORCODEINE AND MORPHINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A novel high-performance liquid chromatographic method for the determination of codeine, norcodeine and morphine in plasma and urine has been developed. The compounds were separated on a cyano column (15 cm×4.6 mm, 5 μm particle size) using a mobile phase of acetonitrile–triethylamine–distilled water (4:0.1:95.9, v/v) pH 3.1 and then determined by fluorescence detection. Calibration curves in the range 5–200 ng/ml for plasma and 0.1–10 μg/ml for urine were linear and passed through the origin. The imprecision and inaccuracy of the assay were less than 10% and the limits of detection were 2 ng/ml for all three compounds in human plasma.

INTRODUCTION

Codeine has been used as an effective analgesic and antitussive agent for over 100 years [1]. Codeine (Fig. 1) is metabolised by O-demethylation to morphine (Fig. 1), N-demethylation to norcodeine (Fig. 1) and glucuronidation to codeine-6-glucuronide, and these are the major metabolites found in urine and plasma [2]. Less than 10% of the dose is recovered unchanged in urine [3]. Whilst administration of codeine produces analgesia, the relative contribution of codeine and its metabolites in producing this pharmacological effect is unknown. However, it is generally considered that "the analgesic effect of codeine may be due to its conversion to morphine" [1]. Numerous an-

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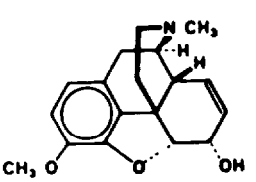
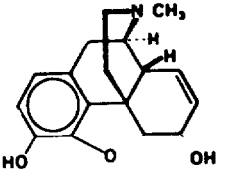
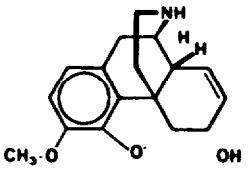


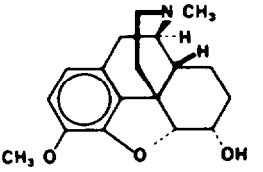
codeine



morphine



norcodeine



dihydrocodeine

Fig. 1. Chemical structures of codeine, morphine, norcodeine and dihydrocodeine.

alytical methods for the determination of codeine in biological fluids have been described. These methods include gas chromatography (GC) [4–8], gas chromatography–mass spectrometry (GC–MS) [9,10], radioimmunoassay (RIA) [11–13] and high-performance liquid chromatography (HPLC) [14–20]. There have been only few reports on the simultaneous determinations of codeine, morphine and norcodeine in plasma or in urine. Cone et al. [21] have measured these compounds and several other "potential" minor metabolites in urine using gas chromatography–mass fragmentography. Posey and Kimble [22] determined codeine, morphine and norcodeine in urine using HPLC, whilst Shah and Mason [23] presented an HPLC assay with electrochemical detection for the determination of codeine, morphine and norcodeine in plasma. This latter assay had a large variability, especially for codeine, resolution was not optimal and a high voltage (1.2 V) was required. Our objective was to develop a precise, sensitive and specific HPLC method for the determination of codeine, norco-

deine and morphine in plasma and in urine, in order to enable studies on the disposition and metabolism of codeine in humans to be conducted.

EXPERIMENTAL

Chemicals

All reagents were of analytical grade and included hydrochloric acid, orthophosphoric acid (BDH, Port Fairy, Australia) and chloroform, triethylamine, anhydrous sodium carbonate and sodium bicarbonate (Ajax, Sydney, Australia). Acetonitrile (Mallinckrodt, South Oakleigh, Australia) was of HPLC grade. Codeine phosphate, morphine sulphate (F.H. Faulding, Adelaide, Australia), norcodeine (Eli Lilly, Indianapolis, IN, U.S.A.) and dihydrocodeine bitartrate (Knoll, Ludwigshafen, F.R.G.) (Fig. 1) were all of British Pharmacopoeial grade quality.

