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Note

Improved method for the simultaneous determination of morphine, codeine and dihydrocodeine in blood by high-performance liquid chromatography with electrochemical detection

K. R. BEDFORD* and P. C. WHITE*
The Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP (U.K.)
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The determination of morphine and other opiates in small samples of blood is a problem commonly encountered by forensic toxicologists. Radioimmunoassay (RIA) can be used for screening samples and quantitation of morphine is possible but the assay is not specific due to cross-reaction with the other opiates. A high-performance liquid chromatographic (HPLC) method utilising electrochemical detection of morphine was developed in this Laboratory several years ago[1]. For accurate quantitation of the opiates complete chromatographic separation is required, but unfortunately this system did not permit resolution between codeine and morphine or dihydrocodeine and the internal standard dextrorphan. Discrimination between the former pair of compounds can be achieved by comparing detector responses at applied potentials of +0.6 V and +0.8 V, but this method is time consuming and requires additional amounts of sample.

To overcome these problems a chromatographic system has been developed to produce complete separation of these compounds. Additional improvements to the original method, which have removed negative peaks and reduced co-extractive levels, are also reported. This modified system also permits the separation of a number of other drugs of toxicological interest.

EXPERIMENTAL

Reagents

Acetonitrile and methanol, both HPLC grade, were obtained from Fisons (Loughborough, U.K.), perchloric acid (60%) from May and Baker (Dagenham, U.K.) and β-glucuronidase Type H5 from Sigma (Poole, U.K.).

The pH 5.0 acetate buffer was prepared by dissolving sodium acetate (5.74 g) and glacial acetic acid (1.74 ml) in distilled water (500 ml). The pH 8.9 borate buffer was prepared by mixing 0.05 *M* borax (70 ml) with 0.2 *M* boric acid (30 ml).

All glassware was silanized with a 5% (v/v) solution of dichlorodimethylsilane in toluene.

* Present address: Chemistry Division, DSIR, P.O. Box 2224, Auckland, New Zealand.

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HPLC conditions

The analyses were performed on a 25 cm × 4.9 mm I.D. stainless-steel column packed with Spherisorb S5W silica (5 μm: Phase Separations, Queensferry, U.K.). To prevent dissolution of the silica in the analytical column a guard column containing 40-μm silica was placed between the pump (Model 400; Applied Chromatography Systems, Macclesfield, U.K.) and the sample injection valve that was fitted with a 20-μl sample loop (Model 7125; Rheodyne, CA, U.S.A.).

The eluent consisted of a mixture of 3 parts of a solution of perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide solution (1.0 *M*), and 7 parts of acetonitrile–methanol (9:1). The pH was measured with a pH meter (Model 6000; Jenway, Essex, U.K.) that had been calibrated against aqueous buffer solutions. Analyses were performed at a flow-rate of 1.5 ml min^{-1}.

The details of the electrochemical cell and associated electronics were similar to those described previously[1]. The glassy carbon working electrode (V25 grade; Le Carbone, Portslade, U.K.) was maintained at an applied potential of +1.1 V *versus* a silver–silver chloride reference electrode.

Extraction procedure

The extraction procedure was similar to that described by White[1]. Blood samples (500 μl) were dispensed into 8-ml screw-topped test-tubes, and the internal standard (50 μl; 5 μg ml^{-1} dextrorphan tartrate in water), acetate buffer (1 ml; 0.1 *M* pH 5.0), and β-glucuronidase (3.8 mg; Type H5) were added. After mixing, four drops of chloroform were added to activate the enzyme, and the mixture was incubated overnight at 37°C. The hydrolysed blood was made basic with borate buffer (1.5 ml; 0.8 *M*, pH 8.9), saturated with sodium chloride and then extracted with ethyl acetate–isopropanol (9:1, 2 × 4 ml) for 10 min on a rotary mixer. After evaporation to a small volume the extract was transferred to an agglutination tube and evaporated to dryness. Prior to analysis the residue was dissolved in methanol (100 μl). For calibration standards blank blood samples were spiked with an aqueous solution (5 μg ml^{-1}) of the opiate to be determined, and then treated as described above. To determine free (unconjugated) as opposed to total (conjugated) opiate levels the addition of the enzyme, and the incubation period are omitted from the procedure.

