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Column-switching high-performance liquid chromatographic detection of pholcodine and its metabolites in urine with fluorescence and electrochemical detection

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ABSTRACT

A sensitive and selective method for the detection of pholcodine and its metabolite morphine in urine using high-performance liquid chromatography is described. It involves on-line clean-up of urine on a trace enrichment column packed with a polymeric strong cation-exchange material. Pholcodine and its metabolites were separated on two analytical columns with different selectivities. Pholcodine was detected by a fluorescence detector and morphine was detected electrochemically. One system, based on reversed-phase chromatography, applied a polystyrene–divinylbenzene column and gradient elution. The other system was based on normal-phase chromatography with a silica column and isocratic elution. Morphine was confirmed to be a metabolite of pholcodine by reversed-phase chromatography and electrochemical detection. Two unidentified metabolites of pholcodine were separated from pholcodine by normal-phase chromatography and detected by fluorescence detection.

INTRODUCTION

The use of unspecific immunological screening methods for drug abuse testing of biological samples has led to a need for specific, sensitive and simple confirmation methods to distinguish illegal from legal use of drugs. The antitussive opiate pholcodine (3-O-morpholinoethylmorphine) has been reported to cross-react when biological samples are screened for opiate abuse by immunological methods [1]. Pholcodine is widely used and is available as a non-prescription medicine. After ingestion of one therapeutic oral dose of pholcodine, a positive opiate response in urine has been measured for 2–6 weeks by radioimmunoassay (RIA) [1] and for 2–4 weeks by the enzyme-multiplied immunoassay technique (EMIT) [1,2]. A high-performance liquid chromatographic (HPLC) method [3] and a capillary gas chromatographic (GC) method [4] have been published for the specific determination of pholcodine in biological samples. The HPLC method [3] involved reversed-phase chromatography with fluorescence detection, and two unidentified metabolites of pholcodine were detected in urine. The capillary GC method [4] with nitrogen–phosphorus detection (NPD) included enzymic hydrolysis of urine. This method was able to detect morphine as a metabolite of pholcodine in hydrolysed urine, showing that pholcodine undergoes 3-O-dealkylation as other opiate drugs. One unidentified metabolite of pholcodine was also detected in urine.

The purpose of the present study was to develop a sensitive and selective HPLC method for the detection of pholcodine and its metabolites in urine in order to confirm these findings. A column-switching procedure was developed for the direct injection of urine samples. A reversed-phase and a normal-phase chromatographic

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method were developed to separate pholcodine and unknown metabolites in urine.

EXPERIMENTAL

Reagents

Pholcodine was purchased from Weiders Farmasøytiske (Oslo, Norway). Morphine hydrochloride was obtained from Norsk Medisinaldepot (Oslo, Norway). Acetic acid and sodium acetate (both p.a. grade) were supplied by E. Merck (Darmstadt, Germany). β-Glucuronidase (*Helix pomatia* type H-1) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC-grade from Fisons (Loughborough, UK). HPLC-grade tetrahydrofuran was from Rathburn (Walkerburn, UK) and HPLC-grade water was obtained by purifying distilled water in a Milli-Q filtration system (Millipore, Bedford, MD, USA).

Preparation of standards

Stock standard solutions of pholcodine (0.2 μmol/ml) and morphine hydrochloride (0.2 μmol/ml) were prepared in 0.001 *M* acetate buffer (pH 6.5). Working standards of pholcodine (2.0–20.0 nmol/ml) and morphine hydrochloride (0.1–2.0 nmol/ml) in 0.001 *M* acetate buffer (pH 6.5) and in urine were prepared by dilution of the standard solutions.

Urine hydrolysis

A 1-ml volume of urine 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 as previously reported [4].

