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DETERMINATION OF MORPHINE AND ITS MAJOR METABOLITE, MORPHINE-3-GLUCURONIDE, IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

The construction of an electrochemical detector flow cell for high-performance liquid chromatography from stainless steel, which also serves as the auxiliary electrode, is described. Glassy carbon is used for the working electrode in a novel configuration based on a hybrid of the wall-jet and thin-layer arrangements.

Application of the detector to the determination of morphine in small blood samples is described. The metabolite, morphine-3-glucuronide, is also determined by hydrolysis with β-glucuronidase prior to extraction. The detector is capable of detecting less than 1 ng of morphine injected.

INTRODUCTION

The determination of therapeutic levels of morphine in small blood samples is a problem commonly encountered by forensic toxicologists. Therapeutic levels of morphine range from a few nanograms to about 1 μg ml^{-1}, and for example in the case of blood taken from impaired drivers only 2 to 3 ml of blood are usually provided for analysis. Samples received in this laboratory are first analysed for alcohol and if absent, or only present at low levels, are then analysed for a wide range of drugs, and any positive results confirmed by another technique. Consequently methods used must be very sensitive and specific. Radioimmunoassay (RIA) is used by us to screen samples for a number of drugs, including morphine[1]. Since the morphine antibody also cross-reacts with the major morphine metabolite, morphine-3-glucuronide, the RIA result gives an estimate of the "total" morphine concentration. However, cross-reaction also occurs with other laevorotatory morphinans which may lead to the occurrence of false positives, especially in the case of codeine which is a widely used analgesic. The only suitable methods for the confirmation of morphine are, (a) gas–liquid chromatography of a derivative with detection by mass spectrometry[2] or electron capture[3], and (b) high-performance liquid chromatography (HPLC) of the dimer, pseudomorphine, with fluorescence detection[4]. Both of these methods detect the morphine indirectly and are either lacking in sensitivity or convenience of analysis.

The use of HPLC with electrochemical detection proved to be a suitable alternative. The sensitivity of this method of detection for phenolic compounds has been demonstrated by Kissinger *et al.*[5], who designed and built a detector, with a carbon paste electrode, capable of detecting picogram quantities of catecholamines. Other workers[6–9], using glassy carbon for the electrode, have built flow cells more suitable for use with non-aqueous eluents. The electrochemical detector described in this paper is capable of detecting less than 1 ng of morphine and was used for the determination of morphine and its major metabolite, morphine-3-glucuronide, in blood. The detector cost less than £ 100 to build and can be used in conjunction with a conventional HPLC system. The method is very sensitive, specific and simple, and has been in routine use in this laboratory for several months.

EXPERIMENTAL

Cell design

The two most common designs of electrochemical detector flow cells are the thin-layer[5] and wall-jet[7] configurations. Several prototype cells based on these designs were built and tested but were either lacking in sensitivity, difficult to construct or unreliable in operation. The use of acrylic plastics and epoxy resin glues for cell construction is undesirable since these materials are attacked by methanolic eluents. Polytetrafluoroethene (PTFE) is too soft to be machined and the harder fluorocarbon-based polymers are very expensive. For the working electrode glassy carbon is generally accepted as being most suitable since it is extremely hard, impermeable and inert. Auxiliary electrodes are usually of platinum or glassy carbon. However, large pieces of platinum are expensive and glassy carbon is difficult to drill and requires special tools.

At the potentials used for electrochemical oxidations it is possible to use stainless steel for the auxiliary electrode. The problems of cell construction therefore were solved by using stainless steel for both the cell body and the auxiliary electrode. The arrangement shown in Fig. 1 was chosen since it made the best use of the available electrode area, was simple to construct and proved to be very sensitive and reliable in operation. Its design is in effect a hybrid of the thin-layer and wall-jet configurations. The eluent impinges on to the centre of the glassy carbon working electrode and then flows radially outwards as a thin film between the auxiliary and working electrodes. The position of the reference electrode is not critical because of the proximity of the working electrode to the auxiliary electrode. With the cell dimensions used the cell volume is only 10 μl which is comparable with commercial UV detectors. However, the dead volume in the concentric waste chamber, downstream of the working electrode, is much larger, and therefore when using the detector in series with other detectors it is best placed last in the line to avoid excessive band spreading.

