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Evaluation of a method for simultaneous quantification of codeine, dihydrocodeine, morphine, and 6-monoacetylmorphine in serum, blood, and postmortem blood

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Summary. A solid-phase extraction and gas chromatographic-mass spectrometric method for the simultaneous determination of codeine, dihydrocodeine, morphine, and 6-monoacetylmorphine in serum, blood or postmortem blood is described. The extraction technique allows the determination of free or total morphine (morphine plus morphine glucuronide). Experiments with spiked blood samples resulted in recoveries of 96.4% ± 4.2% for codeine, 95.8% ± 5.1% for dihydrocodeine, 90.3% ± 7.8% for 6-monoacetylmorphine and 92.5% ± 8.1% for morphine. Excellent linearity was obtained over the range 1–1500 ng/mL. The detection limit for all analytes is less than 1 ng/mL.

Key words: Codeine – Morphine – Blood – Solid-phase extraction – GC/MS-analysis

Zusammenfassung. Eine Festphasen-Extraktion und GC/MS-Methode zur gleichzeitigen Bestimmung von Codein, Dihydrocodein, Morphin und 6-Monoacetylmorphin in Serum, Blut und Leichenblut wird beschrieben. Die Extraktions-Technik ermöglicht die Bestimmung von freiem und Gesamtmorphin (Morphin plus Morphinglucuronid). Versuche mit aufgestockten Blutproben führten zu Wiederfindungsraten von 96.4% ± 4.2% für Codein, 95.8% ± 5.1% für Dihydrocodein, 90.3% ± 7.8% für 6-Monoacetylmorphin und 92.5% ± 8.1% für Morphin. Linearität wurde über den Konzentrationsbereich von 1–1500 ng/mL erhalten. Die Nachweisgrenzen liegen für alle Substanzen unter 1 ng/mL.

Schlüsselwörter: Codein – Morphin – Blut – Festphasen-Extraktion – GC/MS-Analyse

Introduction

Shortly after intake, heroin is metabolized to 6-monoacetylmorphine (MAM) and further hydrolysed to morphine [1]. The morphine itself is deactivated by glucuronidation and the concentration of morphine glucuronide increases in relation to the decrease of free morphine in the blood. However, for toxicological interpretations, blood concentrations of the initial heroin metabolite, MAM, as well as of free and conjugated morphine is of interest. In addition the determination of codeine, which is also metabolized to morphine, and dihydrocodeine indicates a possible intake of pharmaceuticals, which results in positive morphine immunoassays.

Many different analytical techniques are used for the detection of opiates in body fluids, including radioimmunoassay [2–3], enzyme multiplied immunoassay [4–5], thin-layer chromatography [6], high performance liquid chromatography [7–8], and gas chromatography often coupled with mass spectrometry [9–12].

A relatively simple method for the simultaneous quantification of codeine, morphine and MAM in blood has been reported by Schuberth and Schuberth [13] and as a slight modification by Gjerde et al. [14]. This method utilizes a solid-phase extraction procedure combined with a gas chromatographic – mass spectrometric determination of the drugs. The following report offers a modified extraction procedure with a washing step to remove interfering compounds and results in higher recoveries, lower detection limits and allows the simultaneous determination of codeine, dihydrocodeine, morphine, and MAM. The determination of morphine glucuronide according to the procedure of Staub et al. [15] is also ensured.

Materials and methods

Chemicals. Methanol, ethyl acetate (Uvasol, Merck Darmstadt, Germany), water (HPLC grade, Baker Groß-Gerau, Germany); inorganic chemicals: ammonium chloride, boric acid, hydrochloric acid, liquid ammonia, sodium fluoride, sodium hydroxide, sodium tetraborate, trichloroacetic acid (p.a., Merck). Morphine, codeine, dihydrocodeine were obtained commercially, MAM was synthesized from morphine, and 0.1% stock solutions were prepared in methanol. Internal standards d_3-morphine and d_3-codeine were

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purchased from Sigma (Deisenhofen, Germany). Standard solutions contained 10 µg d_3-codeine and d_3-morphine/mL methanol.

Borate buffer (pH9): 835 mL solution A (12.37 g boric acid + 100 mL sodium hydroxide [1 mol/L] with sodium tetraborate [0.05 mol/L] ad 1 L) and 165 mL solution B (hydrochloric acid [0.1 mol/L]). Worldwide Monitoring Clean Up C_{18} end-capped extraction columns (100 mg, 1 mL) were purchased from Amchro (Sulzbach/Taunus, Germany). Blood for the evaluation of the method was obtained from the blood bank of the University Hospital, Düsseldorf. NaF (1%) was added to the blood and stored in glass containers. Blood samples were spiked with methanolic solutions of codeine, dihydrocodeine, morphine, and MAM (1, 10, 50, 100, 500, 1000, 1500 ng/mL).

Instrumentation and chromatographic parameters. The extraction columns were positioned on a Vacuum-Manifold (Amchro). A Model 5890A GC (Hewlett-Packard) with a 5970A Mass Selective Detector (MSD) was used for analysis. Data acquisition and manipulation were performed using standard software supplied by the manufacturer. The instrument was autotuned daily with perfluorotributylamine. For sample analysis the electron multiplier voltage of the detector was set in the range 200–400 V above autotune voltage. A fused silica capillary column OV1 (12 m × 0.2 mm i.d.) was used. The temperature program used with this column consisted of an initial temperature of 150°C, held for 2 min, followed by a linear ramp to 220°C at a rate of 40°C/min. The final temperature was held for 6 min. The split/splitless injector was maintained at a temperature of 260°C.

