Journal of Chromatography, 330 (1985) 323–331
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 791

LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DANSYL DERIVATISATION AND FLUORIMETRIC DETECTION APPLIED TO THE ASSAY OF MORPHINE IN BIOLOGICAL SAMPLES*

F. TAGLIARO* and A. FRIGERIO
Istituto di Medicina Legale e delle Assicurazioni, Università di Verona, Policlinico, 37134 Verona (Italy)
R. DORIZZI
Istituto di Chimica e Microscopia Clinica, Università di Verona, 37134 Verona (Italy)
and
G. LUBLI and M. MARIGO
Istituto di Medicina Legale e delle Assicurazioni, Università di Verona, Policlinico, 37134 Verona (Italy)
(Received April 9th, 1985)

SUMMARY

A simple method employing pre-column dansylation and liquid chromatography is proposed for a very sensitive and specific assay of morphine in biological samples. Nalorphine is used as an internal standard. The detection limit is 0.2 picomol of injected morphine. In the assay of human sera spiked with 150 nmol/l, the intra- and inter-assay coefficients of variation were 3.7% (n = 10) and 4.5% (n = 10), respectively. No interferences were observed from more than 70 opiate and non-opiate drugs. Urine, plasma and total blood were assayed, using different extraction methods, with negligible interference from coextractives.

INTRODUCTION

In recent years, the determination of morphine in biological samples has become almost a routine assay in many toxicology laboratories owing to the spread of the abuse of heroin, which is mainly biotransformed into morphine.

Enzyme immunoassay and radioimmunoassay are the most commonly used techniques for the determination of morphine in biological fluids. These methods are very sensitive (3.5 nmol/l), simple and readily automated, thus allowing a large number of assays to be carried out per day**. However, immunoassays are impaired by

* A preliminary report of this work was presented at Biochemische Analytik 84, Munich, F.R.G., April 10–13, 1984; an abstract appeared in *Z. Anal. Chem.*, 317 (1984) 678.

** Coat-A-Count Kit Morphine, Diagnostic Products, Los Angeles, CA, U.S.A. (sensitivity 1 ng/ml) and Abuscreen for Morphine, Roche Diagnostics, Nutley, NJ, U.S.A. (sensitivity 10–40 ng/ml) were tested in our laboratory.

0021-9673/85/$03.30

specific (cross-reactions of antisera) and non-specific (pH, ion strength) interferences and therefore it is widely accepted that the results must be confirmed by chromatographic methods[1]. The most commonly used chromatographic technique, thin-layer chromatography (TLC), is simple, inexpensive and relatively rapid, but has low sensitivity (1.5–3 μmol/l) compared with immunological methods. Gas–liquid chromatography with electron-capture detection gives detection limits as low as those of immunoassays, but requires a high degree of technical expertise[2,3].

High-performance liquid chromatography (HPLC) has been proposed for the determination of several drugs in biological matrices, many HPLC methods employing normal- or reversed-phase separations and UV (at 210–220 nm), electrochemical or fluorescence detection have been reported[4–11]. Unfortunately, UV detectors do not achieve the sensitivity of immunoassays and, being very non-specific, are affected by interferences due to coextractives.

Fluorimetric detection of morphine, previously pre- or post-column derivatised to the fluorescent dimer pseudomorphine, has higher sensitivity and specificity, but the optimization of the operating conditions is hampered by the relative instability of the reaction product in the presence of the derivatising reagent $K_3Fe(CN)_6$[11]. Very high sensitivity (about 3.5 nmol/l) can be achieved using electrochemical detectors; however, so far they have not been widely used in toxicological laboratories. Moreover, they seem to suffer from poor reliability in routine work, as they are greatly influenced by the mobile phase composition and by contamination of the electrodes.

Dansyl derivatisation in TLC has been proposed[12–14] as a means of increasing the detectability of morphine and for the determination of some alkaloids, including morphine, in pharmaceuticals[15]. Here we propose dansylation for the determination of morphine in biological samples by HPLC with high specificity and sensitivity.

EXPERIMENTAL

Reagents

Dansyl chloride was obtained from Sigma (St. Louis, MO, U.S.A.). Standards of morphine and other opiates were provided by Carlo Erba (Milan, Italy). Standards of other drugs were obtained from Analytical Systems (Laguna Hills, CA, U.S.A.) in the kit Toxi-disc Library, stored dry in glass microfibre discs impregnated with silicic acid. HPLC-grade solvents for liquid chromatography (Carlo Erba) were used.