RESULTS AND DISCUSSION

Optimisation of chromatographic separation

The chromatographic method developed originally in this Laboratory for the detection of morphine in blood was performed on a silica column with methanol–aqueous ammonium nitrate eluents, but failed to separate morphine from codeine and dihydrocodeine from the internal standard dextrorphan[1]. It was considered that greater selectivity could be achieved with this system by the addition of acetonitrile to increase the solvent strength of the eluent. Solvents based upon ammonium nitrate buffer–organic mixtures (10:90) in which the organic component consisted of varying proportions of methanol and acetonitrile were investigated. The buffer was prepared by adjusting the pH of a 0.1 *M* solution of ammonium nitrate to pH 9.5 with ammonia (sp. gr. 0.88). As shown in Fig. 1 separations were obtained by the addition of acetonitrile, and an increase in acetonitrile content increased the separation between morphine and codeine, and dextrorphan and dihydrocodeine.

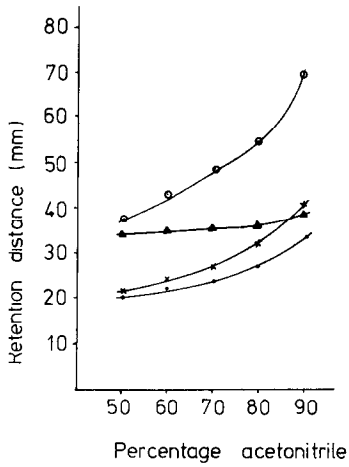

Fig. 1. Effect of changing the percentage of acetonitrile in the organic portion of the eluent on opiate retention. Eluent: 10% ammonium nitrate (0.1 *M*) adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (X : 100 − X). ● = codeine, × = morphine, △ = dextrorphan and ○ = dihydrocodeine.

To achieve optimal conditions, changes in the aqueous content, pH, and ionic strength were studied using eluents containing methanol–acetonitrile (20:80). For an increase in aqueous content from 10 to 20%, retention of all compounds was reduced by 75%, and a level of 10% was considered to be the most favourable in terms of peak shape and overall analysis time.

The effects of changing ammonium nitrate concentration and pH are shown

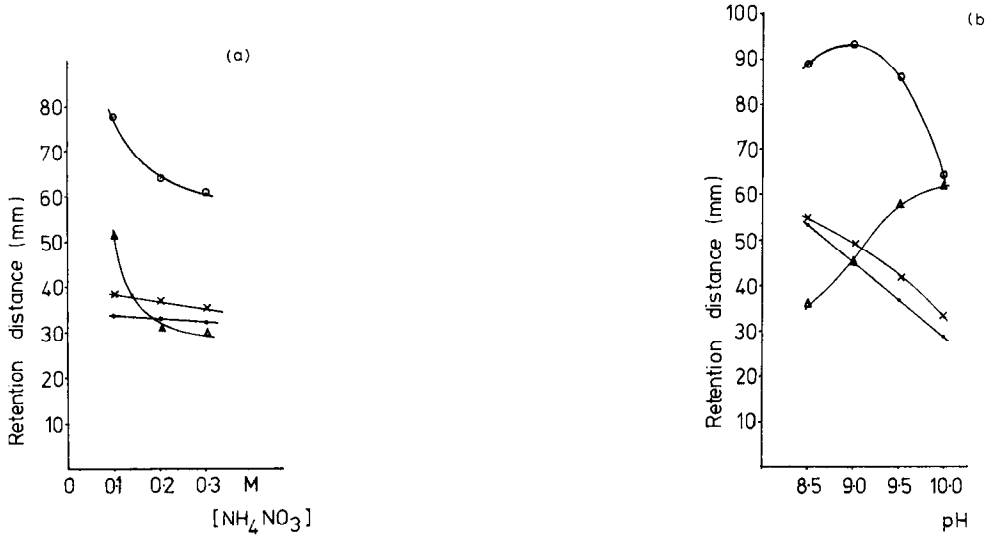

Fig. 2. (a) Effect of changing ammonium nitrate concentration on opiate retention. Eluent: 10% ammonium nitrate adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). (b) Effect of changing pH on opiate retention. Eluent: 10% ammonium nitrate (0.1 *M*) pH varied by addition of ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). ● = codeine, × = morphine, △ = dextrorphan, and ○ = dihydrocodeine.

in Figs. 2a and 2b, respectively. An ammonium nitrate concentration of 0.1 *M* was considered to be most favourable as it produced the best separation between components and still permitted dextrorphan to be used as the internal standard. From the pH study it was evident that optimum separation was produced when the aqueous portion of the eluent was adjusted to pH 9.5.

