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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETECTION OF MORPHINE BY FLUORESCENCE AFTER POST-COLUMN DERIVATISATION

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the fluorometric determination of morphine in biological samples has been developed. Column effluent is mixed with alkaline potassium ferricyanide to produce the fluorescent dimer pseudomorphine. The method provides higher chromatographic specificity and sensitivity than conventional high-performance liquid chromatography. Morphine can be detected at levels of 10 ng on column.

INTRODUCTION

Fluorescent derivatisation for enhanced detection and selectivity has been in use in chromatography for many years, and the availability of fluorescence detection systems for liquid chromatography systems has allowed the application of fluorescent derivatisation to that field. Derivatives may be formed before the sample is introduced (pre-column) or between the column outlet and the detector (post-column). Pre-column derivatisation is necessary if the reaction is slow, but post-column derivatisation is less likely to form artifacts, allows the simultaneous use of different detection systems, *e.g.* series ultraviolet (UV) detection and fluorescence detection, and does not alter the chromatographic properties of the compounds of interest. Post-column techniques have been extensively used for amino acid analyses[1,2] and several post-column methods have been developed for drug analyses[3–6].

Morphine (I, Table I) can be oxidized to the fluorescent dimer, pseudomorphine, XII, by alkaline potassium ferricyanide. This reaction has been extensively used for the detection of morphine in biological samples[7–9], and the reaction has been applied as a pre-column fluorescence derivatisation method for high-performance liquid chromatography (HPLC)[10]. This paper describes a post-column fluorescence derivatisation method suitable for the detection of morphine in biological samples. The technique is simple, involving the use of a second pump to deliver the derivatising reagent to a post-column mixing volume before passing the eluent through a filter fluorometer. It gives both improved sensitivity and selectivity over UV detection. The method is also compared to the pre-column derivatisation method for morphine described by Jane and Taylor[10].

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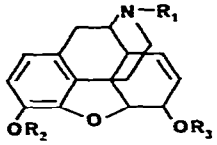
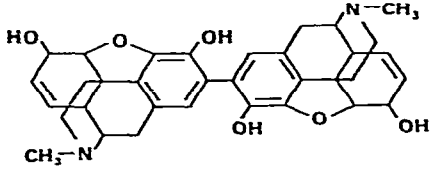
TABLE I

STRUCTURAL FORMULAE OF OPIATES INVESTIGATED FOR OXIDATION BY ALKALINE FERRICYANIDE



Opiate	R_1	R_2	R_3
I Morphine	CH_3	H	H
II Normorphine	H	H	H
III Nalorphine	CH_2–CH = CH_2	H	H
IV Morphine-3-glucuronide	CH_3	$C_6H_9O_6$	H
V Codeine	CH_3	CH_3	H
VI 6-Monoacetylmorphine	CH_3	H	$COCH_3$
VII Diacetylmorphine	CH_3	$COCH_3$	$COCH_3$
VIII Ethylmorphine	CH_3	CH_2CH_3	H
IX Acetylcodeine	CH_3	CH_3	$COCH_3$
X Dihydromorphine*	CH_3	H	H
XI Norcodeine	H	CH_3	H
XII Pseudomorphine			

* Double bond at C-7–C-8 is saturated.

METHODS

Samples and extraction procedures

Ante mortem and post mortem samples arising from forensic inquiries were examined. All extractions of samples were carried out in silanized glassware to avoid loss of drug by adsorption onto the glass surface. The following three extraction procedures were used.

Urine–free morphine. A 100-μl aliquot of a stock solution of nalorphine (0.24 mg/ml) was added to 5 ml of urine. The pH was adjusted to 9 with 2 *M* NaOH, and the urine was buffered with 1.5 ml of *M* K_2HPO_4 and saturated with NaCl (1 g). The urine was then extracted twice with 3 ml of chloroform–2-propanol (9:1) and the organic layer separated by centrifugation. The organic phase was recovered and evaporated to dryness. The residue was then taken up in 100 μl of HPLC eluent, and suitable aliquots were injected onto the HPLC column.

