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Determination of pholcodine and its metabolites in urine by capillary gas chromatography

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ABSTRACT

A sensitive and selective method for the determination of pholcodine and its metabolites in urine using capillary gas chromatography with nitrogen detection is described The procedure includes enzymatic hydrolysis of urine by β-glucuronidase and sample pretreatment on C_2 solid-phase extraction columns Validation of the method showed good sensitivity, precision and reproducibility The method was useful for the study of pholcodine metabolism in man. Pholcodine was found to conjungate with glucuronic acid Morphine was identified as a metabolite and another unidentified metabolite was also detected.

INTRODUCTION

The opiate derivative pholcodine (3-O-morpholinoethylmorphine) is widely used as an antitussive agent and is available as an over-the-counter medicine in several countries Unlike several other opiates this compound lacks analgetic action [1]. Pholcodine has been reported to cross-react when biological samples are screened for opiate abuse by immunological methods [2]. Compared to other opiates pholcodine has a considerably longer elimination half-life [3]. A positive immunological opiate reaction in urine for two to six weeks has been reported, after ingestion of one therapeutic oral dose [1,2]. The widespread use of immunological screening methods for drug abuse testing has introduced the need for specific, sensitive and simple confirmation methods to distinguish illegal from legal use of drugs. The interpretation of positive opiate screening results in forensic toxicology also necessitates studies of the excretory pattern of pholcodine and its possible metabolites. The question then arises if pholcodine, like other opiate drugs, undergoes 3-O-dealkylation to morphine which can be excreted in urine in free form and/or as glucuronide. It can also be expected that pholcodine and other possible metabolites undergo conjugation.

Up to now the pharmacokinetics of pholcodine has mainly been studied using immunological methods that lack the necessary specificity [3]. Chen *et al.* [4] have,

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so far, published the only chromatographic method specific for the determination of pholcodine in biological fluids. However, this method did not include hydrolysis of the biological samples and was unable to detect possible conjugates of pholcodine and its metabolites.

The purpose of this study was to develop a sensitive, specific and simple method for the determination of pholcodine in urine which could also detect other opiate drugs. Enzymic hydrolysis of urine samples was included in the study in order to detect pholcodine and possible metabolites both in free form and as glucuronides. To identify and quantify pholcodine and metabolites, a specific gas chromatographic (GC) method was developed.

EXPERIMENTAL

Chemicals

Pholcodine was purchased from Weiders Farmasøytiske (Oslo, Norway). Morphine hydrochloride, nalorphine hydrochloride and methylnalorphine hydrochloride were obtained from Norsk Medisinaldepot (Oslo, Norway). 6-Monoacetylnalorphine (6-MAN) was synthesized at the National Institute of Forensic Toxicology (Oslo, Norway). Ampoules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were supplied by Supelco (Bellefonte, PA, U.S.A). β-Glucuronidase (*Helix pomatia* type H-1) was purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol were of HPLC grade from Fisons (Loughborough, U.K.). *n*-Butyl acetate was of glass-destilled grade from Rathburn (Walkerburn, U.K.).

Preparation of standards

Stock standard solutions of pholcodine (2 μmol/ml) and methylnalorphine hydrochloride (2 μmol/ml) were prepared in water. Working standards of pholcodine and methylnalorphine hydrochloride in water and urine (0.05–10.0 nmol/ml) were prepared by dilution of the standard solutions. The spiked urine samples were kept at −20°C until analysis.

Gas chromatography

An HP 5880A gas chromatograph (Hewlett Packard, Avondale, PA, U.S.A.) was equipped with a split/splitless capillary HP 7671A autoinjector and a nitrogen–phosphorus detector operated in the nitrogen mode The signals were received on an HP 5880A level 4 Basic integrator. The column was an HP cross-linked methylsilicone capillary column (12 m × 0.2 mm I.D., film thickness 0.33 μm). The samples were injected splitless and the splitter was reopened after 30 s. The detector temperature was set at 300°C and the injection port temperature at 250°C. The initial oven temperature of 120°C was held for 30 s, then increased at 30°C/min to 220°C, at 3°C/min to 260°C and at 30°C/min to 300°C, the final temperature being held for 2 min. Helium was used as carrier gas at a flow-rate of

2 ml/min. The detector gases were hydrogen (4 ml/min) and air (63 ml/min). Helium was used as make-up gas at a flow-rate of 25 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

A VG 12-250 mass spectrometer (Manchester, U.K.) was connected to an HP 5710 gas chromatograph. The capillary column was the same as used for the GC analysis. The mass spectrometer was operated in the electron-impact mode at 70 eV with an ion source temperature of 200°C, and was scanned in the mass range from 40 to 650.

