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Highly sensitive gas chromatographic–tandem mass spectrometric method for the determination of morphine and codeine in serum and urine in the femtomolar range

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Abstract

A sensitive and specific method was developed for the determination of codeine and morphine in human serum and for the determination of trace amounts of endogenous morphine in human urine. The analytes were recovered from serum by a simple liquid–liquid extraction method. Urine samples were hydrolyzed, and purified by two liquid–liquid extraction steps and a solid-phase extraction. Samples were derivatized to the pentafluoropropionic esters and measured by gas chromatography tandem mass spectrometry. Using the deuterated analogues as internal standards a limit of quantification of 20 fmol/ml (5.7 pg/ml) morphine and 500 fmol/ml (150 pg/ml) codeine in human serum and of 2.5 fmol/ml (0.71 pg/ml) morphine in urine was achieved. The method was suitable for the determination of morphine and codeine in pharmacokinetic studies and for the determination of the urinary excretion of endogenous morphine.

Keywords: Codeine; Morphine

1. Introduction

In the eighties morphine, the most important alkaloid in the poppy plant, could be identified as a constituent of various tissues of animals [1–3] and of human cerebrospinal fluid [4]. Since some of the precursors which occur in the poppy plant are also formed enzymatically in mammalian tissues such as liver, it had been proposed that morphine can be formed endogenously [5–7]. In vitro and in vivo experiments suggest a formation of morphine analoguous to that in the poppy plant. The final step in the biosynthesis of morphine is the O-demethylation of codeine, which is mediated in man by CYP2D6. This enzyme exhibits a genetic polymorphism, where 7–10% of a Caucasian population are so called poor metabolizers (PMs) because they do not express this enzyme [8]. After administration of codeine PMs form only trace amounts of morphine [9] and no analgesia is observed [10,11], whereas in the vast majority of the population, the so-called extensive metabolizers (EMs), about 5–15% of a given

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codeine dose is metabolized to morphine. If endogenous morphine is formed from codeine, PMs should form much less morphine than EMs. However, in a previous study, the urinary excretion of endogenous morphine was not different between EMs and PMs [12] raising the possibility that morphine could originate from dietary sources. Indeed it has been reported that morphine is present in cow and human milk at concentrations of 200–500 ng/l and it was also detected in hay and lettuce [13]. An experimental approach to differentiate between endogenous and dietary morphine would be to put volunteers on a diet containing no or only trace amounts <1 pmol/l of morphine. For the determination of low levels of endogenous morphine in former studies a combination of HPLC and RIA has been employed [4]. Other methods, like HPLC or GC–MS are by far not sensitive enough. Since the levels under a morphine free diet were expected to be much lower, an enhancement in sensitivity had to be achieved. Furthermore, we wanted to employ a method with greater selectivity than RIA. We have developed a sensitive GC–MS–MS method for the determination of morphine and codeine in serum after administration of single doses of morphine or codeine during pharmacokinetic studies [11,14]. This assay enables the quantification of low serum levels of morphine obtained after administration of codeine to PMs. However, the sensitivity had to be improved with further purification steps, to quantify very low levels of endogenous morphine in human urine. Both methods, the determination of morphine and codeine in serum after a simple extraction step, and the ultrasensitive determination of endogenous morphine in urine requiring extensive sample clean-up are presented.

2. Experimental

2.1. Chemicals and reagents

Solvents used were of HPLC quality; chemicals were of analytical grade. Morphine hydrochloride trihydrate was obtained from Merck (Darmstadt, Germany), morphine-d_3 hydrochloride trihydrate, codeine and codeine-d_3 hydrochloride dihydrate, were from Sigma (Deisenhofen, Germany). The concentrations given in the text as ng/ml or pg/ml refer to the respective free bases. Pentafluoropropionic anhydride (PFPA) was from Pierce (purchased from KMF, St. Augustin, Germany) and 2,2,3,3,3-pentafluoro-1-propanol was from Fluka (Buchs, Switzerland). Solid-phase extraction (SPE) cartridges, Bond Elut Certify (300 mg) were from Varian (Darmstadt, Germany). Eluent mixture for SPE consisted of dichloromethane–2-propanol–ammonia (25%) (40:10:1; v/v/v). Quality control standard Medidrug Opiate U Level 1 was purchased from Medichem (Stuttgart, Germany).

2.2. Instrumentation and chromatographic conditions

Sample preparation was performed automatically on an ASPEC XL system (Gilson, Abimed, Langenfeld, Germany).

