Report

Determination of Codeine in Plasma by High-Performance Liquid Chromatography

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An automated, sensitive, and selective reverse-phase high-performance liquid chromatographic assay has been developed to measure codeine in plasma. The analysis requires only 1 ml plasma and is accomplished by detection of the fluorescence of codeine following extraction and concentration. The method is simple and rapid, involving a one-step extraction of codeine from alkalinized (pH 10.0) plasma into an organic layer of hexane/dichloromethane, 2/1. The organic layer was evaporated under nitrogen and the residue reconstituted with the mobile phase. The samples were chromatographed on a reverse-phase C-18 column using a mobile phase of acetonitrile–phosphate buffer, 80/20 (pH 5.80). The codeine and internal standard, *N*-allylnorcodeine, peaks were detected using a fluorescence detector. The retention times were 8.6 min for the internal standard and 11.3 min for codeine. Standard curves were linear from 10 to 250 ng/ml. The assay was validated by direct comparison with a gas chromatographic procedure that employed nitrogen–phosphorus detection. The assay has been employed for the analysis of several codeine studies using human, dog, and rat plasma.

KEY WORDS: codeine; measurement of codeine in plasma; high-performance liquid chromatography.

INTRODUCTION

Codeine is a naturally occurring alkaloid which has been used as an analgesic and antitussive for many years. Gas chromatographic (GC) methods have been reported for the determination of codeine in plasma using flame ionization and electron capture detection (1–5). These are limited by the sensitivity of the flame ionization detector, derivatization steps in the sample workup, and/or the need for large sample volumes. A highly selective and sensitive gas chromatographic procedure utilizing a mass spectrometer has been reported (6). However, this instrumentation is not widely available at present.

Several high-performance liquid chromatographic (HPLC) methods for codeine analysis have been reported (7–9). These employ reverse-phase, normal phase, or ion-pair systems. However, some of these methods are not sensitive enough for measuring the usual concentrations of codeine in plasma, while others are not readily adapted to automated sample injection.

An HPLC analysis that could rapidly and accurately determine low concentrations of codeine in plasma after a single oral therapeutic dose was developed. The advantages of this method over previous procedures are that (a) a rapid one-step extraction is employed; (b) only 1 ml plasma is required; and (c) there are no late-eluting peaks, which allows for the automated injection of one sample every 14 min.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile, hexane, and methanol were HPLC grade, and phosphoric acid, potassium bicarbonate, potassium carbonate, and dibasic ammonium phosphate were ACS grade from Fisher Scientific Co., Fairlawn, N.J. Methylene chloride was "distilled-in-glass" grade, from Burdick and Jackson, Muskegon, Mich.

Chromatographic Conditions

The HPLC system was equipped with the following components: an automatic sample injector (WISP, Waters Associates, Milford, Mass.), a solvent delivery system (Waters Associates Model M-6000A), and a fluorescence detector (Schoeffel Model FS-970) operating at a 217-nm excitation wavelength and an emission wavelength cutoff filter of 320 nm. A 25 cm × 4.6-mm (i.d.) analytical column (Whatman Partisil-10 ODS) was used with a 3 cm × 4.6-mm guard column [Brownlee, RP-18 (10 μm)].

The mobile phase was a 77.5:22.5 (v/v) mixture of acetonitrile and 0.005 *M* aqueous dibasic ammonium phosphate, adjusted to a final apparent pH of 5.80 ± 0.05 with 8.5% phosphoric acid. The system was conditioned with at least 60 ml mobile phase at a flow rate of 1.5 ml/min. During sample analysis the flow rate was also 1.5 ml/min. The anal-

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ysis is conducted at room temperature, and under these conditions the retention times for the internal standard, *N*-allylnorcodeine, and codeine were 8.8 and 11.5 min, respectively.

Glassware Treatment

Prior to use, all glassware was soaked in detergent (Micro, International Products, N.J.) for 2 hr, rinsed thoroughly with distilled water, and heat treated for 3 hr at 270°C. Prior to use, polyethylene-lined screw caps (Penn Bottle, Philadelphia, Pa.) were soaked in *n*-heptane for 3 hr and dried in an oven until the solvent was completely evaporated.

