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Toxicological detection of pholcodine and its metabolites in urine and hair using radio immunoassay, fluorescence polarisation immunoassay, enzyme immunoassay and gas chromatography-mass spectrometry*

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Summary. Pholcodine (3-O-(2′-morpholinoethyl)-morphine) is used in many countries as an antitussive without analgesic or addictive properties. It is of forensic relevance that pholcodine interferes with opiate immunoassays. In this paper a gas chromatographic-mass spectrometric (GC-MS) procedure for the precise and sensitive detection of pholcodine and its metabolites in urine and hair, after acid hydrolysis, extraction and acetylation, is presented. Furthermore, detection of pholcodine using radio immunoassay (RIA), fluorescence polarisation immunoassay (FPIA) and enzyme immunoassay (EIA) for opiates is described. Using GC-MS, unmodified pholcodine could be detected in urine samples 4–7 weeks after ingestion of a single therapeutic dose of 50 mg of pholcodine, the desmorpholinohydroxy metabolite for 1–2 weeks and the other metabolites (nor-, nordesmorpholinohydroxy-, hydroxy-, oxo- and noroxo-pholcodine) only during the first few hours. Morphine could also be detected in urine samples for the first few days. It was however mainly formed artificially during acid hydrolysis and only in trace amounts by metabolism. All the immunoassays tested gave positive results in urine samples during the first week taking the cut-off values recommended by the manufacturer into consideration. If values between the cut-off and the detection limit were taken into consideration, RIA and FPIA gave positive results for 2–4 weeks and EIA up to 2 weeks. Pholcodine could also be detected by RIA and GC-MS in samples of head hair clipped 10 weeks after ingestion of 50 mg and in daily shaved samples of beard hair over a period of three weeks after ingestion of three doses of 60 mg. It can be concluded that the widely used immunoassays for opiates show positive results in urine and hair samples for a long time after ingestion of the non-opioid pholcodine and that these results can be confirmed by the GC-MS procedure described in this paper.

Key words: Pholcodine – Urine – Hair – Immunoassay – Gas chromatography-mass spectrometry

Zusammenfassung. Pholcodin (3-O-(2′-Morpholinoethyl)-Morphin) wird in vielen Ländern als Antitussivum ohne analgetische oder suchterregende Potenz verwendet. Es ist aber insofern von forensischer Bedeutung, als es mit Immunoassays für Opiate interferiert. In der Arbeit wird ein gaschromatographisch-massenspektrometrisches (GC-MS) Verfahren beschrieben, das einen spezifischen und empfindlichen Nachweis von Pholcodin in Urin und Haaren nach saurer Hydrolyse, Extraktion und Acetylierung erlaubt. Desweiteren wird die Nachweisbarkeit von Pholcodin mittels Radioimmunoassay (RIA), Fluoreszenzpolarisationsimmunoassay (FPIA) und Enzymimmunoassay (EIA) beschrieben. Mittels GC-MS konnte unverändertes Pholcodin im Urin bis zu 4–7 Wochen nach der Einnahme einer einzigen therapeutischen Dosis von 50 mg nachgewiesen werden. Der Desmorpholinohydroxy-Metabolit konnte 1–2 Wochen, und die anderen Metaboliten (Nor-, Nordesmorpholinohydroxy-, Hydroxy-, Oxo- und Noroxo-Pholcodin) nur in den ersten Stunden erfaßt werden. Morphin konnte innerhalb der ersten paar Tage im Urin nachgewiesen werden. Es wurde jedoch hauptsächlich artefaktisch während der sauren Hydrolyse und nur in Spuren im Stoffwechsel gebildet. Alle getesteten Immunoassays

* Some of these results were reported at the 68. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin, Salzburg (Austria), September 19–23, 1989 (Maurer and Fritz 1990a).
They are part of the M.D. thesis of Ch. F. Fritz (Fritz 1990)

