Determination of Codeine and Morphine in Human Plasma by High Performance Liquid Chromatography with Serial Electrochemical Detection

STEVEN C. HARRIS, M.D.*†, MICHAEL A. MILLER, B.S. †‡ and JACK E. WALLACE, Ph.D.*†

Department of Pathology, *Audie L. Murphy Memorial Veterans Administration Hospital, and †University of Texas Health Science Center, San Antonio, TX 78284

ABSTRACT

A method is described for the determination of codeine and its metabolite, morphine, at low nanogram concentrations in plasma. Analysis is accomplished by high-performance liquid chromatography utilizing a cyanopropyl normal bonded phase (NBP) column in reversed-phase mode and two electrochemical detectors in series configuration. Two internal standards are utilized, ethyl morphine for codeine and nalorphine for morphine. Codeine and morphine concentration data are presented for several patients receiving codeine-containing medications. The lower limit of detectability was 2.00 ± 0.39 ng per mL for codeine and 1.20 ± 0.83 ng per mL for morphine. The patient sample mean within-run coefficients of variation for codeine and morphine (at 10 ng per mL) were less than 10 percent, n = 30. The between-run coefficient of variation for codeine was also less than 10 percent (over a range of two to 190 ng per mL, n = 61), and was approximately 15 percent for morphine (over a range of two to 40 ng per mL, n = 31).

Introduction

Codeine is administered extensively as an analgesic and antitussive agent. The metabolism of codeine has been the topic of many recent publications.1,3,5,8,9,11,12,16,18,21,26,27 Of special interest is codeine’s analgesic properties which may, to a great extent, be due to one of its metabolites, morphine.8,9

In order to conduct metabolic or bioavailability studies for a drug such as codeine, a very sensitive and specific analytical method capable of quantitating low amounts of metabolites in the presence of relatively high quantities of par-
ent drug is necessary. Secondly, the analytical column must provide adequate selectivity for the combined analysis of codeine, morphine, and their internal standards.

Most of the procedures presently available for the quantitation of codeine and morphine in biologic material employ either gas-liquid chromatography or radioimmunoassay (RIA). However, gas chromatographic procedures for opiates require derivatization, particularly if morphine is to be analyzed, even if capillary or megabore columns are utilized. In immunoassays, it may be difficult to obtain a specific antibody which can differentiate among the structurally similar opiate compounds.

Methods which employ high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection may not provide the detection limits for opiates in biologic extracts which are necessary for bioavailability or other pharmacokinetic studies. Nelson et al have published a method combining HPLC for separation and radioimmunoassay (RIA) for quantitation of codeine and morphine and their metabolites. This procedure, however, involves injection of non-extracted serum onto the liquid chromatographic column, thus shortening column life.

In 1980, our group published the first procedure for assaying opiates by HPLC with electrochemical detection. In cases where the drug under investigation can be easily oxidized, as with most natural opiate analogs, it is possible to utilize electrochemical detection for the necessary increased specificity and sensitivity. However, methods that utilize reversed phase octadecylsilane (ODS) columns and electrochemical detection for the analysis of morphine result in inadequate column selectivity when utilized for the combined analysis of codeine and morphine with ethylmorpheine and nalorphine as their respective internal standards.

This current study demonstrates the utilization of an intermediate polarity cyanopropyl normal bonded phase (NBP) column in reversed-phase mode for the assay of codeine and its metabolite, morphine. An increase in selectivity is acquired by using this bonded phase when analyzing the codeine, morphine, ethylmorphine, and nalorphine mixture. The cyanopropyl NBP column offers the advantage of separating compounds with isomeric variations of any polar functionality, e.g., hydroxy, alkoxy, and amine groups. This mode is more applicable to codeine and morphine analysis than is the normal-phase mode as a consequence of the ability to manipulate ionization control in the predominantly aqueous mobile phase. Detection is accomplished with two series-connected electrochemical detectors operating at different oxidative potentials.

Materials and Methods

Apparatus

A model 5030 high-performance liquid chromatograph equipped with a 15 cm CN-5 (5 μm cyanopropyl) column in reversed-phase mode and a 100 μL loop valve injector was used for the separation of codeine and morphine and their internal standards. Detection was accomplished with two series-connected electrochemical detectors. The first in-line detector was a Metrohm EA 1096/2 PCTFE wall-jet detector operated at +0.9 V (oxidation) with a Metrohm E 611 potentiostatic current/voltage measuring instrument. The wall-jet cell was chosen for the first in-line cell because of its extremely low

* Varian Associates, Palo Alto, CA 94303.
† Valco Instruments Co., Inc., Houston, TX 77055.
‡ Metrohm AG, 9100 Herisau, Switzerland.
dead volume, which maintained high resolution for the detection of codeine and ethylmorphine in the second cell. The second in-line detector was a BAS LC-17 equipped with a TL-5A or TL-8A thin-layer transducer operated at +1.2 V (oxidation) by an LC-4A amperometric controller.§

Chromatographic conditions were as follows: mobile phase, 90 percent 0.02 M KH₂PO₄ with 10 mg per L of ethylenedinitrilotetraacetic acid, disodium salt/5 percent methanol/5 percent acetonitrile, degassed with helium; flow rate, 1.0 mL per min.; column at room temperature. The mobile phase was automatically proportioned by the liquid chromatograph from two reservoirs, one pumping buffer, the other a 1:1 methanol/acetonitrile mixture.