Commercially available ready-to-use tubes for alkaloid extraction, Toxi-tubes A, containing buffering salts (Na_2CO_3–$NaHCO_3$, 1:1) and a mixture of organic solvents (CH_2Cl_2–$C_2H_4Cl_2$, 1:1), were supplied by Analytical Systems.

Equipment

The liquid chromatographic apparatus consisted of a Gilson 302 pump (Gilson, Villiers-le-Bell, France), a Gilson 802 manometric module, a Rheodyne 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.), a Gilson Holocrome variable-wavelength UV detector and a Gilson 121 filter fluorimeter, fitted with a 9-μl cell. Filters were checked in order to obtain an excitation wavelength range of 330–380 nm and an emission wavelength range of 410–500 nm. A Spherisorb S3W column (150 × 4.5

mm I.D.) (Phase Separations, Queensferry, U.K.) packed with 3 μm silica was used. The liquid chromatograph was fitted with an HP 3390 A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Mass spectra were obtained with an HP 5987 A quadrupole double-source EI/CI mass spectrometer (Hewlett-Packard).

Methods

Extraction of morphine from biological fluids was carried out using two methods of different specificity and degree of difficulty. The first was a simple one-step procedure employing commercially available Toxi-tubes. Up to 5 ml of sample (urine) and a known amount of nalorphine (internal standard) were added directly to a Toxi-tube. After mixing for 3 min by inversion and centrifuging, the organic phase was transferred into a conical tube and evaporated to dryness under a stream of nitrogen.

A more specific extraction from biological samples, including hydrolysis, was obtained following a partial modification of Felby *et al.*'s procedure[16]. A 10-ml volume of sample (urine, serum, plasma or whole blood) was mixed with 10 ml of water containing a known amount of nalorphine (internal standard), 25 ml of saturated ammonium sulphate solution and 0.5 ml of 12 mol/l hydrochloric acid. Hydrolysis was carried out at 120°C for 30 min, then the mixture was filtered through Whatman No. 1 paper. The pH of the solution was adjusted to 9.0 with 25% (w/v) sodium hydroxide, then the alkaloids were extracted with 125 ml of chloroform–2-propanol (4:1, v/v). The aqueous phase was re-extracted with 50 ml of the same mixture. The pooled organic phases were washed twice with 15 ml of 0.05 mol/l sodium borate solution, then morphine was back-extracted twice with 10 ml of 1 mol/l sulphuric acid. The two acidic fractions were pooled, mixed with 4 ml of saturated ammonium sulphate, adjusted to pH 9.0 with 25% sodium hydroxide and extracted twice with 5 ml of chloroform–2-propanol (4:1, v/v). The pooled organic phases were evaporated to dryness under a stream of nitrogen.

Preparation of derivatives

The dried extracts were dissolved in 50 μl of distilled water and mixed with 100 μl of dansyl chloride in acetone (1:1000, w/v) and 50 μl of 0.2 mol/l sodium carbonate solution. The mixture was kept at room temperature with exclusion of light for at least 3 h; then it was extracted by vortexing for 2 min with 1 ml of toluene (we also tried other solvents as described below) and the organic phase was dried under a stream of nitrogen. The dried extract was redissolved in the chromatographic eluent and up to 20 μl were injected into the chromatograph.

The kinetics of the dansylation of morphine (1 μmol) and nalorphine (1 μmol) under the described conditions were studied, the percentage peak areas relative to the two alkaloids for different incubation times in the range of 15 min–12 h at room temperature being measured. The presence of significant amounts of unreacted morphine or nalorphine at equilibrium was evaluated after adjusting the pH of the incubation mixture to 9.0 with 0.1 mol/l hydrochloric acid, extracting it twice with 5 ml of chloroform–2-propanol (4:1, v/v), evaporating the organic phase to dryness and injecting the extract, previously dissolved in the elution mixture, into the chromatograph. Unreacted morphine and nalorphine, with much higher retention times than the dansyl derivatives, were detected with the UV detector at 214 nm.

Extraction of derivatives

To test alternative extraction solvents for the derivatized alkaloids, we dissolved known amounts of HPLC-purified dansyl-morphine and dansyl-nalorphine in a mixture similar to the derivatizing reaction medium (100 μl of acetone + 100 μl of water + 50 μl of 0.2 mol/l sodium carbonate solution), then extracted by vortexing for 2 min with 1 ml of either benzene, toluene or *n*-hexane. The organic phases were dried, then the extracts were assayed by HPLC. The peak areas were compared with those obtained with unextracted solutions.

