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Note

Simultaneous extraction and chromatographic analysis of morphine, dilaudid, naltrexone and naloxone in biological fluids by high-performance liquid chromatography with electrochemical detection

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High-performance liquid chromatography with electrochemical detection (HPLC–ED) is the method of choice in the analysis of biogenic amines and their metabolites in biological samples [1,2]. In addition to these compounds, HPLC–ED has also been used to measure other biologically important molecules including steroids [3], peptides [4], amino acids [5] and other compounds of pharmacological interest [6]. Efforts have also been made to evaluate the disposition and metabolism of opiate agonists and antagonists using HPLC–ED [7–10]. However, many published accounts describe the need for multiple extraction and complex chromatographic systems to ensure reproducibility and suitable resolution [9]. A simple modification of an existing extraction procedure for morphine [12] is now reported that not only provides improved recovery of morphine from biological fluids but also excellent recoveries of dilaudid, naloxone and naltrexone. The possibility of evaluating opiates in unextracted cerebrospinal fluid (CSF) is also examined. Additionally, an HPLC–ED method has been developed that permits separation and quantitation of these four opiates in a single HPLC run of 15 min.

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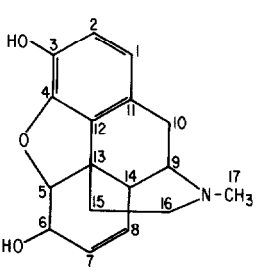

	6*	17*
Morphine	-OH	-H
Dilaudid	=O	-H
Naloxone	=O	-C=CH$_2$ (H)
Naltrexone	=O	-C(CH$_2$)(H)CH$_2$

Fig. 1. Structural representation of the opiate compounds employed in this study.

EXPERIMENTAL

Materials

Morphine, dilaudid, naloxone and naltrexone were obtained from Endolabs (Garden City, NY, U.S.A.). The structures of these compounds are represented in Fig. 1. Stock solutions in molar concentration were prepared for each substance in 0.1 *M* perchloric acid containing 0.01 *M* cysteine and 0.01 *M* EDTA. They were stored at −20°C and diluted when needed in the same solvent mixture. Chloroform, 1-propanol, 2-propanol, ammonium dihydrogenphosphate, orthophosphoric acid and sodium chloride were all analytical-grade chemicals from Fisher Scientific (Pittsburgh, PA, U.S.A.). L-Cysteine was from Sigma (St. Louis, MO, U.S.A.). Octanesulfonic acid (sodium salt) and disodium EDTA were products of Aldrich (Milwaukee, WI, U.S.A.).

Chromatographic separation and quantitation

A number of solvent systems were examined for their capacity to resolve a mixture of morphine, dilaudid, naloxone and naltrexone under isocratic conditions at room temperature (approximately 20°C) on 100 mm×4.6 mm I.D. columns packed with octadecyl-silane (ODS-silica, Ultrex, 3 μm particle size). The column was purchased from Phenomenex (Palos Verdes, CA, U.S.A.). All of the solvent systems contained ammonium dihydrogenphosphate, octanesulfonic acid, $5.4 \cdot 10^{-4}$ *M* disodium EDTA and an alcohol. The concentration of phosphate was varied from 0.05 to 0.2 *M* and the concentration of octanesulfonic acid from $0.9 \cdot 10^{-3}$ to $8.3 \cdot 10^{-3}$ *M*. The solvent systems also differed in the type and concentration of alcohol used. Methanol or ethanol were used at a concentration of 0.65 *M*. 1-Propanol, when employed, was at concentrations that varied from 0.33 to 0.98 *M*. Orthophosphoric acid was used to obtain a range of solvent pH from 3.0 to 5.0. All solvents were filtered using a 0.45-μm polysulfone filter (Gelman, Ann Arbor, MI, U.S.A.) and degassed by a combination of sonication and helium sparging. This procedure ensured a low background noise during analyses.

The HPLC system comprised a Model 396 Milton-Roy mini-pump with

Kenametal pistons (Laboratory Data Control, Ivyland, PA, U.S.A.) and a liquid/sample module preassembled by Rainin (Woburn, MA, U.S.A.). The liquid/sample module consisted of a coil pulse-damper, a Model 7125 injector valve, a prime-purge valve and a dampening-pressure gauge. The system was plumbed with stainless-steel tubing and fingertight fittings (Upchurch Scientific, Oak Harbor, WA, U.S.A.).

