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Determination of morphine and 6-acetylmorphine in plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A method is described for the simultaneous determination of morphine and 6-acetylmorphine in small volumes of human plasma by normal-phase high-performance liquid chromatography using solid-phase extraction, dansyl derivatisation and fluorescence detection. The lower limits of quantitation in a 0.1-ml plasma sample are 10 ng/ml for morphine and 25 ng/ml for 6-acetylmorphine. The method has been applied to determine concentrations of morphine and 6-acetylmorphine in plasma samples from premature babies administered an intravenous infusion of diamorphine.

INTRODUCTION

Morphine and 6-acetylmorphine are the main metabolites of diamorphine, a potent opioid analgesic used for the relief of acute and chronic pain in man. As part of a clinical study to evaluate diamorphine therapy in the neonate [1,2] it was necessary to develop a highly sensitive assay to measure both morphine and 6-acetylmorphine in small volumes (0.1 ml) of neonatal plasma. There are a variety of methods for the determination of morphine in plasma using the techniques of high-performance liquid chromatography (HPLC), gas chromatography and radioimmunoassay [3] but only two HPLC methods have been reported for the determination of 6-acetylmorphine [4,5], neither of which reached the level of specificity and sensitivity required. A fluorescence derivatisation procedure designed to measure low concentrations of morphine in biological samples by HPLC was described by Tagliaro *et al.* [6] and this method had the potential to be used with 6-acetylmorphine and to provide the desired sensitivity and specificity. We have modified the derivatisation and chromatographic stages of this assay method and introduced a new plasma extraction procedure to permit the simultaneous analysis of morphine and 6-acetylmorphine at the required limit of quantitation in human neonatal plasma.

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EXPERIMENTAL

Chemicals and reagents

Morphine hydrochloride was obtained from Boots (Nottingham, U.K.) and 6-acetylmorphine hydrochloride was purchased from Macfarlane Smith (Edinburgh, U.K.). 5-Dimethylamino-1-naphthalenesulphonyl chloride (dansyl chloride) and nalorphine hydrochloride were obtained from Sigma (Poole, U.K.). Sodium carbonate, sodium bicarbonate, 1-pentanesulphonic acid and tetraethylammonium chloride (HPLC grade) were purchased from BDH (Poole, U.K.). Methanol, toluene, 2-propanol, acetone, *n*-hexane and ammonia solution (0.88 SG), all HPLC grade, were obtained from May and Baker (Manchester, U.K.). [1(n)-^{3}H]Morphine (specific activity = 888 MBq/mmol) was obtained from Amersham International (Amersham, U.K.).

Stock solutions

Morphine and 6-acetylmorphine stock solutions at 1 and 0.1 μg/ml (calculated as free base) and nalorphine (internal standard) stock solution at 1.0 μg/ml were prepared in methanol–water (30:70, v/v) and stored at −30°C. Calibration standards were prepared by diluting the stock solutions of morphine and 6-acetylmorphine with blank human plasma. Dansyl chloride stock solution at 1.0 mg/ml was prepared in acetone and stored at −30°C in the dark.

Chromatographic conditions

The HPLC apparatus consisted of two Gilson 305 pumps (Gilson Medical Electronics, Villiers-le-Bel, France), a Gilson 805 Manometric module, a Rheodyne Model 7125 injector with 200-μl loop (Rheodyne, Cotati, CA, U.S.A.), a Merck-Hitachi F 1000 fluorescence detector (Hitachi, Tokyo, Japan), a Kontron 720 LC variable-wavelength ultraviolet detector (Kontron Instruments, Zurich, Switzerland) and a Gilson 712 data handling system. Two types of column were evaluated for the fluorescence method: a Spherisorb S3W silica column (150 mm × 4.6 mm I.D.) and a Spherisorb 3CN (150 mm × 4.6 mm I.D.), both packed with 3-μm silica (Phase Separations, Queensferry, U.K.). A silica precolumn (3 μm particle size, 10 mm × 4.6 mm I.D.) was placed between the injector and the main analytical column. The mobile phase was a mixture of *n*-hexane–2-propanol–ammonia (95:5:0.25) and the flow-rate was 1.5 ml/min. The fluorescence detector was set to an excitation wavelength of 340 nm and an emission wavelength of 500 nm.

