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Note

Determination of pholcodine in biological fluids by high-performance liquid chromatography with fluorescence detection

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Pholcodine (3-O-morpholinoethylmorphine, Fig. 1) is a codeine-like antitussive agent reported to lack significant analgesic action [1]. It is widely used as a cough suppressant in paediatric and adult medicine, and is available as an over-the-counter medicine in many countries.

There are almost no data on the pharmacokinetics of pholcodine in humans, mainly because of the lack of a specific, sensitive and convenient assay. Previous reports on pholcodine disposition in man were based upon radioimmunoassay (RIA) [2] and ^{14}C-labelled drug [3]. Svenneby et al. [4] reported that pholcodine interfered with the immunoassay for opiates in urine. The pharmacokinetics and metabolism of pholcodine have also been studied in the rat using RIA [5]. In this study, morphine was a minor metabolite of pholcodine. We are not aware of any reports in the literature on an assay for pholcodine in biological fluids using specific chromatographic methods. Only one study, analysing a cough mixture, involved high-performance liquid chromatography (HPLC) [6]. We report here a rapid, specific and sensitive HPLC method with fluorescence detection for the determination of pholcodine in plasma, saliva and urine.

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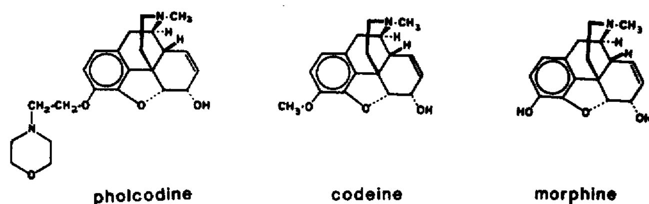

Fig. 1. Chemical structures of pholcodine, codeine and morphine.

EXPERIMENTAL

Reagents

All reagents were of analytical grade and included diethyl ether, hydrochloric acid and orthophosphoric acid (BDH Chemicals, Port Fairy, Australia), chloroform, pentan-1-ol, triethylamine, methanol, anhydrous sodium carbonate and sodium bicarbonate (Ajax Chemicals, Sydney, Australia). Acetonitrile (Mallinckrodt, South Oakleigh, Australia) was of HPLC grade. Pholcodine BP, codeine phosphate and morphine sulphate (F.H. Faulding, Adelaide, Australia) were all of British Pharmacopoeial grade quality.

Apparatus

The HPLC system consisted of an SP 8770 isocratic pump (Spectra-Physics, San Jose, CA, U.S.A.), an LS-5 luminescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.), a WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm$\times$4.6 mm I.D. stainless-steel column was homepacked with Spherisorb 5-μm ODS-2 packing material (Phase Separations, Queensferry, U.K.). The final composition of the mobile phase was acetonitrile–triethylamine–distilled water (16:0.035:83.965, v/v/v), adjusted to pH 3.5 with orthophosphoric acid. The flow-rate through the column at ambient temperature was 1 ml/min which produced a back-pressure of 7.93 MPa. The excitation and emission wavelengths of the detector were set at 230 and 350 nm, respectively, and the excitation and emission slits were set at 10 and 20 nm, respectively.

Stock solutions

Pholcodine was made up as a 1 mg/ml stock solution in distilled water and was diluted to concentrations ranging from 2 to 200 ng/ml in blank plasma and saliva, and from 0.2 to 10 μg/ml in blank urine. Codeine, the internal standard (1 mg/ml in distilled water) was diluted in distilled water to 5 μg/ml for plasma and saliva analysis and 100 μg/ml for urine analysis.

Sample preparation

Plasma and saliva. Plasma (or saliva) (1 ml) was pipetted into a 10-ml screw-capped, tapered plastic tube (Mallinckrodt) to which were added 10 μl of the 5 μg/ml internal standard solution and 0.5 ml of bicarbonate buffer (pH 9.6). The

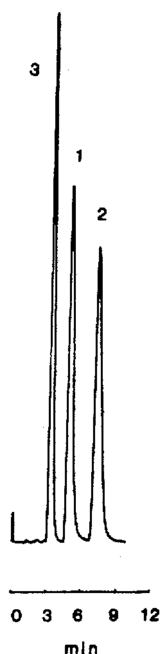

Fig. 2. Chromatogram of a standard solution of pholcodine (1), codeine (2) and morphine (3).