To minimize background, a pulse dampener was installed in a “tee” configuration prior to the analytical column. The pulse dampener was necessary for the Bioanalytical Systems electrochemical cell, a design which is more sensitive to flow fluctuations. Pulsation noise was thus considerably reduced at high sensitivity settings.

REAGENTS

Codeine phosphate and morphine sulfate were obtained.‖ A 1 g/L solution (corrected to free base) of each drug was prepared in methanol.

Ethylmorphine hydrochloride§ and nalorphine hydrochloride were obtained.** A 1 g per L solution (corrected to free base) of each compound was prepared in methanol. Aliquots of these stock solutions were diluted with methanol to give a working solution collect-

† Bioanalytical Systems Inc., West Lafayette, IN 47906.
‡ A. H. Robins Co., Richmond, VA 23220.
** Merck, Sharp and Dohme Research Lab., West Point, PA.

tively containing one μg per mL nalorphine and four μg per mL ethylmorphine. An appropriate volume of the working solution was added to the vessel in which the 10 percent isobutanol in chloroform extracting solvent was prepared to yield a concentration of one ng per mL nalorphine and four ng per mL ethylmorphine.

Acetonitrile and monobasic potassium phosphate were purchased (Cat. No. A-998 and P-286, respectively).†† Both were HPLC grade.

Chloroform, ethyl acetate, isopropanol and methanol were purchased, Cat. Nos. CX1054, EX241, PX1834 and MX488, respectively).‡‡ All were HPLC grade.

Isobutanol was purchased (Cat. No. 2986) and was spectrophotometric grade. §§

The pH 8.9 borate buffer used to adjust the pH of the serum prior to the initial extraction consisted of 0.5 M boric acid and 0.043 M sodium borate. The pH 10 dibasic potassium phosphate wash buffer was prepared by adding 35 g of K₂HPO₄ to 100 mL of deionized H₂O.

Sodium carbonate (Cat. No. 7521)§§ and sodium bicarbonate (Cat. No. SX0320-1)‡‡ were used in a 1:2 w/w mixture to adjust the pH of the aqueous layer after extraction.

The water utilized for chromatography was distilled and passed through a carbon column, a 0.5 μm filter, and an ion-exchange resin.

DOsing

Each of twenty-four subjects was given two 755 mg tablets composed of 400 mg methocarbamol, 325 mg acetaminophen, and 30 mg codeine phosphate. Each subject ingested both tablets during the morning following a

‡‡ MCB Manufacturing Chemists, Inc., Cincinnati, OH 45212.
§§ Mallinckrodt, Inc., St. Louis, MO 63147.
minimum eight-hour fast. The tablets were swallowed with approximately four oz of water followed with four oz of orange juice.

Sample Collection

Venous blood samples were drawn in Venoject® tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. The 15 mL samples were drawn at 10, 20, 30, and 45 minutes, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 18, and 24 hours after dosing. All blood samples were centrifuged, and the plasma was transferred into storage tubes supplied by the sponsor, labeled with a code number, and frozen until assayed. During freezing, the tubes were set at an angle (15 to 75°) to minimize breakage.

Procedure

Plasma standards utilized in the procedure were prepared by adding appropriate amounts of diluted stock solutions of codeine and morphine to screw-cap tubes, drying under a stream of air, adding an appropriate amount of plasma or serum, and vortexing.