For GC–MS–MS analysis a TSQ 700 mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a 5890 II gas chromatograph (Hewlett Packard, Waldbronn, Germany) was used. GC was performed on a DB-5 capillary column (25 m, 0.25 mm I.D., dimethylpolysiloxane with 5% phenyl groups, 0.25 μm film thickness, J&W Scientific, Fisons, Mainz, Germany) in the splitless mode, carrier gas helium at an inlet pressure of 100 kPa. Injections were carried out automatically at 280°C with an A200S autosampler (CTC Analytics, Zwingen, Switzerland). The initial oven temperature of 150°C was held for 1 min, then increased by 35°C/min to 250°C, this temperature held for 4 min and then increased by 30°C/min to 300°C. Mass spectrometry was performed in the negative ion chemical ionization (NICI) mode. MS conditions were: source temperature 150°C; methane CI gas pressure 75 Pa; electron energy 120 eV; emission current 200 μA; argon collision cell pressure 133 mPa; collision energy 15 eV.

The $[M\text{-}20]^-$ ions were used as parent ions for morphine (m/z 557), morphine-d_3 (m/z 560), codeine (m/z 425), and codeine-d_3 (m/z 428). The daughter ions used were m/z 499 for morphine and morphine-d_3, and m/z 128 for codeine and codeine-d_3.

2.3. Preparation of standard solutions

Stock standard solutions (1 mg/ml) of morphine, morphine-d_3, codeine and codeine-d_3 were prepared in water from the respective salts. Working standard solutions were prepared from the stock solutions. All standard solutions were kept at −30°C.

2.4. Biological samples

Human serum samples were obtained from volunteers administered morphine or codeine during pharmacokinetic studies. Urine samples collected in 24 h fractions were obtained from volunteers which had their normal diet for 4 days and a liquid diet substitution for 5 days. All study protocols were approved by the local ethics committee. Written informed consent was obtained from each participant.

2.5. Extraction and derivatization

2.5.1. Serum samples from pharmacokinetic studies

To 1 ml of serum 20 μl of internal standard solution (1.25 nmol/ml morphine-d_3 and 2.5 nmol/ml codeine-d_3 in water) was added. After mixing for 15 min, pH was adjusted to 9.6 with saturated carbonate buffer and the samples were extracted with 6 ml of dichloromethane–2-propanol (9:1; v/v). The organic phase was evaporated to dryness in a stream of nitrogen and the pentafluoropropionyl (PFP) derivatives prepared by treatment with 30 μl of PFPA, 20 μl of acetonitrile and 10 μl of pentafluoro-1-propanol for 20 min at 80°C. The derivatizing reagent was removed (N_2) and the residue dissolved in 30 μl of acetonitrile. Aliquots (2 μl) were subjected to GC–MS–MS analysis.

2.5.2. Urine samples for determination of endogenous morphine

From each urine sample two aliquots of 5 ml were treated as follows. To each glass tube containing 5 ml of urine 5 pmol (1.44 ng) of morphine-d_3 (5 μl of a 1 nmol/ml solution) was added as internal standard. The samples were hydrolyzed with 1 ml of hydrochloric acid (37%) for 30 min at 125°C in a heating block. After cooling to room temperature samples were extracted with 1 ml of dichloromethane–2-propanol (9:1; v/v) for 10 min and then centrifuged at 1500 *g* for 5 min. The aqueous phase was transferred to a clean tube, 600 μl of 10 *M* potassium hydroxide and 2 ml of saturated carbonate buffer (pH 9.6) were added and each sample divided into 2 aliquots of about 5 ml, which were pipetted into clean tubes. Then, 4.5 ml of dichloromethane–2-propanol were added to each tube. After extraction for 10 min and centrifugation at 1500 *g* for 5 min, the upper aqueous phase was aspirated and discarded. The organic phases of all four tubes belonging to one urine sample were combined in a clean tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 2 ml of Tris buffer (2 *M*, pH 8.1) and transferred to sample tubes for the ASPEC. Solid phase extraction was performed with Bond Elut Certify (300 mg) cartridges. Each cartridge was at first conditioned with 2 ml of methanol and 2 ml of water at 3 ml/min. After application of the samples at 1 ml/min, the cartridges were washed with 2 ml of water, 2 ml of acetate buffer (50 m*M*, pH 4) and 1 ml of methanol. Then, the cartridges were dried by applying 10 ml of air and the analytes eluted with 3 ml of eluent mixture. Samples were evaporated and the PFP derivatives prepared as described for the serum samples.

