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Direct determination of codeine, norcodeine, morphine and normorphine with their corresponding O-glucuronide conjugates by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the detection, separation and measurement of codeine and its metabolites norcodeine, morphine and normorphine, with their glucuronide conjugates. The glucuronidase *Escherichia coli* type VIIA hydrolyses codeine-6-glucuronide completely and is used for the construction of the calibration curves of codeine-6-glucuronide. Enzymic hydrolysis of codeine-6-glucuronide depends on the specific activity of the glucuronidase applied. Examples are shown of a volunteer who is able to form morphine from codeine and one who is unable to do so.

INTRODUCTION

The morphinomimetic drug codeine is used as an analgesic and antitussive agent. Codeine is metabolized by conjugation with glucuronic acid to codeine-6-glucuronide, and to a minor extent via N-demethylation to norcodeine and via O-demethylation to morphine. The possible metabolites generated by these three metabolic pathways are shown in Fig. 1. To correlate the observed analgestic or antitussive effects with the corresponding plasma concentration of the active agent, most if not all analytical methods have been developed for codeine, norcodeine and morphine [1–8]. Formation of morphine may contribute to the development of dependence with chronic codeine administration. Recently it has become more and more obvious that glucuronidation of morphine at the 6-position does not reduce the analgestic effect: morphine-6-glucuronide has a stronger analgesic effect than morphine [9–15], and its plasma concentration is much high-

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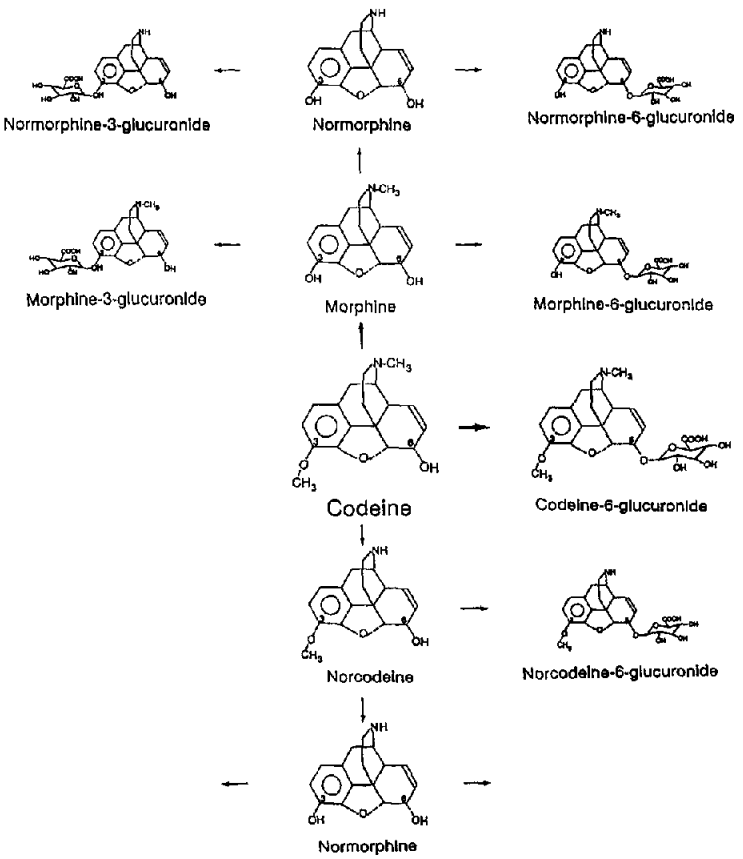

Fig. 1. Structures of codeine and its metabolites.

er than that of morphine [11]. By analogy with morphine, codeine-6-glucuronide must possess a similar activity to codeine itself. Reports of direct high-performance liquid chromatography (HPLC) of codeine-6-glucuronide are limited [16,17]. The aim of this investigation was to develop a precise, sensitive and selective HPLC method for the determination of codeine, norcodeine and morphine, and their possible glucuronides, in plasma and urine, in order to study the pharmacokinetics of codeine in humans.