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lieferten unter Berücksichtigung der von den Herstellern empfohlenen Cut-Off-Werte in den Urinproben der ersten Woche positive Befunde. Unter Berücksichtigung von Meßwerten zwischen Cut-Off und Nachweisgrenze lieferten RIA und FPIA 2–4 Wochen und der EIA bis zu 2 Wochen "positive" Ergebnisse. Pholcodin wurde mittels RIA und GC-MS auch in Kopfhaarproben nachgewiesen, die 10 Wochen nach der Einnahme abgeschnitten worden waren. In Barthaaren, die nach der Einnahme von 3mal 60 mg Pholcodin bei der täglichen Rasur gesammelt worden waren, konnte Pholcodine drei Wochen lang nachgewiesen werden. Zusammenfassend kann festgestellt werden, daß die weitverbreiteten Immunoassays für Opiate in Urin und Haaren selbst lange Zeit nach der Einnahme des Nicht-Opioids Pholcodin in Urin und Haaren positive Ergebnisse liefern und daß alle diese Ergebnisse mit Hilfe des vorgestellten GC-MS-Verfahrens bestätigt werden können.

Schlüsselwörter: Pholcodin – Urin – Haare – Immunoassay – Gaschromatographie-Massenspektrometrie-Kopplung

Introduction

Pholcodine (3-O-(2′-morpholinoethyl)-morphine) was first described in 1950 (Chabrier et al. 1950) and since then has been widely used as a potent antitussive agent in many countries. Several studies have been performed to investigate the antitussive properties, which were found to be equivalent or superior to those of codeine (Cahen 1961; Eddy et al. 1970). In contrast to codeine, pholcodine was reported to lack significant analgesic and addictional potency (Eddy et al. 1970; Findlay 1988). This may due to the fact that O-desalkylation of pholcodine to morphine is hindered by the bulky morpholinoethyl substituent (Butz et al. 1983). Nevertheless, pholcodine is of forensic relevance because it interferes with opiate immunoassays (Svenneby et al. 1983). A study of the metabolism of pholcodine in man was a prerequisite to be able to confirm immunological results by gas chromatography-mass spectrometry (GC-MS) (Maurer and Fritz 1990b).

A gas chromatographic-mass spectrometric (GC-MS) procedure is presented for the precise and sensitive detection of pholcodine and its metabolites in urine and hair according to the procedure for opioids of Maurer and Pfleger (1984). Furthermore, detection of pholcodine using a radio immunoassay (RIA), a fluorescence polarisation immunoassay (FPIA) and an enzyme immunoassay (EIA) for opiates is described and the efficiency of the GC-MS procedure for confirmation of the immunological results is discussed.

Materials and methods

Urine and hair samples. After beeing informed according to the declaration of Helsinki, and after obtaining their written consent, three healthy volunteers received a single oral dose of 50 mg of pholcodine hydrochloride. Urine samples from the volunteers were collected over an 8 week period and stored at −20°C before analysis. Samples of head hair of the three volunteers were clipped at the root 10 weeks after ingestion. Samples of beard hair were collected over a 4 week period during the daily shave of one volunteer who had been administered three oral doses of 60 mg of pholcodine hydrochloride on one day. Blank samples of urine and hair were collected before the application of drugs to control whether the samples were free of interfering compounds.

Sample preparation of urine. A 10 ml volume of urine was refluxed with 3 ml of 30% hydrochloric acid for 15 min. Following hydrolysis, approximately 3 g of potassium hydroxide pellets was added and the resulting solution was mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with 10 ml of a dichloromethane/isopropanol/ethyl acetate mixture (1:1:3). After phase separation by centrifugation the organic layer was evaporated to dryness and the residue derivatized by acetylation with 100 µl of acetic acid anhydride/pyridine (3:2) for 30 min at 60°C. After evaporation of the derivatization mixture, the residue was dissolved in 100 µl of methanol and 0.5–2 µl were injected into the gas chromatograph.

Sample preparation of hair. One gram of head hair or 60 mg of beard hair was washed with acetone to eliminate drugs which could have contaminated the hair via perspiration. After drying, the hair was cut into small pieces and then pulverized. The resulting powder was suspended in 15 ml of Sörensen buffer for two days at ambient temperature (Möller 1989). This sample was used for the radio-immunological tests. For the GC-MS determination, the sample was hydrolyzed, extracted and derivatized as described in the preceding section.