A reversed-phase HPLC–ultraviolet detection method was used to determine the recovery of morphine, 6-acetylmorphine and nalorphine during evaluation of the plasma extraction procedure. The column was a Spherisorb ODS 2 (250 mm × 4.6 mm I.D.) packed with 5-μm particles (Phase Separations). The mobile phase was distilled water–acetonitrile (74:26) with 6.1 g/l 1-pentanesulphonic acid and 0.1% (v/v) tetraethylammonium chloride dissolved in the mixture, the aque-

ous phase being adjusted to pH 2.1 with orthophosphoric acid. The flow-rate was 1.0 ml/min and a wavelength of 210 nm was used for detection.

Assay methodology

A flow chart of the complete assay procedure is shown in Fig. 1. The plasma sample or calibration standard (0.1 ml) was transferred to a 1.5-ml polypropylene tube. To each tube was added nalorphine as the internal standard solution (20 μl

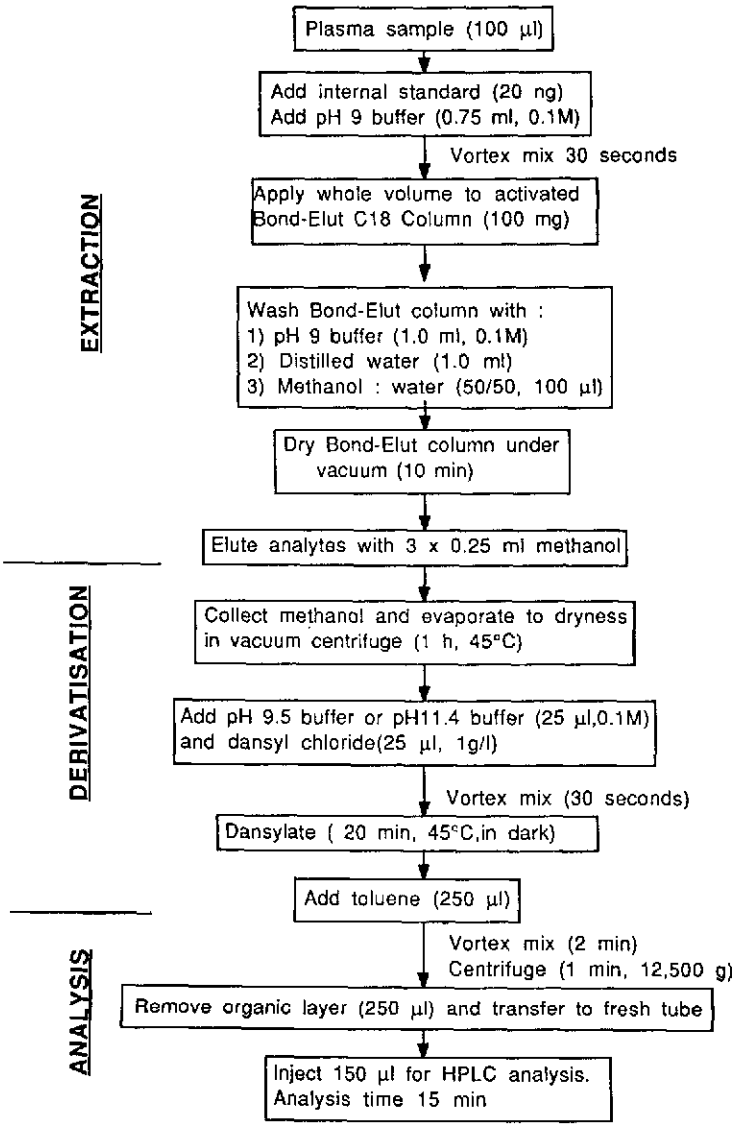

Fig. 1. Flow chart for plasma assay for morphine and 6-acetylmorphine.