Extraction and derivatization. Internal standard (10 µL) was added to spiked serum, blood (1 mL) or postmortem blood (1 g). Spiked blood samples were mixed with 2 mL acetone, vortexed and centrifuged for 5 min at 1000 g. The supernatant was evaporated to dryness and dissolved in 2 mL borate buffer. Spiked serum samples were prepared for solid-phase extraction by adding 1 mL borate buffer. To determine the total amount of morphine (morphine and morphine glucuronide) in authentic cases, 0.5 mL 10% trichloroacetic acid and 1.5 mL hydrochloric acid (2 M) were added to 1 mL aliquots. The whole sample was vortexed and centrifuged (5 min, 1000 g). The supernatant was transferred to screw-top tempered glass tubes, hydrolysed by incubating at 100°C for 45 min and adjusted to pH 9 by adding 1 mL saturated ammonium chloride and 0.3–0.4 mL 25% liquid ammonia. The whole volume was applied to an extraction column.

The extraction columns were conditioned by washing with 2 mL methanol followed by 2 mL water and 1 mL borate buffer. Prepared samples were applied to the columns and drawn through by a vacuum with a flow rate of approximately 1 mL/min. The columns were washed with 1 mL water followed by 1 mL 25% methanol in water and dried by centrifugation of the columns (5 min, 1000 g). The opiates were eluted with 2 × 0.5 mL methanol and collected in a vial. The eluate was evaporated to dryness at 50°C under a stream of nitrogen.

To form the pentafluoropropionyl derivatives, 40 µL pentafluoropropionyl anhydride (PFPA) and 10 µL pentafluoropropanol (PFPOH) were added to the dried extract, the sample was mixed by vortexing, incubated at 70°C for 30 min, evaporated to dryness at 50°C under a stream of nitrogen and reconstituted in 20 µL ethyl acetate. A volume of 2 µL was injected into the GC/MS.

Table 1. Diagnostic mass fragments and retention times

	m/z	Retention time (min)
Codeine	119, 282, 449	8.20
Dihydrocodeine	119, 282, 447	7.92
Morphine	119, 414, 577	7.61
6-Monoacetylmorphine	204, 414, 473	9.20
d_3-Codeine	119, 285, 452	8.15
d_3-Morphine	119, 417, 580	7.59

Results and discussion

For evaluation of the procedure blood samples were spiked with different concentrations of codeine, dihydrocodeine, MAM and morphine. Electron impact (EI) mass spectra of the compounds were recorded by total ion monitoring. Retention times and characteristic mass fragments were recorded and the chosen diagnostic mass fragments were monitored for each compound in the selected ion monitoring (SIM) mode (Table 1). The mass fragments m/z 119, 282 and 449 were chosen to monitor for the presence of codeine, m/z 119, 285 and 452 were used for d_3-codeine, m/z 119, 282 and 447 were used for morphine, m/z 119, 417 and 580 for d_3-morphine, and the mass fragments selected for MAM were m/z 204, 414 and 473. For quantification the peak area ratios of substance and internal standard were calculated as a function of the concentration of the substance. The following peak area ratios were used: ion 445 (codeine) / ion 448 (d_3-codeine), ion 447 (dihydrocodeine) / ion 448 (d_3-codeine), ion 414 (morphine) / ion 417 (d_3-morphine) and ion 473 (MAM) / ion 417 (d_3-morphine). Excellent linearity was obtained over the range 1–1500 ng/mL blood for all drugs investigated. The coefficients of correlation for the calibration curves ranged from 0.993 to 0.997. Recoveries and reproducibility of the method were measured by analyzing 5 replicates of 2 blood samples assayed to contain 10 ng and 500 ng drug/mL (Table 2). The recoveries (means ± SD) determined with external standards ranged from 90.3% ± 7.8% to 96.4% ± 4.2% for the high concentration samples and from 90.1% ± 6.8% to 96.2% ± 5.1% for the low concentration samples. The day-to-day precision CV's ranged

Table 2. Precision data determined with spiked blood samples (n = 5)

	Codeine		Dihydrocodeine		Morphine		MAM	
Added (ng/ml)	10	500	10	500	10	500	10	500
Found (ng/ml) ± SD	9.6 0.55	480 26	9.7 0.61	473 33	9.4 0.81	471 30	10.7 0.80	542 40
CV (%)	5.5	5.2	6.1	6.6	8.2	7.2	8.4	8.0
Recovery (%) ± SD	96.2 5.1	96.4 4.2	95.6 5.3	95.8 5.1	91.3 7.2	92.5 8.1	90.1 6.8	90.3 7.8

from 4.2% to 8.1% at the higher levels and from 5.1% to 7.2% at the lower levels. Using the routine method described above, the minimal detectable drug concentrations were less than 1 ng/mL blood.

The present method is a slight modification of the method published by Schuberth and Schuberth [13] and evaluated by Gjerde et al. [14]. Precipitation of the sample before application to a C_{18} sample preparation column is only used for blood and not for serum. Also hydrolyzed samples adjusted to pH 9 can be used for solid-phase extraction. To produce highly purified extracts the columns were first washed with distilled water, then with 25% aqueous methanol to remove interfering components. These washing steps did not reduce the recoveries of drugs but improved the purity of the extracts better than washing with water alone. The recoveries were higher than previously reported. Detection limits less than 1 ng/mL were ensured because background fluctuations were reduced through the additional washing step.

In summary an efficient extraction and GC/MS analysis of codeine, dihydrocodeine, morphine and 6-monoacetylmorphine from serum, blood or postmortem blood is reported. Determination of total morphine after hydrolysis can also be easily performed. This procedure, established as a routine method in our laboratory, is relatively simple, reproducible and sensitive and produces results within 45 min.

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