EXPERIMENTAL

Drugs

Codeine was obtained from the Hospital Pharmacy. Norcodeine, normorphine, morphine-3-glucuronide and morphine-6-glucuronide were obtained from Sigma (St. Louis, MO, USA). Morphine was obtained from Diosynth (Oss, Netherlands). Codeine-6-glucuronide was identified in human plasma and urine.

Chromatography

A Spectroflow 400 high-performance liquid chromatograph (Kratos, Rotterdam, Netherlands) equipped with an electrochemical detector (Model 5100 A, ESA, Kratos) was used.

A stainless-steel column (250 mm × 4.6 mm I.D.; Chrompack, Middelburg, Netherlands) packed with CP-tm-Spher C_8, particle size 8 μm (Chrompack) was used. The injection volume was 50 μl.

The mobile phase consisted of 0.25 g of heptanesulphonic acid (HSA), 0.68 g of KH_2PO_4 and 5.5 ml of H_3PO_4 (25% in water) adjusted to a total volume of 425 ml, with 20 ml of methanol and 60 ml of acetonitrile added. It was degassed with a gentle stream of helium. The flow-rate was 2.0 ml/min at a pressure of 15.2 MPa. The temperature of the column was 40°C. The capacity factors of codeine and its metabolites are given in Table I.

For sample injection a Marathon autosampler (Interscience, Breda, Netherlands) was used.

The analytical cell (Model 5010, ESA) consisted of two units, which can be regulated separately. The potentials of detector cell 1 and detector cell 2 were +0.65 V and +0.85 V, respectively, for the measurement of morphine-3-glucuronide, codeine-6-glucuronide, norcodeine, and codeine. For the measurement of morphine-6-glucuronide, normorphine, and morphine, the potentials of the cells were +0.25 V and +0.35 V, respectively. Both cells were connected to a two-channel Chromjet integrator (Spectra Physics, Eindhoven, Netherlands).

Sample preparation

Plasma. A 0.6 ml plasma sample was extracted with the Baker-10 extraction system (Baker Chemicals, Cat. No. 70180, Deventer, Netherlands) fitted with

TABLE I

CAPACITY FACTORS OF CODEINE AND ITS METABOLITES

Compound	Capacity factor (k')	k' ratio[a]		
		OCH_3	6gluc/aglycone	NCH_3
Morphine-3-glucuronide	2.2			
Morphine-6-glucuronide	3.6		0.57	
Normorphine	6.0			
Morphine	6.3			1.05
Codeine-6-glucuronide	9.9		0.52	
Norcodeine	17.4	2.90		
Codeine	19.2	3.05		1.10

[a] OCH_3 = effect of the 3-methoxy group *versus* 3-OH group; NCH_3 = effect of the N-methyl group *versus* NH_2 group; 6gluc = effect of the 6-O-glucuronide group *versus* 6-OH group.

1-ml disposable extraction columns packed with reversed-phase octadecylsilane (C_{18}) bonded to silica gel (Cat. No. 7020-01). The extraction column was conditioned with two 1-ml volumes of methanol, two 1-ml volumes of water and 1 ml of 500 m*M* diammonium sulphate (pH 9.3). Plasma (0.6 ml) diluted with an additional 0.5 ml of 500 m*M* diammonium sulphate buffer (pH 9.3), was placed into of the column. The column was washed with two 1-ml volumes of 5 m*M* diammonium sulphate (pH 9.3). The sample was eluted with 400 μl of a mixture of 0.01 *M* KH_2PO_4 with 10% acetonitrile and 10% H_3PO_4 (25%).

Urine. A 0.2-ml sample of urine and 0.5 ml of 500 m*M* diammonium sulphate (pH 9.3) were placed into the extraction column. The urine was processed in the same way as plasma, except that the column was washed four times with 1 ml of 5 m*M* diammonium sulphate (pH 9.3). The sample was eluted with 1 ml of 0.01 *M* KH_2PO_4–10% acetonitrile 10% H_3PO_4 (25%).