Standard Curves

A stock solution of 1 mg/ml of codeine (free base) was prepared in methanol. From dilutions of the stock solution, two standard solutions were prepared that contained 10 and 1 ng/μl codeine in methanol. Appropriate volumes of these two solutions were used to seed plasma samples to construct the nine-point standard curve, which ranged from 10 to 250 ng/ml.

Two hundred microliters of a methanolic stock solution of *N*-allylnorcodeine (1 ng/μl free base) was added to each 1-ml plasma sample prior to analysis.

Procedure

To citrated human plasma (1.0 ml) in 15-ml disposable screw-cap bottles was added the internal standard and 0.5 ml 0.2 *M* bicarbonate/carbonate buffer (pH 10.9). Standard codeine concentrations were 10, 25, 50, 75, 100, 125, 150, 200, and 250 ng/ml. Standards were prepared by seeding control plasma with methanolic codeine solutions. A 6.0-ml aliquot of hexane:methylene chloride (2:1, v/v) was added. The bottles were shaken on a tabletop shaker for 15 min and centrifuged at 800*g* for 15 min. Five milliliters of the upper organic layer was transferred to a 15-ml conical glass centrifuge tube and evaporated to dryness under a stream of nitrogen at room temperature. The sides of the tubes were then rinsed with approximately 500 μl methanol. The methanol was again evaporated to dryness. The residue was reconstituted with 150 μl mobile phase, all of which was then transferred to a WISP limited-volume insert. One hundred microliters of each sample was injected into the liquid chromatograph. The detector sensitivity was 0.5-μA response full scale, and the photomultiplier was set at 720 V.

Quantitation and Data Handling

Standard curve data were generated by analyzing a series of nine plasma standards (10–250 ng/ml). Three separate experiments were conducted by weighing and diluting drug and internal standard and analyzing duplicate standards each day for 3 consecutive days. The data were analyzed by linear regression analysis using the reciprocal of the variance of the peak height ratios as the weighting factor. Concentrations of codeine in unknown plasma samples were determined using the calculated peak height ratios and the linear regression equation.

A Hewlett-Packard 3354C Lab Automation System was used for automatic data acquisition, temporary data storage, linear regression analysis, and report generation.

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma and plasma spiked with codeine and the internal standard are shown in Fig. 1. The blank plasma sample is free of fluorescent peaks after 6 min. The retention times for the internal standard and codeine are 8.8 and 11.5 min, respectively. Caffeine and acetaminophen as well as a metabolite norcodeine do not interfere with codeine quantitation using this method. A good correlation was obtained between the peak height ratios of drug/internal standard and the theoretical codeine plasma concentrations. Linear regression analysis of the data gave a correlation coefficient (r) of 0.998, a slope (±SD) of 0.004 (0.00003), and a Y intercept (±SD) of −0.004 (0.0006). The standard curve was reproducible (less than ±10% variation) from day to day. The variability of the peak height ratios was less than 10% at each of the nine concentrations, indicating a good interday precision. The back-calculated values of the standards in the range of 10 to 250 ng/ml were within 5.6% of their theoretical values, indicating a good accuracy.

The percentage recoveries for both codeine and the internal standard were determined at the 100 ng/ml concentration by comparison of the peak heights of extracted samples with the direct injection of an equivalent amount of compound. The mean (±SD) recoveries were 51.8 (5.1)% and