High-performance liquid chromatography

Two pumps, a Model LC-9A with a gradient module 5 CV-9AL (Shimadzu, Kyoto, Japan) and a Model 709 (LDC/Milton Roy, Riviera Beach, CA, USA) isocratic pump were used in combination with a Model 7000 (Rheodyne, Berkeley, CA, USA) six-port switching valve to perform column-swithching. A three-way slider valve (Model 5302, Rheodyne) connected between two flushing eluent reservoirs and the isocratic pump allowed washing of the trace enrichment column with 0.001 *M* acetate buffer (pH 6.5) and methanol. The injector was a Model 7125 (Rheodyne) with a 100-μl loop. The trace enrichment column (10 mm × 2 mm I.D.) from Chrompack (Middelburg, Netherlands) was packed with 20 μm polystyrene–divinylbenzene strong cation-exchange (SCX) material (Dynospheres, Dyno Particles, Lillestrøm, Norway).

Reversed-phase chromatography. The polystyrene–divinylbenzene column obtained from Polymere Labs. (Church Stretton, UK) was a 5 μm PLRP-S, 100 Å (150 mm × 4.6 mm I.D.). The mobile phase was acetonitrile–tetrahydrofuran–0.08 *M* borate buffer (pH 10.0) (8:9:83) during the first 5 min. After 5 min a linear gradient was applied. The final mobile phase composition, acetonitrile–tetrahydrofuran–0.08 *M* borate buffer (pH 10.0) (13:14:73), was reached after 1 min and kept constant during the rest of the analysis. The flow-rate was automatically changed from 0.4 to 0.7 ml/min, 8 min after the analysis was started.

Normal-phase chromatography. The analytical silica column was a 5 μm (250 mm × 4.6 mn I.D.) Whatman Partisil 5 from Alltech Assoc. (Deerfield, IL, USA). The mobile phase was methanol–2 *M* NH_3–1 *M* NH_4NO_3 (90:6.7:3.3) (pH 9.6), and the flow-rate was 1.0 ml/min.

The analytical columns were always flushed with the mobile phase at 0.100 ml/min overnight and during weekends, without recycling.

Detection

A Model RF 535 (Shimadzu) fluorescence detector and a Model L-ECD-6A (Shimadzu) electrochemical detector were coupled in series. Pholcodine was detected by fluorescence detection and the fluorescence detector was operated at an excitation wavelength of 230 nm and an emission wavelength of 350 nm as previously reported [3]. Morphine was detected electrochemically at a glassy carbon electrode at an oxidation potential of 350 mV *vs.* an Ag/AgCl reference electrode as recently reported [7]. Chromatograms were recorded on a Chromatopac C-R4A (Shimadzu) integrator.

Column switching

The sequence of switching events is given in Table I; the flow-rate of the flushing eluents was 0.5 ml/min.

TABLE I

SEQUENCE OF COLUMN-SWITCHING PROCEDURE

Time after injection (min)	Event
0	Sample is injected on to the trace enrichment column with 0.001 *M* acetate buffer (pH 6.5). Column-switching valve is in the load position.
3	The three-way slider valve is switched, and the trace enrichment column is washed with methanol.
11	The tree-way slider valve is switched, and the trace enrichment column is washed with 0.001 *M* acetate buffer (pH 6.5).
12	The column-switching valve is switched to the inject position, and the trace enrichment column is back-flushed with HPLC mobile phase.
14	The column-switching valve is switched to the load position. The trace enrichment column is re-equilibrated with 0.001 *M* acetate buffer (pH 6.5).
20	Fresh injection into the trace enrichment column.

Calibration and validation

The quantitation of pholcodine and morphine were based on peak-height measurements. The recoveries from urine were determined by comparing peak heights with those obtained from aqueous standards injected into the analytical column. The calibration curves were obtained by least-squares regression of the concentration of pholcodine and morphine added to drug-free urine *versus* peak heights. The linearity of the calibration curves was tested in the concentration range 2.0–20.0 nmol/ml for pholcodine and 0.1–2.0 nmol/ml for morphine. The coefficient of variation (C.V.) was calculated after analysis of six replicates of each concentration.