Solid-phase extraction

A Vac Elut™ vacuum manifold and Bond Elut™ columns from Analytichem International (Harbor City, CA, U.S.A.) were used to clean up samples prior to GC analysis. The urine samples were extracted on 1-ml Bond Elut solid-phase extraction (SPE) tubes packed with 100 mg of C_2. The tubes were first conditioned with two 1-ml aliquots of methanol, and residual methanol was washed from the packing with 1 ml of 1 *M* ammonia buffer (pH 9.0) A 500-μl aliquot of the urine sample containing 8 nmol/ml methylnalorphine (internal standard, I.S.) was then mixed with 500 μl of 1 *M* ammonia buffer (pH 9.0) and added to each column. The mixture was drawn through the column using the vacuum manifold. The column was washed with 1 ml of 1 *M* ammonia buffer (pH 9.0) and then with two aliquots of purified water. The compounds were eluted with 700 μl of methanol and collected in glass vials. The eluate was then dried under nitrogen in an electrical heating block at 70°C. The dry residue was immediately derivatized.

Derivatization

To the dry extracts were added 80 μl of BSTFA–acetonitrile (1:2, v/v). The capped tubes were heated at 60°C for 20 min. The reaction mixtures were evaporated to dryness under nitrogen (60°C) and dissolved in 100 μl of *n*-butyl acetate; 1 μl was injected into the gas chromatograph.

Urine hydrolysis

Urine (1 ml) containing 8 nmol of methylnalorphine (I.S.) was mixed with 500 μl of 0.05 *M* acetate buffer (pH 4.5) and 5000 U of β-glucuronidase. The samples were incubated at 56°C for 48 h. The hydrolysates were then extracted using solid-phase extraction as previously described.

RESULTS AND DISCUSSION

Solid-phase extraction

SPE tubes packed with 100 mg of C_{18}, C_8 and C_2, respectively, were tested, and methylnalorphine, nalorphine and 6-MAN were investigated as internal standards for the determination of pholcodine. The recovery after elution of these

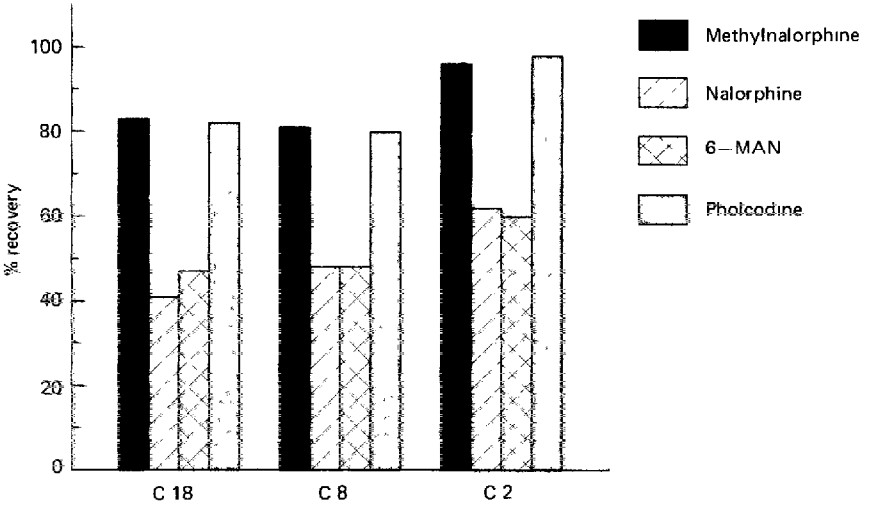

Fig 1 Recovery of methylnalorphine, nalorphine, 6-MAN and pholcodine from C_{18}, C_8 and C_2 solid-phase extraction columns after elution with 700 μl of methanol (concentration 8 nmol of opiate per ml of urine).