of 1.0 μg/ml solution) and pH 9.0 sodium bicarbonate buffer (0.75 ml, 0.1 *M*). The tube contents were vortex-mixed for 10 s. The Bond-Elut extraction columns (Analytichem International, Harbor City, CA, U.S.A.) were activated by drawing through, under vacuum, methanol (1.0 ml), distilled water (1.0 ml) and pH 9.0 sodium bicarbonate buffer (1.0 ml). Care was taken to ensure that the columns did not dry out during this procedure. The prepared sample was applied to the Bond-Elut column and drawn through under vacuum. The extraction column was washed with pH 9.0 sodium bicarbonate buffer (1.0 ml, 0.1 *M*) followed by distilled water (1.0 ml) and methanol–water (50:50, 0.1 ml). The Bond-Elut column was allowed to dry under vacuum (10 min) and the analytes eluted from the column using 3 × 0.25 ml of methanol. The methanolic extract was evaporated to dryness in a vacuum centrifuge (1 h, 45°C). To the dried extract was added pH 11.4 aqueous sodium carbonate solution (25 μl, 0.1 *M*) or pH 9.5 aqueous sodium bicarbonate solution (25 μl, 0.1 *M*) and dansyl chloride solution (25 μl, 1.0 g/l in acetone). The tube contents were vortex-mixed, the tubes capped and allowed to stand at 45°C for 20 min in the dark. To the dansylated derivatives was added toluene (0.25 ml) and the tube contents were vortex-mixed for 2 min and centrifuged (1 min, 12 500 *g*). The organic (upper) layer was transferred to a glass vial and 100–200 μl were injected for HPLC analysis.

Sample preparation

The recovery of morphine during the sample preparation procedure was assessed by the use of [^{3}H]morphine as a tracer. Plasma calibration standards (0.1 ml) containing 200 ng/ml morphine and 2.0 kBq/ml [^{3}H]morphine were subjected to the sample preparation procedure and the recovery of [^{3}H]morphine was assessed by liquid scintillation counting of samples taken at each stage of the procedure. The recovery of 6-acetylmorphine and nalorphine was determined by HPLC analysis of the plasma extracts with ultraviolet detection before the dansylation stage. The effect of washing the Bond-Elut column with buffers of pH 2, 7 and 9 on the recovery and selectivity of the extraction of the analytes was investigated by the use of HPLC analysis with ultraviolet or fluorescence detection.

Dansyl derivatisation

The dansyl derivatisation reaction conditions for morphine previously reported in the literature [6–8] were assessed for their suitability for the simultaneous derivatisation of morphine, nalorphine and 6-acetylmorphine. The recovery of morphine was determined by the use of [^{3}H]morphine (with 20 ng per sample of added non-radiolabelled morphine) and that of nalorphine and 6-acetylmorphine was measured by HPLC with fluorescence detection. The stability of 6-acetylmorphine during the dansyl derivatisation procedure was assessed over a range of pH values (pH 9–11.4) by the measurement of dansyl 6-acetylmorphine and dansyl morphine (the degradation product) in the reaction mixture. Stability of the dansyl derivatives in the toluene extract from the dansyl reaction mixture was

assessed at room temperature for up to 24 h and at 4°C for up to one week by HPLC analysis with fluorescence detection.

Stability of 6-acetylmorphine in solution and in human plasma

The stability of 6-acetylmorphine (100 μg/ml) in aqueous solution was assessed over a range of alkaline pH values (pH 7–11) at temperatures of 20, 45 and 60°C. The decrease in 6-acetylmorphine concentration and the increase in morphine concentration were monitored with time using HPLC analysis with ultraviolet detection. The plasma stability of 6-acetylmorphine was determined at room temperature (20°C) for up to 3 h and at −30°C for one week by adding known amounts of the drug to human plasma and assaying aliquots at time intervals using HPLC analysis with fluorescence detection.

RESULTS AND DISCUSSION

Stability of 6-acetylmorphine

The ester bond of 6-acetylmorphine is known to be unstable under alkaline conditions [9] but there is no information on how the rate of hydrolysis varies with pH and temperature. Because the assay procedure has three stages at which alkaline conditions are encountered it was necessary to ensure that any hydrolysis of the ester bond of 6-acetylmorphine was kept to a minimum. The effect of pH and temperature on the rate of hydrolysis of 6-acetylmorphine is shown in Fig. 2. A decrease in 6-acetylmorphine concentration in each experiment was accompanied by a corresponding increase in the hydrolysis product morphine confirming that the reduction was due to hydrolysis alone and not due to dimer formation or binding to glassware. Stability experiments were performed for a duration of 8 h