RESULTS AND DISCUSSION

Trace enrichment

Both pholcodine and morphine are basic compounds and are charged in acidic solutions. A polymeric SCX material was chosen as the packing material in the trace enrichment columns to extract cations selectively from urine. The polymer is stable in both alkaline and acidic solutions. Retention of cations from urine by the polymeric SCX material is facilitated by solvents of low ionic strength at a pH where the isolates are charged. However, the amount of impurities that can be extracted from urine is reduced by increased pH and ionic strength of the solvent. In this investigation 0.001 *M* acetate buffer (pH 6.5) was selected as the flushing eluent. This eluent gave nearly complete retention of pholcodine and morphine and a satisfactory clean-up of the urine samples. After a washing with acetate buffer, the trace enrichment column was washed with methanol. Methanol elutes impurities that are retained by the polymeric SCX material solely by hydrophobic interaction. No breakthrough of the analytes was observed when 4 ml of methanol were pumped through the trace enrichment column after the injection of 100 μl of urine. After injection of a total of 2 ml of urine, an increased back-pressure was observed and the trace enrichment column was repacked after twenty injections of 100 μl of urine.

Liquid chromatographic systems

It has been shown that electrochemical detection of morphine is enhanced in alkaline solutions [7] so, in order to detect trace amounts of morphine, alkaline mobile phases were used. Two systems, a reversed-phase and a normal-phase system, were optimized for the separation of pholcodine and its metabolites and for the desorption of the analytes from the trace enrichment column.

Reversed-phase chromatography. A polystyrene–divinybenzene analytical column was used because of its pH stability. During the development of the method, ammonia, carbonate and borate buffers in combination with acetonitrile and tetrahydrofuran were investigated as mobile phase additives. Borate buffer was preferred because it gave the highest number of theoretical plates. The final composition was optimized for quantitative desorption of pholcodine and its metabolites from the trace enrichment column. Elution of the isolates from the SCX material is promoted by a mobile phase of high ionic strength and a pH where the isolates are neutral. Complete elution was obtained with 0.08 *M* borate buffer (pH 10) mixed with acetonitrile and tetrahydrofuran. Lower concentrations of borate buff-

er and a pH value below 10 gave incomplete elution of pholcodine and morphine. Precipitation of borate may occur if a higher concentration of the borate buffer is used during gradient elution. Gradient elution was applied to decrease the analysis time owing to the large difference between the retention times of pholcodine and morphine.

Normal-phase chromatography. A normal-phase system was used as an alternative system to confirm the results of the reversed-phase system. It had previously been shown that efficient HPLC separations of basic drugs can be obtained on unmodified silica columns with aqueous methanol eluents [5,6]. Methanol–water eluents provide stable yet flexible systems for the analysis of basic drugs, and the effect of alterations in eluent pH and ionic strength can be predicted [5]. A mobile phase of methanol–2 *M* NH_3–1 *M* NH_4NO_3 (90:6.7:3.3) (pH 9.6) eluted pholcodine and morphine quantitatively from the trace enrichment column and separated pholcodine and morphine on the silica column.

Detection

The limit of detection in urine at a signal-to-noise ratio of 2 was 0.1 nmol/ml for pholcodine by fluorescence detection and 6 pmol/ml for morphine by electrochemical detection.

Validation of the procedure

The column-switching procedure gave nearly complete recovery of pholcodine and morphine from urine (Table II). The standard curves were linear in the concentration range 0.1–2.0 nmol/ml for morphine and 2.0–20.0 nmol/ml for pholcodine, with correlation coefficients of $r = 0.999$ for morphine and $r = 0.998$ for pholcodine. Satisfactory intra-assay coefficients of variation of 1.5–4.0 and 0.95–4.0% were obtained for pholcodine and morphine, respectively.