Cell construction

The cell body is made from 25.4 mm (1 in.) diameter stainless-steel rod (free cutting; grade unknown), drilled and tapped to take a 6.2 mm (1/4 in.) diameter stainless steel rod through the centre and secured at one end by means of a 1/4 in. compression fitting (Swagelok) whilst the other end is ground flush with the cell body and then polished with a fine silicon carbide abrasive paper (600 grit). This end of the

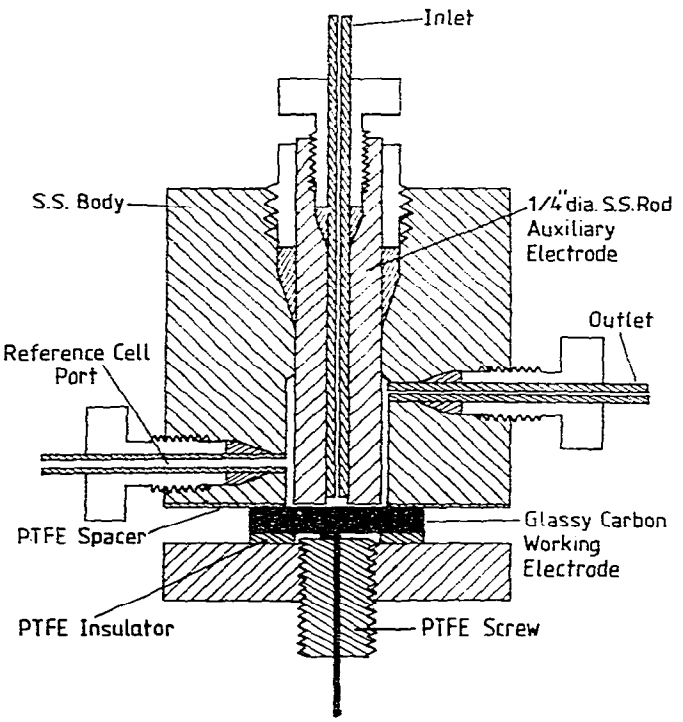

Fig. 1. Electrochemical detector flow cell. The four bolts for holding the glassy carbon electrode in place are not shown. All ferrules were of PTFE.

cell body is drilled out to a diameter of 7.1 mm (9/32 in.) and a depth of about 10 mm to provide a concentric space around the inner rod for the eluent waste. The inlet, outlet and reference ports are drilled and tapped to take 1/16 in. O.D. PTFE tubing with compression fittings (Waters Assoc.). The working electrode is a piece of glassy carbon measuring 12 × 12 × 2 mm (Le Carbone UK Ltd.; grade V10) which is ground flat and polished to a mirror finish with a diamond paste. It is separated from the cell body by means of a 254 μm (0.01 in.) thick PTFE gasket and is held in place by means of a 4.5 mm thick aluminium disc clamped to the cell body by four 6 BA bolts (not shown). A second PTFE gasket on the back of the glassy carbon acts as an insulator and prevents damage to the electrode. Electrical contact is made by a platinum wire inside a PTFE screw.

The reference electrode (Fig. 2) is a silver–silver chloride electrode (SSCE) and consists of a silver wire in a 3 *M* sodium chloride solution saturated with silver chloride by the addition of a drop of 10% silver nitrate solution. The reference cell is made from 10 mm diameter Perspex (I.C.I. Plastics) rod drilled and tapped to take a 1/16 in. compression fitting at one end and an 0 BA threaded Perspex electrode holder at the other end. Connection to the main cell is made via a piece of porous PTFE trapped in one end of a 50 mm length of 1/16 in. O.D. wide bore (0.032 in. I.D.) PTFE tubing.