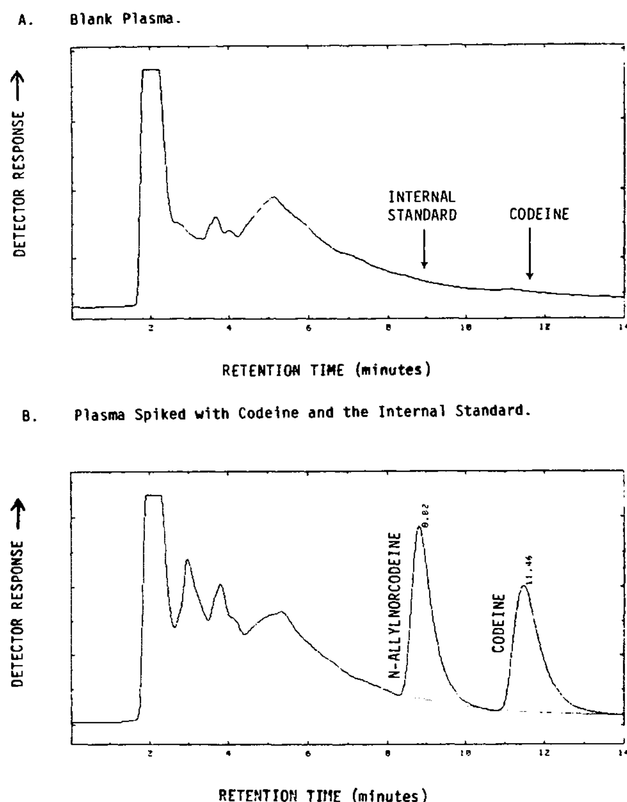

Fig. 1. HPLC assay of codeine in human plasma. Chromatograms of (A) blank plasma and (B) plasma spiked with 150 ng/ml of codeine and 200 ng/ml of *N*-allylnorcodeine.

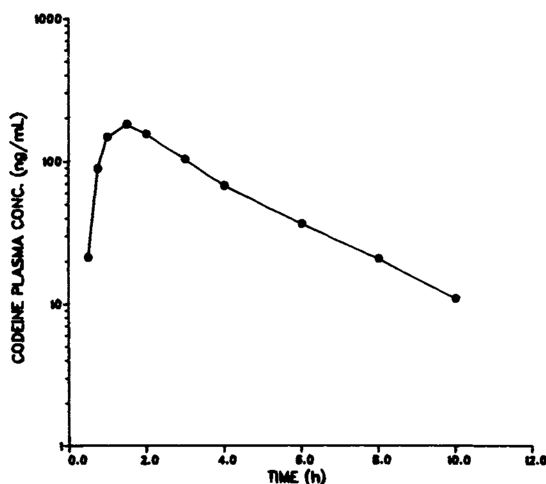

Fig. 2. Plasma concentration versus time profile of codeine following the oral administration of 60 mg of codeine phosphate to a healthy volunteer.

54.0 (5.0)% for codeine and *N*-allylnorcodeine, respectively ($N = 6$). Extraction efficiencies at codeine concentrations of 50 and 200 ng/ml were not significantly different. Analysis of control samples indicated that recovery was unchanged in samples stored up to 3 months at −20°C. Using 1 ml plasma, the minimal detectable concentration of codeine was found to be 4 ng/ml. This was the smallest codeine peak height which could be separated from background noise by the HP3354 laboratory automation system. The minimum quantitation limit is 10 ng/ml, using a 1-ml plasma sample. The relative standard deviation at 10 ng/ml was 2.9%, indicating a good precision at the limit of quantitation.

The utility of this method was demonstrated by the analysis of codeine plasma concentrations of volunteers following the oral administration of 60 mg of codeine phosphate. Shown in Fig. 2 is a plasma concentration–time curve for one subject. The peak concentration was 184 ng/ml at 1 hr, and the elimination half-life was 2.45 hr. These values are similar to those previously reported for codeine.

The HPLC assay was validated by comparison with a GC method which utilized nitrogen–phosphorous detection (5). Plasma samples ($N = 22$) from subjects who received 60 mg codeine phosphate were analyzed by both procedures. The range of codeine concentrations varied from 10 to 150 ng/ml. There was a good correlation ($R = 0.997$) between the HPLC and the GC methods (a slope of 0.97 and a Y intercept of 1.6 ng/ml).

This method provides an efficient, specific, and sensitive procedure for the rapid determination of codeine plasma concentrations and is adapted to an autosampler. It has been employed in a number of bioavailability studies in humans as well as in codeine disposition studies in animals.

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