Chemicals. All chemicals used were obtained from E. Merck, Darmstadt (FRG) and were of analytical grade.

Gas chromatography-mass spectrometry. Pholcodine and its metabolites were separated and identified in the acetylated extracts of urine and hair using a Hewlett-Packard (HP, Waldbronn, FRG) Series 5890 gas chromatograph combined with an HP MSD Series 5970 mass spectrometer and an HP Series 59970 C workstation. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m × 0.2 mm I.D.), cross-linked methylsilicone, 0.33 µm film thickness; column temperature, programmed from 100°C to 310°C at 30°C/min, initial time 3 min, final time 8 min; injection port temperature, 270°C; carrier gas, helium, flow rate 1 ml/min. The MS conditions were as follows: scan mode; ionization energy, 70 eV; ion source temperature, 220°C, capillary direct interface heated at 260°C.

Immunoassays. Native urine samples and hair samples prepared as described above were used for immunological determination. The following assays for opiates were used: RIA: Abuscreen system of Hoffmann-La Roche (Nutley, NJ); FPIA: TD_x system of Abbott (Irving, TX); EIA: EMIT-dau system of Syva (Palo Alto, CA). The cut-off values and the detection limits (ng/ml) recommended by the manufacturers were as follows: Abuscreen: 300/10, TD_x: 200/25 and EMIT-dau: 300. A detection limit of the EMIT system cannot be given. For determination of the cross reactivities, blank urine samples were spiked with pholcodine in concentrations of between 50 and 1000 ng/ml.

Results and discussion

Sample preparation

For sample preparation, the procedure was used that has proved to be successful for opioids (Maurer and Pfleger

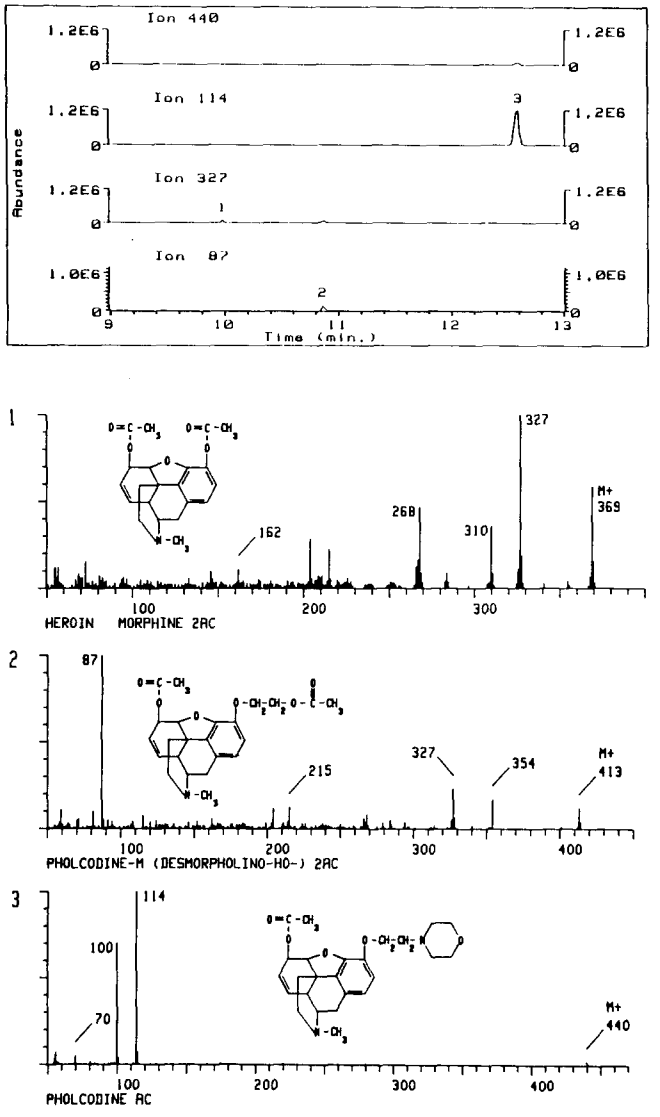

Fig. 1. Typical mass chromatograms indicating the presence of acetylated morphine (hydrolysis artifact of pholcodine) *(1)*, acetylated desmorpholinohydroxy-pholcodine *(2)* and acetylated pholcodine *(3)* in a urine sample collected 12 h after ingestion of 50 mg of pholcodine *(top)*. The mass spectra and structures of these compounds are shown for the precise identification *(bottom)*

