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Note

Determination of codeine in plasma and urine by reversed-phase high-performance liquid chromatography

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Several methods have been reported for the determination of codeine, a narcotic analgesic and antitussive drug. Gas chromatographic procedures involving flame-ionization detection of the derivatized drug have been commonly used [1–5]. A method has also been reported using nitrogen-selective detection without the need for derivatization of codeine [6]. The sample preparation, however, required several solvent extraction steps. Combined gas chromatography–mass spectrometry has also been reported for the determination of codeine [7, 8]. Radioimmunoassay procedures [9, 10] and normal-phase high-performance liquid chromatography (HPLC) [11–13] have also been reported. All of these procedures lacked the required sensitivity for measurement of the drug at therapeutic concentrations, involved tedious sample preparation procedures, required sophisticated instrumentation or were not specific for the drug itself.

Several reversed-phase HPLC procedures have also been described. Ultraviolet absorption at low non-specific wavelengths [14–19] was the most common detection method used. Because of the basic nature of codeine, several methods reported the use of basic mobile phases [14, 20–22] with reversed-phase HPLC columns to obtain satisfactory chromatograms. In our experience this has led to rapid column degradation. Other workers have reported the use of high flow-rates [23, 24] to overcome peak tailing when codeine is chromatographed on reversed-phase columns. Several methods of sample clean-up have been described. They were based on solvent extraction [15, 25] and were tedious and time-consuming.

The present method utilizes reversed-phase HPLC with an acidic mobile phase and fluorescence detection. It uses a 5-cm column and the retention

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times for codeine and the internal standard are 1.5 and 2.8 min, respectively. Excellent peak shapes are obtained for both species. The sample clean-up uses a C_2 extraction cartridge and the resulting chromatograms are essentially free from interfering endogenous compounds for both plasma and urine. The method is extremely simple for routine analysis of both plasma and urine samples. The limits of detection are ca. 3 ng/ml for plasma and ca. 0.25 μg/ml for urine, which are adequate for measuring drug concentrations following a therapeutic dose of codeine (30 or 60 mg).

EXPERIMENTAL

Materials

All of the reagents and solvents used were reagent grade and HPLC grade unless otherwise specified and were supplied by Fisher (Pittsburgh, PA, U.S.A.). Glusulase® was purchased from DuPont (Wilmington, DE, U.S.A.) and human control plasma from Sera-Tec Biologicals (North Brunswick, NJ, U.S.A.). Codeine phosphate standard was obtained from Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.) and the internal standard, N-allylnorcodeine, was a gift from McNeil Pharmaceuticals (Spring House, PA, U.S.A.). C_2 (100 mg) disposable extraction cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Equipment

This assay has been run successfully on a Hewlett-Packard 1090 and a Varian LC-5000 HPLC system. Both of these instruments were equipped with a heated column compartment. The HP-1090 was equipped with an autosampler and the LC-5000 used a Waters WISP Model 710B autosampler. The HPLC column used was a C_{18} Sepralyte (5 cm × 4.6 mm, 3 μm packing) supplied by Analytichem International. Both HPLC systems were fitted with a Schoeffel FS-970 fluorescence detector and a Spectra-Physics Model 4270 computing integrator was used to measure peak areas. The other equipment used was a Thermolyne maxi-mix vortex mixer, a Tecan DB3 dry-block heater and an IEC Model 7R centrifuge.

Preparation of standard solutions and reagents

Glusulase working solution was prepared by diluting stock solution (1 ml) with sodium acetate buffer (0.1 *M*, pH 5.2, dilute to 100 ml). The aqueous part of the mobile phase was 0.2 *M* sodium perchlorate–0.1 *M* phosphoric acid. The organic modifier was methanol. Stock solutions of codeine phosphate (1 mg/ml codeine free base) and the internal standard (1 mg/ml) were prepared in phosphoric acid (0.1 *M*). Working standards of codeine were prepared at 1.5, 1, 0.5, 0.25 and 0.15 μg/ml for plasma assays and 40, 20, 10, 4, 2 and 1 μg/ml for urine. This gave standard line ranges of 15–150 ng/ml in plasma and 0.5–20 μg/ml in urine. Working standards for the internal standard were prepared at 2 μg/ml for plasma and 40 μg/ml for urine analysis

HPLC and detector conditions

The column temperature was maintained at 50° C and the flow-rate used was

1.5 ml/min. The mobile phase was aqueous buffer–methanol (84:16). The injection volume used was 50 μl. The fluorescence detector excitation wavelength was set at 220 nm and the emission wavelength at 355 nm using a band-pass optical filter. The sensitivity of the detector was set at 1.