compounds with methanol from the different columns is shown in Fig. 1. All compounds gave the highest recovery on SPE tubes packed with C_2. The recovery of nalorphine and 6-MAN was low, 60 and 58%, respectively, while the recovery of pholcodine and methylnalorphine was nearly complete, 98 and 96%, respectively. Methylnalorphine was therefore chosen as the internal standard. Optimization of the washing and elution steps on the C_2 solid-phase material was carried out with spiked urine samples. No interfering compounds were observed in the extracts after washing the SPE tubes with 1 *M* ammonia buffer (pH 9.0) and water followed by elution with methanol. To minimize the evaporation time other eluents such as acetonitrile, dichloromethane, chloroform, diisopropyl ether and mixtures of methanol and chloroform were tested. However, methanol was the only eluent giving acceptable recovery of both pholcodine and methylnalorphine. Evaporation of the solvent at 70°C gave dry extracts after 20 min. The recovery of morphine extracted from urine according to this procedure was 41 ± 8% (n = 5).

Gas chromatography

Fig. 2 shows a chromatogram of a urine spiked with morphine, methylnalorphine and pholcodine. The trimethylsilyl (TMS) derivatives of pholcodine and methylnalorphine gave sharp symmetrical peaks by capillary GC. No compound interfering with morphine, methylnalorphine or pholcodine was observed. The calibration curve obtained by plotting peak-height ratios of pholcodine and I S. *versus* concentration of pholcodine in urine was linear over the concentration range 0.1–10 nmol/ml with r = 0.9992. The within-run coefficient of variation

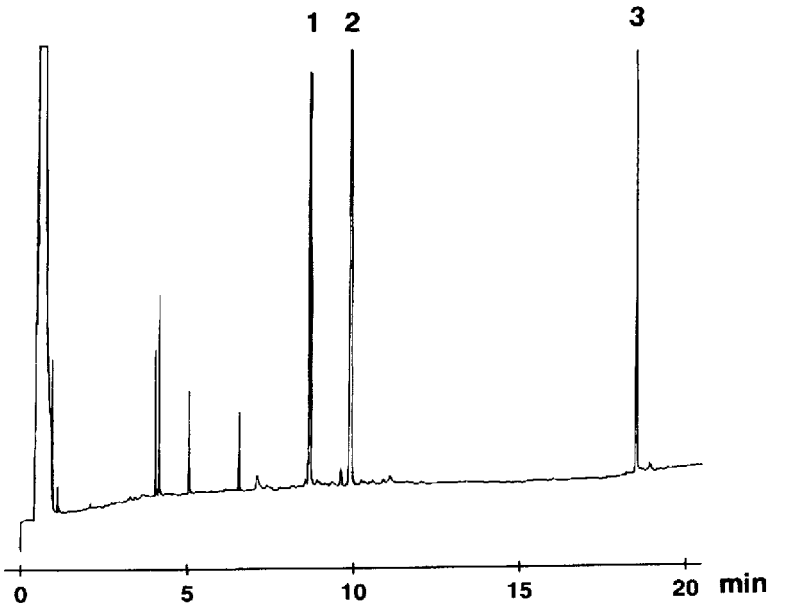

Fig 2 Chromatograms of a urine spiked with 8 nmol/ml pholcodine, 8 nmol/ml morphine and 8 nmol/ml methylnalorphine. Peaks 1 = morphine; 2 = methylnalorphine; 3 = pholcodine.

(C.V., $n = 5$) for pholcodine was 7.2% for 2 nmol/ml and 4.9% for 8 nmol/ml. The between-run C.V. ($n = 5$) was 7.7 and 5 8% for 2 and 8 nmol/ml, respectively. The detection limit in urine at a signal-to-noise ratio of 2 was 0.1 nmol/ml for pholcodine and 0.04 nmol/ml for morphine.

Urine hydrolysis

Combie *et al.* [5] evaluated β-glucuronidases for routine hydrolysis of glucuronic acid conjugates of drugs, and recommended an optimum pH of 4.5 for morphine and an optimum temperature of 56°C for *Helix pomatia* type H-1. The conditions for enzymic hydrolysis in this study were tested on urine samples spiked with morphine and codeine glucuronides and on authentic samples containing morphine and codeine glucuronides. The hydrolysis temperature, time and enzyme concentration were optimized for both morphine and codeine glucuronides. Fig. 3 shows the rate of hydrolysis of codeine glucuronide and morphine glucuronide at 37 and 56°C. At 37°C, 25% of the morphine and codeine glucuronides were hydrolysed as compared to hydrolysis at 56°C. These results are in agreement with the results reported by Chen *et al.* [6] for hydrolysis of codeine glucuronide. After incubation for 48 h at 56°C the hydrolysis of morphine and codeine glucuronides was complete. Raising the enzyme concentration from 5000 to 10 000 U per ml of urine increased the rate of hydrolysis. The yields after 48 h were, however, the same as the ones obtained for 5000 U of enzyme per ml of

