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Simultaneous determination of dihydrocodeine and dihydromorphine in serum by gas chromatography–tandem mass spectrometry

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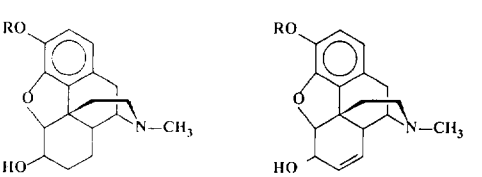
Abstract

A sensitive and specific method was developed for the determination of dihydrocodeine and its metabolite dihydromorphine in human serum using codeine and morphine as internal standards. Measurement is performed with GC–tandem MS after one simple extraction step and derivatization to the pentafluoropropionic esters. Sensitivity of the method is excellent and allows for the reproducible quantification of dihydrocodeine and dihydromorphine with limits of quantification of 2 ng/ml and 40 pg/ml serum, respectively. The method is therefore well suited for investigation of the pharmacokinetics and the metabolism of dihydrocodeine.

1. Introduction

The opioid dihydrocodeine, which was first synthesized by Skita and Franck in 1911 [1] is frequently used as antitussive and analgesic drug. Furthermore, increasing numbers of drug addicts are treated with dihydrocodeine in Germany. There are however only very limited data on the pharmacokinetics and metabolism of this opioid [2,3]. It has been proposed that O-demethylation of dihydrocodeine might lead to formation of the active metabolite dihydromorphine (Fig. 1). Due to its structural similarity to codeine it is likely that this metabolic step is catalyzed by the cytochrome P450 CYP2D6. This enzyme exhibits a genetic polymorphism where 7–10% of a Caucasian population are so called poor metabolizers because they do not express this enzyme [4]. We intend to investigate this possible cause of the interindividual variability in dihydrocodeine disposition in drug addicts and in healthy volunteers. In order to determine dihydrocodeine and dihydromorphine in serum



R = H dihydromorphine morphine
R = CH3 dihydrocodeine codeine

Fig. 1. Structures of dihydrocodeine, dihydromorphine, codeine and morphine.

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we had to focus on two major issues: (1) the method had to be very sensitive, since dihydromorphine concentrations were expected to be in the range below one pmol/ml for poor metabolizers and (2) it had to be very selective because other opioids (e.g. heroin, codeine) are frequently consumed by drug addicts in addition to dihydrocodeine treatment.

Only few methods for the determination of dihydrocodeine in plasma or serum have been described, using GC with nitrogen–phosphorous detection [5], HPLC with UV detection [2], fluorescence detection [5] or electrochemical detection [6]. Having a limit of quantification of at best 20 ng/ml these methods are not sensitive enough; moreover they lack specificity. For the determination of dihydromorphine in biological fluids only two methods exist, e.g. HPLC for quantification in urine or bile [7] and GC–MS for quantification in urine [8]. The sensitivity of these methods is absolutely insufficient for the determination of dihydromorphine in serum since the limits of quantification achieved are only 20 and 10 ng/ml, respectively.

We have developed a specific and sensitive GC–MS–MS method which enables the quantification of dihydrocodeine and dihydromorphine in serum for a detailed study of the pharmacokinetics after a single oral dose of 60 mg of dihydrocodeine bitartrate.

2. Experimental

2.1. Chemicals

Solvents used were of HPLC quality; chemicals were of analytical grade. Dihydrocodeine bitartrate was obtained from Merck (Darmstadt, Germany), dihydromorphine hydrochloride was a generous gift from Mundipharma (Limburg, Germany), codeine, codeine-d_3 hydrochloride dihydrate, morphine sulfate pentahydrate and morphine-d_3 hydrochloride trihydrate were from Sigma (Deisenhofen, Germany). Pentafluoropropionic anhydride (PFPA) was supplied by Aldrich (Steinheim, Germany).