TABLE II

RECOVERY AND REPRODUCIBILITY FOR PHOLCODINE AND MORPHINE

Compound	Concentration added (nmol/ml)	Mean recovery ($n = 6$) (%)	C.V. (%)
Pholcodine	2.0	93.5	4.0
	10.0	94.5	3.6
	20.0	94.5	1.5
Morphine	0.1	95.0	4.0
	0.5	99.0	3.5
	2.0	97.5	0.95

Analysis of urine samples

In an earlier study, pholcodine metabolism was examined by analysis of urine samples by capillary GC [2]. The peak concentration of pholcodine in urine was 12–18 nmol/ml, measured by capillary GC 12 h after administration of a single oral dose of 50 mg of pholcodine. Pholcodine was found to conjugate with glucuronic acid, and 15% of the pholcodine dose was excreted in urine as the glucuronide, and 29% as unconjugated pholcodine. Only 0.5–1% of pholcodine was

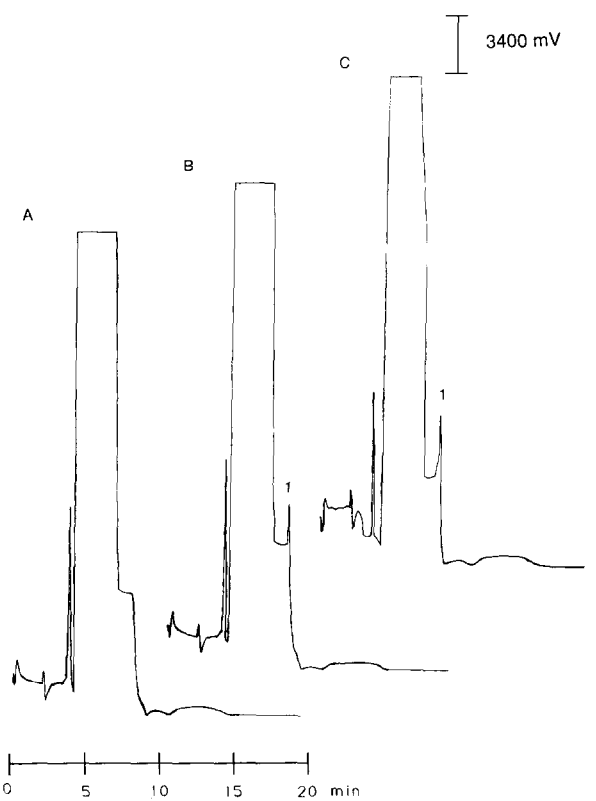

Fig. 1. Reversed-phase chromatography and electrochemical detection of (A) hydrolysed blank urine, (B) unhydrolysed urine collected 12 h after administration of 50 mg of pholcodine and (C) hydrolysed urine collected 12 h after administration of 50 mg pholcodine. Peak 1 = morphine.

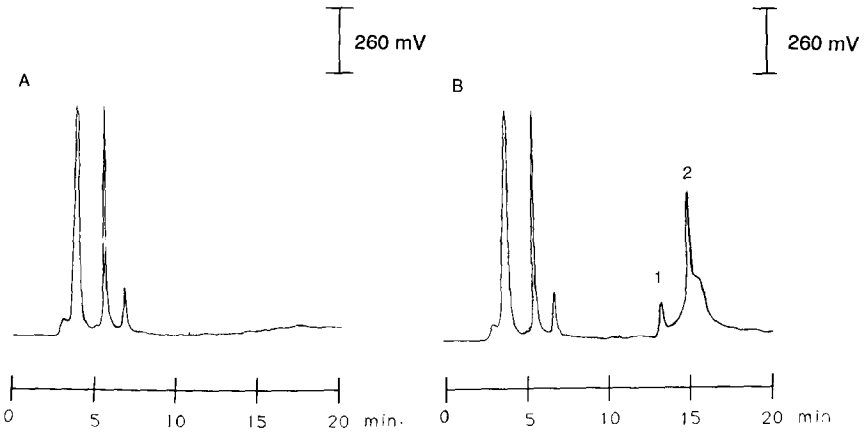

Fig. 2. Reversed-phase chromatography and fluorescence detection of (A) hydrolysed blank urine and (B) hydrolysed urine collected 12 h after administration of 50 mg of pholcodine. Peaks: 1 = unknown metabolite; 2 = pholcodine.

found to be metabolized to morphine and excreted in urine as morphine glucuronide.