Urine–total morphine. A 100-μl aliquot of nalorphine stock solution was added to 1 ml of urine. To this was added 1 ml of 70% hydrochloric acid, and the solution was digested in a boiling water bath for 30 min. After cooling, the solution was adjusted to pH 9 with ammonia solution, then buffered and extracted as described for free morphine in urine.

Blood. Specimens of blood (10 ml) were digested with acid, filtered and made alkaline as described for total morphine in urine. The solution was then extracted with 50 ml of $CHCl_3$–propan-2-ol (9:1), and the organic phase was separated and back-extracted with 0.1 *M* H_2SO_4. The aqueous phase was separated, made alkaline with ammonia solution, and extracted with chloroform–2-propanol (9:1). The organic phase was separated and evaporated to dryness in preparation for HPLC analysis.

Chromatography

A Waters Model 6000A HPLC pump, Model U6K injector and 440 UV detector (254 nm) were used in normal HPLC configuration. A Metering Pumps Model HM reciprocating piston pump was used as the second pump to introduce the derivatising reagent. The fluorescent response was determined using an Amino Fluorocolorimeter set in the fluorescent mode with an 85-W mercury lamp, a Corning 7-54 excitation filter and a Wratten 2A emission filter and equipped with a 70-μl flow cell. A dual pen recorder was used to monitor the UV and the fluorescent response.

Three columns were used in this study: a 100 × 4.0 mm I.D. Partisil 10 ODS, a 100 × 4 mm I.D. Zorbax ODS (8 μm), and a 200 × 4.0 mm I.D. Kieselgel 60 HPLC (5 μm). The columns were slurry-packed with 2-propanol into glass-lined stainless-steel tubing with 6.35 mm ($\frac{1}{4}$ in.) to 1.56 mm ($\frac{1}{16}$ in.) stainless-steel Swagelok reducing unions as end-fittings, with the 6.35-mm end being drilled out to minimise dead volume. Sintered stainless-steel frits were used to retain the packing. These columns were all operated at ambient temperature. The mobile phase used for the pre-column derivatisation using the Kieselgel 60 column was methanol–2 *M* NH_4OH–1 *M* NH_4NO_3 (30:20:10). The flow-rate was 2.0 ml/min, and the analytical procedure used was as described by Jane and Taylor[10]. The mobile phase for the post-column der-

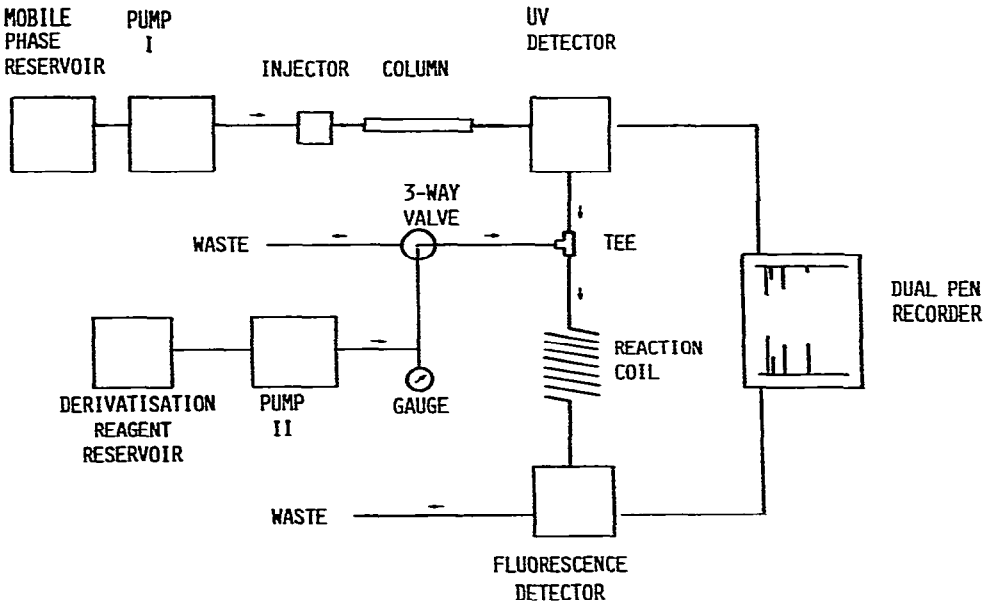


Fig. 1. HPLC system for morphine using post-column derivatisation and fluorescence detection.

ivatisation systems using the reversed-phase columns was methanol–0.1 *M* KBr (12.5:87.5) adjusted to pH 3 with phosphoric acid. The post-column derivatising reagent was 50 mg of $K_3Fe(CN)_6$ in 250 ml of 4 *M* NH_4OH.