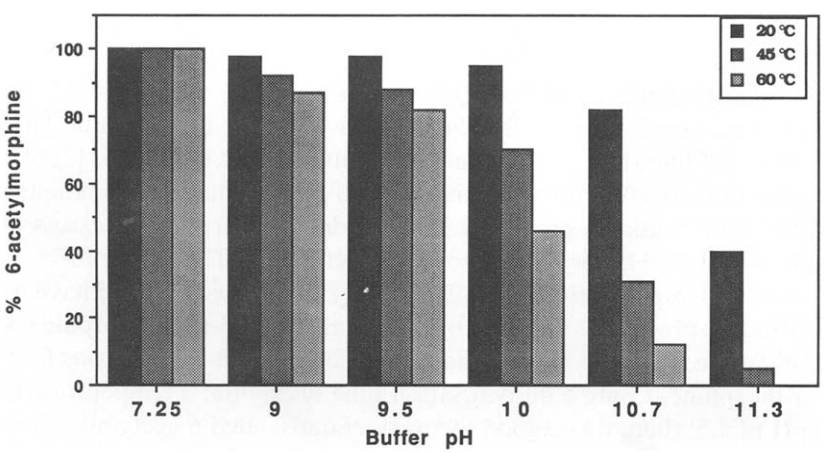

Fig. 2. Effect of pH and temperature on the stability of 6-acetylmorphine in aqueous solution (30-min incubation time).

and representative data after 30 min of incubation are shown in Fig. 2. From the stability data it can be seen that the brief contact of 6-acetylmorphine with alkaline medium during the extraction stage and during chromatographic analysis would cause negligible hydrolysis. Hence it was not necessary to modify these stages from the conditions used for morphine. However, 6-acetylmorphine was completely hydrolysed under the strongly alkaline conditions normally used in the dansyl derivatisation of morphine (pH 11.4) and it was necessary to change the derivatisation buffer to pH 9.5 to minimise this degradation when both morphine and 6-acetylmorphine were to be analysed.

6-Acetylmorphine is metabolised to morphine in man, but appears to be stable in human plasma [10,11], though this has not been studied in detail. Any breakdown of 6-acetylmorphine in plasma samples, during sample collection or storage, would lead to an overestimation of morphine concentration. The results of the experiments on the stability of 6-acetylmorphine in plasma showed that there was no significant degradation at room temperature for up to 3 h or at − 30°C for up to one week. These results are in agreement with previously published data [10,11] and suggest that there should be no errors introduced into the assay by the enzymic hydrolysis of 6-acetylmorphine during sample collection and storage.

Extraction methods

The recovery of morphine in the extraction procedure, determined by the use of [^{3}H]morphine, was 98.3 ± 0.6% (n = 6). The recoveries of morphine, 6-acetylmorphine and nalorphine in the extraction procedure, determined by HPLC analysis, were 93.6 ± 4.7% (n = 6), 98.0 ± 6.3% (n = 6) and 95.9 ± 5.8% (n = 6), respectively. The use of a pH 9.0 wash of the plasma sample on the Bond-Elut column resulted in a significant reduction in the number of interfering substances extracted with the analytes without significant reduction in recovery. However, a pH 2.0 wash resulted in a reduction in recovery of the analytes and was not used.

Preparation of dansyl derivative of 6-acetylmorphine

Various reaction conditions for the dansyl derivatisation of morphine have been reported in the literature [7–9]. The conditions used by Frei *et al.* [7] of a 20-min reaction time at 60°C using a mixture of 0.1 M sodium carbonate and acetone (50:50) were found to give a recovery of dansyl morphine in excess of 90% as determined by [^{3}H]morphine recovery experiments. The longer reaction time of 3 h at room temperature used by Tagliaro *et al.* [6] was found to have no advantages over this procedure. Because of the instability of 6-acetylmorphine at alkaline pH it was necessary to modify the dansyl derivatisation conditions from that used for morphine. Using a derivatisation time of 20 min, a temperature of 45°C and a pH of 9.5, there was a good recovery of dansylated 6-acetylmorphine with minimum hydrolysis (Fig. 3). The recovery of dansylated morphine and nalorphine was slightly reduced under these conditions but was acceptable. The