2.6. Standardization

Calibration samples for serum were prepared by adding increasing amounts of morphine (0.05–50 pmol/ml; 0.014–14.3 ng/ml) and codeine (0.5–1000 pmol/ml; 0.15–299 ng/ml) to control serum.

Calibration samples for urine were prepared by adding increasing amounts of morphine (25 fmol–10 000 fmol; 7.1–2853 pg) to 10 ml of urine–water (1:25 v/v).

Standard curves were evaluated by weighte ($1/x^2$) linear regression analysis based on internal standard calibration and were obtained by plotting peak-area ratios against the amount of the substance. The respective deuterated substances were used as internal standards.

2.7. Assay validation

To determine assay variability in serum samples, quality control samples were prepared by adding known amounts of morphine and codeine to 25 ml of drug-free serum, which was divided into 1.1-ml aliquots and stored at −20°C.

Quality control urine samples were prepared like the calibration samples. Additional quality control samples were obtained by dilution of Medidrug Opiate U Level 1 quality control samples (containing 0.1 mg/l of morphine and 0.5 mg/l morphine-3-glucuronide, i.e. total morphine after hydrolysis 409 ng/ml or 1434 pmol/ml) with water.

Quality control samples were analysed always together with the unknown samples.

3. Results and discussion

3.1. Sample preparation

The use of tandem MS enhances the sensitivity and enabled the substances to be measured in serum after a single extraction step. In urine morphine is mainly conjugated with glucuronic acid and sulphate requiring hydrolysis for the recovery of total morphine. In preliminary experiments we have tested several hydrolysis conditions including hydrolysis with concentrated hydrochloric acid or with different β-glucuronidases. Acidic hydrolysis at 125°C for 30 min resulted in complete cleavage of morphine-3-glucuronide and morphine-6-glucuronide without significant degradation of morphine. One problem encountered with urine analysis is the high background, which is even increased by hydrolysis. Therefore, extensive cleaning procedures were required to obtain sufficient sensitivity. Too much dirt in the sample lead to a decrease in the yield of the derivatization reaction or the mass spectrometric response and required frequent cleaning of the GC inlet port and the ion source. With one further liquid–liquid extraction step and one solid-phase extraction the samples were clean enough to obtain high signal to noise ratios even at very low concentrations.

3.2. Derivatization and GC–MS–MS analysis

PFP derivatives are widely used for GC–MS determination of morphine and codeine. Addition of PFPOH increased the yield of the derivatives, a reaction time of 20 min at 80°C proved to be sufficient.

For analysis of the PFP derivatives of opiates very often the electron impact mode is chosen but in the negative ion chemical ionization (NICI) mode the sensitivity is much higher. With MS–MS additional improvement of sensitivity can be achieved. Optimization of MS–MS conditions was carried out and the daughter processes with the highest signal intensities were chosen for detection [15] leading to limits of quantification of 0.02 or 0.5 pmol/ml (5.7 or 150 pg/ml) serum for morphine or codeine, respectively.

Typical chromatograms of serum samples are shown in Fig. 1. In the blank serum sample (Fig. 1A) no interfering peaks can be detected. The small peak for codeine is caused by a small amount of undeuterated codeine present in the internal standard d_3-codeine (0.5%). Chromatograms of a serum sample from a poor metabolizer 25 h after treatment with 170 mg of codeine phosphate are shown in Fig. 1B.

Chromatograms of urine samples from a volunteer participating in the diet study are shown in Fig 2. (Fig. 2A before the study, normal diet, Fig. 2B after 5 days of liquid diet substitution.)

3.3. Validation

The method has good linearity over the entire range measured: 0.02–50 pmol/ml (5.7–14265 pg/ml) for morphine and 0.5–1000 pmol/ml (0.15–299 ng/ml) for codeine in serum and 2.5–1000 fmol/ml (0.71–285 pg/ml) for morphine in urine. Reproducibility was determined by repeatedly analyzing aliquots of serum samples or diluted urine samples spiked with known amounts of analytes.

The intra- and interassay variabilities for the determination of morphine and codeine in serum are given in Tables 1 and 2. Even at the lowest morphine concentration of 0.02 pmol/ml (5.7 pg/ml) the reproducibility is still sufficient with a coefficient of variation of 16.9%. For codeine the coefficient of variation is below 10% at all concentrations.