The column was connected by a high-pressure PTFE tubing (3 cm × 0.5 mm I.D., Rainin) to a thin-layer electrochemical cell (TL4) fitted with an RE-3 reference electrode and a 3 mil (1 mil = $2.54 \cdot 10^{-4}$ cm) PTFE gasket (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The electrochemical cell was connected to a potentiostat modified from a design by Gerhardt and Adams [13] that converted current signal to voltage and amplified this signal. Voltage output from the detector was displayed and recorded on a two-channel Kipp and Zonen strip-chart recorder (LKB Biotechnology, Piscataway, NJ, U.S.A.). Peak responses were measured by peak height for direct conversion to nanoamperes. Standard curves of peak height in nA versus concentration in ng/ml were constructed for each of the test compounds. Capacity factors were measured from elution time of the injection artifact (t_0) to the point of maximum peak height of the analyte (t_R) and used to identify sample components. Capacity factors were used to estimate the capacity constant (k') for each substance using the formula $k' = (t_R - t_0)/t_0$ [15].

Several factors influence the selection of the applied potential of the electrochemical cell. Obviously a maximum response is desired for each of the compounds of interest in any given analysis. However, a loss of sensitivity may be encountered if compounds polymerize at high potentials. This loss of sensitivity was found to be particularly significant with the analysis of opiates and required an optimum potential setting based on transducer stability and compound response [12,16]. An optimal potential was determined for our transducer by running hydrodynamic voltammograms of standard mixtures containing all four of the opiate compounds (50 ng/ml). The working potential of the cell was increased in 0.05-V increments from +0.5 V to +1.05 V measured against the Ag/AgCl reference electrode (RE-3), and the responses were recorded in each case. A potential of +0.85 V was selected because in addition to a sensitive response it provided a reproducibility that was not found at higher potentials. In the course of this experiment we also found that running the electrode for 30 min at −0.5 V would restore sensitivity and reduce the downtime compared to polishing the electrode, an alternative procedure routinely used to restore sensitivity.

Extraction of opiates from biological sources

An extraction procedure for isolating opiates from tissues and plasma was developed. This procedure was modified from one originally described for morphine [14] by employing smaller volumes and documenting the recovery of

dilaudid, naloxone and naltrexone using standards prepared in buffer, urine and plasma at concentrations of 100 ng/ml. A 100-μl aliquot of the test mixture was placed in a 1.5-ml Eppendorf tube, and 100 μl of 0.1 *M* ammonium dihydrogenphosphate solution, pH 8.6 were added. The tube was capped and briefly mixed. A 500-μl volume of chloroform–2-propanol (9:1, v/v) was added, and the phases were agitated for 30 s. A brief centrifugation (11 000 *g*, 10 min, room temperature) separated the two phases. The lower organic phase contained the opiates. The upper aqueous phase was aspirated, and 400 μl of the organic phase were carefully transferred to a clean Eppendorf tube. The contents of this tube were evaporated to dryness under a stream of nitrogen at room temperature (20–30 min) and resuspended in the solvent used for column separation prior to chromatographic analysis. With plasma and urine, a proteinaceous suspension formed at the interface of the two phases and care was needed to avoid contamination of the sample when removing the material from the organic phase. A volume of 50 μl of the resuspended sample in solvent was used to load a 20-μl sample loop. Our biological samples were urine and plasma samples from male Long-Evans rats (265–340 g) given subcutaneous implants of naloxone pellets (5 mg per day for fifteen days; Innovative Research of America, Gaithersburg, MD, U.S.A.). The urine and plasma samples were acidified by the addition of one tenth final volume of 1.0 *M* perchloric acid containing 0.1 *M* cysteine and 0.1 *M* EDTA. Several acidified urine samples and reference standard preparations of opiates in 0.1 *M* perchloric acid were allowed to undergo hydrolysis at 20°C for 16 h to evaluate stability and in the case of urine samples to liberate opiates from their conjugates. An evaluation was also made of opiate levels in samples of CSF obtained from patients receiving intraspinal administration of morphine. This involved chromatographic analysis performed on unextracted CSF using the methods described here. Coombs et al. [17] have already published an evaluation of the same samples, but these authors employed a liquid–solid phase extraction and a different chromatographic procedure. Their report also provides clinical, experimental and post-mortem data for the study. Values obtained for the samples using our methods were compared with those obtained by Coombs et al. [17].