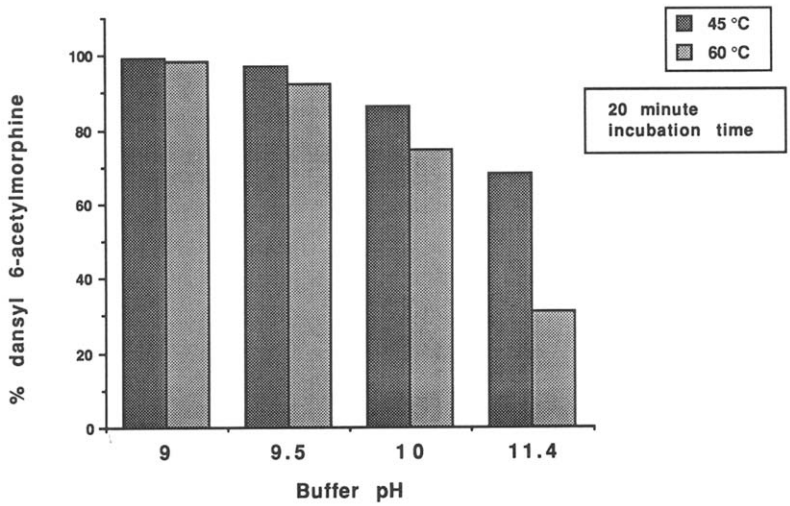

Fig. 3. Effect of pH and temperature on the stability of 6-acetylmorphine during dansyl derivatisation.

dansyl derivatives were stable for a minimum of 24 h when stored at 20°C in the dark, and for up to one week when stored at 4°C.

Chromatographic separation

Some difficulty was encountered in the separation of the dansyl derivatives of morphine, 6-acetylmorphine and nalorphine from interfering components resulting from the derivatisation reaction. These interfering components were more noticable at a dansyl derivatisation pH of 9.5 used for 6-acetylmorphine and were reduced under pH 11 derivatisation conditions. The use of the highest purity of solvents and reagents reduced the problem to a manageable level.

The use of an underivatised silica column was found to result in long delays in equilibration and a resulting slow drift in retention times during analysis. This problem was overcome by changing to a CN-bonded HPLC column which was used in the normal-phase mode. The CN-bonded column gave a short equilibrium time of 20–30 min and reproducible retention times.

The mobile phase was saturated with silica by the use of an in-line guard column placed between the pump and the injector. This was effective in reducing the rate of degradation of the silica column. The chromatographic efficiency was found to be reduced when certain batches of HPLC stationary phase were used and this was believed to be related to the greater susceptibility of these particular batches to alkaline attack.

The dansyl derivatives of morphine, 6-acetylmorphine and nalorphine were successfully separated by this method with retention times of 5.7, 3.3 and 2.8 min respectively. Typical chromatograms of blank plasma and plasma spiked with morphine, 6-acetylmorphine and internal standard are shown in Figs. 4 and 5, respectively.

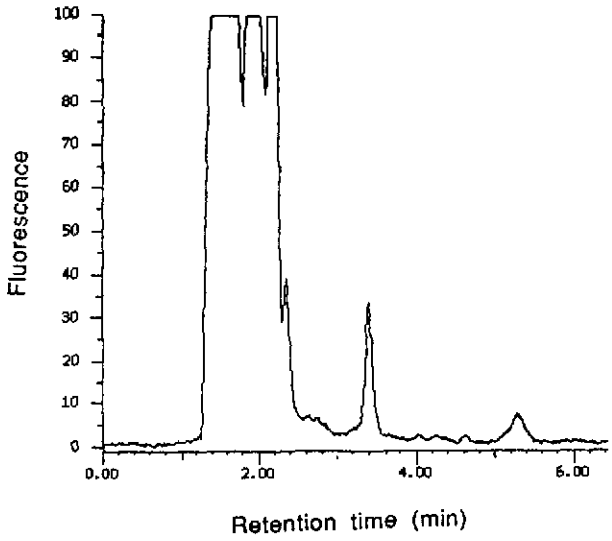

Fig. 4. Chromatogram of a blank plasma extract.