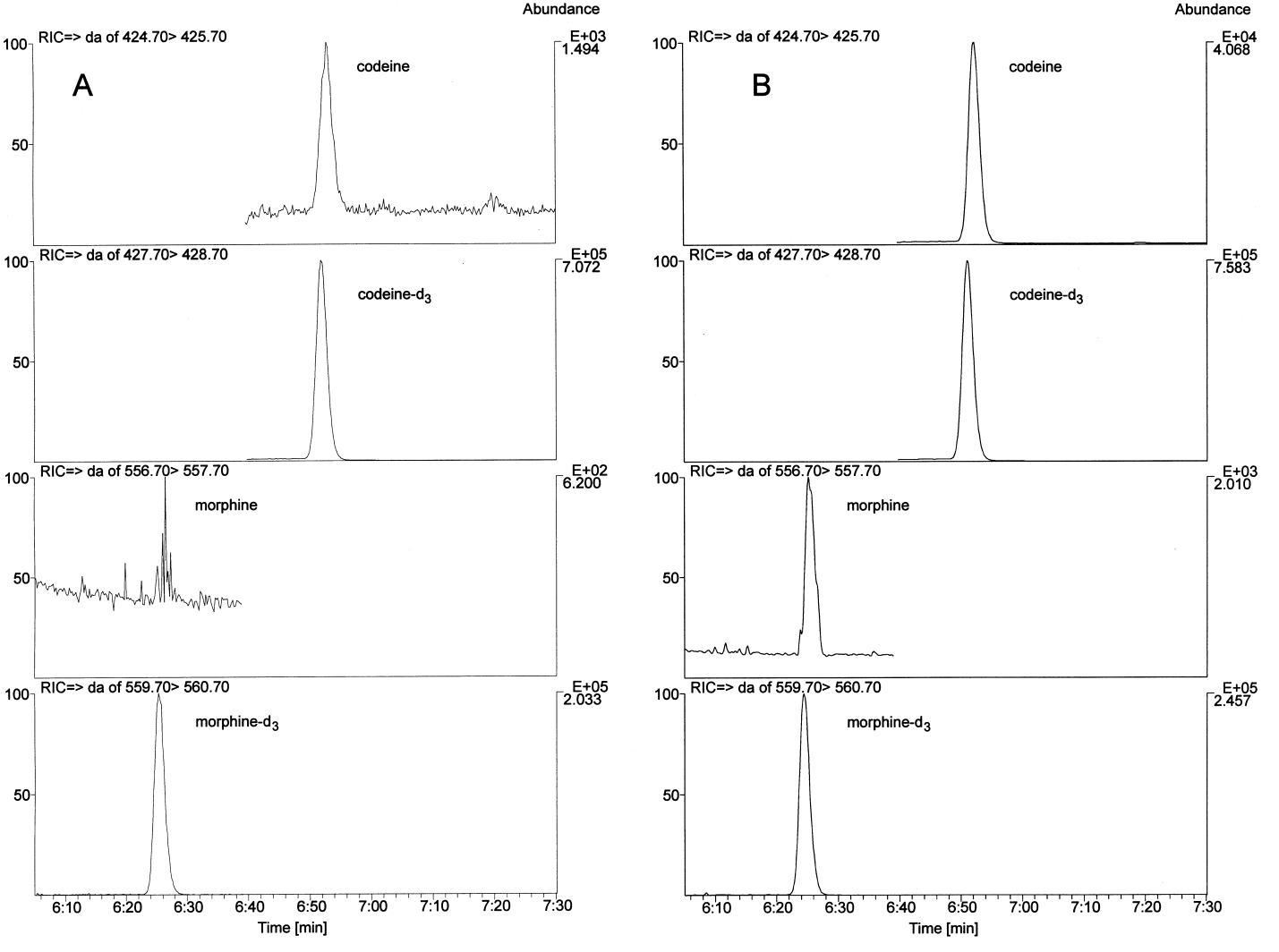

Fig. 1. Daughter-ion chromatograms of derivatized extracts from (A) blank human serum spiked with 25 pmol/ml (7.2 ng/ml) morphine-d_3 and 50 pmol/ml (15.1 ng/ml) codeine-d_3 as internal standards, (B) serum from a volunteer 25 h after administration of 170 mg of codeine phosphate containing 0.071 pmol/ml (20.3 pg/ml) morphine, 5.4 pmol/ml (1617 pg/ml) codeine and internal standards (25 pmol/ml (7.2 ng/ml) morphine-d_3 and 50 pmol/ml (15.1 ng/ml) codeine-d_3).

