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ORIGINAL ARTICLE

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Evaluation of a solid-phase extraction procedure for the simultaneous determination of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine in plasma and whole blood by GC/MS

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Abstract Different procedures of solid-phase extraction were re-examined and a new solid-phase extraction procedure was developed using gas chromatography-mass spectrometry for the simultaneous detection and quantitation of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine in plasma and whole blood. The effects of different types of sorbent and buffer solutions on the recoveries and purity of the extracts were also studied. Some preparation techniques on whole blood samples were also investigated. The method developed using Chromabond C18 (100) with spiked plasma samples had good recoveries for all opiates of interest: morphine 93.1% ± 7.4%, 6-monoacetylmorphine 68.0% ± 6.7%, codeine 77.0% ± 8.3% and dihydrocodeine 67.9% ± 8.4%. The detection limit of all compounds was less than 5 µg/L. The blank plasma showed no interfering peaks in the GC/MS-analysis.

Key words Morphine · Codeine · Solid-phase-extraction · Mixed mode solid-phase extraction · GC/MS analysis

Introduction

The selective determination and quantification of opiates has great importance in forensic toxicology. The detection of morphine alone is of no use to differentiate between the sources of morphine. Morphine is detectable after the intake of codeine, morphine, heroin and poppy seeds and pharmaceuticals such as codeine and dihydrocodeine can give positive results for morphine immunoassays. A correct interpretation of the results for forensic purposes is based on the selective detection of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine and the comprehension of their metabolism. Shortly after intravenous injection heroin is deacetylated to 6-monoacetylmorphine with a half-life of 9 min, which is further deacetylated to morphine [12, 15]. Free morphine is deactivated by glucuronidation resulting in morphine-3-glucuronide and in lower concentrations of morphine-6-glucuronide. About 5% is demethylated to normorphine [9]. 6-Monoacetylmorphine is a unique metabolite of heroin and not metabolized from morphine or codeine [6]. Codeine, often used as substitute for heroin, is transformed to norcodeine by N-demethylation and to morphine by O-demethylation [1]. The genetic variation of cytochrom -450 oxidase permits no interpretation of the codeine/morphine ratio as sometimes codeine is completely excreted while morphine is still detectable [7]. Dihydrocodeine is metabolized in a similiar way by O-demethylation and glucuronidation. In contrast to codeine the unmetabolized part of dihydrocodeine is 3 times higher and is therefore detectable for a longer period of time [1]. It's detection has gained importance since more and more physicians tend to use this antitussive as a substitution for drug addicts.

Since the 1940s liquid-liquid extraction is the most commonly used method for the extraction of opiates, especially codeine. The experiments with bonded phase extractions in the 1970s brought great improvements in reproducibility. The use of hydrophobic phases such as XAD and C18 allowed selective extraction of the compounds of interest although many interfering substances occurred in the eluates. Since 1980 ion-exchange columns have permitted more selectivity and a greater purity. The final step in the evolution of the solid-phase extraction was the development of copolymeric phases in 1986, which consist of C8-chains and ion-exchange groups. Many publications in the 1990s tried to reduce background noise and show the wide range of chemical compounds which can be separated [20]. Several methods for the detection of opiates in body fluids with C18-columns have been reported. Fehn and Megges [8] reported a simple method for the determination of 6-monoacetylmorphine in urine, which was modified by Hanisch and von Meyer [10]. Schuberth and Schuberth [17] described a procedure for the simultaneous quantification of morphine, 6-monoacetylmorphine and codeine in blood. A

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modified extraction procedure is given in the report of Musshoff and Daldrup [14], by which dihydrocodeine can additionally be determined by GC/MS-analysis. Since 1990 many of new extraction procedures have been performed using copolymeric phases. Some publications of Chen et al. [2–5] offered the first attempts to use these new sorbents for systematical use especially for the extraction of morphine. Huang et al. [11] and Wu et al. [19] also used the advantages of a more effective column washing after stabilizing the binding of the compounds by a pH-switch to a more acidic environment that creates ion-bindings. This investigation compares the different procedures using the sorbents XAD2, C18, C18 end-capped and Certify and offers a simplified C18-extraction method of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine which is optimised with regard to sorbents, pH-value and blood sample preparation.