For analysis, 0.5 or 1.0 mL of serum or plasma, one or two mL, respectively, of borate buffer and 25 mL of 10 percent isobutanol in chloroform (containing 0.5 ng per mL nalorphine and two ng per mL ethylmorphine for a 1.0 mL assay) are placed into a 50 mL screw-cap plastic tube (Falcon #2070). The tubes are shaken for 15 min on an Eberbach mechanical shaker and then centrifuged for five min at 2000 rpm. The upper aqueous layer is removed by aspiration and the remaining organic layer is washed twice by shaking for 15 min with five mL portions of pH 10 phosphate wash buffer, centrifuging for five min at 2000 rpm, and removing the aqueous layer by aspiration. Five mL of 0.5 N HCl are added to the tube containing the washed organic layer. The tube is shaken for 15 min on an Eberbach shaker and then centrifuged for five min at 2000 rpm and 4.5 mL of the aqueous HCl layer are next transferred to a 16 × 125 mm glass screw-cap tube (teflon-lined cap). The aqueous solution is adjusted to a pH of 8.7 ± 0.2 by the addition of one level spoon (Coors #02) of a 2:1 w/w mixture of sodium bicarbonate:sodium carbonate. Five mL of 10 percent isopropanol in ethyl acetate are added to the tube. The contents of the tube are shaken for 15 min on an Eberbach shaker and then centrifuged for five min at 2000 rpm; 4.5 mL of the organic layer is removed to a separate clean tube and dried in a heating block at 50°C under a stream of air. The residue in the tube is reconstituted in 200 μL of mobile phase and vortexed; 100 μL of resulting solution are injected onto the liquid chromatograph. Quantitation is based upon the peak height ratios of codeine to ethylmorphine and morphine to nalorphine.

Results

As illustrated in figure 1, the extraction scheme and electrochemical detection utilized in this procedure provided very clean chromatograms free of interferences from endogenous plasma constituents, while the mobile phase in conjunction with a five μm cyanopropyl column in reversed-phase mode resulted in excellent separation of codeine, morphine, and their internal standards. The total elution time for all four chromatographed components was less than nine minutes.

Two lots of plasma controls spiked with codeine and morphine, in the range of one to 125 ng per mL, and their internal standards ethylmorphine (0.5 ng per
PLASMA CODEINE/MORPHINE BY HPLC/DUAL ECD

Figure 1. Typical chromatographic separation of morphine (M), codeine (C) and their internal standards nalorphine (N, 0.5 ng per mL) and ethylmorphine (E, 2 ng per mL), respectively, from the system described: (A) first in-series cell operating at +0.9 V of 25 ng per mL (left) and 2.5 ng per mL (right) morphine plasma controls. (B) second in-series cell operating at +1.2 V of 50 ng per mL (left) and 30 ng per mL (right) codeine plasma controls.

And 2, respectively) and 7.81 percent (n = 5), respectively.

The between-run CV’s, averaged over a range of two to 40 ng per mL, for morphine’s 0.5 mL and 1.0 mL assays were 6.34 percent and 4.18 percent (n = 4), respectively, while those for codeine, averaged over a range of two to 125 ng per mL, were 11.28 percent and 9.28 percent (n = 6), respectively. Spiked controls below the two ng per mL level were below the lower limit of detectability.

The mean within-run CV, at 10 ng per mL concentration, for codeine was 7.91 percent with a 95 percent confidence interval (95 percent CI) of ±3.37 percent (n = 30), and 8.87 ± 3.08 percent (95 percent CI, n = 26) for morphine. Figure 2 illustrates a plot of the linearity of the assays.

The patient sample between-run CV values for codeine and morphine covered a concentration range of two to 190 ng per mL and two to 40 ng per mL, respectively. The mean CV for codeine was 9.76 ± 1.21 percent (95 percent CI, n = 61), and for morphine was 15.69 ± 0.34 percent (95 percent CI, n = 31). While acetaminophen and morphine were well resolved at low acetaminophen concentrations, at high acetaminophen concentrations the acetaminophen peak broadened so as to partially mask the morphine peak.

The lower limit of detectability for codeine was 2.00 ± 0.39 ng per mL (95 percent CI, n = 61) and for morphine was 1.20 ± 0.83 ng per mL (95 percent CI, n = 31). This corresponds to 0.5 ± 0.1 ng of codeine and 0.30 ± 0.21 ng of morphine detected on the column after extraction.

The corrected absolute recoveries from plasma specimens reinforced with 25 ng per mL of codeine and morphine were 56.8 ± 1.20 percent and 39.3 ± 3.7 percent, (mean ± 1 SD) respectively. For these determinations, no
internal standards were added to the plasma samples prior to extraction, and calculations were based on peak areas obtained from assayed plasma samples relative to those obtained from buffer standards of the same concentration.

The composite bioavailability profile for the 24 patients studied, figure 3, demonstrates codeine's pharmaco-kinetic activity during absorption, distribution, and elimination after a single oral dose of two tablets each containing 30 mg of codeine. The lowest quantifiable level observed during absorption, 10 min after ingestion, for 10 of the 24 patients monitored was $7.27 \pm 2.74$ ng per mL (95 percent CI, n = 10). A peak level of $147.34 \pm 19.15$ ng per mL (95 percent CI, n = 24) was attained one hour after initial ingestion. The lowest quantifiable level during elimination was $5.25 \pm 2.27$ ng per mL (95 percent CI, n = 4) which occurred 18 hours after ingestion.