1984) as well as for several hundred other toxicologically relevant drugs and over 1000 of their metabolites (Maurer 1988). Although pholcodine is not metabolized to conjugates (Maurer and Fritz 1990b), rapid acid hydrolysis was performed before isolation and derivatization in order to also simultaneously detect conjugated metabolites of other opioids which interfere with opiate immunoassays. However, small amounts of pholcodine were altered to morphine during hydrolysis. The analytical recovery of pholcodine was 64,7 ± 10%. Variation of the pH and/or the mixture of solvents did not lead to a better recovery. Derivatization was essential for sensitive detection of the weakly polar pholcodine and its metabolites. The preparation of hair samples has also proved successful for opiates, methadone, cocain, cannabis, barbiturates and benzodiazepines (Möller et al. 1989).

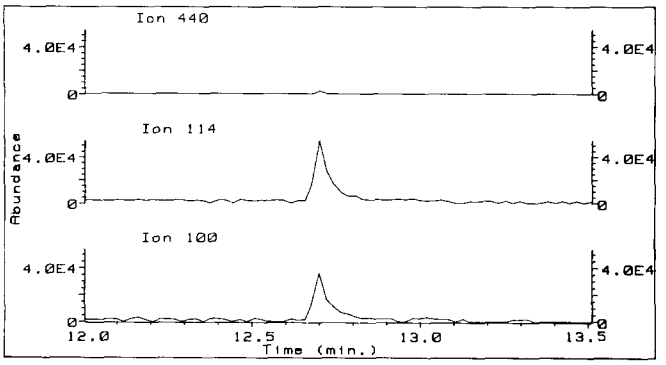

Fig. 2. Typical mass chromatograms indicating the presence of acetylated pholcodine in a beard hair sample collected on the 10th day after ingestion of 3 × 60 mg of pholcodine

Detection by gas chromatography-mass spectrometry

The full mass spectra recorded during temperature-programmed gas chromatography were evaluated using mass chromatography. In urine samples, the selective ions m/z 440 and 114 were used for indication of the presence of acetylated pholcodine, ion 327 for acetylated morphine (hydrolysis artifact of pholcodine) and ion 87 for the acetylated desmorpholinohydroxy metabolite (Fig. 1, top). Positive signals in the reconstructed mass chromatograms were identified by visual (Fig. 1, bottom) or computerized (Pfleger et al. 1990a) comparison of the peak underlying full mass spectra with reference spectra. The spectra of the less abundant metabolites are to be presented elsewhere (Maurer and Fritz 1990b; Pfleger et al. 1990a, b). Because of the mass spectral identification, interferences by other drugs are improbable. The detection limit of pholcodine in urine was 3 ng/ml. Therefore, the method is also suitable for the detection of low therapeutic concentrations of pholcodine.

In hair samples, the ions m/z 440, 114 and 100 were used for indication of acetylated pholcodine (Fig. 2). Because the molecular ion m/z 440 is of minor abundance, it was not indicated in beard hair samples containing very low concentrations of pholcodine. Therefore, it is preferable to also use the two most abundant ions m/z 114 and 100. Because no other compounds were found which interfere with these ions at the retention time of acetylated pholcodine (Pfleger et al. 1990b), this detection mode is sufficient for confirmation of the immunological results. Since small amounts of pholcodine were altered during acid hydrolysis to morphine, traces of (acetylated) morphine could also be found in hair samples containing relatively high concentrations of pholcodine. In addition, it should be remarked that small amounts of pholcodine were also altered to morphine by alkaline hydrolysis.