Materials and methods

Chemicals

Methanol (prepsolv, Merck Darmstadt, Germany), dichlormethane, ethyl acetate, ammonia, ortho-phosphoric acid, boracic acid (pro analysi, Merck Darmstadt, Germany) and water (Aqua ad iniectabilia B. Braun Melsungen, Germany).

Buffer solutions were prepared as follows:

Sörensen-phosphate-buffer: buffer solution pH 3.3 from potassium dihydrogen phosphate 1/15 M (pro analysi, Merck Darmstadt, Germany) and disodium hydrogen phosphate 1/15 M (pro analysi, Merck Darmstadt, Germany).

Borate-buffer: 1.54 g 0.05 M boracic acid in 500 mL water, adjusted to pH 8.7, 9.0 and 9.2 by adding 2 M sodium hydroxide.

Pharmaceuticals

Morphine-sulfate, codeine-hydrochloride, dihydrocodeine-hydrochloride and methaqualone as internal standard were purchased from Sigma Aldrich (Deisenhofen, Germany). Stock solutions of 1 mg/L, 10 mg/L and 100 mg/L were prepared in ethanol (Merck Darmstadt, Germany) and stored at 4° C.

Extraction columns

Amberlite XAD2 200 mg (Biochemical Diagnostics, N.Y.), Bond Elut Certify 300 mg (Varian, Harbor City), Chromabond C18 and C18 ec, 100 mg and 200 mg (Macherey-Nagel Düren, Germany). To enlarge the volume up to 10 mL for all columns a special reservoir was plugged into the top of the columns.

Samples

Blood samples for the evaluation were obtained from the blood bank of the University Hospital of Dermatology, Munich, bovine plasma samples were obtained from ICN Biomedicals (Nockenheim, Germany). The samples were stored at 4° C and spiked with 1–1000 ng/mL each day.

Instrumentation and chromatographic parameters

The extraction columns were positioned on a 10-place vacuum manifold (ICT Analytichem International, Harbor City). For the analysis of the eluates a Model 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) with a 5971A mass selective detector (Hewlett-Packard) was used. Data acquisition and manipulation were performed by using standard software supplied by the manufacturer. The stationary phase was a fused silica column (30 m × 25 mm), the separating layer was DB-1 (0.25 µm film thickness). Helium was used as carrier gas with a flow rate of 30 m/min and an unregulated pressure of 3 PSI. The following temperature programme was used: injector 280° C, oven 100° C increased by 20° C/min up to 300° C, and interface 300° C. The injector was 1 min splitless and then in split function. The electron source voltage was 70 eV and the electron multiplier voltage was 1364.7 kV in single ion monitoring function of the MS.

Derivatisation

To form the propionic acid anhydride derivatives the eluates were dried under a stream of nitrogen and 50 µL propionic acid anhydride (PAA) was added to the dried extract. After incubating the mixture for 45 min in a 100° C heating block, it was dried again and reconstituted in 50 µL ethyl acetate with 5% PAA. A volume of 1 µL was injected into the GC/MS.

Extraction procedure

Internal standard 10 µL of (10 mg/L methaqualon in ethanol) was added to 1 mL plasma or whole blood. The samples were vortexed and blood samples were centrifuged for 15 min at 12500 g in 1.5 mL Eppendorf tubes. The supernatant was decanted in a 10 mL glass tube dissolved in 9 mL borate buffer pH 9.2 and the whole volume was applied to an activated extraction column. The extraction columns were conditioned by washing with 2 × 1 mL methanol, 2 × 1 mL water and 1 mL borate buffer pH 9.2 (flow rate 1–2 mL/min). The samples were drawn through the prepared columns by a vacuum at approximately 1 mL/min. The washing step was performed at low vacuum with 1 mL water and 1 mL 20% methanol in water (v/v). The column was then dried under a strong vacuum for several minutes. The opiates were eluted by passing 1 mL methanol through the column, with a flow rate of 1 mL/min, and transferred to the derivatisation tubes.