2.2. Preparation of standard solutions

Stock standard solutions (1 mg/ml) of dihydrocodeine, dihydromorphine, morphine and codeine were prepared in water from their respective salts. Working standard solutions were prepared from the stock solutions. All standard solutions were kept at −30°C. All concentrations given refer to the respective salts.

2.3. Extraction and derivatization

To 1 ml of serum 10 ng of morphine (10 μl of a 1 ng/μl solution in water) and 20 ng of codeine (20 μl of a 1 ng/μl solution in water) were added as internal standards. After mixing for 15 min, the 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 20 μl of PFPA for 30 min at 60°C. The derivatizing reagent was removed (under nitrogen) and the residue dissolved in 30 μl of acetonitrile. Aliquots (2 μl) were subjected to GC–MS–MS analysis.

2.4. Instrumentation and chromatographic conditions

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 and W Scientific, Fisons, Mainz, Germany) in the splitless mode. The carrier gas was 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 was 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 10 eV.

The $[M^-]^-$ ion, *m/z* 579, was used as parent ion for dihydromorphine, the $[M-20]^-$ ions were used as parent ions for morphine (*m/z* 557), dihydrocodeine (*m/z* 427) and codeine (*m/z* 425). The daughter ions used were *m/z* 413 for dihydromorphine, *m/z* 499 for morphine, *m/z* 321 for dihydrocodeine and *m/z* 128 for codeine.

2.5. Standardization

Calibration samples were prepared by adding increasing amounts of dihydromorphine (0.01–20 ng) and dihydrocodeine (0.5–500 ng) to control serum. Standard curves were based on internal standard calibration and were obtained by plotting peak-area ratios against the amount of the substance.

2.6. Assay validation

To determine assay variability, quality control samples were prepared by adding known amounts of dihydromorphine and dihydrocodeine to 20 ml of drug-free serum, which was divided into 1.2-ml aliquots and stored at −20°C. Quality control samples were analyzed always together with the serum samples. The accuracy over the entire concentration range was determined by adding various amounts of the analytes to drug-free serum and measuring the concentration.

3. Results and discussion

The method described allows for the simultaneous determination of dihydrocodeine and dihydromorphine in serum using codeine and morphine as internal standards. Excellent sensitivity could be achieved by combination of an appropriate derivative (PFP-ester) and measurement in the NICI mode. NICI mass spectra are

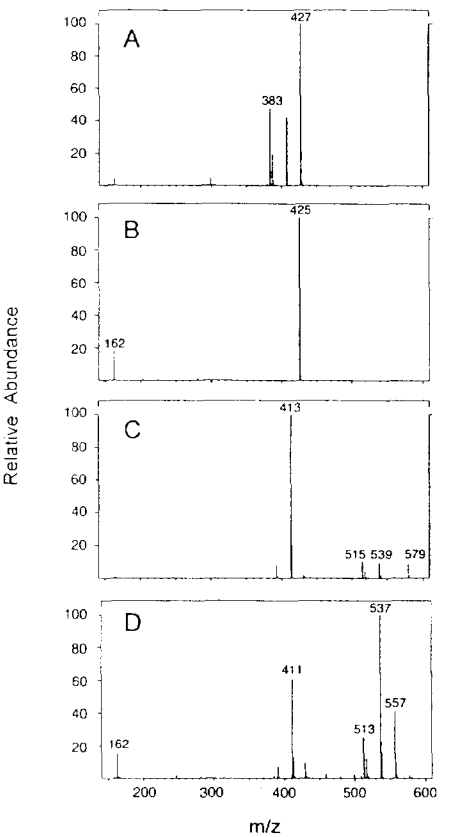

Fig. 2. Negative-ion chemical-ionization mass spectra of the PFP derivatives of (A) dihydrocodeine ($C_{21}H_{22}F_5NO_4$, M_r 447.4), (B) codeine ($C_{21}H_{20}F_5NO_4$, M_r 445.4), (C) dihydromorphine ($C_{23}H_{19}F_{10}NO_5$, M_r 579.4) and (D) morphine ($C_{23}H_{17}F_{10}NO_5$, M_r 577.4).