Detection by immunoassays

The cross reactivity of the tested opiate immunoassays with pholcodine was as follows: Abuscreen: 85–105%, TD_x: 72–97% and EMIT-dau: 47–102%. Therefore, these immunoassays showed positive results in biosamples after ingestion of pholcodine which was in accordance with the studies of Svenneby et al. (1983).

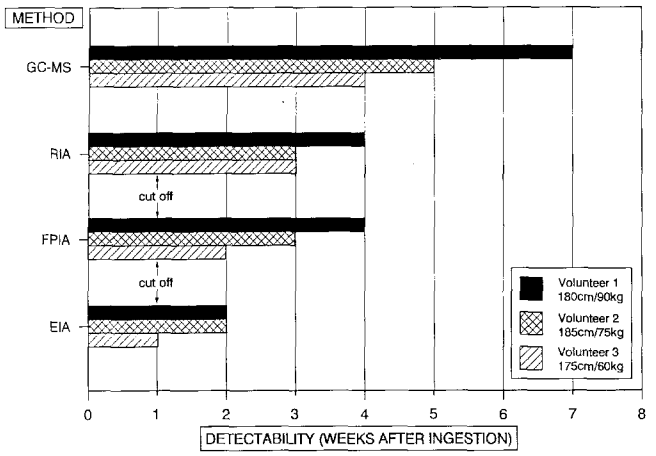

Fig. 3. Detectability of pholcodine (weeks after ingestion) by different methods (GC-MS, RIA, FPIA, EIA) in urine samples after ingestion of 50 mg of pholcodine

Detectability of pholcodine in urine by GC-MS, RIA, FPIA and EIA

Using GC-MS, unchanged pholcodine could be detected in urine samples 4–7 weeks after ingestion of a single therapeutic dose of 50 mg of pholcodine (Fig. 3). The desmorpholinohydroxy metabolite was detectable for 1–2 weeks and the other metabolites (nor-, nordesmorpholinohydroxy-, hydroxy-, oxo- and noroxo-pholcodine) only during the first few hours. Morphine was detected in urine during the first days when the concentration of pholcodine in urine was relatively high (>1000 ng/ml).

As shown in Fig. 3, all the tested immunoassays showed positive results in urine samples during the first week after ingestion of 50 mg of pholcodine, considering the cut-off values recommended by the manufacturer. If values between the cut-off and the detection limit were taken into consideration, RIA and FPIA showed positive results for 2–4 weeks and EIA up to 2 weeks. Because a defined detection limit of the EMIT system cannot be given, values were used which were above those of the blank urine determined in any series. All the immunological results could be confirmed by GC-MS because it was much more sensitive (Fig. 3). The deviation in the time of excretion of pholcodine may be caused by inter-individual differences e.g. relative body mass and/or renal efficiency.

Detectability of pholcodine in hair by RIA and GC-MS

The opiate RIA showed positive results in samples of head hair clipped 10 weeks after ingestion. The RIA was also positive in samples of beard hair from the 5th to the 20th day after ingestion. The RIA values ranged from 0.5 to 0.6 ng/mg of head hair and between 0.6 and 1.7 ng/mg of beard hair, while opiate concentrations in hair samples of fixers or codeine abusers are between 1 and 6 ng/mg (Möller et al. 1989).

Using GC-MS, pholcodine could be detected in all the immunologically positive hair samples. Therefore, the immunological results in hair samples could also be confirmed by GC-MS.

Conclusions

The GC-MS procedure described have allowed the precise and sensitive detection of pholcodine and its metabolites in urine and hair samples after therapeutic doses of pholcodine. Other opiates and potent analgesics (Maurer and Pfleger 1984) could be detected in urine using the same procedure. In accordance with Svenneby et al. (1983), immunoassays such as RIA, FPIA and EIA which are widely used for screening of opiates showed positive results in biosamples after ingestion of the non-opioid pholcodine. All the positive results in urine and hair samples could be confirmed by the described GC-MS procedure, so that forensic misinterpretation could be excluded. Because pholcodine is slowly metabolised and excreted (Maurer and Fritz 1990b), it could be detected in urine and hair for a long time after ingestion.

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