Liquid chromatography

HPLC analysis of the derivatives was carried out in the normal-phase mode. The mobile phase was *n*-hexane–2-propanol–ammonia (97:2.7:0.3); the stationary phase was 3 μm silica packed in a 150 × 4.5 mm I.D. stainless-steel column. The flow-rate was 1.5 ml/min. Injection volumes were up to 20 μl. Results were calculated by internal standardization on the basis of peak areas.

RESULTS

Extraction methods

Urine samples spiked with known amounts of morphine and nalorphine were assayed by the first extraction method. Recoveries were calculated by comparing the peak areas of samples with those of standard solutions. Mean recoveries ($n = 5$) in the range 300 nmol/l–120 μmol/l were 43.9% (C.V. = 7.0%) for morphine and 63.8% (C.V. = 5.0%) for nalorphine.

Plasma and whole blood samples spiked with standard morphine and nalorphine were extracted using the second method and assayed as described above. Mean recoveries ($n = 5$) in the range 150 nmol/l–140 μmol/l were 72.8% (C.V. = 8.5%) for morphine and 80.1% (C.V. = 6.9%) for nalorphine.

Preparation of derivatives

The kinetics of dansylation at room temperature are shown in Table I. At equilibrium, the proportion of unreacted morphine and nalorphine was less than 10% for up to 70 nmol of total added alkaloids; larger amounts were not investigated.

TABLE I

KINETICS OF MORPHINE AND NALORPHINE DANSYLATION AT ROOM TEMPERATURE

Time	*Relative peak areas (%) (12 h = 100%)*	
	Morphine	*Nalorphine*
15 min	74.4	85.5
50 min	83.7	88.4
90 min	84.2	88.7
3 h	99.0	99.2
6 h	99.1	99.7
12 h	100.0	100.0

Extraction of alkaloid derivatives

A 1-nmol amount of dansyl-morphine or dansyl-nalorphine was extracted using three different solvents: benzene, toluene and *n*-hexane. The recoveries were, respectively, 88.5% (C.V. = 5.1%), 89.9% (C.V. = 4.9%) and 77.2% (C.V. = 5.8%) for dansyl-nalorphine (n = 5) and 92.5% (C.V. = 5.0%), 92.2% (C.V. = 4.5%) and 74.4% (C.V. = 6.0%) for dansyl-morphine (n = 5).

Stability of derivatives

The stored derivatives dried at 4°C with exclusion of light, remained stable for 15 days.

Mass spectrometry

Mass spectra of dansyl-morphine, extracted from the reaction mixture with toluene, were obtained using a direct injection probe with a temperature gradient from 60 to 260°C at 30°C/min. Either electron impact or chemical ionization (methane) was used (Fig. 1).

Only one hydroxy group reacted with dansyl chloride, as shown by the mass spectra. Moreover, we observed that codeine, substituted on the phenolic hydroxy group, cannot be derivatised. These data are in agreement with the hypothesis that only the morphine hydroxy group at C-3 reacts with dansyl chloride.

Liquid chromatography

Under the described conditions, dansyl-morphine and -nalorphine were eluted within 10 min, depending on the ammonia concentration, but good separations can be obtained with retention times of 2–3 min by using shorter columns. The efficiency of the chromatographic column used was about 9000 plates without a significant decrease in performance even after 200–300 assays.

The morphine/nalorphine (peak areas) response factor was 0.94 ± 0.05 (standard deviation). The calibration graph was linear from 3.3 nmol/l to 1 μmol/l of morphine in plasma. The detection limit, with a signal-to-noise ratio of 2, was 0.2 pmol of injected dansyl-morphine.

Interferences

Aqueous solutions of many opiate and non-opiate drug (10 mg/l) were extracted using Toxi-tubes, dansylated and chromatographed in order to establish any peaks not resolved from those of morphine or nalorphine. In Table II are listed more than 70 drugs that do not interfere in the HPLC determination of morphine.

Assay of biological samples

Ten urine samples spiked with 300 nmol/l of morphine and nalorphine and ten samples spiked with 10-fold higher amounts of alkaloids were extracted using Toxi-tubes, dansylated and assayed by HPLC. Within-run of C.V. values of 4.3% and 3.8%, respectively, were obtained for the two different concentrations. Ten urine samples containing 300 nmol/l of the two alkaloids were also assayed on different days, giving a C.V. of 5.9%.