Electrostatic interference is eliminated by housing the flow cell and reference compartment in an aluminium die-cast box which is connected to earth. The inlet and

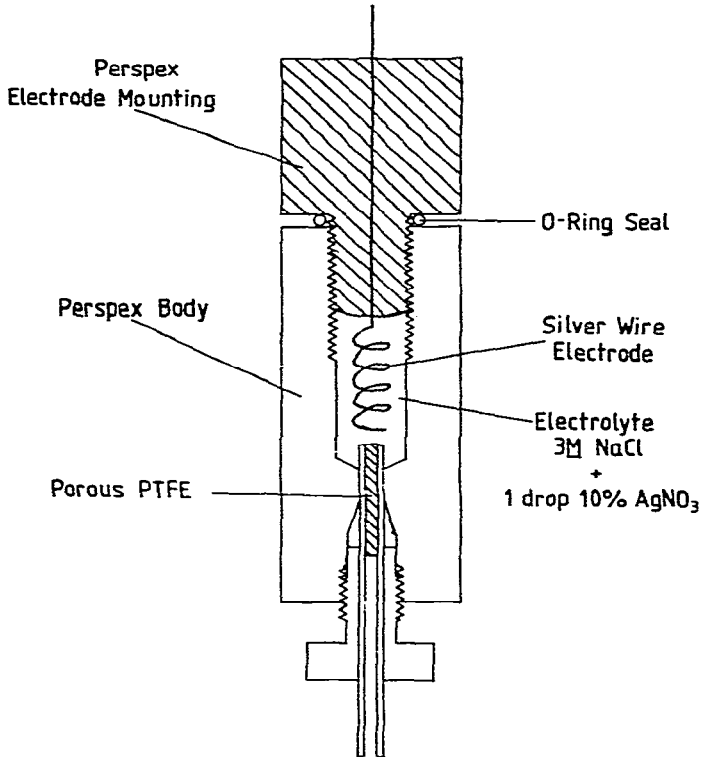

Fig. 2. Silver–silver chloride reference electrode connected to the reference cell port of the flow cell by a 50 mm length of 0.032 in. I.D., 1/16 in. O.D., PTFE tubing.

outlet tubes are also earthed by the insertion of low dead volume straight couplings which are then connected to the screening box at the points of entry and departure.

Electrode equilibration

One problem encountered with glassy carbon electrodes is the slow rate at which the background current reaches a steady state[10]. This is due to the existence of various surface oxide groups which undergo electrochemical reactions. Since these solid state surface reactions are slow, it takes a long time to reach redox equilibrium. However, for low sensitivity operation this is not a problem as most of the background current decays within about 0.5 h. At maximum sensitivity several hours equilibration may be required before a steady background current is reached. One way of speeding up this process is to set the electrode potential initially some 200 mV higher than the required operating potential. After about 1.5 h, when the background current is falling relatively slowly, the electrode potential is reduced to the operating potential. A short period (approx. 15 min) of re-equilibration then follows after which the background current is reasonably stable, even at high sensitivity. For the detection of morphine the operating potential was +0.60 V *vs.* SSCE and the equilibration potential was +0.80 V *vs.* SSCE.

The inconvenience of electrode equilibration can if necessary be avoided if the detector, when not in use, is left switched on with a low flow rate of recycled eluent passing through the cell.

Electronics

The classification and description of circuits for electroanalytical applications have been described by Schwarz and Shain[11]. The circuit used was similar to those used by Kissinger *et al.*[5] and Lankelma and Poppe[8]. Additional filtering was provided by a second 0.47 μF capacitor placed between the auxiliary and reference electrodes, as close to the cell as possible. The maximum sensitivity was 50 nA V^{-1}, with additional amplification being provided by the recorder. Connections to the cell were made with screened leads and 5-pin DIN connectors. A Princeton Applied Research model 174A polarographic analyser (EG & G Brookdeal Electronics) was also used during the development of the detector.