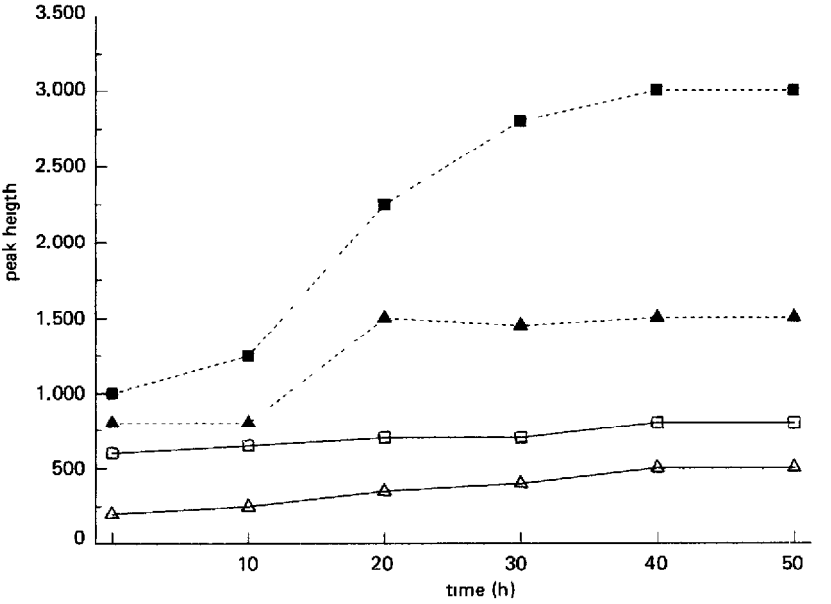

Fig. 3. Rate of hydrolysis of codeine glucuronide (□, ■) and morphine glucuronide (△, ▲) at 37°C (□,△) and at 56°C (■,▲)

urine For economical reasons hydrolysis was carried out with 5000 U of enzyme per ml of urine at 56°C for 48 h.

Metabolites of pholcodine

Findlay *et al.* [3] describing the disposition of pholcodine in man concluded that pholcodine appeared to undergo little conjugation. This study was based on plasma analysis using an immunological method, and morphine was not detected after pholcodine intake. The only pharmacokinetic study of pholcodine using a specific chromatographic method was published by Chen *et al.* [7]. This method was based on high-performance liquid chromatography with fluorescence detection. Enzymic hydrolysis was included in the assay, but the hydrolysis conditions were not optimized. The method was unable to detect morphine. However, two unidentified metabolites were detected. The procedure reported in this paper was used in a metabolism study of pholcodine in three healthy subjects receiving a single oral dose of 50 mg pholcodine [8]. Urine was collected as long as a positive opiate test was measured with the enzyme-multiplied immunoassay technique (EMIT). A peak concentration of 12–18 nmol/ml pholcodine in urine was measured by capillary GC 12 h after administration, and a positive EMIT response was measured 16-26 days after the oral dose. Fig. 4 shows typical chromatograms of a urine sample collected 12 h after administration before and after enzymatic hydrolysis. Pholcodine was found to be conjugated, and *ca.* 15% of the pholco-

dine was excreted in urine as the glucuronide. Morphine was found to be another metabolite of pholcodine showing that pholcodine undergoes 3-O-dealkylation. Morphine was detected after hydrolysis of urine samples from all three subjects