Deconjugation

Deglucuronidation was carried out with 100 μl of urine, 25 μl of β-glucuronidase and 125 μl of 0.02 *M* KH_2PO_4 (pH 6.8) for 17 h at 37°C. Thereafter, 0.7 ml of 500 m*M* diammonium sulphate was added and the sample was extracted. The urine samples containing codeine-6-glucuronide were taken from a volunteer after oral intake of 30 mg of codeine phosphate. Four different glucuronidase and sulphatase enzymes (A–D) were used: (A) 0.2 *M* phosphate buffer (pH 5.0) and 100 000 U/ml β-glucuronidase type B1 from bovine liver (Sigma, Cat. No. G-0251); (B) 0.2 *M* phosphate buffer (pH 5.0) and 107 200 U/ml β-glucuronidase plus 4500 U/ml arylsulphatase *Helix pomatia* type H2 (Sigma, Cat. No. G-0876); (C) 0.2 *M* phosphate buffer (pH 6.8) and 20 000 U/ml β-glucuronidase *Escherichia coli* type VIIA (Sigma, Cat. No. G-7646); (D) 0.2 *M* phosphate buffer (pH 3.8) and 100 000 U/ml β-glucuronidase plus 893 U/ml arylsulphatase type LII lyophilized powder from limpets *P. vulgata* (Sigma, Cat. No. G-8132).

Calibration curves for glucuronide

The increase in the concentration of codeine in urine after deconjugation represented the concentration of the conjugate. A calibration curve was constructed with the help of the following formula:

$$[\text{codeine-6-gluc}] = d[\text{codeine}] \cdot M_{\text{codeine-gluc}}/M_{\text{codeine}}$$

where d[codeine] is the difference between the concentrations of codeine before and after deconjugation, and M is the relative molecular mass. Thereafter, calibration curves for codeine-6-glucuronide were constructed by spiking urine with known concentrations of the compound.

Calibraton curves for plasma codeine-6-glucuronide were obtained by spiking 1 ml of plasma with known (measured after deglucuronidation) concentrations of the glucuronides in urine [18–20].

Subjects

Two human volunteers (one male, one female) took an oral dose of 30 mg of codeine phosphate (tablets obtained from the Hospital Pharmacy). The study had the approval of the Hospital Ethics Committee.

Sampling

Blood samples were collected at regular time intervals after administration for 24 h and centrifuged at 2600 *g*. The plasma was separated and stored at −20°C until analysis. Urine was collected on spontaneous voiding for 48 h, the total volume and the time of void were recorded, and an aliquot was stored at −20°C until analysis.

Recovery and reproducibility

The calibration curves were prepared by adding a variable amount of stock solution to blank plasma/urine.