Although the noise levels of the two instruments were similar (approximately 50 pA at +0.60 V *vs.* SSCE), ours, owing to its relatively simple design, was less stable and gave rise to some baseline drift as shown by the chromatograms in Figs. 4 and 5.

Chromatographic system

The column used was of stainless steel, 20 cm × 4.6 mm I.D. (1/4 in. O.D.), slurry packed with silica (Syloid 74, W.R. Grace, London, Great Britain; fractionated by sedimentation to give a nominal particle size of 7 μm in diameter). The top of the column was fitted with a stainless-steel frit and septum injector. The pump was a Waters Assoc. model M-6000, complete with both high and low pressure pulse dampers. A Cecil Instruments CE212 variable wavelength UV detector was used in series with the electrochemical detector. Chromatograms were recorded on Servoscribe 1S flat bed recorders (Smiths Industries). The eluent was methanol–pH 10.2 ammonium nitrate buffer (9:1) with a flow-rate of 1 ml min^{-1} at 1050 p.s.i. A stock solution of buffer was made from 1.25 *M* ammonia and 0.25 *M* nitric acid (2:1). Degassing of the eluent to remove dissolved oxygen was performed immediately before use.

Glassware

All glassware was silanised using a 5% solution of dichlorodimethylsilane in toluene.

Extractions

After all extractions the phases were separated by centrifugation and the organic layer removed with a Pasteur pipette. Evaporations were performed on a water-bath at 60° under a stream of nitrogen.

Reagents

HPLC grade methanol, double distilled water and "Aristar" grade chemicals were used for the eluent. Ethyl acetate, isopropanol and ether were redistilled. The β-glucuronidase solution was a crude solution from *Helix pomatia* (Type H-2 from Sigma, London, Great Britain). All other chemicals were normal reagent grade.

Extraction procedure

To 0.5 ml of blood in an 8-ml screw-topped test-tube was added 20 μl of a solution of internal standard (10 ng ml^{-1} dextrorphan tartrate in water), 1 ml of

0.1 *M* acetate buffer (pH 5.0), and 20 μl β-glucuronidase solution. After mixing, 4 drops of chloroform were added to retard bacterial action and activate the enzyme. The mixture was then incubated at 37° overnight (18 h). The hydrolysed blood was basified with 1.5 ml of 0.8 *M* borate buffer (pH 8.9), saturated with sodium chloride (approximately 1.5 g) and then extracted twice with 4 ml of ethyl acetate–isopropanol (9:1) for 10 min on a rotary mixer. The combined extracts were transferred to a 10-ml tapered centrifuge tube and evaporated to a small volume (approximately 0.5 ml). The concentrated extract was transferred to an agglutination tube and evaporated to dryness. The residue was redissolved in 50 μl of 0.1 *M* hydrochloric acid, saturated with sodium chloride, and then washed twice with 0.5 ml of diethyl ether using a vortex mixer. The ether washings were discarded. The residual aqueous solution was basified with 100 μl of 0.8 *M* borate buffer (pH 8.9), resaturated if necessary with sodium chloride, and then extracted twice with 0.5 ml of ethyl acetate–isopropanol (9:1) using a vortex mixer. The combined extracts were transferred to another agglutination tube and evaporated to dryness. The residue was redissolved in 20 μl of 0.02 *M* hydrochloric acid and a portion injected on to the column. The efficiences of the extraction procedure for morphine and dextrorphan were approximately 81% and 46% respectively.

Out of date transfusion blood was used as a blank and one spiked with 100 ng ml^{-1} of morphine was also analysed. If several samples with widely differing morphine levels were being analysed, several spiked bloods with different morphine concentrations were run and the amount of internal standard added to each adjusted proportionately.