All data were examined by analysis of variance, one-way with multiple comparisons for extraction using buffer, urine and plasma as the independent variable and two-way for comparison of medium and hydrolysis as independent variables. The dependent variables were the nanoampere responses for the individual opiates. Standard regression analysis was used to develop and examine the linearity of the electrochemical responses to varying concentrations of each of the opiates. Comparison between CSF values obtained by the different techniques was made using correlational analysis (Pearson's Product-moment). Both statistical procedures were available through a commercial software package (Crunch, Interactive Software, San Francisco, CA, U.S.A.). Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Chromatographic solvents comprising 0.1 *M* ammonium dihydrogenphosphate and $0.9 \cdot 10^{-3}$ *M* octanesulfonic acid at a pH of 4.5 were found to provide optimal conditions with respect to those variables for the separation of the opiates. For these salt and pH conditions, the effects of alcohol chain-length at a fixed concentration of 0.65 *M* are shown in Fig. 2. For all of the compounds examined there was an inverse relationship between capacity factor and the number of carbons in the alcohol. Although a similar resolution could be achieved by using differing concentrations of ethanol, methanol or 1-propanol in the solvent mixture, much lower concentrations of 1-propanol were required to provide a suitable separation. The employment of 10% (v/v) 1-propanol in the chromatographic solvent allowed us to reduce run times to 15 min. A chromatogram representing a typical separation of the opiates with this 1-propanol buffer system is shown in Fig. 3. The electrochemical characteristics found in this study for morphine and related opiates (Fig. 4) agree favorably with those already reported [11]. Morphine was seen to be the most electrochemically labile, a property that is likely attributable to the two oxidisable hydroxyl groups present in its structure (Fig. 1), while the other compounds are less electrochemically active because they have substitutions at these positions. The column capacity factors for these opiates and for other related opioid compounds appeared to be principally related to the group present at position 17 (Fig. 1). The addition of carbons as substitutions on the amine group at this position increased the capacity factor. The initially selected potential of +0.95 V vs. Ag/AgCl in our study was found to provide a high sensitivity, but under these conditions the response of the electrode decreased rapidly, often after as few as ten to twelve samples had been injected. On the other hand, a potential of

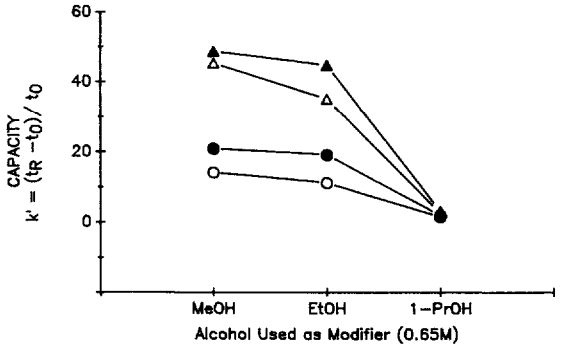

Fig. 2. Capacity factors for morphine (○), dilaudid (●), naloxone (△) and naltrexone (▲) in mobile phase containing either methanol, ethanol or 1-propanol as the organic modifier (all alcohols are at a concentration of 0.65 *M*). The buffer consisted of 0.1 *M* $NH_4H_2PO_4$ and 0.02% (w/v) octane sodium sulfate, at pH 4.5. Flow-rate, 1.0 ml/min.

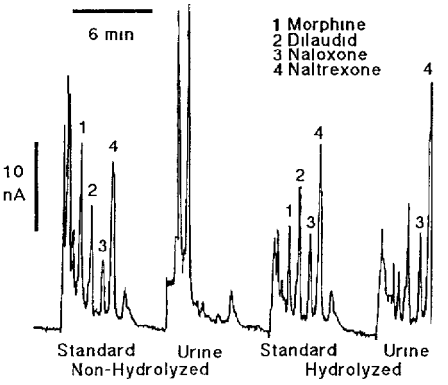

Fig. 3. Chromatograms of standard preparations and urine samples before and after acid hydrolysis for 16 h at 20°C. Chromatographic conditions: same as for Fig. 2 but mobile phase is made 10% (v/v) in 1-propanol.