Results and discussion

For the evaluation of the extraction procedure some important parameters had to be investigated in detail. The most important was the selection of an appropriate sorbent that allows good recoveries and a low background noise. Furthermore the pH-value of the column was exactly adjusted to the best recoveries in a range pH 8.7–9.2. In contrast to the very simple preparation of plasma samples, the procedure for whole blood is more difficult. The benefits of some precipitation techniques and a technique without precipitation were compared.

Electron impact mass spectra were formerly recorded by total ion monitoring (TIC). Retention times and characteristic mass fragments were recorded and for each compound two diagnostic mass fragments were monitored in the single ion monitoring technique (SIM). The selected mass fragments were m/z 235 and 250 for the presence of methaqualone, m/z 300 and 357 for dihydrocodeine, m/z 282 and 355 for codeine, m/z 327 and 383 for 6-monoacetylmorphine and m/z 341 and 397 for morphine. Retention times were monitored as follows: methaqualone 6.27 min, dihydrocodeine 8.10 min, codeine 8.25 min, 6-

monoacetylmorphine 8.75 min and morphine 9.25 min. Methaqualone, used in our routine laboratory with excellent results, was chosen as internal standard, even if deuterium-derivatives of opiates are generally more precise and mainly used nowadays. The quantification of the opiates was performed first by adjusting the counts of the internal standard and then comparing the peak area ratios with those of an Extrelut extraction of the same sample. Absolute recoveries of this reference procedure were determined as dihydrocodeine 91.7%, codeine 100%, 6-monoacetylmorphine 80.0%, morphine 97.0% and allowed the calculation of absolute recoveries for the solid-phase extraction procedures. These recoveries reflect the accuracy because all values were adjusted with counts of the internal standard.

The comparison of XAD2, C18 and Certify columns with extraction procedures similiar to Musshoff and Daldrup [14] or Chen et al. [5] gave the best results for the silica sorbents. Except for lower recoveries, especially for codeine and dihydrocodeine on XAD2 and Certify, the strong chemical environment on the Certify column resulted in a loss of 27% of 6-monoacetylmorphine compared to using C18. With respect to the interfering peaks which occurred in the mass spectra for m/z 300 and 357 at the same time as dihydrocodeine after XAD2 and Certify extraction, C18 columns were chosen for the method. A change from C18 to C18 ec showed better recoveries for dihydrocodeine (+28%), codeine (+13%) and 6-monoacetylmorphine (+13%) and a minimal deterioration for morphine (–3%). The lower stability of this endcapped sorbent at a pH higher than 9.0 resulted in damage of the GC capillary column by eluted silica particles. With respect to these results the C18 columns were the best choice for the performance in our laboratory. According to most publications the optimal pH on the column is about 9 [8, 10, 14, 16–18]. Studies with borate buffer ranging from pH 8.7 to 9.2 yielded better recoveries with the more alkaline environment. In comparison to the results of extractions at pH 8.7 , the recoveries at pH 9.0 were nearly the same, but at pH 9.2 codeine and dihydrocodeine especially showed an improvement of +18% and +10% (Fig. 1). To establish this procedure as a routine method, extractions should be made with whole blood in the same way as with plasma. Many previous publications discussed the possible advantages of protein precipitation [5, 17]. This step can be done by adding acetone [14], acetonitrile [13, 16] or ethanol [17]. We analysed four different methods. Method A was performed by centrifuging the 1 mL whole blood sample for 10 min at 12 500 U without precipitation. In method B 0.7 mL acetone was added and the mixture was evaporated to dryness after centrifugation. Method C was similiar to method B but without the evaporating step and in method D 0.7 mL ethanol was used without evaporation after centrifugation (Fig. 2). The results supported the presumption that any precipitation is combined with a loss of recovery through the inclusion of the opiates in the protein precipitates. Method A had the best results, lower recoveries resulted from methods B and D (both –15% to –35%) and the enormous loss (about –50%) of method C indicates that an acetone precipitation without evaporation of the alcoholic part of the mixture disturbs the hydrophobic binding of the opiates to the C18 chains.