The flow diagram for the HPLC post-column derivatisation system is shown in Fig. 1. The outlet from the UV detector was connected to the reagent outlet from the second pump and the reaction coil (5 m of 1.6 mm PTFE tubing of 0.3 mm I.D.) by a 1.6 mm Swagelok tee. The post-column reagent at a flow-rate of 0.4 ml/min, was mixed with mobile phase eluting at a flow-rate of 2.0 ml/min. The reaction coil was kept at room temperature, and its outlet connected to the flow cell of the fluorescence detector.

Quantitative work

The method described by Jane and Taylor[10] was used to determine morphine using pre-column derivatisation and adsorption chromatography, except that nalorphine was used as the internal standard rather than dihydromorphine. For the reversed-phase HPLC and post-column derivatisation, pure drug standards (Table I) were prepared using the mobile phase as solvent, and aliquots were injected onto the column to determine retention times and the fluorescent response after derivatisation.

The post-column reagent flow was optimised by maintaining a constant mobile phase flow-rate and varying the reagent flow-rate while monitoring the fluorescent response of standards. Calibration curves over the range from 10 ng to 2 μg (on column) were obtained for the standards.

The efficiency of the derivatisation was estimated from the calibration curves for morphine and pseudomorphine on the Zorbax ODS column. Quenching of the fluorescent response was estimated by comparing the calibration curves obtained for pseudomorphine with the $K_3Fe(CN)_6$ derivatising reagent, and with this reagent replaced by 4 *M* NH_4OH. The reliability of the whole procedure was determined by extracting morphine-free urine and whole blood samples to which known amounts of morphine and nalorphine had been added, and determining the drug levels. Apart from studies on the derivatisation efficiency and quenching, the Partisil 10 ODS column was used throughout.

RESULTS AND DISCUSSION

The structures of the opiates examined, and their chromatographic properties and fluorescent response determined using the post-column derivatisation technique, are listed in Tables I and II. The oxidation of morphine cogeners by alkaline potassium ferricyanide to form fluorescent pseudomorphine-like dimers is influenced by three structural features: the presence of a free hydroxy group at the O-3 position; the absence of a carbonyl group at the C-6 position; and the presence of the furan oxygen bridge[11]. Thus compounds substituted at the O-3 position (codeine, morphine-3-glucuronide, etc.) or lacking the furan oxygen bridge (pentazocine, levallorphan, etc.) do not form fluorescent derivatives. Optimum conditions for this oxidation in the HPLC system described were selected by varying the derivatising reagent flow-rate while maintaining a constant mobile phase flow-rate through the HPLC column. The results obtained for a number of opiates oxidized by ferricyanide are given in Table III, and the optimal reagent flow-rate was 0.4 ml/min. The five opiates listed in Table

TABLE II

RETENTION, RELATIVE TO MORPHINE, AND FLUORESCENT RESPONSE, RELATIVE TO PSEUDOMORPHINE, OF OPIATES AFTER HPLC SEPARATION ON PARTISIL 10 ODS

Opiate	*Relative retention time*	*Relative fluorescence*
Morphine-3-glucuronide	0.74	nil
Normorphine	0.80	89
Morphine	1.0	100
Dihydromorphine	1.10	81
Nalorphine	1.40	62
Norcodeine	1.48	nil
Codeine	1.83	nil
6-Monoacetylmorphine	2.23	7.5
Ethylmorphine	3.12	nil
Acetylcodeine	7.8	nil
Diacetylmorphine	8.7	nil

III all exhibit an excitation maximum of 323 ± 1 nm and an emission maximum of 432 ± 2 nm. The excitation filter, a Corning 7-54, and the emission filter, a Wratten 2A, are both cut-off filters and allow greater light transmission than band-pass filters, and thus gave greater sensitivity than band-pass filters on the fluorescence detector.