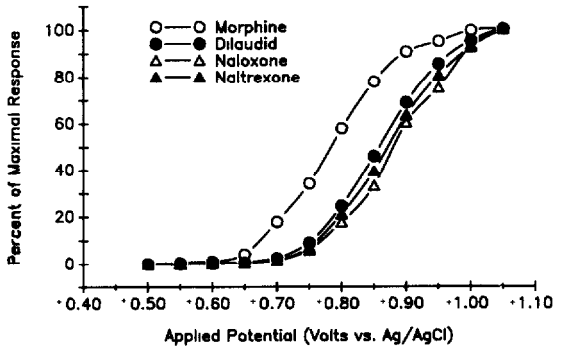

Fig. 4. Hydrodynamic voltammogram of the opiates (50 ng/ml each) in saline. Three injections of the mixture were made at each voltage setting. Chromatographic conditions: same as for Fig. 3.

+0.85 V gave 50–90% of the sensitivity of the higher voltage and reproducible responses through at least three times the number of analyses. When the sensitivity of the detector decreased it could be reestablished simply by running the transducer at −0.5 V. The relationships between amount of opiate and nanoamperes generated at +0.85 V demonstrated the linearity of our system ($r > 0.99$ for all four compounds) over a thousand-fold range of concentration for each opiate. These relationships have slopes of 0.045, 0.013, 0.008 and 0.010 ng/ml·nA for morphine, dilaudid, naloxone and naltrexone, respectively, with corresponding intercepts of 0.323, −0.069, 0.011 and −0.007.

The recoveries of opiates from saline and plasma extracted with chloroform–2-propanol are shown in Fig. 5. Extraction efficiency for the reduced volume used was greater than 88%, with recoveries highest for naloxone and naltrexone. Similar results were obtained for opiate recoveries from urine and CSF

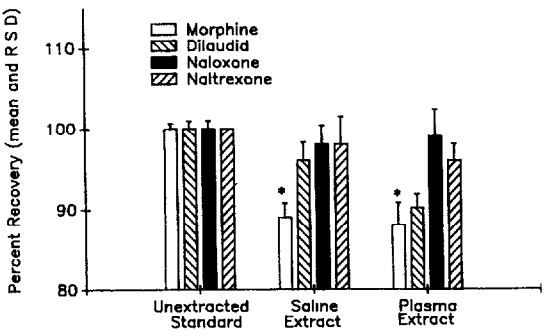

Fig. 5. Recoveries of the opiates (50 ng/ml) prepared in saline or plasma, compared to unextracted standard.

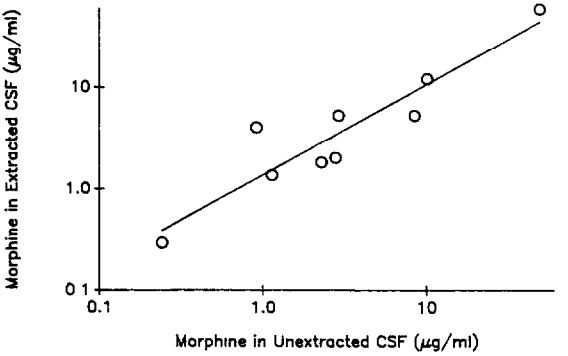

Fig. 6. Correlation of morphine values in extracted versus unextracted CSF samples from patients; $n=9$, $r=0.935$, $p<0.001$.

extracted with chloroform–2-propanol. Of particular significance was the finding that these compounds could be suitably measured by our HPLC–ED method in unextracted CSF. In fact, our determinations for morphine using unextracted CSF from cancer patients who received central infusion of this substance as part of their treatment demonstrated a strong correlation with those determinations published by Coombs et al. [17] who evaluated column extracts from the same CSF samples (Fig. 6). Paired t-test analysis revealed that there was no significant difference between these determinations ($r=0.981$, $p=0.3204$, $F=8$, $t=1.0591$). Additionally, hydrolysis of urine samples at 20°C successfully increased the peak of non-conjugated naloxone in urine samples (Fig. 3), while this procedure did not measurably influence the stability of reference standards of opiates. It confirms that appreciable amounts of administered opiates become conjugated as previously noted [11,15] and that they can be readily liberated to allow quantitation by acid hydrolysis.

Our results, therefore, indicate that the methods described can be readily

used to estimate the levels of opiates in biological fluids of patients being treated with these compounds or to screen subjects for possible abuse.

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