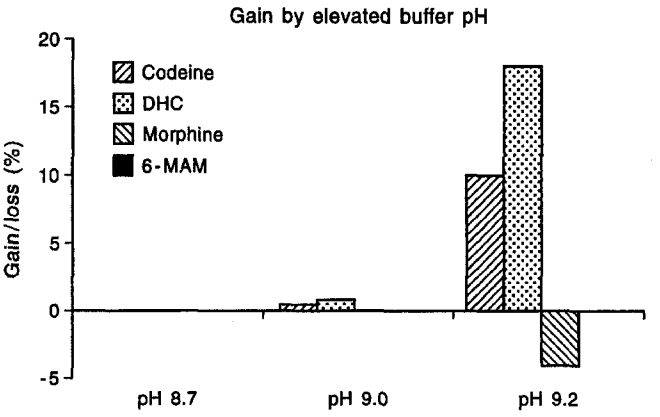

Fig. 1 Gain loss by different buffer pH-values

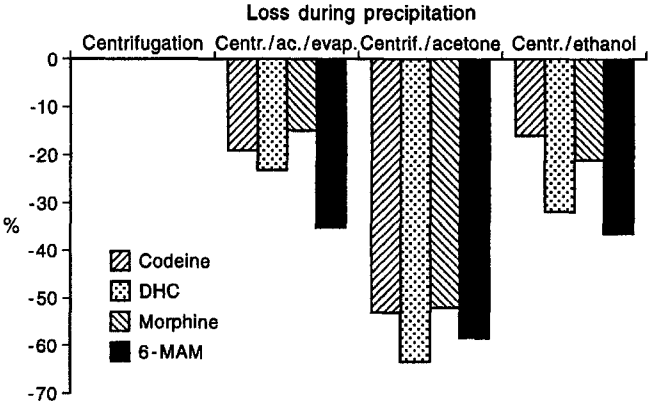

Fig. 2 Gain loss by different precipitation techniques

Table 1 Recoveries standard deviations and detection limits using the modified method of solid-phase extraction

	DHC	Codeine	Morphine	6-MAM
Concentration	500 µg/L	500 µg/L	1000 µg/L	100 µg/L
Samples	$n = 2$	$n = 2$	$n = 2$	$n = 2$
Recovery	67.9%	77.0%	93.1%	68.0%
Concentration	100 µg/L	100 µg/L	100 µg/L	100 µg/L
Samples	$n = 6$	$n = 6$	$n = 6$	$n = 5$
SD	± 8.4%	± 8.3%	± 7.4%	± 6.7%
Detection limit	< 5 µg/L	< 5 µg/L	< 5 µg/L	< 5 µg/L
Peak : noise ratio	4 : 1	3 : 1	4 : 1	4 : 1

Washing the column with 20% methanol in water (v/v) did not reduce the recoveries compared to cleaning only with water while washing with 25% methanol (v/v) resulted in a loss of 8% of the morphine recovery.

Recoveries and reproducibility of the described method were measured by analysing spiked serum samples. The recoveries were determined in a typical blood concentration range of the substance ($n = 2$) and all standard deviations measured at a concentration of 100 µg/L ($n = 6$). The

results are shown in Table 1. Recoveries ranged from 67.9% (dihydrocodeine 500 µg/L) to 93.1% (morphine 1000 µg/L). The standard deviations (SD) as precision data were between 6.7% and 8.4%. For our purposes the minimal detectable drug concentrations were satisfactory. With a peak to noise ratio greater than 3:1 all substances were detectable in concentrations less than 5 ng/mL. Blank plasma samples showed no interfering peaks in the mass spectra.

In conclusion a method for the simultaneous determination of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine in plasma and blood using gas chromatographic/mass spectrometric analysis is presented. The procedure is very simple and reproducible, and shows satisfactory recoveries and a low detection limit.