shown in Fig. 2. MS–MS conditions were optimized with regard to both high sensitivity and selectivity. For the measurement of dihydrocodeine and its internal standard codeine the respective $[M-20]^-$ ions (*m/z* 427 for dihydrocodeine and *m/z* 425 for codeine) were used as parent ions. These fragments result from the abstraction of hydrogen fluoride (HF) from the molecular ion of the respective PFP derivative and represent the base peak with more than 30% of the total ion current. The most intense daughter ions, *m/z* 321 for dihydrocodeine and *m/z* 128 for codeine, were used for detection in the selected-reaction monitoring (SRM) mode (Fig. 3). The parent ions chosen for measurement of dihydromorphine (*m/z* 579) and the

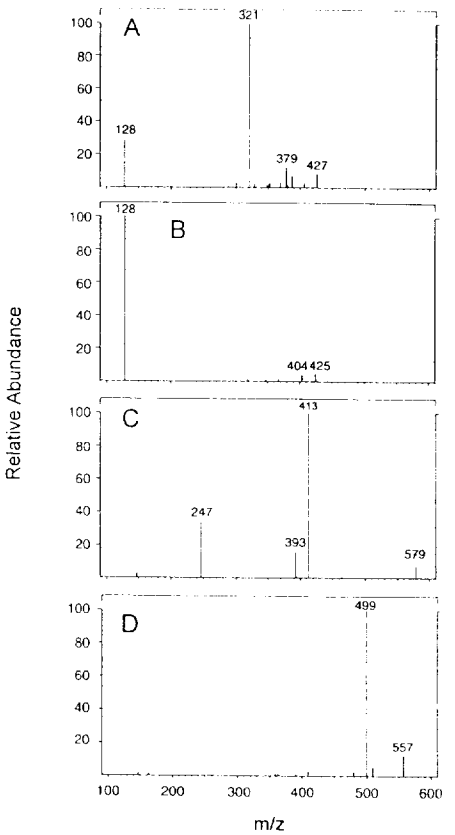

Fig. 3. Daughter-ion MS–MS spectra of (A) 6-PFP-dihydrocodeine (parent ion 427), (B) 6-PFP-codeine (parent ion 425), (C) 3,6-di-PFP-dihydromorphine (parent ion 579) and (D) 3,6-di-PFP-morphine (parent ion 557).

appropriate internal standard morphine (m/z 557) have an abundance of only 10 and 20%, respectively, but show simple daughter spectra (Fig. 3) with m/z 413 (dihydromorphine) or m/z 499 (morphine) as the most abundant daughter ions.

The use of tandem MS enhanced the selectivity compared to single-stage MS and enabled the substances to be measured after a single extraction step. In serum samples from drug addicts deuterated morphine and codeine were used as internal standards. Therefore concomitant illicit drug use (naltrexone, heroin, cocaine, phenobarbital, flunitrazepam) did not interfere with the present method. Moreover, monitoring of heroin intake was possible by the determination of its metabolite morphine (Fig. 4A).

The sensitivity achieved is appropriate for the measurement of dihydrocodeine and dihydromorphine in serum up to 25 h after a single oral dose of 60 mg of dihydrocodeine bitartrate even in poor metabolizers (Fig. 4B). Concentrations of dihydromorphine of 0.01 ng/ml could be measured with a signal-to-noise ratio of 5. In drug-free sera no dihydromorphine was detectable and dihydrocodeine was below ca. 0.1 ng/ml. The method has good linearity over the entire range measured: 0.01–20 ng/ml for dihydromorphine and 0.5–500 ng/ml for dihydrocodeine. A typical standard curve for dihydrocodeine is $y = 0.004x + 0.0056$ ($r > 0.9992$, response factor 0.077 ± 0.0206) and for dihydromorphine $y = 0.0358x + 0.0013$ ($r > 0.9994$, response factor 0.241 ± 0.051). The accuracy of the assay is shown in Table 1. There is a good correlation between the concentration added and that measured both for dihydrocodeine ($y = 1.08x + 0.38$, $r = 0.9979$) and for dihydromorphine ($y = 0.96x + 0.01$, $r = 0.9999$).