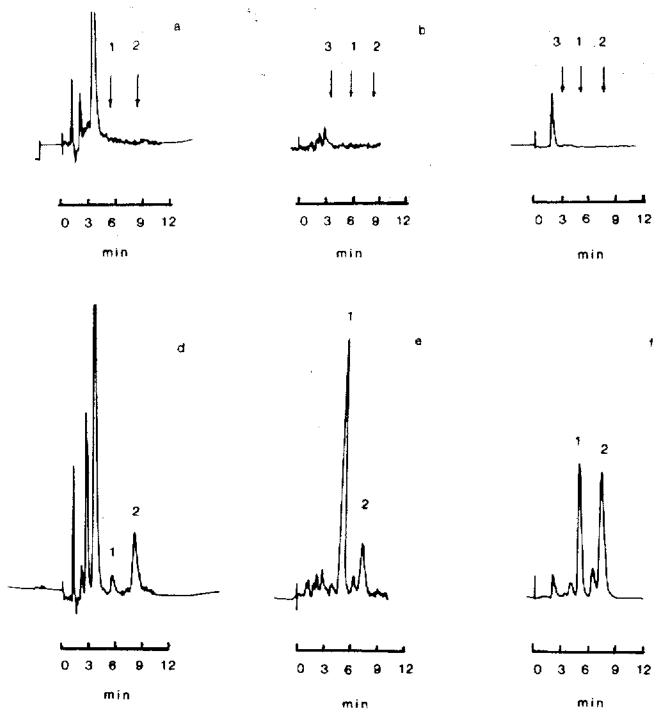

Fig. 3. Chromatograms from an extract of (a) a blank plasma sample, (b) a blank saliva sample, (c) a blank urine sample, (d) a plasma sample from a subject 0.5 h after ingestion of 60 mg pholcodine (concentration 15 ng/ml), (e) a saliva sample from the same subject 3 h after ingestion of 60 mg pholcodine (concentration 196 ng/ml) and (f) a urine sample from the same subject during 48–72 h (concentration 0.72 μg/ml). Peaks: 1 = pholcodine; 2 = codeine (internal standard); 3 = morphine.

TABLE I

ASSAY REPRODUCIBILITY OF PHOLCODINE IN PLASMA, SALIVA AND URINE

	Concentration (ng/ml)	Coefficient of variation (%)
Intra-day (n=10)		
Plasma	10	3.92
	50	3.42
Saliva	10	4.17
	50	3.62
Urine	200	2.45
	1000	1.89
Inter-day (n=6)		
Plasma	5	9.31
	200	7.78
Saliva	5	9.47
	200	7.11
Urine	500	4.89
	10000	2.27

TABLE II

DRUGS SHOWN NOT TO INTERFERE WITH THE PHOLCODINE ASSAY

Tablet preparations and pure drugs tested		Plasma from patients on multiple-drug therapy	
Amphetamine	Dihydrocodeine	Amiloride	Lithium
Cocaine	Dipyridamole	Amoxycillin	Minoxidil
Dextromethorphan	Ethylmorphine	Carbamazepine	Paracetamol
Dextrorphan	Naloxone	Digoxin	Phenindione
Dextropropoxyphene	Pentobarbital	Flucloxacillin	Salbutamol
		Frusemide	Sorbide dinitrate
		Hydrochlorothiazide	Theophylline
		Ibuprofen	Verapamil

mixture was briefly vortexed, 4 ml of diethyl ether–chloroform–pentan-1-ol (2:1:1, v/v/v) were added and the tubes were placed on a rotary mixer for 10 min. The organic and the aqueous phases were separated by centrifugation at 2000 *g* for 10 min. The upper organic phase was transferred to a clean 10-ml screw-capped, tapered plastic tube containing 100 μl of 0.1 *M* hydrochloric acid. The tubes were vortex-mixed briefly and then placed on a rotary mixer for 10 min. The two phases were separated by centrifugation at 2000 *g* for 5 min. The organic layer was discarded and the tubes were then placed in hot water for 10 min to remove all traces of organic solvents. An aliquot (10–30 μl) was injected onto the column via the automatic injector. Peak heights were measured manually and the peak-height ratio of pholcodine to codeine was then calculated.

Urine. A 0.5-ml aliquot of urine was pipetted into a 10-ml screw-capped plastic tube to which were added 25 μl of a 100 μg/ml internal standard solution. The samples were then handled in exactly the same manner as the plasma samples.

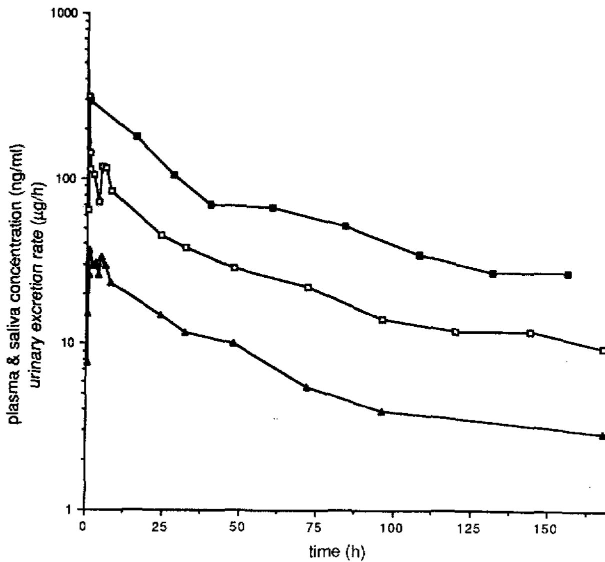

Fig. 4. Semilogarithmic plot of plasma (△) and saliva (□) concentration, and urinary excretion rate (■) versus time for pholcodine after a single 60-mg oral dose in a human volunteer.