If sufficient sample was available the unconjugated morphine was also determined by omission of the enzyme and incubation period. With very small samples the method can be incorporated into a general drug extraction procedure. After analysing for acidic and neutral drugs the blood is hydrolysed with β-glucuronidase and then extracted as described above. The morphine fraction can then also be used for the analysis of other basic drugs which may be suspected.

RESULTS AND DISCUSSION

Steady-state voltammetry of morphine

The current-voltage curve for morphine is shown in Fig. 3. Contributions from the background current and other residual currents which arise in scanning voltammetry are conveniently avoided by plotting peak height for morphine against electrode potential to obtain the voltammogram.

The first wave at $E_{\frac{1}{2}} = +0.44$ V *vs.* SSCE is probably due to a one electron oxidation of morphine followed by dimerisation of the free radical to pseudomorphine[12]. The second wave at $E_{\frac{1}{2}} = +0.70$ V *vs.* SSCE may be due to further oxidation of the pseudomorphine[12], or a two electron oxidation of morphine to an intermediate phenoxonium ion[13].

The choice of electrode potential for the detection of morphine depends on two factors. Firstly, although increasing the potential increases the response, the background current and consequently the noise level are also increased. Secondly, increasing the potential decreases the specificity of the detector. For morphine a value of +0.60 V *vs.* SSCE was found to be most suitable.

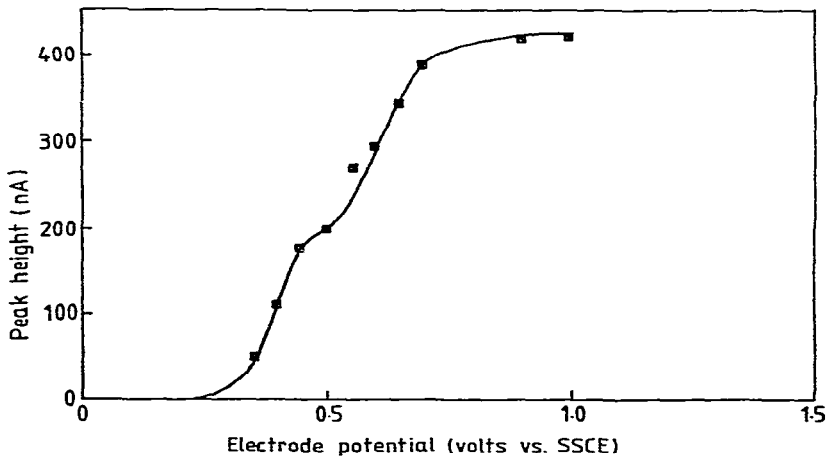

Fig. 3. Current–voltage curve for morphine (500 ng injections).

Linearity

The eluent used for the chromatography of morphine has a specific conductance of $8.0 \times 10^{-4}\ \Omega^{-1}\ cm^{-1}$, which with the cell dimensions used gives a calculated cell resistance of 107 Ω. Therefore even with currents as high as 1 μA the voltage drop (iR) is only about 0.1 mV. As a result of this very low iR drop the detector has a very wide linear range. Plots of peak height against amount of morphine injected gave good straight lines over the range 0.5 ng to 500 ng.

Non-linearity in electrochemical detectors is caused mainly by an increase in the iR drop when a compound is detected. In a two electrode cell this iR drop decreases the potential of the working electrode and therefore decreases the detector response by an amount depending on the slope of the current–voltage curve for that compound at the electrode potential used. There are two approaches to detector design which can be used to minimise this iR drop: (i) Incorporation of a reference electrode as close as possible to the working electrode. Although this method is frequently used by electrochemists it is not easily applied to low volume flow cells and can lead to contamination of the working electrode by the reference cell electrolyte. (ii) Use of a thin-layer cell where the auxiliary electrode is placed close to the working electrode.