Chromatography

The HPLC system consisted of an M6000A pump (Waters Assoc., Milford, MA, U.S.A.), a fluorescence detector (LS-5 luminescence spectrometer, Perkin-Elmer, Beaconsfield, U.K.), a WISP 710B automatic injector (Waters Assoc.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm×4.6 mm I.D. stainless-steel column was packed with Spherisorb, 5 μm particle size, cyano (CN) packing material (Phase Separations, Queensferry, U.K.). The composition of the mobile phase was acetonitrile–triethylamine–distilled water (4:0.1:95.9, v/v) adjusted to pH 3.1 with 2 *M* orthophosphoric acid. The flow-rate through the column at ambient temperature was 1 ml/min which produced a back-pressure of 12.4 MPa. The excitation and emission wavelengths of the detector were 230 and 350 nm, respectively, and the excitation and emission slits were set at 15 and 20 nm, respectively.

Stock solutions

Codeine, norcodeine and morphine were made up as 1 mg base per ml stock solution in distilled water and were diluted to concentrations ranging from 5 to 200 ng/ml in drug-free plasma and from 0.1 to 10 μg/ml in drug-free urine. Dihydrocodeine, the internal standard, was diluted in distilled water to a concentration of 6.6 μg/ml for plasma analysis and 134 μg/ml for urine analysis.

Sample preparation

Plasma. A 1-ml aliquot of plasma was pipetted into a 10-ml screw-capped, tapered plastic tube (Mallinckrodt) to which were added 10 μl of the 6.6 μg/ml internal standard solution and 0.5 ml of 0.2 *M* bicarbonate buffer (pH 9.6). The sample was briefly vortex-mixed, 5 ml of chloroform were added and the tubes were placed on a rotary mixer for 10 min. The organic and aqueous

phases were separated by centrifugation at 1500 *g* for 10 min. The upper aqueous phase was removed by aspiration and discarded and the organic phase (at least 4 ml) was transferred to a clean 10-ml screw-capped, tapered plastic tube containing 100 μl of 0.1 *M* hydrochloric acid. The tubes were briefly mixed on a vortex and then placed on a rotary mixer for 10 min. The two phases were separated by centrifugation at 1500 *g* for 5 min. An aliquot (10–50 μl) of the upper aqueous phase was injected onto the column via the automatic injector.

Urine. A 0.3-ml aliquot of urine was pipetted into a 10-ml screw-capped plastic tube to which were added 25 μl of the 134 μg/ml internal standard solution. The samples were then handled in exactly the same manner as the plasma samples.

Statistical analysis

Peak heights were measured manually and the peak-height ratios of the three compounds (codeine, norcodeine and morphine) to the internal standard (dihydrocodeine) were calculated. Standard curves were plotted as peak-height ratio versus drug concentration. Linear regression analysis was performed to determine the slope, intercept, their variability and the strength of the correlation.

Precision was evaluated by spiking codeine, norcodeine and morphine to achieve concentrations of 10 and 200 ng/ml in plasma and 0.1 and 10 μg/ml in urine. Analyses were performed with eight or nine samples intra-assay and five or six samples inter-assay. Accuracy was assessed by spiking codeine, norcodeine and morphine to achieve concentrations of 10 and 200 ng/ml in plasma and 0.1 and 10 μg/ml in urine. These were assayed eight times (for plasma 200 ng/ml) and nine times (for plasma 10 ng/ml and for urine 0.1 and 10 μg/ml), and the estimated concentrations from concurrently run standard curves were calculated.

Stability

Codeine, norcodeine and morphine were spiked to achieve concentrations from 10 to 200 ng/ml in drug-free plasma and from 0.1 to 10 μg/ml in drug-free urine to allow standard curves to be constructed and were stored at −20°C. Stability was assessed by comparison of the slopes from six standard curves evaluated over a nine-month period. To determine if hydrolysis of codeine-6-glucuronide to codeine occurred during storage, plasma and urine containing known concentrations of codeine-6-glucuronide were stored at −20°C for twelve weeks. Aliquots of these were assayed for codeine over this period.