Reproducibility was determined by repeatedly analyzing aliquots of serum samples spiked with known amounts of dihydrocodeine and dihydromorphine. The intra-assay and inter-assay variabilities are given in Table 2 for dihydrocodeine and in Table 3 for dihydromorphine. Intra-assay reproducibility was better than 10%. The day-to-day variation is acceptable even at the lowest concentrations (19.6% at the limit of quantification for dihydrocodeine and 14.4% for 70 pg/ml dihydromorphine). The limits of quantification were 2.0 ng/ml for dihydrocodeine (Table 2) and 0.04 ng/ml for dihydromorphine (Table 3); for these concentrations both variability and relative error were better than 20%. For lower concentrations reproducibility was still sufficient but the relative error increased to 25–30%.

The method described has been used to determine dihydrocodeine and dihydromorphine in serum samples from volunteers administered a 60-mg dose of dihydrocodeine bitartrate. A typical serum concentration–time curve of a poor metabolizer is shown in Fig. 5. Maximum serum concentrations (C_{max}) ranged between 166 and 455 ng/ml for dihydrocodeine and between

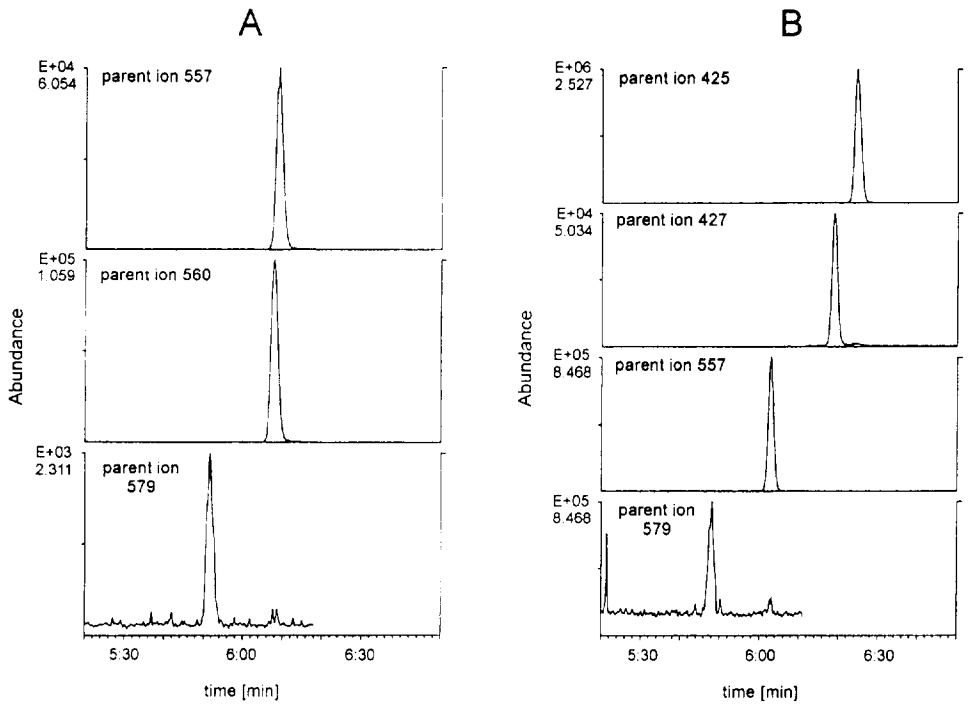

Fig. 4. Daughter-ion chromatograms of derivatized extracts from (A) serum from a drug addict with 0.9 ng/ml dihydromorphine (parent ion 579) and about 30 ng/ml morphine (parent ion 557), internal standard d_3-morphine (parent ion 560), (B) serum from a volunteer 25 h after administration of 60 mg of dihydrocodeine bitartrate containing 0.07 ng/ml dihydromorphine, 10 ng/ml internal standard morphine, 4.1 ng/ml dihydrocodeine (parent ion 427) and 20 ng/ml internal standard codeine (parent ion 425).