The latter arrangement was chosen as it simplified the construction and enabled low cell volumes to be obtained. A reference electrode was also incorporated so that the working electrode could be set at any desired potential and, since the iR drop is negligible its position was not critical.

Reliability and maintenance

The flow cell has now been in use for several months without any deterioration of performance. This may be due to the high methanol concentration in the eluent preventing deactivation of the electrode by adsorption. Deactivation, however, can occur if the polarity of the cell is inadvertantly reversed. If this is the case the stainless steel becomes corroded and a deposit of iron is formed on the glassy carbon. The cell is easily restored to its original condition by dismantling and repolishing the glassy carbon and stainless steel surfaces.

The porous PTFE junction is not ideal for separating the eluent from the reference electrolyte and some leakage does occur. The resultant mixing of eluent with reference electrolyte appears to cause degassing to occur in the reference cell which can give rise to sporadic fluctuations of the electrode potential and consequently of the background current. This problem can be avoided in two ways: (i) At the beginning of each day the PTFE junction is purged with eluent to remove any bubbles and then the reference electrolyte is renewed, or (ii) for routine use at a constant electrode potential the reference electrode is eliminated. This can be simply done by short circuiting the reference electrode terminal to that of the auxiliary electrode. In this mode of operation the value set on the electrode potential control will be the actual voltage applied to the cell. If a particular electrode potential is required then the corresponding cell voltage must first be measured using a very high impedance millivoltmeter and with the reference electrode in normal operation.

The use of a continuous flow injection port and pulse free pump is desirable for operation at maximum sensitivity since the detector is sensitive to variations in the flow-rate. A septum injector was used since this was inexpensive and already available; however, it is not ideal since septa debris accumulates on top of the stainless-steel frit and has to be periodically removed.

Specificity

Blood samples from persons not suspected of having taken morphine (or heroin) have been analysed and no peaks having the same retention time as morphine were observed. Relative retention data for a large number of drugs were measured. Phenolic compounds (Table I) are most susceptible to electrochemical oxidation and therefore have similar detection limits to morphine. Indoles and phenothiazines (Table II) are oxidised less readily and consequently have a lower detector response relative to morphine. In general they also have high extinction coefficients in the ultraviolet spectrum and are therefore easily distinguished from simple phenolic compounds by using a UV detector in series with the electrochemical detector. Codeine and other compounds with much higher redox potentials (Table III) give no significant response at $+0.60$ V *vs.* SSCE and therefore are only detected at very high concentrations or at higher electrode potentials.

The only compounds found to have similar or the same retention times as morphine are cyclazocine, etilefrine, profadol, methyldesorphine, and normetanephrine. Although the latter is one of the metabolites of adrenaline it was not detected in control blood samples. None of the other possible interferences are at present prescribed in Great Britain and are unlikely to be encountered illicitly. If the method is used in conjuction with RIA then methyldesorphine is the only known compound likely to interfere with both methods. Further discrimination can if necessary be achieved by running a second chromatogram using a different eluent pH which will change the relative retention time of morphine.

Although many other coextractives, *e.g.* butylated hydroxytoluene and 2-thiobenzothiazole (originating from sample containers), are detected electrochemically only those containing a basic nitrogen atom are retained under the chromatographic conditions used. Furthermore the extraction procedure used preferentially extracts only basic and some amphoteric compounds.

TABLE I

RETENTION OF NITROGEN-CONTAINING PHENOLIC DRUGS RELATIVE TO DEXTRORPHAN (15.0 ml)

Conditions are as described in text.