Detection of morphine

Morphine was confirmed to be a metabolite of pholcodine in humans by reversed-phase chromatography and electrochemical detection, as shown in the chromatograms of unhydrolysed and hydrolysed urine collected 12 h after intake of a single oral dose of 50 mg of pholcodine (Fig. 1). The normal-phase system was unable to detect morphine owing to interfering compounds in urine. Morphine was conjugated with glucuronic acid, and the free morphine concentration was estimated to 0.1 nmol/ml before hydrolysis and 0.2 nmol/ml after hydrolysis. This was in agreement with the earlier study. The capillary GC method was, however, unable to detect morphine in unhydrolysed urine.

Detection of pholcodine and other metabolites

Pholcodine and other metabolites were detected by fluorescence detection. The chromatograms of drug-free urine showed no presence of interfering compounds and there was no significant difference between unhydrolysed and hydrolysed drug-free urine. Fig. 2 shows the reversed-phase separation of pholcodine from possible metabolites in hydrolysed urine collected 12 h after administration of a single oral dose of pholcodine. This system was, however, unable to separate pholcodine satisfactorily from these metabolites. The chromatogram shows, however, a possible presence of two unidentified metabolites of pholcodine. These two metabolites are sepa-

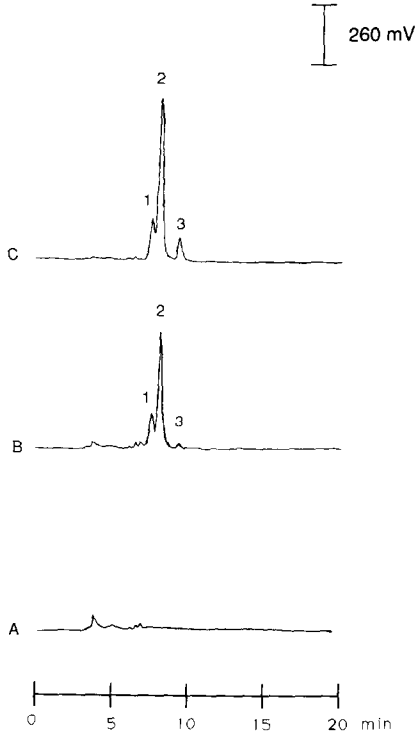

Fig. 3. Normal-phase chromatography and fluorescence detection of (A) hydrolysed blank urine, (B) unhydrolysed urine collected 12 h after administration of 50 mg of pholcodine and (C) hydrolysed urine collected 12 h after administration of 50 mg of pholcodine. Peaks: 1 = unknown metabolite; 2 = pholcodine; 3 = unknown metabolite.

rated from pholcodine by the normal-phase system, as shown in Fig. 3. Both these metabolites seem to be conjugated with glucuronic acid, as shown by an increased peak height after hydrolysis. Glucuronides present in unhydrolysed urine were probably not retained by the SCX trace enrichment column and therefore could not be detected. The presence of two unidentified metabolites is in agreement with previously reported results using HPLC and fluorescence detection [3]. Only one unidentified metabolite of pholcodine was, however, detected by capillary GC and nitrogen detection [4]. The concentrations in urine collected 12 h after administration were 16 and 22 nmol/ml in unhydrolysed and hydrolysed urine, respectively. This is in the same concentration range as reported earlier [2].

CONCLUSION

Pholcodine and its metabolites can be analysed in urine by HPLC in a column-switching system using a SCX extraction column. Morphine was detected as a metabolite of pholcodine by reversed-phase chromatography and electrochemical detection. A normal-phase system with fluorescence detection was able to show the presence of two unidentified metabolites of pholcodine in urine.

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