RESULTS AND DISCUSSION

Fig. 2 shows chromatograms of a human plasma sample containing codeine,

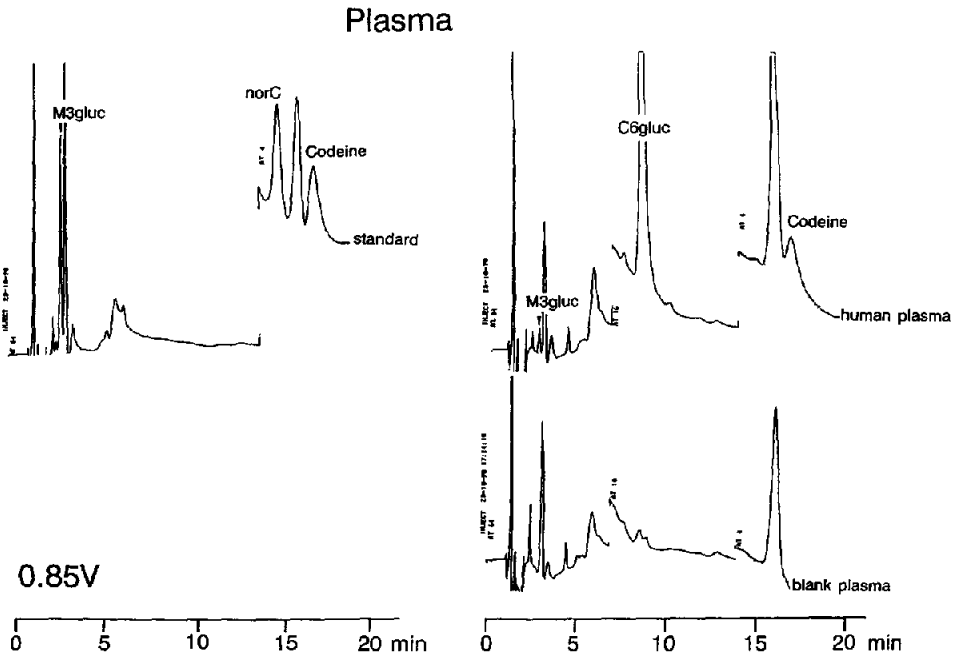

Fig. 2. Chromatograms of codeine (C), its metabolites norcodeine (norC), its glucuronides codeine-6-glucuronide (C6gluc) and morphine-3-glucuronide (M3gluc) in a water standard, in a human plasma sample and in a blank human plasma sample. The water standard contained 123 ng/ml codeine, 119 ng/ml norC and 523 ng/ml M3gluc. The shifts in the baseline are the result of a change in the attenuation.

its metabolite norcodeine, and the conjugates codeine-6-glucuronide and morphine-3-glucuronide. Fig. 3 shows chromatograms of a human urine sample containing codeine, its metabolites norcodeine, morphine and normorphine, and the conjugates codeine-6-glucuronide and morphine-3-glucuronide. The peaks are well separated from each other. Table I lists the capacity factors. As with other HPLC methods in which the parent drug and the glucuronide conjugate are measured, there is a big difference in capacity factors between glucuronide and aglycone [18–24]. 6-O-Glucuronidation of codeine and morphine reduces the capacity factor by *ca.* 50%, 3-O-methylation of morphine increases the capacity factor by a factor of 3, and N-methylation of norcodeine and normorphine affects the capacity factor minimally. The nearly constant ratio between the capacity factors of compounds differing by one functional group can be used as an additional tool in identifying structural analogues [23,24]. In this way, it could be decided that norcodeine-6-glucuronide, normorphine-3-glucuronide and normorphine-6-glucuronide were not present in the plasma and urine of humans.