Statistical analysis

Standard curves were plotted as peak-height ratio versus pholcodine concentration. Linear regression analysis was performed to determine the slope, intercept, their variability and the strength of the correlation. Precision was evaluated by spiking plasma and saliva with pholcodine to concentrations ranging from 5 to 200 ng/ml, and urine from 0.2 to 10 μg/ml, performing replicate analysis intra- and inter-day and determining coefficients of variation.

Assay application

A pilot pharmacokinetic study was conducted to confirm the utility of the assay. A young male volunteer (aged 28 years, weight 55 kg) took 60 mg pholcodine in linctus form (Pectolin®, F.H. Faulding) as a single dose. Immediately before and at intervals for one week after the dose, blood, saliva and urine were collected for measurement of pholcodine concentrations.

RESULTS AND DISCUSSION

Fig. 2 is a representative chromatogram from an injection of a solution containing a mixture of pholcodine, morphine and codeine each at a concentration of 2 μg/ml. Morphine had a retention time of 3.5 min and a capacity factor of 1.75, pholcodine had a retention time of 5.5 min and a capacity factor of 3.25, whereas codeine had a retention time of 7.5 min and a capacity factor of 4.92. After evaluation of ultraviolet and electrochemical detectors, the fluorescence detector was chosen. Ultraviolet detection, although providing sufficient sensitivity, was unacceptable because of interference from endogenous substances. Electrochemical detection provided sufficient sensitivity for morphine and co-

deine but not for pholcodine. Unlike the others, the fluorescence detection gave acceptable sensitivity and selectivity from interfering endogenous substances. The effects, on the retention time of the three compounds, of pH and composition of mobile phase were investigated. Firstly, increasing the pH from 2 to 5 resulted in an increase in retention time for all three compounds. Secondly, increasing the triethylamine concentration from 0.025 to 0.1% reduced the retention time for all three. Lastly, increasing the acetonitrile concentration from 12 to 30% resulted in a marked reduction in the retention time for codeine, a moderate reduction in retention time for morphine and a modest increase for pholcodine. As a result of the above, the final mobile phase of 16% acetonitrile and 0.035% triethylamine adjusted to pH 3.5 was chosen because it permitted the baseline separation of the three compounds with reasonable analysis time and selectivity. Calibration curves showed good linearity between peak-height ratios and concentrations from 2 to 200 ng/ml for pholcodine in plasma and saliva ($r=0.9998$) and from 0.2 to 10 μg/ml for pholcodine in urine ($r=0.9992$). For plasma, saliva and urine standard curves, the 95% confidence intervals of the intercepts included the origin. The standard errors of the slopes were less than 5% for all three.

The assay showed good precision at low and high pholcodine concentrations in plasma, saliva and urine. Table I shows the intra- and inter-day assay variability. Calculated concentrations were within 95% of added concentrations. The recovery of pholcodine averaged 80% and of codeine 95%. The 95% confidence intervals of the slopes of standard curves, prepared from the same samples (plasma, saliva and urine) stored at −20°C and assayed eleven weeks apart, overlapped indicating no loss of pholcodine from the biological media.

Interference by other drugs was studied by preparing and chromatographing aliquots of pure drugs and by analyzing samples from patients on multiple-drug therapy (Table II). None of the drugs in Table II interfered with the assya of pholcodine. Fig. 3 shows a chromatogram from blank plasma, saliva and urine, a plasma sample from the volunteer 0.5 h after pholcodine ingestion, a saliva sample 3 h after ingestion and a urine sample collected between 48 and 72 h. Especially in urine and saliva samples two additional peaks not found in any blank samples were observed at retention times of 4.5 and 6.5 min. It is likely that these are metabolites of pholcodine and studies are now in progress to elucidate their structures.

Fig. 4 illustrating the utility of the assay, shows plasma and saliva concentrations and urinary excretion rate versus time for the volunteer. The terminal half-lives were similar from all three fluids and averaged 48.6 h. Morphine was not detected in the urine and saliva samples.

In summary, an original method has been developed for the assay of pholcodine in plasma, saliva and urine. The method shows good precision, accuracy, specificity and sensitivity and is currently being used to determine the pharmacokinetics of pholcodine in patients.

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