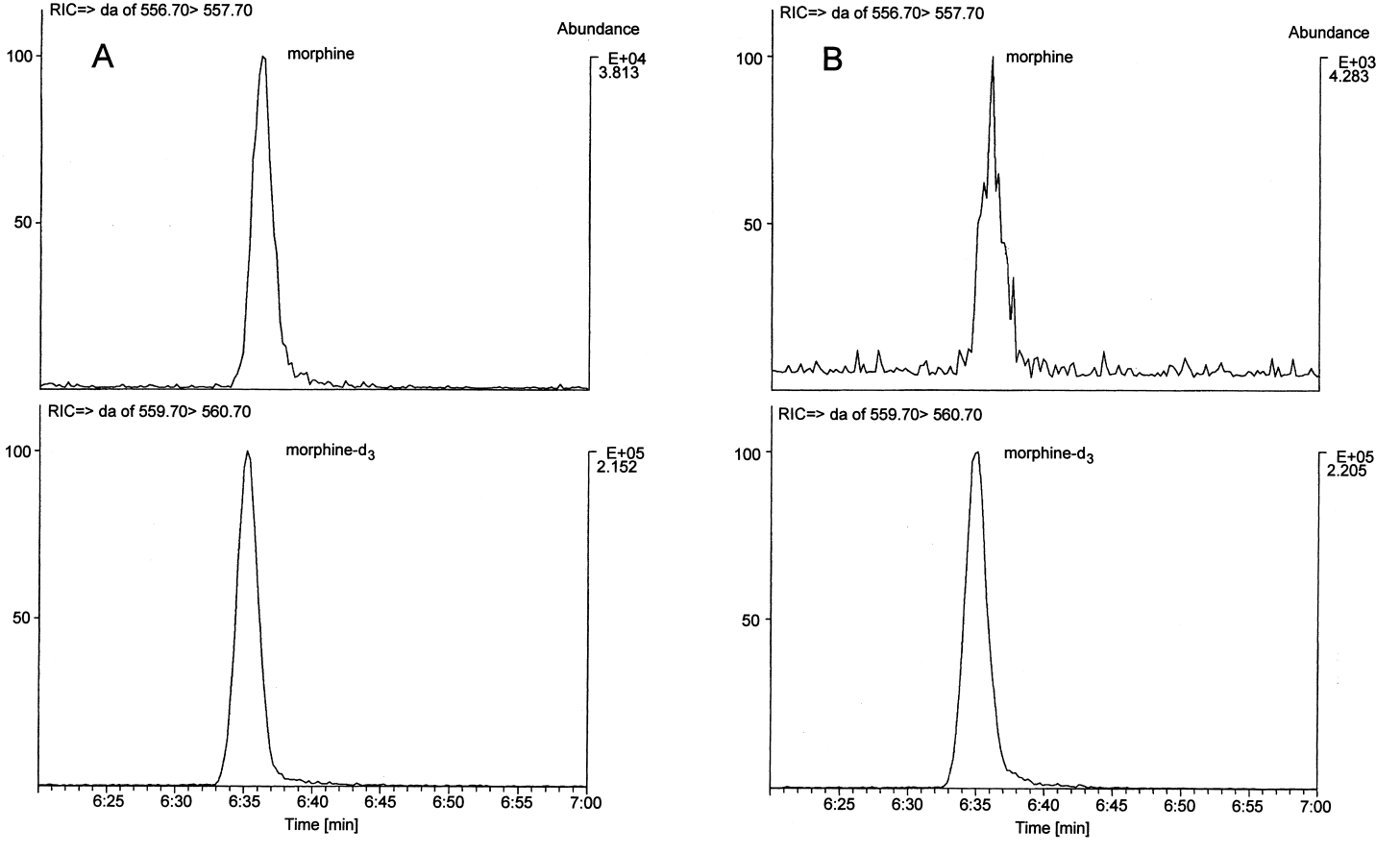

Fig. 2. Daughter-ion chromatograms of derivatized extracts from (A) urine of a volunteer under normal diet containing 186 fmol/ml (53.1 pg/ml) morphine and 1 pmol/ml (288 pg/ml) internal standard morphine-d_3, (B) urine of the same volunteer under a liquid diet substitution on day 5 containing 20 fmol/ml (5.7 pg/ml) morphine and 1 pmol/ml (288 pg/ml) internal standard morphine-d_3

Table 1
Intra-assay and inter-assay precision for the determination of morphine in serum

Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
Intra-assay				
0.020	5	0.0216±0.0034	0.8	16.9
0.050	5	0.0488±0.0056	−2.3	11.4
0.268	5	0.296±0.017	10.4	5.7
2.68	6	2.62±0.08	−2.2	2.9
13.4	6	13.03±0.21	−2.8	1.7
Inter-assay				
0.050	22	0.057±0.0069	14.6	12.1
0.268	23	0.293±0.015	9.5	5.1
2.68	26	2.57±0.19	−4.0	7.6
13.4	29	13.1±1.1	−1.9	8.1
50.0	28	50.0±3.1	0.0	6.2

Assay variabilities for the determination of morphine in urine are given in Table 3. To achieve the limit of quantification of 2.5 fmol/ml (0.71 pg/ml) an amount of 10 ml of urine had to be used. For urine analysis a commercially available quality control sample (Medidrug Opiate U Level 1) was included that also contains morphine glucuronides. This quality control sample was used for checking the hydrolysis. The small bias of −6.9 or 5.8% intra- or inter-day shows the completeness of the acid hydrolysis method.

3.4. Application

The method described has excellent sensitivity both for serum and urine samples which is not reached by other methods. The following examples show the application of the method to pharmacokinetic studies and to a study examining the influence of diet on the urinary excretion of endogenous morphine.

Table 2
Intra-assay and inter-assay precision for the determination of codeine in serum

Concentration added added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
Intra-assay				
1.00	6	1.10±0.09	10.2	8.1
2.00	6	2.24±0.16	12.1	7.1
66.8	6	70.8±1.2	6.0	1.7
668	6	586±39	−12.2	6.6
Inter-assay				
0.50	20	0.573±0.054	14.5	9.5
66.8	25	71.5±3.0	7.1	4.3
500	28	496±40	−0.8	8.0
2000	28	1807±178	−9.7	9.8

Table 3
Intra-assay and inter-assay precision for the determination of endogenous morphine in urine

Concentration added (fmol/ml)	*n*	Concentration found (fmol/ml)	Bias (%)	C.V. (%)
Intra-assay				
2.50	5	2.80±0.29	12.0	10.4
5.0	5	5.54±0.08	10.8	1.4
100	6	104.8±0.7	4.8	0.7
100.4[a]	6	93.5±3.1	−6.9	3.3
Inter-assay				
100.4[a]	4	106.3±7.3	5.8	6.9

[a] Obtained by dilution of Medidrug Opiate U Level 1 quality control.

The assay has been used to determine codeine and morphine in serum samples from volunteers administered codeine or morphine during clinical studies. A typical serum concentration time curve of a poor metabolizer administered 170 mg codeine phosphate is shown in Fig. 3. After 25 h a codeine concentration of 5.4 pmol/ml (1617 pg/ml) and a morphine concentration of 0.071 pmol/ml (20.3 pg/ml) was determined.

Urinary excretion of morphine was investigated

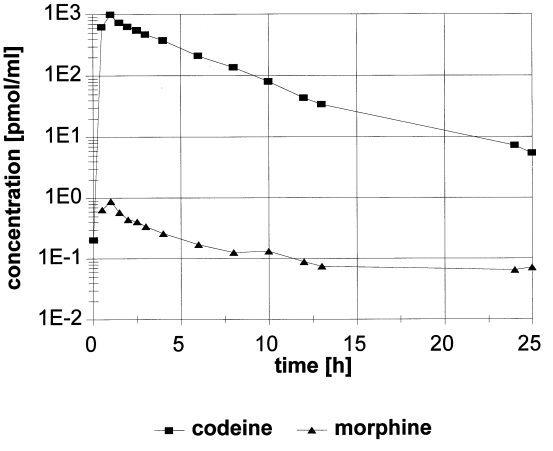

Fig. 3. Serum concentration–time curve of codeine and morphine in a poor metabolizer of CYP2D6 administered 170 mg of codeine phosphate.

Table 4
Urinary excretion of morphine of a volunteer under normal dietary conditions and under a liquid diet substitution

	Day	Morphine concentration (fmol/ml)	Urine volume in 24 h (ml)	Morphine excretion (pmol/24 h)
Normal diet	1	518	1430	741
	2	145	1600	233
	3	104	1435	149
	4	118	1350	154
Liquid diet substitution	5	49	2345	115
	6	33	1730	58
	7	50	1185	59
	8	25	1520	38
	9	27	1505	41

under normal dietary conditions and under a morphine-free liquid diet substitution. Table 4 shows the values of one volunteer participating in the study. During the liquid diet substitution a significant reduction of morphine excretion is observed.

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