Analogous tests performed by using the alternative more complex extraction procedure on plasma samples spiked with 150 nmol/l and 1.5 μmol/l of drugs gave

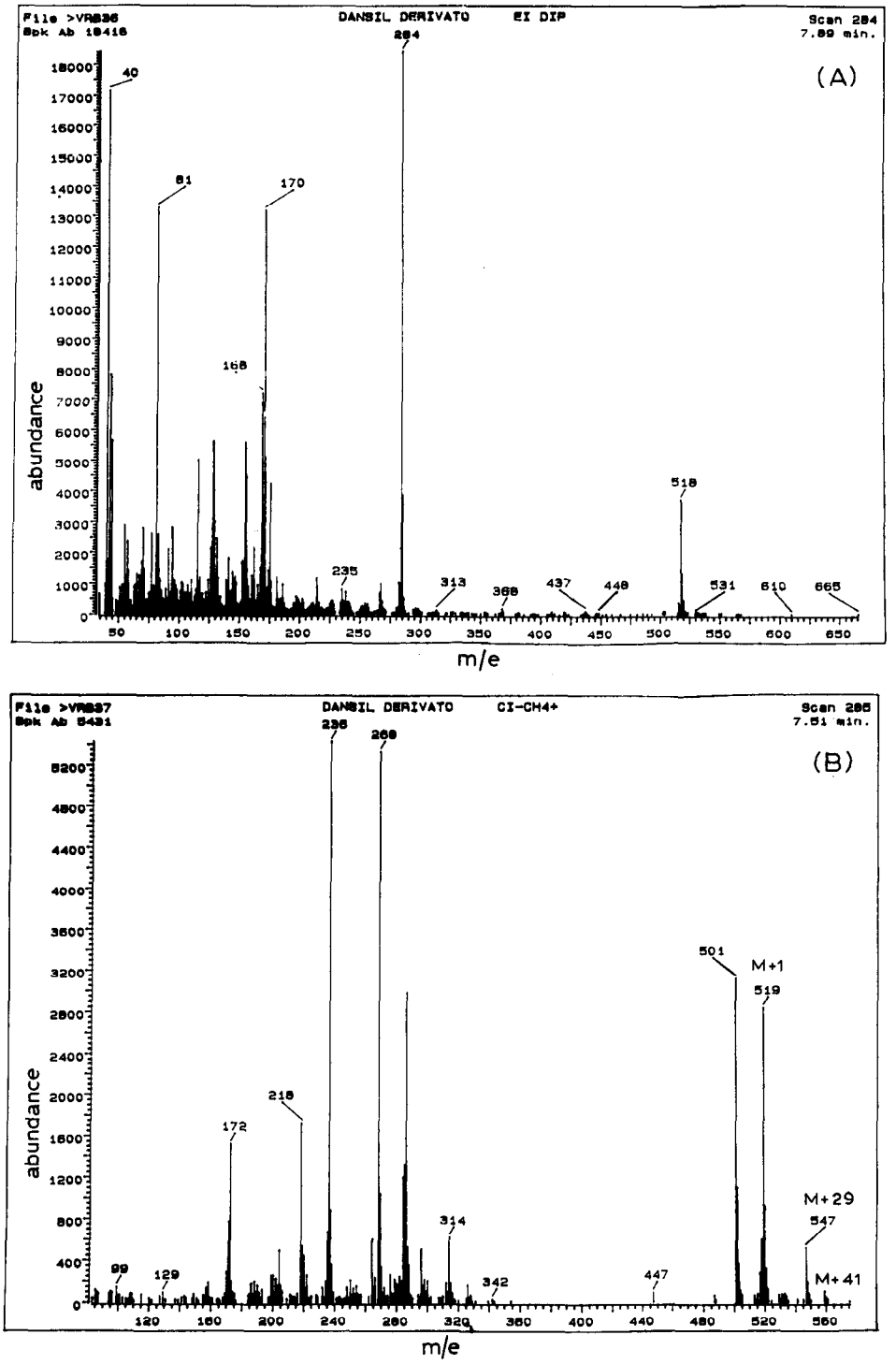

Fig. 1. Mass spectra of dansyl-morphine (*m/e* = 518), obtained either by (A) electron impact or (B) chemical ionisation (methane), demonstrating that only one hydroxy group of morphine reacts with dansyl chloride.

TABLE II

DRUGS INVESTIGATED IN ORDER TO EXCLUDE INTERFERENCES IN MORPHINE DETERMINATION

No interferences were observed using up to 10 mg/l of the drugs listed.