The chromatograms obtained for morphine and nalorphine using the Partisil 10 ODS column are shown in Fig. 2. Trace a was obtained after post-column derivatisation using fluorescent detection as described above, and trace b was obtained using UV detection at 254 nm.

The extent of the conversion of morphine into pseudomorphine in the reaction coil was estimated by comparing the calibration curves obtained for morphine and pseudomorphine using the Zorbax ODS column. On this column, pseudomorphine had a retention time relative to morphine of 1.2 compared with 3.9 on the Partisil 10 ODS column. Assuming that a small difference in the retention times of these com-

TABLE III

EFFECT OF POST-COLUMN REAGENT FLOW-RATE ON RELATIVE FLUORESCENT INTENSITY

*Compound**	*Normalised fluorescent intensity*			
	Reagent flow-rate (ml/min)			
	0.2	*0.4*	*0.6*	*0.8*
Normorphine	52	100	85	74
Morphine	74	100	80	64
Dihydromorphine	73	100	82	73
Nalorphine	76	100	83	62
6-Monoacetylmorphine	74	100	59	56
Pseudomorphine**	86	65	42	35

* All results determined on the Partisil 10 ODS column with an eluent flow-rate of 2 ml/min.

** Maximum response was observed when $K_3Fe(CN)_6$ was omitted from the reagent.

Fig. 2. HPLC separation of morphine (1) and nalorphine (2) on Partisil 10 ODS using (a) fluorescence detection and (b) UV detection under conditions described in the text.

pounds on the Zorbax ODS column should not significantly affect their fluorescent response, the comparison of calibration curves showed morphine to have 53% of the response of pseudomorphine and thus the derivatisation can be estimated as 50% complete. Quenching of the fluorescent signal by the derivatising reagent was also examined using the Zorbax ODS column by preparing calibration curves for pseudomorphine with ferricyanide present and omitted from the derivatising reagent. These curves showed a reduction of 40% in the fluorescent response when the ferricyanide was present (see also Table III).

The combined effect of incomplete derivatisation and quenching of the fluorescent response by the ferricyanide appeared to reduce the optimum response of the system by *ca.* 80%. A similar reduction in response was shown by comparing the on-column detection limit for morphine (10 ng) with that for pseudomorphine when $K_3Fe(CN)_6$ was omitted from the derivatising reagent (2 ng).

The results obtained from biological samples using the post-column method, described here, and the pre-column method, described by Jane and Taylor[10], are listed in Table IV. Agreement between the two methods is good, and using the post-column method replicate determinations of morphine in extracts obtained from a urine sample (3, Table IV) gave a standard deviation of 11% ($n = 9$). Replicate

TABLE IV

MORPHINE LEVELS DETECTED IN BIOLOGICAL SAMPLES BY DERIVATISATION HPLC

Sample	*Method**	*Morphine (μg/ml)*	
		Free	*Total*
1** Urine	A	30	148
	B	34	120
Blood	B	n.d.***	0.03
2 Urine	A	2.4	18
	B	2.0	12
3 Urine	A	14	37
	B	10	35
4 Blood	A	n.d.	0.09
	B	n.d.	0.04
5 Gastric lavage	A	n.d.	26

* A, post-column derivatisation; B, pre-column derivatisation.
** Samples from 1 were post mortem.
*** n.d. = Not determined.

determinations of the peak height ratio of morphine to nalorphine in extracts from a blood sample to which morphine and nalorphine had been added gave a standard deviation of 2.9% ($n = 17$). The recoveries of both morphine and nalorphine from urine were 80%.

Typical chromatograms obtained from the same urine extract are shown in Fig. 3. Trace a was obtained using UV detection; trace b using fluorescence detection without derivatisation and trace c using fluorescence detection with derivatisation. Peaks 1 and 2 in trace c correspond to morphine and nalorphine.

