Liquid Chromatography of Codeine in Plasma with Fluorescence Detection

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This analytical method for easier determination of codeine in human plasma is based on “high-performance” liquid chromatography for separation and the natural fluorescence of codeine for detection. Codeine is extracted from alkalized plasma with a mixture of hexane and dichloromethane, and the extract is further purified and chromatographed. The method can be used for routine assay of codeine at the concentrations of 10 μg/L or greater in human plasma. As little as 4 μg/L can be detected. Coefficients of variation for the assay of codeine in the concentration range of 10 to 100 μg/L were 2.2–7.4% (n = 6). We used this method to establish a concentration/time profile for plasma from a human volunteer after a 60-mg oral dose of codeine sulfate.

Additional Keyphrases: drug assay • drug bioavailability

A simple, sensitive, and specific analytical method for the measurement of codeine in human plasma is needed for studying the bioavailability of codeine from oral formulations containing a therapeutic dose of codeine sulfate. Several methods of analysis for codeine in human plasma have been reported. Gas-chromatographic methods involving flame-ionization detection for the determination of codeine and its trimethylsilyl derivative have been described (1–4), but their sensitivities are often limited by the flame-ionization detector used. In addition, with methods involving direct assay of codeine with a gas chromatograph, we encountered problems associated with the absorption of the compound by column packings. Thin-layer chromatography combined with nitration of in situ codeine has been used for the quantitation of nanogram amounts of codeine in plasma (5). This method, however, is tedious.

A method based on gas chromatography/mass spectrometry has also been described (6), but the expensive and sophisticated instrumentation required is not found in many clinical laboratories, and the technique is not suitable for analysis of a large number of samples. More recently, a sensitive radio-immunoassay procedure has been developed for the assay of codeine in plasma (7, 8).

Here we describe a sensitive and rapid assay for codeine in human plasma by “high-performance” liquid chromatography (HPLC), with fluorescence detection.

Materials and Methods

Chemicals: All solvents used were “distilled in glass” grade (Burckard and Jackson Labs, Muskegon, MI 49442). Codeine phosphate, USP reference standard, was obtained from U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852. The internal standard, N-isopropylcodeine phosphate, and [3H]codeine (spec. acty. 200 Ci/mol) were synthesized by the Institute of Organic Chemistry, Syntex Research. Phosphoric acid (85%), monobasic sodium phosphate, and sodium hydroxide were all of AR grade (Mallinckrodt, Inc., St. Louis, MO 63147).

Apparatus: We used a “high-performance” liquid chromatograph equipped with a M6000 liquid chromatography pump, a U6K injector (Waters Associates, Milford, MA 01757) and a Model 7130A recorder (Hewlett-Packard, San Diego, CA 92106). A Model FS970 fluorescence detector (Schoeffel Instrument Corp., Westwood, NJ 07675) was operated at an excitation wavelength of 213 nm and an emission cutoff filter of 320 nm. Detector sensitivity was set at 0.05 μA. We used a 3.9 mm × 30 cm C18 μ-Bondapak column (Waters Associates) and a 4 mm × 8 cm Co-Pell ODS guard column (Whatman, Inc., Clifton, NJ 07014) for chromatographic separation. Test tubes of 15-mL capacity, fitted with Teflon-lined screw caps, were used for extraction.

Solutions: Phosphate buffer solution (50 mmol/L, pH 8), was prepared by dissolving 7 g of monobasic sodium phosphate in 1 L of water and adjusting the pH to 8 with a 1 mol/L solution of NaOH.

A stock solution of codeine was prepared by dissolving the amount of codeine phosphate equivalent to 1 mg of codeine in 10 mL of methanol. The stock solution was further diluted with an equilibrium mixture of methanol and water to yield a solution containing 1 μg of codeine per milliliter. This solution was used for supplementary drug-free samples of plasma. A stock solution and a working solution of internal standard were prepared in the same way.

Procedure: Pipet 2 mL of plasma into a 15-mL test tube and add 200 ng of the internal standard. Alkalinate the plasma with 2 mL of the 50 mmol/L phosphate buffer solution, and extract twice with 6-mL portions of hexane/dichloromethane (2:1 by vol) by manually shaking the mixture for 2 min, then centrifuging. Combine the organic extracts and wash with 1 mL of NaOH, 50 mmol/L, to remove any potentially interfering substances. Transfer the extract to a 15-mL conical test tube and evaporate under a gentle stream of nitrogen to about 1 mL. Wash down the inside wall of the test tube with 1 to 2 mL of methanol and evaporate the entire contents of the test tube to dryness under nitrogen. Dissolve the residue in 200 μL of HPLC mobile phase (see below). Place the test tube in an ultrasonic bath for 30 s, then vigorously vortex-mix. Inject a 50-μL aliquot onto the chromatographic column.

Chromatographic conditions: The mobile phase was composed of methanol/water (21:79 by vol), containing 1.5 g of phosphoric acid per liter. Flow rate was set at 2 mL/min and columns were operated at ambient temperature. Column pressure was maintained between 1000 to 2000 psi (6.89 × 106 to 13.7 × 106 Pa). The recorder was used at a chart speed of 5 mm/min.

Results and Discussion

The establishment of the bioavailability parameters of a drug formulation requires monitoring of low concentrations of drug in biological fluids after a single oral dose. Therefore, a sensitive, rapid, yet specific method is required to measure codeine concentrations in humans after administration of a formulation containing codeine.