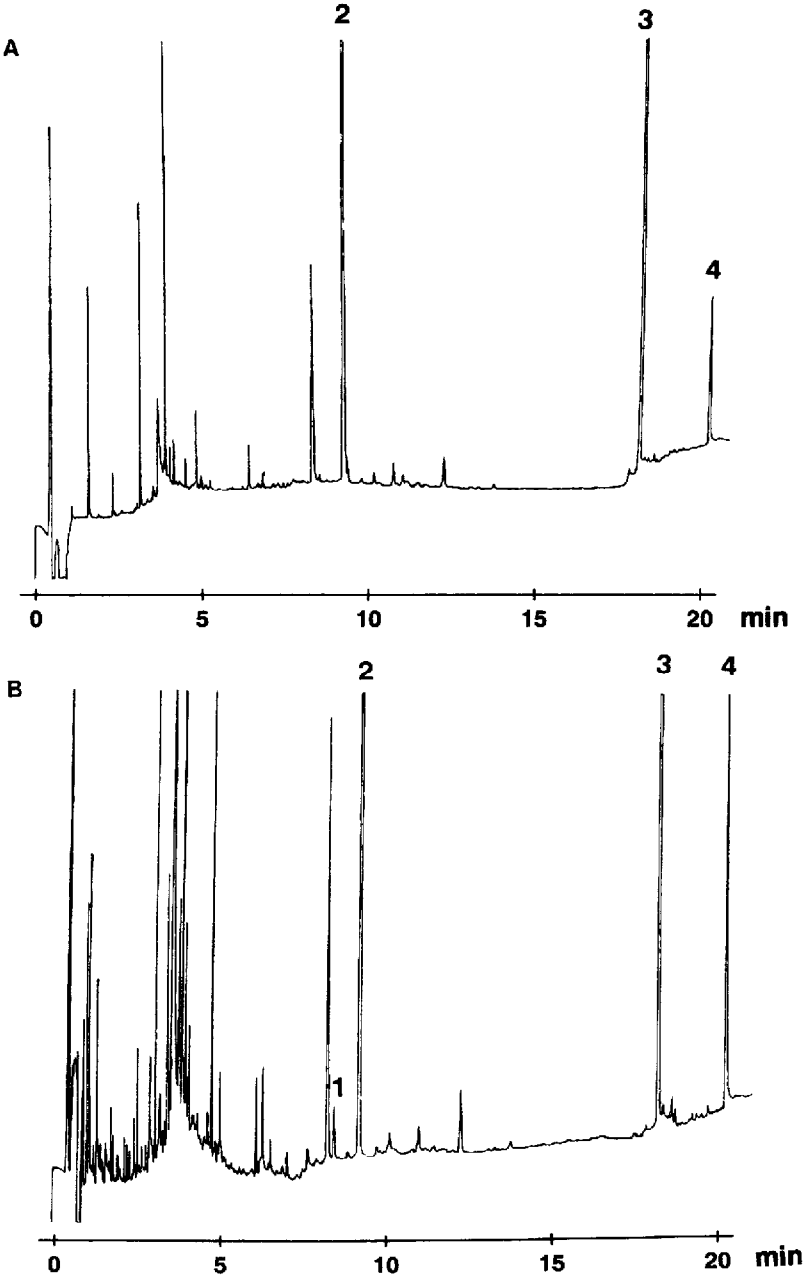

Fig 4 Chromatograms of a urine sample (A) before hydrolysis and (B) after hydrolysis with β-glucuronidase. Peaks 1 = morphine, 2 = methylnalorphine; 3 = pholcodine, 4 = unidentified metabolite

except in the urine collected during the last three days of the study. It was impossible to detect free morphine before hydrolysis, as shown in Fig. 4A. Only 0.5–1% of pholcodine was metabolized to morphine. The low levels excreted is possibly the reason why morphine has not been detected in other studies. The identity of morphine was confirmed by GC–MS as the pentafluoropropionic anhydride (PFPA) derivative and as the TMS derivative in full-scan mode. The mass spectra of the PFPA derivate of morphine included the fragments *m/z* 414 and 577 and the mass spectra of the TMS derivate of morphine included the fragments *m/z* 236 and 429. The possibility of artefact formation upon enzymatic hydrolysis was controlled by analysis of hydrolysed urine samples spiked with pholcodine. No morphine was detected in these samples. Another unidentified metabolite of pholcodine with a longer retention time than pholcodine is also shown on the chromatogram in Fig. 4. This metabolite also undergoes conjugation, but the identity of this metabolite has, however, not been verified.

CONCLUSION

The method developed in this study has been used successfully in a metabolism study of pholcodine in man. The method showed good accuracy and precision. Pholcodine was shown to undergo conjugation and morphine was identified as a metabolite of pholcodine.

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