These studies indicated that the eluent with the following composition was the most appropriate, *viz.* 10% ammonium nitrate (0.1 *M*) adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). An excellent separation of a standard containing these compounds was observed under these conditions, but when a blank blood sample was analysed, a negative peak with a retention time similar to that of morphine was detected. Following further studies it was established that when a UV detector was used to monitor the separations, no negative peaks were observed. These results indicate that with the eluent–electrochemical system described, the negative peak effect is similar to that generated and used in indirect photometric detection[2].

When the aqueous portion of the eluent was prepared from either sodium acetate or nitrate solutions buffered with sodium hydroxide, no negative peaks were observed in chromatograms of blood extracts. However, with these eluents a further problem of variable retention of the opiates was observed, and this was attributed to their poor buffering capacity. The phenomena of negative peaking and variable retention time data were finally overcome by buffering the eluent with an aqueous solution of perchloric acid and sodium hydroxide.

To provide optimum resolution of the opiates with the perchloric acid mobile phase some modifications to the eluent described earlier were required, and the final conditions were; a mixture of 3 parts perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide (1.0 *M*) and 7 parts of acetonitrile–methanol (9:1). A chro-

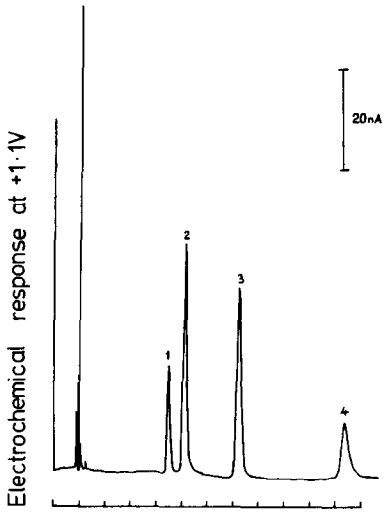

Fig. 3. Illustration of the separation of a mixed opiate standard using the finalised eluent conditions; 30% of a solution of perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide (1.0 *M*) and 70% acetonitrile–methanol (90:10). 1 = Codeine, 2 = morphine, 3 = dextrorphan and 4 = dihydrocodeine. Scale graduations represent 2-min intervals and all other conditions are as described in the text.

matogram showing the separation of a standard containing the opiates analysed with the eluent is shown in Fig. 3.

With this eluent reproducible retention data were obtained from freshly prepared columns after pumping eluent for a period of 24 h. Thereafter columns performed with no loss in performance on systems that were run continually for one month. Retention data for the opiates and some possible interfering compounds obtained with this chromatographic method are given in Table I.

TABLE I

RETENTION TIME DATA FOR OPIATES AND POSSIBLE INTERFERENCES

NR = Not retained.

Compound	*Relative retention time**	*Compound*	*Relative retention time**
Buprenorphine	NR	Codeine	0.88
Dextromoramide	NR	Morphine	1.00
Diphenoxylate	NR	Dextromethorphan	1.34
Nalorphine	0.31	Dextrorphan	1.42
Dextropropoxyphene	0.33	Normorphine	1.58
Pentazocine	0.37	Pholcodeine	1.63
Methadone	0.48	Dihydrocodeine	2.22
Acetylcodeine	0.48	Dihydromorphine	2.49
6-Monoacetylmorphine	0.54		

* Relative to morphine (retention time = 10 min).

Detection conditions

The background current, and hence sensitivity of any electrochemical detector is very dependent on the eluent used and the potential applied to the working electrode. With the introduction of acetonitrile to the eluent it was found that large variations in background currents were observed with various grades produced by several suppliers. At an applied potential of +1.1 V background currents varied from greater than 1000 nA for reagent grade down to 85–95 nA for the UV grade. The HPLC grade supplied by Fisons, which is not recommended for low-wavelength UV detection, produced a level of 110 nA. Based upon these values and the relative costs, the latter material was found to be the most acceptable.