Preparation of plasma standards

Frozen blank human plasma was thawed at room temperature, vortex-mixed (30 s) and centrifuged (15 min at 2000 *g*). Aliquots of the supernatant (500 μl) were used for the preparation of the standard line. The working solutions of codeine (50 μl) were added to separate aliquots of blank plasma followed by working internal standard solution (50 μl). Sodium carbonate (0.1 *M*, 500 μl) was added to each tube and the contents were vortex-mixed. A C_2 extraction cartridge was washed with methanol (1 ml) and sodium carbonate (0.1 *M*, 1 ml). The plasma standards were loaded onto respective cartridges. A vacuum was then applied and the eluates were discarded. Each cartridge was further washed with sodium carbonate (0.1 *M*, 1 ml) and methanol–sodium carbonate (30:70, 1 ml) and the eluates were discarded. Each cartridge was then eluted with methanol–sodium carbonate (80:20, 200 μl) and the eluate collected. Perchloric acid (0.2 *M*, 400 μl) was added to each tube and the total contents were vortex-mixed. An aliquot (50 μl) was injected for HPLC analysis.

Preparation of plasma samples

The procedure described for plasma standards was followed substituting patients' plasma (500 μl) for blank plasma and phosphoric acid (0.1 *M*, 50 μl) for the codeine working standards.

Preparation of urine standards

Frozen blank human urine was thawed completely at room temperature and vortex-mixed. Aliquots (100 μl) of this urine were placed in separate tubes and used for the preparation of the standard line. Codeine working standards (50 μl) were added to respective tubes followed by working internal standard solution (50 μl). Glusulase working solution (300 μl) was added to each tube, which was then incubated (1 h at 37°C). Sodium carbonate (0.2 *M*, 500 μl) was added to each tube. A C_2 extraction cartridge was washed as described for plasma. The respective standards were loaded onto the extraction cartridges which were then subjected to vacuum and the eluates were discarded. Each cartridge was washed with sodium carbonate (0.1 *M*, 3 × 1 ml) and methanol–sodium carbonate (30:70, 3 × 1 ml) and the washings were discarded. The cartridges were then eluted with methanol–sodium carbonate (80:20, 300 μl) and the eluates collected. Perchloric acid (0.2 *M*, 750 μl) was added to each tube, the contents were mixed and an aliquot (50 μl) was injected for HPLC analysis.

Preparation of urine samples

The procedure described for urine standards was followed substituting patients' urine (100 μl) for blank urine and phosphoric acid (0.1 *M*, 50 μl) for codeine working standards.

RESULTS AND DISCUSSION

This method used reversed-phase HPLC with fluorescence detection. Codeine and the internal standard, N-allylnorcodeine, are both naturally fluorescent and can be detected in plasma and urine at therapeutic concentrations with minimal interference from endogenous compounds and metabolites. The method has been successfully applied to the analysis of plasma and urine samples from a clinical study where 60 mg of codeine phosphate were administered.

A C_2 extraction cartridge is activated with methanol and then washed with sodium carbonate (0.1 *M*). Under these basic conditions codeine and the internal standard are well retained. A further washing with methanol–sodium carbonate (30:70 for plasma and urine) will not elute these compounds, but will remove polar and many acidic compounds that would otherwise interfere with the chromatographic analysis. Methanol–sodium carbonate (80:20) was found to be the best eluting solvent for these compounds from the extraction cartridges. It gave high recoveries of both drug and internal standard and minimal elution of interfering endogenous compounds. Interfering compounds were eluted from the cartridge using either methanol or methanol–acid mixtures.

Urine samples were first treated with Glusulase to hydrolyze the glucuronide

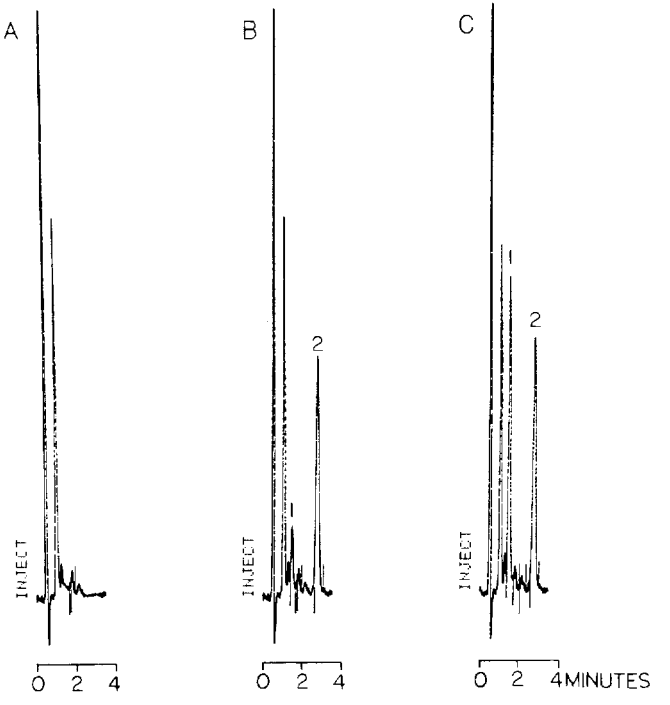

Fig. 1. Typical chromatograms for codeine in plasma. (A) Blank plasma; (B) blank plasma containing 25 ng/ml codeine and 200 ng/ml internal standard; (C) patient plasma containing 108 ng/ml codeine and 200 ng/ml internal standard. Peaks: 1 = codeine; 2 = internal standard.