Fig. 4. shows chromatograms of human urine before and after deglucuronida-

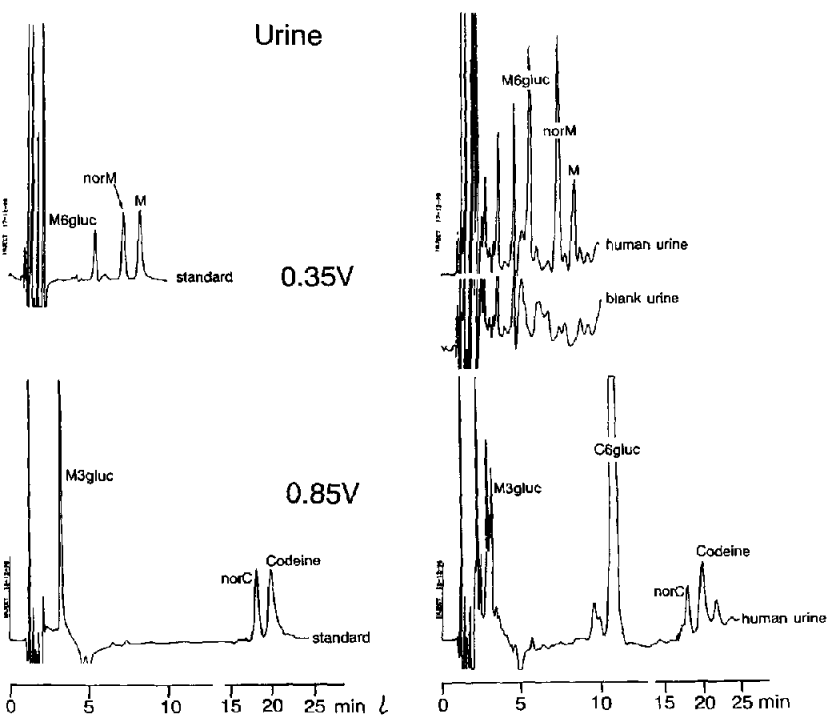

Fig. 3. Chromatograms of codeine (C), it metabolites norcodeine (norC), normorphine (norM) and morphine (M), and the glucuronides codeine-6-glucuronide (C6gluc), morphine-3-glucuronide (M3gluc) and morphine-6-glucuronide (M6gluc) in a water standard, in a human urine sample and in a blank human urine sample. The water standard contained 760 ng/ml codeine, 550 ng/ml norC, 3200 ng/ml M3gluc, 262 ng/ml M6gluc, 797 ng/ml norM and 760 ng/ml M.

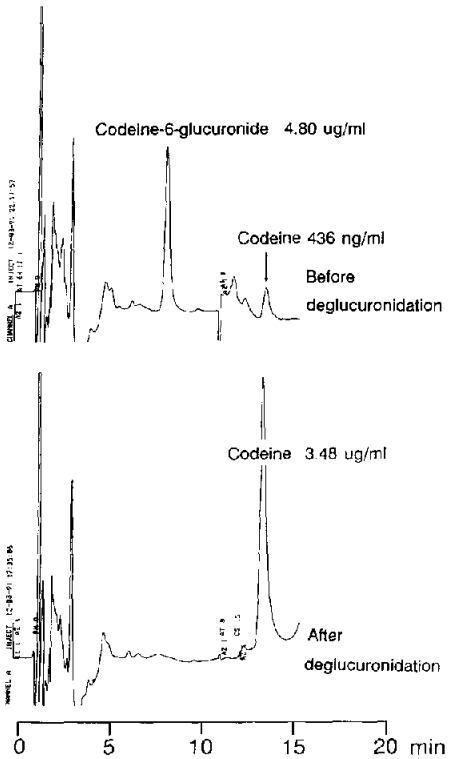

Fig. 4. Chromatograms of codeine and its glucuronide codeine-6-glucuronide in a human urine sample before (upper panel) and after (lower panel) deglucuronidation with *E. coli* type VIIA glucuronidase (system C).

tion with system C (*E. coli* type VIIA). The systems C and D both reached 100% deglucuronidation of codeine-6-glucuronide. System C was preferred to system D because the chromatograms were almost clear of interfering peaks from the glucuronidase solution. The systems A and B reached only 34 and 74% deconjugation. Incomplete hydrolysis (56–81%) with *H. pomatia* H1 was reported by Chen *et al.* [16]. An inhibitory effect of urine constituents on the hydrolysis process was assumed. Our system B, with *H. pomatia* H2 glucuronidase, also gives incomplete hydrolysis (74%). The hydrolysis of the substrate codeine-6-glucuronide in urine depends on the activity of the glucuronidase enzyme, as reported earlier for the hydrolysis of a series of sulphonamide-N_1-glucuronides [18–20].

The correlation coefficients were 0.999 or more for morphine-6-glucuronide, morphine-3-glucuronide, normorphine, morphine, codeine, norcodeine and codeine-6-glucuronide.

The recoveries from the extraction procedure were: for morphine, 92%; for morphine-3-glucuronide, morphine-6-glucuronide and codeine-6-glucuronide, 100%; for codeine and norcodeine, 85%.

The detection limit for codeine, codeine-6-glucuronide and norcodeine is 5 ng/ml in plasma and 25 ng/ml in urine. The detection limit for morphine is 5 ng/ml plasma and 20 ng/ml in urine, for morphine-3-glucuronide 10 ng/ml in plasma and 70 ng/ml in urine, and for morphine-6-glucuronide 5 ng/ml in plasma and 50 ng/ml in urine (signal-to-noise ratio of 3).

Table II lists the inter-day and the intra-day variations in plasma, Tables III and IV, respectively, the intra- and inter-day variations in urine.