To determine the optimum applied potential for the low-level detection of codeine, morphine, dihydrocodeine and dextrorphan these compounds were analysed over a range of potentials from +0.6 to +1.3 V, and the detector response and eluent background currents were compared. An applied potential of +1.1 V was considered to offer the best sensitivity, and it was also observed that for the same concentration of each of the opiates very similar responses were obtained. Under these conditions detection limits based upon the amount of material injected were, 250 pg for morphine and codeine and 500 pg for dihydrocodeine (signal-to-noise ratio = 3).

Analysis of blood samples

Hydrolysed and unhydrolysed blood samples were analysed for opiate content following the extraction method that was originally developed in this Laboratory, except that the acid back-extraction was omitted. Experimental results have shown that when using the new eluent, there were no advantages to be gained by including this stage in the procedure.

The type of β-glucuronidase enzyme preparation used to hydrolyse opiate conjugates was found to influence the final chromatogram. Several of these preparations produced compounds which eluted under the described chromatographic conditions, and in some instances they produced a strong electrochemical response. A comparative study of six enzyme preparations was conducted, and based upon hydrolysis efficiency and extract purity, it was found that the Type H5 preparation gave the most satisfactory results. A comparison of chromatograms of the same blood sample containing a very high level of morphine (free = 2.0 μg ml^{-1}, total = 5.6 μg ml^{-1}) hydrolysed with Type H2 and H5 β-glucuronidases is shown in Fig. 4.

An example of the use of the system to detect opiate mixtures in a single chromatographic run is shown in Fig. 5. This hydrolysed blood sample was found to contain a high therapeutic level of codeine (290 ng ml^{-1}) and morphine (95 ng ml^{-1}), and the morphine level in this sample was attributed to the *in vivo* metabolism by the person who has been prescribed codeine.

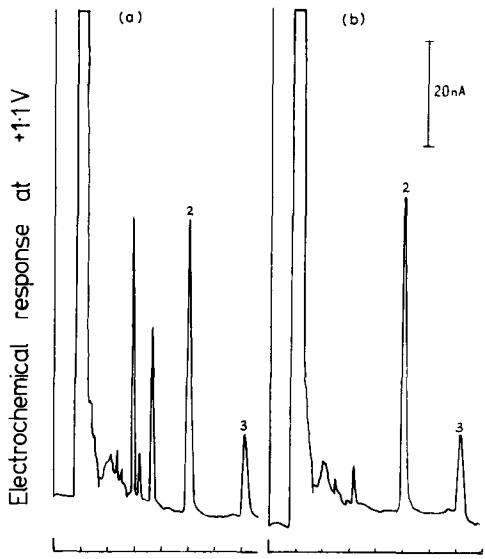

Fig. 4. A casework blood sample containing morphine hydrolysed with (a) Sigma Type H2 β-glucuronidase and (b) Sigma Type H5 β-glucuronidase. Eluent conditions as described in Fig. 3 and scale graduations represent 2-min intervals.

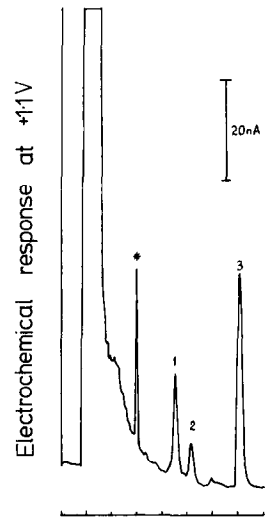

Fig. 5. A casework blood sample containing codeine and morphine after hydrolysis with the Type H5 β-glucuronidase. Eluent conditions as described in Fig. 3 and scale graduations represent 2-min intervals. 1 = Codeine, 2 = morphine, 3 = dextrorphan and * = unidentified.

CONCLUSIONS

The described chromatographic method permits excellent separation and quantitation of the opiates commonly encountered in blood samples. Through the judicious choice of HPLC reagents and β-glucuronidase employed, negative peaks are not detected and interference problems can be minimised, thereby increasing the sensitivity and selectivity of the technique.

REFERENCES

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