Compound	*Relative retention volume*	*Compound*	*Relative retention volume*
Brocresine	0.21	Hydroxyamphetamine	0.56
Isoxuprine	0.22	Hordenine	0.60
Etorphine	0.23	Bufotenine	0.65
Naloxone	0.23	Phenylephrine	0.67
Diprenorphine	0.24	Tyramine	0.68
Levophenacylmorphan	0.24	Oxedrine	0.74
Phenomorphan	0.24	Desomorphine	0.75
Buphenine	0.26	Methyldihydromorphine	0.75
Hydroxypethidine	0.33	*p*-Hydroxyephedrine	0.77
Pentazocine	0.33	Serotonin	0.78
Bamethan	0.34	Phentolamine	0.79
Oxymorphone	0.37	Metopon	0.81
Metaraminol	0.39	Hydromorphone	0.84
6-Monoacetylmorphine	0.40	Dihydromorphine	0.88
Ketobemidone	0.41	Metanephrine	0.90
Cephaeline	0.42	Pholedrine	0.95
Cyclazocine	0.48	Metazocine	0.98
Etilefrine	0.50	Levorphanol	1.00
Profadol	0.50	Dextrorphan	1.00
Methyldesorphine	0.51	Normorphine	1.06
Morphine	0.51	Oxymetazoline	1.60
Normetanephrine	0.51	Norlevorphanol	1.84
Psilocin	0.55		

TABLE II

RETENTION OF INDOLE AND PHENOTHIAZINE TYPE DRUGS RELATIVE TO DEXTRORPHAN (15.0 ml)

Conditions are as described in text.

Compound	*Relative retention volume*
Thiopropazate	0.23
LSD (lysergide)	0.24
Perphenazine	0.26
Dimethothiazine	0.28
Methotrimeprazine	0.31
Trimeprazine	0.32
Promethazine	0.34
Chlorpromazine	0.35
Prochlorpromazine	0.36
Thioridazine	0.43
Promazine	0.45
Dimethyltryptamine	0.62
Diethyltryptamine	0.68
Tryptamine	0.74

TABLE III

EXAMPLES OF COMPOUNDS SHOWING NO SIGNIFICANT RESPONSE AT THE ELECTRODE POTENTIAL USED FOR MORPHINE

Conditions: electrode potential, +0.60 V *vs.* SSCE; sensitivity, 100 nA f.s.d.; injection, 500 ng.

Allopurinol	Fluphenazine
Amphetamine	Hydroxyquinoline
Caffeine	Mebhydrolin
Chlorprothixene	Methylamphetamine
Clopenthixol	Nicotine
Codeine	Pericyazine
Diamorphine (heroin)	Phenindamine
Dothiepin	Thiothixene
Flupenthixol	Trifluoperazine

Application

The method is used for the confirmation and quantitation of morphine in those blood samples which give positive results for the morphine RIA. Typical chromatograms are shown in Figs. 4 and 5. Peaks 3 and 4 are impurities in the enzyme