Assay application

A young male subject (aged 20 years, weight 58 kg) ingested a single 30-mg codeine phosphate tablet, and through an indwelling catheter, kept patent with a stylet (Jelco™, Critikon, Tampa, FL, U.S.A.), placed in a forearm vein,

multiple blood samples were collected for 12 h and all urine for 48 h. These samples were assayed for codeine, norcodeine and morphine concentrations. This procedure was approved by the Human Ethics Committee of Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

RESULTS AND DISCUSSION

Fig. 2 is a representative chromatogram from an injection of a solution containing a mixture of codeine, norcodeine and morphine each at a concentration of 1 μg/ml and dihydrocodeine at a concentration of 3.3 μg/ml. The limit of detection was 2 ng/ml in plasma defined as three times the baseline noise level. Morphine had a retention time of 3.4 min and a capacity factor (the ratio of the times spent in the stationary and in the mobile phase) of 1.1, norcodeine had a retention time of 5.0 min and a capacity factor of 2.1, dihydrocodeine had a retention time of 6.1 min and a capacity factor of 2.8 and codeine had a retention time of 7.2 min and a capacity factor of 3.5.

The CN column was chosen after evaluation of C_{18} and CN columns. Although a C_{18} column gave adequate separation of the four compounds, morphine eluted too early and was interfered with by endogenous compounds in plasma. Alterations in mobile phase composition and pH did not solve this problem. Substitution with a CN column was effective. Variations in the composition and pH of the mobile phase were then explored. Increasing the percentage of acetonitrile and/or triethylamine resulted in a decrease of the retention time of all four compounds and increasing the pH of the mobile phase

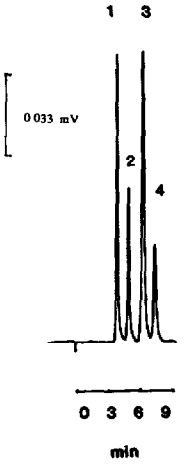


Fig. 2. Chromatogram of a standard solution of 1 μg/ml morphine (1), 1 μg/ml norcodeine (2), 3.3. μg/ml dihydrocodeine (3) and 1 μg/ml codeine (4).

TABLE I

ASSAY PRECISION FOR MORPHINE, NORCODEINE AND CODEINE IN PLASMA AND URINE

Compound	Plasma concentration (ng/ml)	Coefficient of variation (%)	n
Intra-assay			
Morphine	10	7.7	9
	200	2.2	8
Norcodeine	10	6.3	9
	200	1.3	8
Codeine	10	6.3	9
	200	2.6	8
Inter-assay			
Morphine	10	5.7	6
	200	10.2	6
Norcodeine	10	7.7	6
	200	6.2	6
Codeine	10	5.9	6
	200	1.4	6
	Urine concentration (μg/ml)	Coefficient of variation (%)	n
Intra-assay			
Morphine	0.10	8.4	9
	10.00	2.5	9
Norcodeine	0.10	4.9	9
	10.00	2.5	9
Codeine	0.10	6.4	9
	10.00	2.9	9
Inter-assay			
Morphine	0.10	9.8	5
	10.00	6.0	5
Norcodeine	0.10	7.8	5
	10.00	2.9	5
Codeine	0.10	2.2	5
	10.00	3.4	5

increased the retention time of these four compounds. The mobile phase chosen resulted in optimal separation of the peaks within a convenient time scale.

Recovery was determined by comparing the peak heights of extracted plasma samples with the peak heights of standards of the same concentrations. The recovery (mean $\pm$ S.D., $n=5$) at the concentration of 0.1 μg/ml was $75.4 \pm 1.3\%$ for codeine, $92.4 \pm 1.4\%$ for norcodeine, $60.0 \pm 0.7\%$ for morphine and $72.2 \pm 1.8\%$ for dihydrocodeine. The pH of the initial extraction step appears