Type	*Drugs*			
Opiates and antagonists	Meperidine	Methadone	Hydrocodone	Terpin hydrate
	Oxicodone	Dextrometorphan	Propoxyphene	Dihydrocodeine
	Ethylmorpohine	Hydromorphone	Codeine	Diphenoxilate
	Papaverine	Naloxone		
Central nervous system active drugs	Amphetamine	Metamphetamine	Phenmetrazine	Phentermine
	Amitriptylene	Nortriptyline	Doxepin	Imipramine
	Protriptylene		Cocaine	
	Loxapine	Thioridazine	Thiothixene	Trifluperazine
	Triflupromazine	Chlorprothixene	Chlorpromazine	
	Flurazepam	Diazepam	Nordiazepam	Prazepam
	Carbamazepine	Diphenylhydantoin		Caffeine
	Phencyclidine		Strychnine	Methylphenidate
Miscellaneous	Lidocaine	Benzoilecgonine		Atropoine
	Trihexyphenidyl		Hydrocortisone	
	Methocarbamol	Carisoprodol	Orphenadrine	
	Procainamide	Disopyramide	Nicotine	
	Phenacetin	Acetaminophen		Cimetidine
	Pentazocine	Glutethimide		Propranolol
	Pyrilamine	Cyclizine	Hydroxyzine	Doxylamine
	Tripelennamide	Metapyrilene		Phenolphthalein
	Trimethoprim	Emetine	Quinine	Erythromycin
	Triamterene	Spironolactone		Trimetobenzamide

a within-run C.V. of 3.7% (n = 10) for the lower and 3.0% (n = 10) for the higher concentration. For a level of 150 nmol/l the between-day C.V. was 4.5% (n = 10).

Fig. 2 shows typical chromatograms of (A) blank whole blood and (B) whole blood spiked with 30 nmol/l of morphine, extracted using Felby *et al.*'s modified method[16].

DISCUSSION

It is well known that dansyl chloride reacts with primary amono and phenolic hydroxy groups. The morphine molecule contains a tertiary amino group, an alcoholic hydroxy group (on C-6) and a phenolic hydroxy group (on C-3). Only the last group reacts with dansyl chloride, according to Garrett and Gukan's[13] and our mass spectrometric data.

The results obtained here indicate that dansylation gives a highly fluorescent product that can be easily separated by HPLC and detected by simple and relatively inexpensive filter fluorimetric detectors with high sensitivity. A simple one-step extraction provides sufficiently rapid sample preparation for the assay of free morphine in urine (Fig. 3). Nevertheless, in our experience this method is not useful for assaying

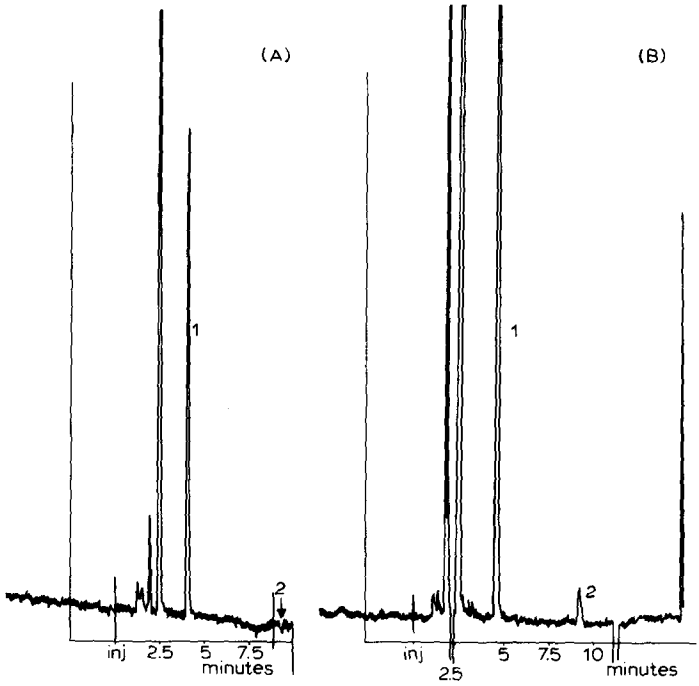

Fig. 2. Chromatograms of (A) blank whole blood and (B) whole blood spiked with 30 nmol/l of morphine extracted according to Felby *et al.*'s method[16]. Peaks: 1 = nalorphine; 2 = morphine.