References

1. Bruchhausen F von, Ebel S, Frahm A, Hackenthal E (1993) Hagers Handbuch der Pharmazeutischen Praxis, 5. Aufl, Bd 7 und 8. Springer, Berlin Heidelberg New York
2. Chen XH, Wijsbeek J, van Veen J, Franke JP, de Zeeuw RA (1990) Solid-phase extraction for the screening of acidic, neutral and basic drugs in plasma using a single column procedure on Bond Elut Certify. J Chromatogr 529:161–166
3. Chen XH, Wijsbeek J, Franke JP, de Zeeuw RA (1992) A single-column procedure on Bond Elut Certify for systematic toxocological analysis drugs in plasma and urine. J Forensic Sci 37:61–71
4. Chen XH, Franke JP, Wijsbeek J, de Zeeuw RA (1992) Isolation of acidic, neutral and basic drugs from whole blood using a single mixed-mode solid-phase extraction. J Anal Toxicol 16: 351–355
5. Chen XH, Hommerson ALC, Zweipfennig PGM, Franke JP, Harmen-Boverhof CW, Ensing K, de Zeeuw RA (1993) Solid phase extraction of morphine from whole blood by means of Bond Elut Certify columns. J Forensic Sci 38:668–676
6. Cone EJ, Welch P, Mitchell JM, Paul BD (1991) Forensic drug testing for opiates: I. Detection of 6-monoacetylmorphine in urine as an indicator of recent heroin exposure; Drug and assay considerations and detection times. J Anal Toxicol 15:1–7
7. Cone EJ, Welch P, Paul BD, Mitchell JM (1991) Forensic drug testing for opiates: III. Urinary excretion rates of morphine and codeine following codeine administration. J Anal Toxicol 15: 161–166
8. Fehn J, Megges G (1985) Detection of O-6-monoacetylmorphine in urine samples by GC/MS as an evidence for heroin use. J Anal Toxicol 9:134–138
9. Forth W, Henschler D, Rummel W, Starke K (1992) Allgemeine und Spezielle Pharmakologie und Toxikologie. 6. Auflage BI Wissenschaftsverlag, Mannheim Wien Zurich
10. Hanisch W, Meyer L von (1993) Determination of the heroin metabolite 6-monoacetylmorphine in urine by high-performance liquid chromatography with electrochemical detection. J Anal Toxicol 17:48–50
11. Huang W, Andollo W, Hearn W L (1992) A solid phase extraction technique for the isolation and identification of opiates in urine. J Anal Toxicol 16:307–310
12. Inturrisi CE, Max MB, Foley KM, Schultz M, Shin SU, Houde RW (1984) The pharmacokinetics of heroine in patients with chronic pain. N Engl J Med 310:1213–1217
13. Musch G, Massart D L (1988) Isolation of basic drugs using solid-phase extraction with a cyanopropyl bonded phase. J Chromatogr 432:209–222
14. Musshoff F, Daldrup T (1993) Evaluation of a method for simultaneous quantification of codeine, dihydrocodeine, morphine and 6-monoacetylmorphine in serum, blood and postmortem blood. Int J Legal Med 106:107–109
15. Nakamura GR, Thornton JI, Noguchi TT (1975) Kinetics of heroin deacetylation in aqueous alkaline solution and in human serum and whole blood. J Chromatogr 110:81–89
16. Papadoyannis I, Zotou A, Samanidou V, Theodoridis G, Zougrou F (1993) Comparative study of different of different solid-phase extraction cartridges in the simultaneous RP-HPLC analysis of morphine and codeine in biological fluids. J Liquid Chromatogr 16:3017–3040
17. Schuberth J, Schuberth J (1989) Gas-chromatographic mass-spectrometric determination of morphine, codeine and 6-monoacetylmorphine in blood extracted by solid phase. J Chromatogr 490:444–449
18. Svensson JO, Rane A, Säve J, Sjöqvist F (1982) Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high performance liquid chromatography. J Chromatogr 230:427–432
19. Wu AHB, Onogbinde TA, Wong SS, Johnson KG (1992) Evaluation of full-scanning GC/ion trap MS analysis of NIDA drugs-of-abuse urine testing in urine. J Anal Toxicol 16:202–206
20. Worldwide Monitoring (1994) Bonded Phase Extraction Sorbents. United Chemical Technologies, Bristol/PA, USA