**Discussion**

The extraction process utilized in this procedure is essentially the same as described in an earlier publication from this laboratory. There is, however, an important modification. Owing to the amphoteric properties of morphine and nalorphine, their extraction from an aqueous medium into an organic solvent must be accomplished within a narrow pH range. For this reason, combinations of sodium bicarbonate and sodium carbonate were explored for the neutralization and buffering of 4.5 mL of 0.5 N HCl to a pH of 8.7 ± 0.2. One level spoon (Coors #02) of a 2:1 w/w mixture of sodium bicarbonate:sodium carbonate provided the necessary buffering capacity to achieve the desired pH.

Since codeine and morphine differ both in chemical and electrochemical properties, two internal standards were used for their quantitation. Ethylmorphine exhibits structural, chemical, and electrochemical properties which are similar to those of codeine, while nalorphine has properties similar to those of morphine. The use of these two compounds provided an excellent means of internal standardization for the quantitation of codeine and morphine.
The separation of the four components under investigation was attempted on a 30 cm ODS (10 μm) reversed-phase column as well as on a 15 cm cyanopropyl (5 μm) intermediate polarity column, both in combination with a variety of mobile phases. It was observed that the selectivity of the reversed phase (ODS) column was not sufficient for the separation of these four components.

However, with the cyanopropyl column, excellent separation was achieved when the mobile phase consisted primarily of dilute monobasic potassium phosphate solution with a small quantity of acetonitrile. While the chromatography was exemplary with this particular mobile phase, problems associated with electrochemical detection were encountered. The glassy carbon electrode (operating at +1.2 V) would lose sensitivity after approximately five injections of a solution containing codeine, morphine, ethylmorphine, and nalorphine. To eliminate the rapid coating of the glassy carbon surface, methanol was added to the mobile phase (90:5:5 0.02 M KH₂PO₄:MeCN:MeOH). Although this modification resulted in maintaining a clean glassy carbon surface, a new problem of interfering leachate peaks occurred. Disodium ethylenedinitrilotetraacetic acid (EDTA) was added to the mobile phase (10 mg per L of 0.02 M KH₂PO₄) in order to chelate to the stainless steel tubing and injection valve surfaces, and its addition eliminated the occurrence of the leachate peaks.

Another problem which was encountered during the development of this methodology was traced to the step involving the drying of the isopropanol/ethyl acetate extract. Several small interfering peaks appeared on all chromatograms, whether they were of serum extracts or solvent standards. These peaks were found to be due to components (perhaps plasticizers) which were leaching from the rubber tubing through which air passed for sample drying purposes. When teflon tubing was substituted for the rubber tubing, these peaks were absent from the chromatograms. For this reason, it is suggested that the air being used for drying come into contact only with metal pipe, teflon, or glass.

Although morphine and nalorphine can be oxidized at a potential of +0.9 V, initial studies during the development of the proposed methodology were performed using a single electrochemical detector, operating at +1.2 V. This seemed to be the most straightforward means of detection for all four components under investigation. Under these conditions, analysis for codeine met stringent quantitative criteria. However, morphine analysis was complicated when relatively large amounts of acetaminophen and methocarbamol were added to the spiked sera. Methocarbamol, with its high plasma concentration, electrochemical sensitivity, and chromatographic proximity to morphine, often obscured the analysis of morphine. Since methocarbamol does not oxidize appreciably at a potential of +0.9 V, the decision was made to perform the analysis with two series-connected electrochemical cells, one operating at a potential of +0.9 V and the other at +1.2 V. This would allow detection of morphine and nalorphine on one electrochemical cell and codeine and ethylmorphine on the other, with no interference from methocarbamol. Acetaminophen, at high plasma concentrations in patient samples gave electrochemical activity at nearly all workable oxidation potentials, and with its chromatographic proximity to morphine tended to mask the morphine peak, compromising parts of the pharmacokinetic profile.

The low recovery values reported previously clearly demonstrate why internal standardization was essential when applying this method to bioavailability.
Conclusions

This report demonstrates that cyano-propyl columns in reversed-phase mode combined with dual electrochemical detectors operating in series configuration offer the selectivity, specificity, and sensitivity required for low nanogram concentration simultaneous quantitation of codeine and morphine with their internal standards nalonephine and ethylmorphine, respectively. This procedure has sufficient sensitivity to be utilized in a bioavailability or pharmacokinetic study without employing expensive equipment. It can also be utilized as an intermediate assay between screening tests and the more elaborate gas chromatography/mass spectrometry (GC/MS) procedures so as to protect the GC/MS laboratory from being overwhelmed with confirmatory tests.

Acknowledgments

The assistance of Asha Modak, Lance R. Hall, Tracey Totem, Michael E. Wallace, and Ginny Wolfe is greatly appreciated. This investigation was sponsored by the Veterans Administration and A. H. Robins Company Research Laboratories, Inc., Richmond, VA.

References


26. Wright, J. A., Base