Fig. 5 shows the plasma concentration–time curves and renal excretion rate–time profiles of codeine and its metabolites in a human volunteer after an oral dose of 30 mg of codeine phosphate. The codeine plasma concentration is low and only 3.9% of the compound is excreted in the urine. The main metabolite in plasma and urine is codeine-6-glucuronide, which accounts for 77.3% of the dose administered. Morphine and its metabolites morphine-3-glucuronide and morphine-6-glucuronide are formed and excreted as *ca.* 3.3% of the dose. Fig. 6 shows the kinetic data of codeine in a volunteer who is unable to O-demethylate codeine into morphine.

TABLE II

INTRA-DAY AND INTER-DAY COEFFICIENTS OF VARIATION OF CODEINE AND ITS METABOLITES IN HUMAN PLASMA

	Concentration (μg/ml)		
	M3gluc	C6gluc	Codeine
Intra-day			
Mean	312	1042	899
S.D.	13.6	83.0	2.3
C.V. (%)	4.3	7.9	2.6
Mean	123	244	31.1
S.D.	5.6	19	3.0
C.V. (%)	9.0	3.3	10.7
Mean	31.0	28.7	7.5
S.D.	2.8	0.96	0.80
C.V. (%)	9.0	3.3	10.7
Inter-day			
Mean	324	1092	87.3
S.D.	20.0	43.4	3.9
C.V. (%)	6.1	4.0	4.5
Mean	121	267	31.1
S.D.	7.8	20.5	1.0
C.V. (%)	6.4	7.7	3.2
Mean	29.3	30.4	7.7
S.D.	1.5	2.2	0.2
C.V. (%)	5.1	7.2	2.6

TABLE III

INTRA-DAY VARIATION OF CODEINE AND ITS METABOLITES IN HUMAN URINE

	Concentration (μg/ml)						
	C6gluc	norC	Codeine	Morphine	norM	M3gluc	M6gluc
Mean	115.2	5.2	13.5	2.31	3.67	10.08	1.78
S.D.	7.18	0.213	0.248	0.208	0.213	0.68	0.155
C.V. (%)	6.2	4.0	1.8	9.0	7.7	6.8	8.7
Mean	12.48	0.56	1.34	0.265	0.421	1.25	0.188
S.D.	0.24	0.010	0.022	0.0071	0.0065	0.032	0.0020
C.V. (%)	1.9	1.9	1.7	2.7	1.6	2.6	1.1
Mean	1.33	0.055	0.128	0.023	0.0407	0.155	[a]
S.D.	0.036	0.0049	0.0046	0.0010	0.0005	0.0055	–
C.V. (%)	2.7	8.9	3.6	4.3	1.3	3.5	–

[a] Below detection limit.

TABLE IV

INTER-DAY VARIATION OF CODEINE AND ITS METABOLITES IN HUMAN URINE

	Concentration (μg/ml)						
	C6gluc	norC	Codeine	Morphine	norM	M3gluc	M6gluc
Mean	122.8	5.6	13.4	2.45	3.85	10.21	1.88
S.D.	5.67	0.192	0.135	0.095	0.160	0.25	0.199
C.V. (%)	4.6	3.4	1.0	3.9	4.2	2.5	10.6
Mean	12.57	0.56	1.29	0.255	0.422	1.09	0.0200
S.D.	0.456	0.0166	0.0166	0.016	0.0065	0.093	0.0265
C.V. (%)	3.6	3.0	1.3	6.2	1.5	8.5	13.3
Mean	1.32	0.055	0.126	0.022	0.040	0.120	–[a]
S.D.	0.055	0.0035	0.0059	0.0004	0.0017	0.020	–
C.V. (%)	4.1	6.4	4.6	2.0	4.1	16.6	–

[a] Below detection limit.