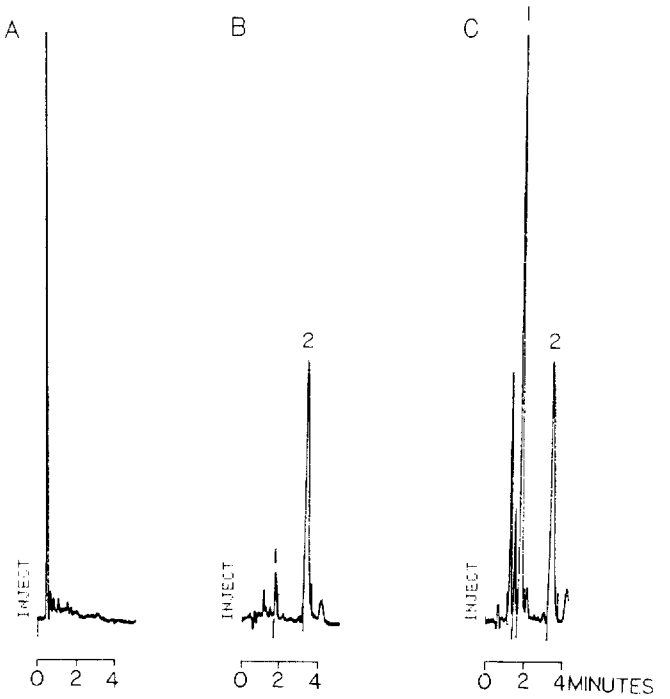

Fig. 2. Typical chromatograms for codeine in urine. (A) Blank urine; (B) blank urine containing 0.5 μg/ml codeine and 20 μg/ml internal standard; (C) patient urine containing 7.4 μg/ml codeine and 20 μg/ml internal standard. Peaks: 1 = codeine; 2 = internal standard.

TABLE I

INTER-DAY REPRODUCIBILITY FOR THE DETERMINATION OF CODEINE IN PLASMA AND URINE

Concentration standard	Mean ratio drug/ internal standard	S.D.	R.S.D. (n = 5) (%)
Plasma (ng/ml)			
15	0.1062	0.0064	6.0
25	0.1668	0.0178	10.6
50	0.3064	0.0190	6.2
100	0.6102	0.0332	5.4
150	0.9415	0.0469	5.0
Urine (μg/ml)			
0.5	0.0418	0.0016	3.8
1.0	0.0941	0.0120	12.7
2.5	0.2336	0.0123	5.3
5.0	0.4673	0.0118	2.5
10	1.0486	0.0138	1.3
20	2.1103	0.0088	0.4

metabolites. The samples were then alkalinized and taken through a similar clean-up procedure to that described for plasma.

Typical chromatograms from patient plasma are shown in Fig. 1 and the corresponding chromatograms for urine samples are shown in Fig. 2. The peak

TABLE II

QUALITY CONTROL RESULTS FOR CODEINE IN PLASMA AND URINE

Plasma			Urine		
Date	Low region (target = 25 ng/ml)	High region (target = 100 ng/ml)	Date	Low region (target = 1 μg/ml)	High region (target = 10 μg/ml)
1/29/85	28.8	106.8	2/5/85	1.17	9.95
1/30/85	25.2	106.6	2/6/85	0.99	10.02
2/11/85	24.7	109.6	2/7/85	1.03	10.18
2/12/85	27.3	106.5			
Mean	26.5	107.4	Mean	1.06	10.05
S.D.	1.9026	1.4886	S.D.	0.0945	0.1179
R.S.D. (%)	7.2	1.4	R.S.D. (%)	8.9	1.2

shapes in both cases are excellent and the chromatograms are essentially free from endogenous interference.

Standard lines were run from 15 to 150 ng/ml for plasma and from 0.5 to 20 μg/ml for urine. The limits of detection for the assays by this method were approx. 3 ng/ml for plasma and 0.25 μg/ml for urine. The linearity of each standard line was confirmed by plotting the ratios of drug/internal standard versus concentration for each point. The precision of the assay was checked by comparing the inter-day variability of each standard point. The data are summarized in Table I for plasma and urine. The accuracy of the method was established by preparing quality control samples of known concentrations at low and high regions of the plasma and urine standard lines. These samples were frozen before any of the patient samples were assayed. On any given day standard lines were run that included quality control samples with the patient samples. The data are summarized in Table II.

The retention times for codeine and the internal standard were 1.5 and 2.8 min, respectively. This very short analysis time has proved to be very useful for the assay of large numbers of clinical samples. The sample clean-up procedure was also very simple to perform and gave very clean chromatograms. The assay has proved to be very rugged. In excess of 500 clinical samples (plasma and urine) have been assayed on a single HPLC column with no increase in column back-pressure or loss of chromatographic resolution.

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