The compounds eluting later than morphine and nalorphine were not affected by stopping the flow of derivatising agents, indicating that they had a native fluorescence and were not related to morphine. No attempt was made to identify these peaks, and similar chromatograms were obtained from all the urine samples examined. Blood extracts did not contain any significant fluorescent coextractives. Morphine and nalorphine could not be detected in the urine extracts using UV detection because of interference caused by coextractives (trace a). The non-appearance of morphine peaks when $K_3Fe(CN)_6$ was omitted from the derivatising reagent was used as a confirmation of morphine in biological extracts.

The use of a solvent system incorporating halide ions is not generally recommended by HPLC pump manufacturers. However, such use is being increasingly reported[12–16] and corrosion problems can be avoided if adequate precautions are taken. Our procedures involved adequate flushing of the entire chromatographic system with distilled water after a run had been concluded. Solvents containing salts are not allowed to remain in the system unless there is a solvent flow. It is also recommended that the HPLC system, without the column, is passified regularly by pumping 200 ml of 50% HNO solution through it[17].

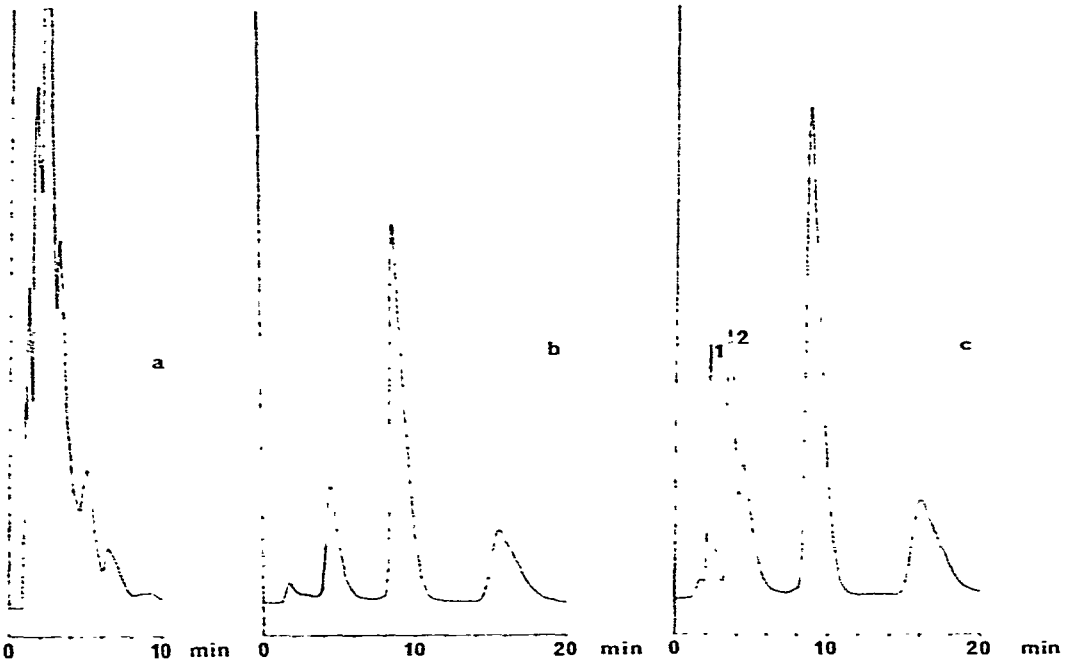


Fig. 3. HPLC of urine extract containing morphine and nalorphine (internal standard) on Partisil 10 ODS under conditions described in the text. (a) UV detection at 254 nm; (b) fluorescence detection, no derivatisation; (c) fluorescence detection with derivatisation. Peaks: 1 = morphine; 2 = nalorphine.

CONCLUSIONS

A reversed-phase HPLC system involving post-column derivatisation has been developed for the detection of morphine. The derivatisation to pseudomorphine followed by fluorescent detection gives the method both added sensitivity and specificity over UV detection, and it is suitable for the detection of morphine in post mortem fluids.

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