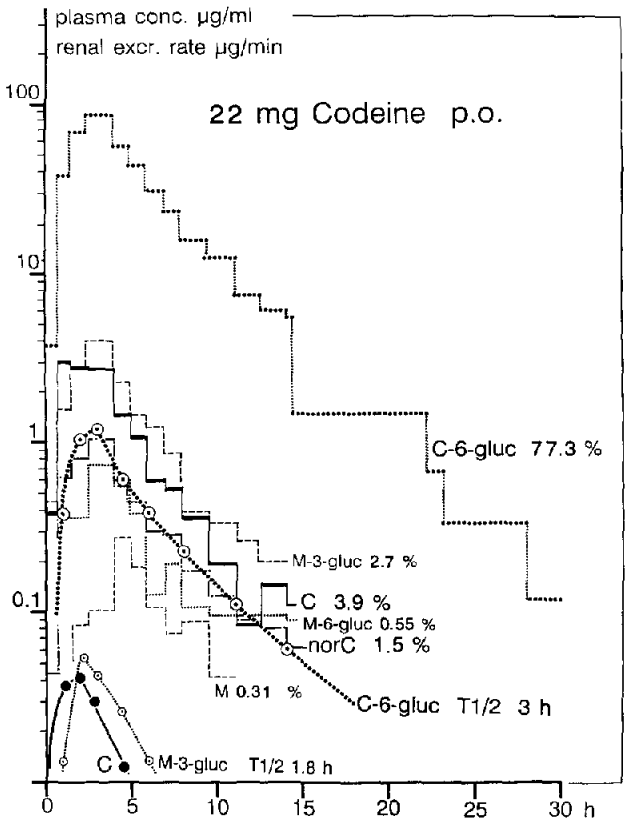

Fig. 5. Plasma concentration–time curves and renal excretion rate–time profiles of codeine (C), its metabolites norcodeine (norC) and morphine (M), and the glucuronides codeine-6-glucuronide (C-6-gluc), morphine-3-glucuronide (M-3-gluc) and morphine-6-glucuronide (M-6-gluc) in a volunteer after an oral dose of 30 mg of codeine phosphate (=22 mg of codeine).

This method enables the direct measurement of codeine, its metabolites and its O-glucuronides. It is comparable with and an alternative to published methods that included the glucuronide conjugates. Differences between the present and earlier reported methodologies are found in the detection, such as UV detection [17], fluorescence detection [15,16], electrochemical detection [1,25,26] and enzymic hydrolysis [16].

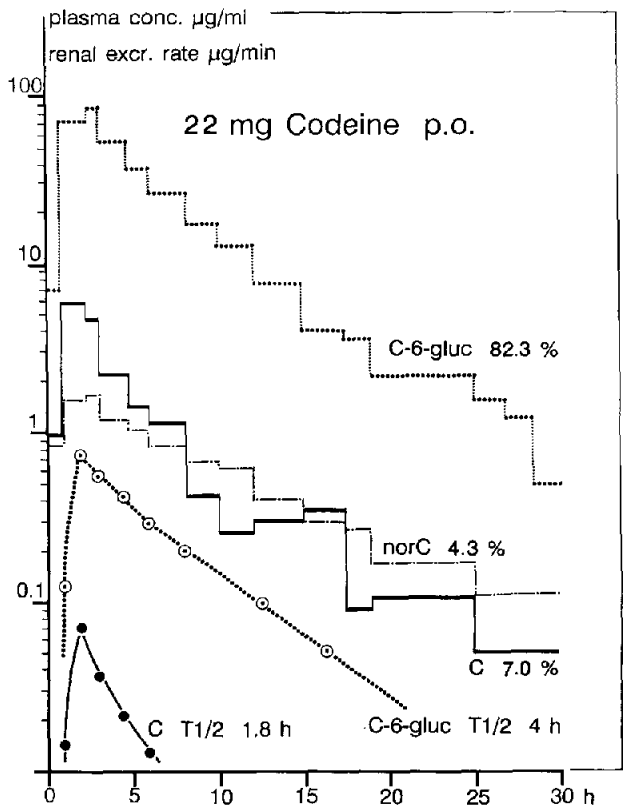

Fig. 6. Plasma concentration–times curves and renal excretion rate–time profiles of codeine (C), its metabolites norcodeine (norC) and morphine (M), and the glucuronides codeine-6-glucuronide (C-6-gluc), morphine-3-glucuronide (M-3-gluc), morphine-6-glucuronide (M-6-gluc) in a volunteer after an oral dose of 30 mg of codeine phosphate (=22 mg of codeine). This volunteer is unable to O-demethylate codeine into morphine.

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