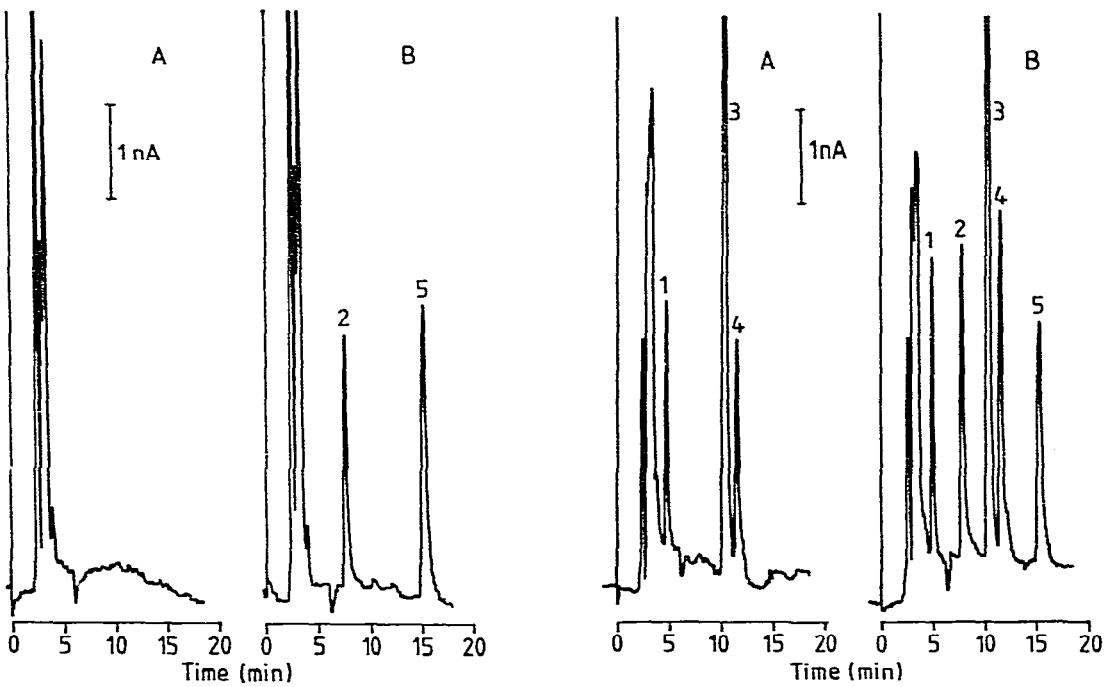

Fig. 4. Chromatograms of unhydrolysed blood. (a) Blank blood. (b) Blood spiked with 50 ng ml^{-1} morphine and 400 ng ml^{-1} dextrorphan tartrate internal standard. Conditions as described in text. Injection, 2.5 μl (1/8 of extract of 0.5 ml blood). Peaks: 2 = morphine; 5 = dextrorphan.

Fig. 5. Chromatograms of enzyme hydrolysed blood. (a) Blank blood. (b) Blood spiked with 100 ng ml^{-1} morphine-3-glucuronide (equivalent to 62 ng ml^{-1} morphine base) and 400 ng ml^{-1} dextrorphan tartrate internal standard. Conditions as described in text. Injection, 2.5 μl (1/8 of extract of 0.5 ml blood). Peaks: 1, 3, 4 = unknowns present in the crude enzyme solution; 2 = morphine; 5 = dextrorphan.

preparation and although not identified their retention parameters are indicative of tyramine and tryptamine, respectively. The cause of the negative response at approximately 6.2 min is unknown, but it appears to occur with all injections, including distilled water.

To estimate the concentration of morphine in a suspect's blood the peak height ratio of morphine to internal standard is compared with that obtained from a spiked blood containing the same concentration of internal standard and a known concentration of morphine. The results obtained have been in good agreement with those of RIA, except in one case where a result of about 30 ng ml^{-1} by RIA gave a negative result by the HPLC method. Since there was no suspicion of morphine having been taken, it is likely that the RIA result was due to cross-reaction with codeine. The possibility of confirming codeine by demethylation to morphine[14] is at present being investigated.

Measurement of the ratio of free to conjugated morphine provides useful toxicological data on metabolism, although large numbers of samples will have to be analysed before any significance can be attached to such data. Possible uses may include estimation of time of administration of the drug, time of death after administration, and degree of habituation.

The analysis of urine samples by this method is not at present feasible, since large quantities of interfering compounds are also extracted. The electrochemical detector should also be useful for the detection of traces of morphine in injection syringes and on other items associated with illicit drug use.

CONCLUSIONS

HPLC with electrochemical detection has proved to be a sensitive and reasonably specific method for the detection and quantitation of morphine in blood. The detector also has the advantage of being relatively inexpensive, simple to use and reliable in operation. The use of stainless steel for its construction and for the auxiliary electrode is a significant development in the design of electrochemical detectors.

The low cost, high sensitivity and unique range of specificity of electrochemical detectors is likely to lead to their application in many more areas of HPLC analysis.

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