TABLE II

ASSAY ACCURACY FOR MORPHINE, NORCODEINE AND CODEINE IN PLASMA AND URINE

Sample No.	Calculated concentration					
	Morphine		Norcodeine		Codeine	
Plasma (ng/ml)						
1	9.2	195.4	10.8	198.9	10.0	196.0
2	9.5	184.0	9.8	195.8	9.3	200.5
3	10.6	185.0	9.6	198.6	11.2	210.6
4	11.3	189.0	10.4	200.0	9.9	202.5
5	9.1	194.3	10.4	203.2	9.9	205.1
6	10.1	192.6	10.2	203.6	9.7	200.9
7	9.9	189.4	9.6	198.6	10.7	200.0
8	9.1	188.9	8.9	198.2	9.9	193.5
9	10.4	-	10.8	-	11.0	-
Mean	9.9	189.8	10.1	199.6	10.2	201.1
S.D.	0.8	4.1	0.6	2.6	0.6	5.3
Nominal	10.0	200.0	10.0	200.0	10.0	200.0
Mean accuracy (%)	99.0	94.9	101.0	99.8	102.0	100.5
Urine (μg/ml)						
1	0.11	9.2	0.09	9.4	0.10	9.9
2	0.11	9.7	0.10	9.9	0.10	9.9
3	0.10	9.9	0.10	10.0	0.11	10.5
4	0.10	9.9	0.09	10.1	0.10	10.7
5	0.12	9.7	0.10	10.0	0.11	10.5
6	0.11	10.1	0.11	10.3	0.10	10.0
7	0.09	10.0	0.10	10.0	0.09	10.5
8	0.09	9.6	0.10	9.8	0.11	10.2
9	0.10	9.7	0.09	10.0	0.10	10.5
Mean	0.10	9.8	0.10	9.9	0.10	10.3
S.D.	0.01	0.3	0.01	0.3	0.01	0.3
Nominal	0.10	10.0	0.10	10.0	0.10	10.0
Mean accuracy (%)	100.0	98.0	100.0	99.0	100.0	103.0

to be critical for morphine. Other workers have used an extraction pH for morphine of between 8.9 and 10.0 [20–22]. Our optimal extraction pH of 9.6 is in accord with these previous reports. However, codeine and norcodeine are more fully extracted from more alkalinised plasma.

Extracting the analytes from 4–5 ml chloroform into 100 μl of 0.1 *M* hydrochloric acid and removing only the aqueous phase is potentially technically difficult. Alternatively a larger acid volume could be used and a larger volume injected on-column. Adding diethyl ether to chloroform, to ensure that the organic phase is the upper layer, resulted in unacceptable lowering of the recovery of norcodeine (about 20%) but not of codeine and morphine.

TABLE III

DRUGS SHOWN NOT TO INTERFERE WITH THIS ASSAY

Drugs in patients plasma	Pure drug preparations[a]
Amiloride	Codeine-6-glucuronide
Beclomethasone dipropionate	Dextromethorphan
Captopril	Dextropropoxyphene
Cimetidine	Dextrorphan
Chlorothiazide	Ethylmorphine
Digoxin	Morphine-3-glucuronide
Frusemide	Morphine-3-sulphate
5-Fluorouracil	Morphine-6-glucuronide
Glyceryl trinitrate	Naloxone
Metoclopramide	Oxycodone
Multivitamins	Pholcodine
Nitrazepam	
Paracetamol	
Polystyrene sulphonate	
Prednisolone	
Pseudoephedrine	
Quinine bisulphate	
Salbutamol	
Spironolactone	
Temazepam	
Terbutaline sulphate	
Theophylline	
Tolbutamide	

[a]Concentrations were 0.5–2 μg/ml.

Calibration curves showed good linearity between peak-height ratios and concentrations from 5 to 200 ng/ml for codeine, norcodeine and morphine in plasma (morphine, $y=0.0182x+0.0388$, $r=0.9959$; norcodeine, $y=0.0144x+0.0083$, $r=0.9999$; codeine, $y=0.0075x+0.0282$, $r=0.9994$) and from 0.1 to 10 μg/ml in urine (morphine, $y=1.0822x-0.0467$, $r=0.9986$; norcodeine, $y=0.9621x-0.1122$, $r=0.9997$; codeine, $y=0.6542x-0.1429$, $r=0.9992$). For plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin, and the standard errors of the slopes were less than 5%. The assay showed good precision at low and high concentrations in plasma and urine and Table I shows the intra- and inter-assay precision, which in most cases was less than 10%. The accuracy of the assay for morphine, norcodeine and codeine in plasma and urine is shown in Table II. Except for morphine at 200 ng/ml in plasma (reason unclear), accuracy was greater than 95%. In samples stored at $-20\,^{\circ}C$ for nine months, there was no loss ($<5\%$) of codeine, norcodeine or morphine. There was less than 1% hy-