Table 1
Accuracy of the determination of dihydrocodeine and dihydromorphine in serum by GC–MS–MS

Dihydrocodeine			Dihydromorphine		
Concentration added (ng/ml)	Concentration found (ng/ml)	Found/ added (%)	Concentration added (ng/ml)	Concentration found (ng/ml)	Found/ added (%)
190.0	195.0	102.6	20.00	19.20	96.0
175.0	195.0	111.4	17.00	16.00	94.1
3.0	3.1	103.3	0.20	0.17	85.0
13.0	11.8	90.8	0.60	0.59	98.3
65.0	72.9	112.2	9.00	8.80	97.8
90.0	107.3	119.2	13.00	12.60	96.9
0.5	0.34	68.0	0.010	0.012	120.0
1.0	0.69	69.0	0.020	0.022	110.0
2.0	1.77	88.5	0.050	0.051	102.0
5.0	4.3	86.4	0.10	0.10	100.0
10.0	9.5	94.9	0.50	0.47	94.0
20.0	23.0	115.0	1.00	0.92	92.0

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

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Bias (%)	C.V. (%)
Intra-assay				
2.0	6	1.630 ± 0.035	−18.5	2.2
4.0	5	3.42 ± 0.32	−14.4	9.3
20.0	5	18.9 ± 0.5	−9.5	2.8
200.0	5	208 ± 9.0	4.0	4.3
Inter-assay				
2.0	15	2.30 ± 0.45	15.0	19.6
20.0	16	20.5 ± 2.1	2.5	10.0
200.0	18	199 ± 15	0.5	7.7

Values are given as mean ± S.D.; C.V. = coefficient of variation.

Table 3
Intra-assay and inter-assay precision for the determination of dihydromorphine in serum

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Bias (%)	C.V. (%)
Intra-assay				
0.02	5	0.025 ± 0.001	27.0	3.5
0.04	6	0.047 ± 0.001	18.7	2.2
0.07	5	0.078 ± 0.006	11.4	8.1
0.90	5	0.92 ± 0.05	2.2	4.9
5.0	5	4.47 ± 0.11	−10.6	2.4
Inter-assay				
0.07	9	0.074 ± 0.011	5.7	14.4
0.10	12	0.116 ± 0.014	16.0	11.9
1.0	12	1.10 ± 0.09	10.0	8.3
5.0	12	5.36 ± 0.40	7.2	7.5

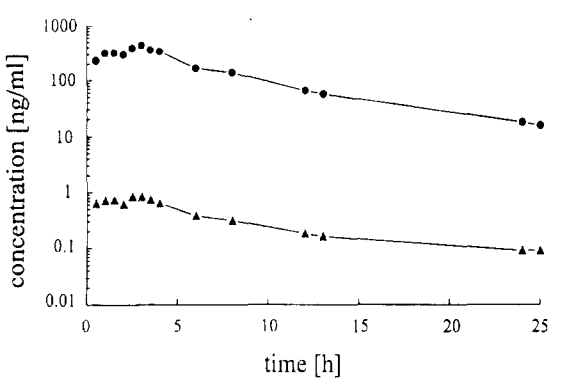

Fig. 5. Typical serum concentration–time curve of (●) dihydrocodeine and (▲) dihydromorphine in a poor metabolizer administered 60 mg of dihydrocodeine bitartrate.

0.24 and 6.49 ng/ml for dihydromorphine. Dihydrocodeine concentrations measured in serum samples 25 h after drug intake were between 2 and 18 ng/ml, dihydromorphine concentrations were between 0.03 and 0.39 ng/ml. The pharmacokinetics of dihydrocodeine and its active metabolite dihydromorphine can therefore be characterized reliably.

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