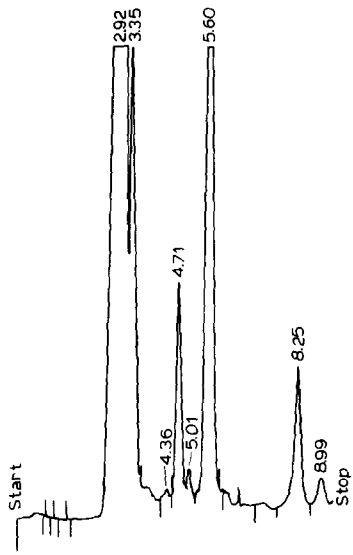

Fig. 3. Chromatogram of urine spiked with 300 nmol/l of morphine and nalorphine extracted using Toxitubes. Retention times: morphine, 4.7 min; nalorphine, 8.25 min.

hydrolysed samples because acid hydrolysis liberates reactive substances that give rise to many interfering peaks. Therefore, when hydrolysis is needed or when complex matrices, such as whole blood, are processed, a more specific and complex extraction such as Felby *et al.*'s[16] procedure must be used. Extracted morphine is easily dansylated and the product can be extracted from the reaction mixture with toluene. Toluene seems to be more suitable than benzene, used by other workers, owing to the comparable recoveries obtained and the toxicity of benzene. Dansyl-morphine and -nalorphine can be chromatographed by HPLC with simple isocratic elution. Because of the well known quenching of dansyl fluorescence caused by polar solvents, we checked the normal-phase mode, which allows a low-polarity eluent mixture such as *n*-hexane–2-propanol–ammonia (95:4.5:0.5) to be used. This eluent permitted good performances to be obtained with modern HPLC columns, with a reasonable column life, in spite of high pH of the eluent.

We tested more than 70 drugs without finding peaks that coelute with morphine and the internal standard. The inter-assay and the between-day reproducibility were comparable to those of other HPLC methods. The sensitivity was comparable to that obtained with electrochemical detectors and higher than that of methods employing derivatisation of morphine to pseudomorphine[10,11].

Hence the method proposed here could be a valid alternative to other HPLC methods employing electrochemical or fluorimetric detectors, and could result in a useful sensitive assay of morphine contained in complex matrices such as biological samples, thus allowing the results of the most sensitive radioimmunoassays to be validated.

ACKNOWLEDGEMENTS

We thank Dr. V. Raverdino and Dr. R. Bortolazzi (Hewlett-Packard) for measuring the mass spectra.

REFERENCES

1 F. Black, in J. F. B. Stuart (Editor), *Methods of Morphine Estimation in Biological Fluids and the Concept of Free Morphine*, Royal Society of Medicine, London, 1983, p. 29.
2 B. Dahlstrom and L. Paalzow, *J. Pharm. Pharmacol.*, 27 (1975) 172.
3 P. O. Edlund, *J. Chromatogr.*, 206 (1981) 109.
4 J. G. Umans, T. S. K. Chiu, R. A. Lipman, M. F. Schultz, S.-U. Shin and C. E. Inturrisi, *J. Chromatogr.*, 233 (1982) 213.
5 B. L. Posey and S. N. Kimble, *J. Anal. Toxicol.*, 7 (1983) 241.
6 J.-O. Svensson, A. Rane, J. Säwe and F. Sjöqvist, *J. Chromatogr.*, 230 (1982) 427.
7 J. E. Wallace, S. C. Harris and M. W. Peek, *Anal. Chem.*, 52 (1980) 1328.
8 J. A. Owen and D. S. Sitar, *J. Chromatogr.*, 276 (1983) 202.
9 R. D. Todd, S. M. Muldoon and R. L. Watson, *J. Chromatogr.*, 232 (1982) 101.
10 I. Jane and J. F. Taylor, *J. Chromatogr.*, 109 (1975) 37.
11 P. E. Nelson, S. L. Nolan and K. R. Bedford, *J. Chromatogr.*, 234 (1982) 407.
12 H. H. Loh, I. K. Ho, T. M. Cho and W. Lipscomb, *J. Chromatogr.*, 76 (1973) 505.
13 E. R. Garrett and T. Gurkan, *J. Pharm. Sci.*, 67 (1978) 1512.
14 R. Wintersteiger, *Analyst (London)*, 107 (1982) 459.
15 R. W. Frei, W. Santi and M. Thomas, *J. Chromatogr.*, 116 (1976) 365.
16 S. Felby, H. Christensen and A. Lund, *Forensic Sci.*, 3 (1974) 77.