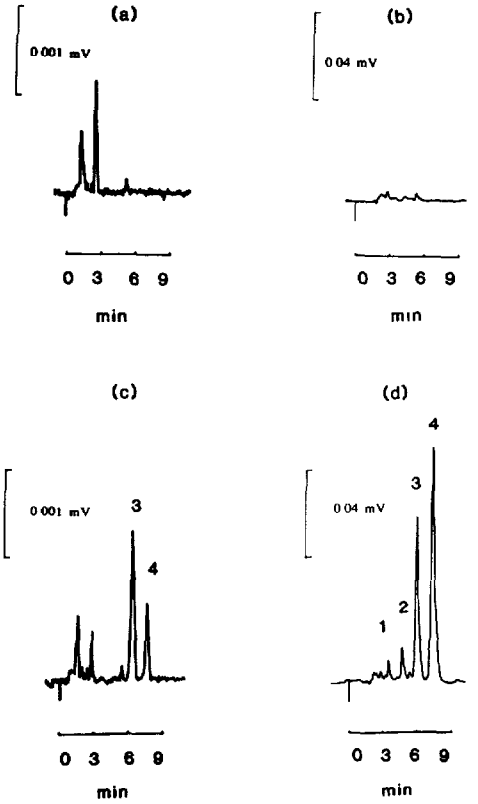


Fig. 3. Chromatograms from an extract of (a) drug-free plasma, (b) drug-free urine, (c) a plasma sample from the subject 1.25 h after ingestion of 30 mg codeine phosphate (codeine concentration 39 ng/ml) and (d) a 0–12 h urine sample from the same subject (concentration: codeine, 14.5 μg/ml; norcodeine, 2.1 μg/ml; morphine, 0.8 μg/ml, sample diluted 1:2). Peaks: 1 = morphine; 2 = norcodeine; 3 = dihydrocodeine (internal standard); 4 = codeine.

drolysis of codeine-6-glucuronide to codeine in plasma and urine over a twelve-week period.

Interference was studied by chromatographing aliquots of solutions of pure drug and by analyzing plasma and urine samples from patients on multiple drug therapy. Drugs shown in Table III were found not to interfere with this assay.

Fig. 3 shows a chromatogram from drug-free plasma and urine, a plasma sample from the subject 1.25 h after codeine ingestion and a sample of the 0–12 h urine collection. There is a small peak from plasma and urine samples which elutes near, but is resolved from, the norcodeine peak in the chromatogram. In this subject, who is representative of other healthy volunteers receiving a single dose of 30 mg codeine phosphate (unpublished), morphine and norcodeine could not be detected in plasma (limit of sensitivity 2 ng/ml).