Previous reports (3, 9) indicate that codeine concentrations declined to low microgram per liter values in plasma after a therapeutic dose regimen. The method reported here achieved the desired sensitivity to measure microgram per liter concentrations in plasma by taking advantage of the natural fluorescence of codeine, which was observed in neutral, acidic,
and basic media. The uncorrected excitation and emission spectra of codeine in methanolic solution are shown in Figure 1. Fluorescence of codeine in methanolic solution was maximum at the excitation wavelength of 213 nm with an emission band from 300 to 400 nm. Few endogenous substances fluoresce under the described assay conditions. As a result, by using fluorescence detection, we were able to minimize sample purification before HPLC analysis.

Tritiated codeine was used to establish the extraction efficiency and total recovery for this procedure. The radiochemical purity of $[^3H]$codeine was first established by thin-layer chromatography. The $R_f$ of codeine in a solvent system of acetone/dichloromethane/methanol/ammonium hydroxide (30/10/5/1 by vol) on a silica gel plate was 0.31. $[^3H]$Codeine was added to drug-free plasma and the radioactivity was monitored through each step of the procedure. The extraction efficiency of codeine from alkalized plasma by the hexane/dichloromethane extraction solvent was 80%. The total recovery of codeine subjected to the entire extraction and clean-up procedure, up to and just before HPLC, was 75%.

Adding a small amount of phosphoric acid to the mobile phase results in the formation of an ion-pair with codeine. This improved peak shapes and allowed the detection of codeine at nanogram quantities.

To obtain better reproducibility with this procedure, we included a structural analog of codeine, $N$-isopropylcodeine, as an internal standard. Codeine and the internal standard were extracted similarly and resolved well under the described chromatographic conditions.

Figure 2(a) shows a chromatogram of drug-free human plasma processed through the entire assay procedure. There was no interference from blank plasma at the retention times of codeine or internal standard. Representative chromatograms obtained from the analysis of plasma fortified with codeine at 10 and 100 μg/L are shown in Figures 2(b) and 2(c), respectively.

To study the precision of the method, we fortified drug-free plasma with codeine at concentrations of 10, 25, 50, 75, or 100 μg/L and assayed six sets of fortified samples on six separate days. Between-assay coefficients of variation (CV) at each concentration are shown in Table 1.

Calibration curves obtained by analyzing plasma samples fortified with codeine in the concentration range of 10 to 100 μg/L were subjected to regression analysis (Table 2). The statistics of the data from six calibration curves indicated the linearity and reproducibility of the assay in this concentration range.

The lower limit of quantitation for this method was set at 10 μg/L. At this concentration the CV for the assay of codeine in plasma was 7.4%, and the signal-to-noise ratio for the codeine peak was approximately 6. From this we estimated that a signal-to-noise ratio of 2 would be obtained for a sample containing 4 μg of codeine per liter, and this concentration was set as the minimum detectable limit of the method.

To investigate the possibility that drugs administered with codeine or metabolic products of codeine might interfere with the measurement of codeine, we injected selected compounds onto the HPLC under the described conditions and determined the retention times for the compounds (Table 3). Of all the compounds investigated, only $N$-norcodeine (retention

<p>| Table 1. Reproducibility of Results for Codeine Determination in Drug-Free Plasma Fortified with Codeine |
|------------------------------------------|------------------------------------------|------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Codeine added, μg/L</th>
<th>Peak height ratio (codeine/int. std.)</th>
<th>Mean (and SD)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.17, 0.21, 0.20, 0.19, 0.19, 0.18</td>
<td>0.190 (0.014)</td>
<td>7.4</td>
</tr>
<tr>
<td>25</td>
<td>0.44, 0.48, 0.45, 0.47, 0.48, 0.46</td>
<td>0.463 (0.016)</td>
<td>3.5</td>
</tr>
<tr>
<td>50</td>
<td>0.91, 0.98, 0.96, 1.06, 1.02, 1.00</td>
<td>0.988 (0.052)</td>
<td>5.2</td>
</tr>
<tr>
<td>75</td>
<td>1.36, 1.52, 1.41, 1.53, 1.51, 1.55</td>
<td>1.480 (0.076)</td>
<td>5.2</td>
</tr>
<tr>
<td>100</td>
<td>1.97, 2.02, 1.98, 2.09, 1.98, 1.99</td>
<td>2.005 (0.045)</td>
<td>2.2</td>
</tr>
</tbody>
</table>
time 3.3 min) might interfere with the measurement of codeine under these experimental conditions. However, only trace amounts (<10 μg/L) of N-norcodeine have been reported (3) to be present in plasma of some subjects who had been dosed with codeine. This trace amount, which was present in plasma only at the peak codeine concentration time (3), was not considered to be significant enough to affect the assay of codeine in plasma. Metabolically generated morphine was not detected by this method. Because of the relatively low polarity of the extraction solvent, morphine was not extracted with codeine from plasma. We therefore cannot use this method for the simultaneous detection of morphine and codeine.

All of the chromatograms from plasma extracts had a negative baseline deflection between 8 and 9 min after injection. We later confirmed that this negative baseline deflection was due to the presence of caffeine (5 mg/L or more) in plasma from subjects who had consumed caffeine-containing beverages. Therefore, it is critical that the HPLC columns and the mobile phase used can separate caffeine (retention time, 9 min) from internal standard (retention time, 7 min).

To demonstrate the applicability of the developed method, a human volunteer ingested 60 mg of codeine sulfate. Codeine concentrations in plasma of this subject were similar to previously reported values (5): at 20 and 40 min and 1, 2, 4, 8, and 10 h after the dose, the codeine concentrations were 11, 90, 101, 84, 43, 17, and 13 μg/L, respectively. The peak concentration in plasma occurred 1 h after the dose and the rate of decrease of drug concentration indicated a half-life of 2.65 h. Between 10 and 24 h after the dose, codeine concentration in the plasma decreased to less than the limit of quantitation for the method (10 μg/L).

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References