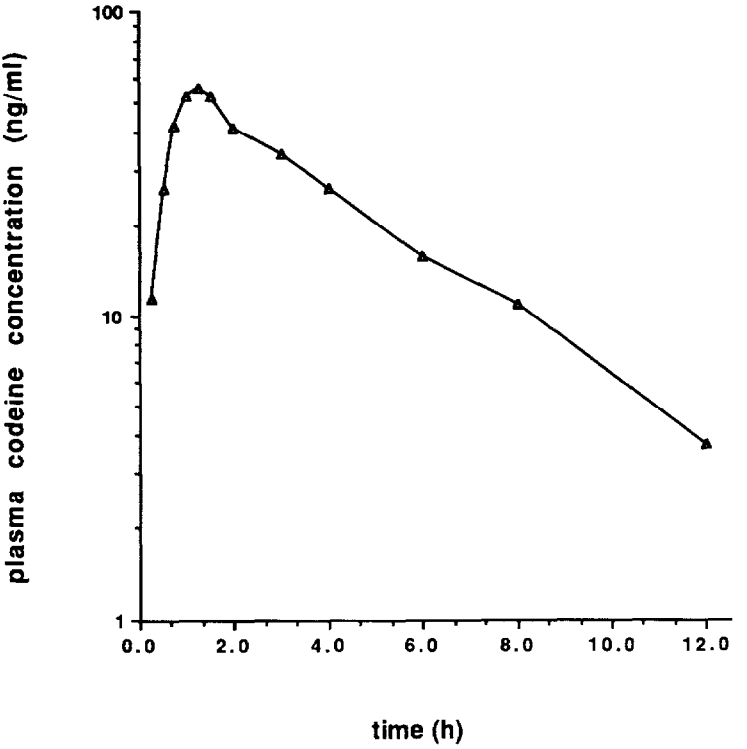


Fig. 4. Semilogarithmic plot of plasma concentration versus time profile for codeine after a single oral dose of 30 mg codeine phosphate in a human volunteer.

However, in urine they could be quantitated. It should be noted that, especially in urine, glucuronide conjugates of codeine, norcodeine and morphine are also present (refs. 1–3 and unpublished observations).

Fig. 4 shows plasma concentrations of codeine following a single 30-mg dose of codeine phosphate. The terminal half-life of codeine was 3.1 h. Plasma concentrations of morphine and norcodeine were below 2 ng/ml. All three compounds were detected in urine. The cumulative urinary excretion of unchanged codeine was 14%, unconjugated norcodeine 5% and unconjugated morphine 1% (Fig. 5). Codeine-6-glucuronide has been reported to be the major metabolite of codeine in man. The urinary excretion of this metabolite may account for up to 50% of the dose (ref. 3 and unpublished observations), and an assay for the direct determination of codeine-6-glucuronide in plasma and urine is currently being developed in our laboratory.

Other methods for the simultaneous determination of the three compounds in urine [22] or in plasma [23] have been reported. Unlike these methods, the present assay can be used for both plasma and urine determinations.

In summary, an original method has been developed for the assay of codeine,

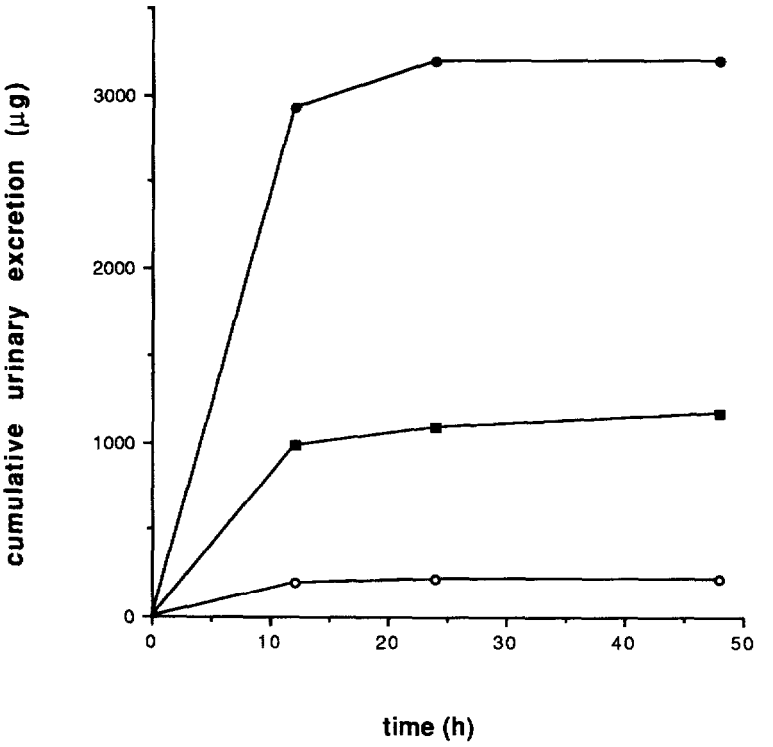


Fig. 5. Cumulative urinary excretion of codeine (●), morphine (○) and norcodeine (■) after a single oral dose of 30 mg codeine phosphate in a human volunteer.

norcodeine and morphine in plasma and urine. The method shows good precision, accuracy, selectivity and sensitivity and is currently being used to determine the pharmacokinetics and metabolism of codeine in humans.

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