Assay validation

A linear response was obtained in the plasma calibration graphs from 10 to 250 ng/ml for morphine and from 25 to 250 ng/ml for 6-acetylmorphine. The limit of quantitation for a 0.1-ml plasma sample was 10 ng/ml for morphine and 25 ng/ml for 6-acetylmorphine. The within-day reproducibilities (expressed as

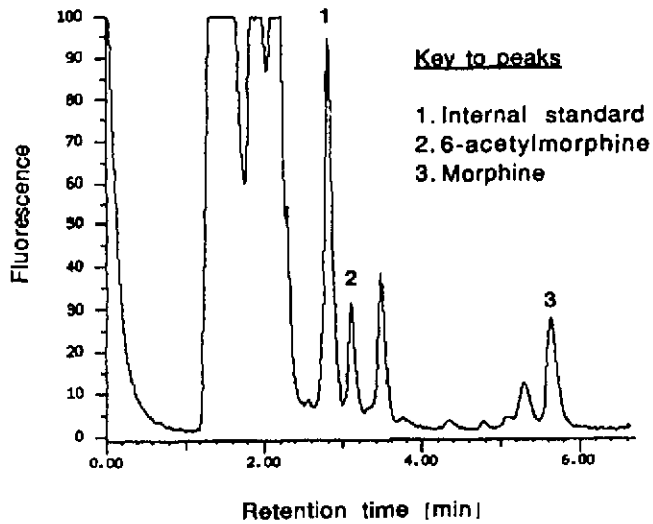

Fig. 5. Chromatogram of a blank plasma extract spiked with morphine (75 ng/ml), 6-acetylmorphine (75 ng/ml) and internal standard (200 ng/ml).

coefficient of variation of six replicate samples) were 15.9, 5.9 and 6.2% at morphine concentrations of 10, 75 and 250 ng/ml, and 10.8, 10.9 and 5.6% at 6-acetylmorphine concentrations of 25, 75 and 250 ng/ml, respectively. The between-day reproducibility at 75 ng/ml was 5.7% (n = 12) for morphine. The accuracy of the assay was within 10% of the expected values over the range of calibration standards. There was no interference with the assay from endogenous compounds nor from a selection of drugs which were co-administered with diamorphine during the neonatal study. These assay validation data relate to a 0.1-ml plasma volume; for larger sample volumes an improvement would be expected.

Assay of neonatal plasma samples

Following the intravenous infusion of diamorphine to ten premature neonates it was possible to detect morphine in plasma samples from all subjects, but 6-acetylmorphine was detected in only two subjects. A typical assay chromatogram of a study plasma sample in shown in Fig. 6 and a plot of morphine plasma concentration *versus* time from one patient is shown in Fig. 7. The inability to detect 6-acetylmorphine in the majority of the study subjects may be due to a lack of sufficient sensitivity of the assay or to a difference in metabolism of diamorphine between adults and premature newborn babies. Inturrisi *et al.* [12] in a study of diamorphine kinetics in adults reported plasma levels of 6-acetylmorphine in excess of 25 ng/ml after an intravenous infusion of the drug.

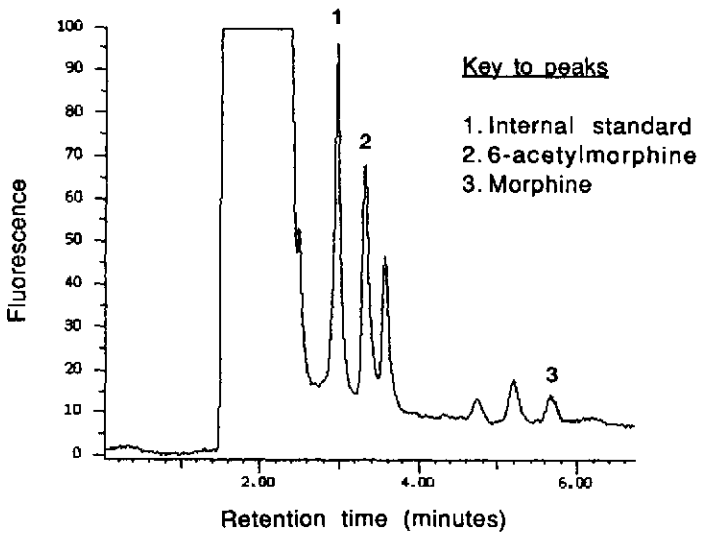

Fig. 6. Chromatogram of a plasma extract from a patient during diamorphine infusion at 15 μg/kg/h.

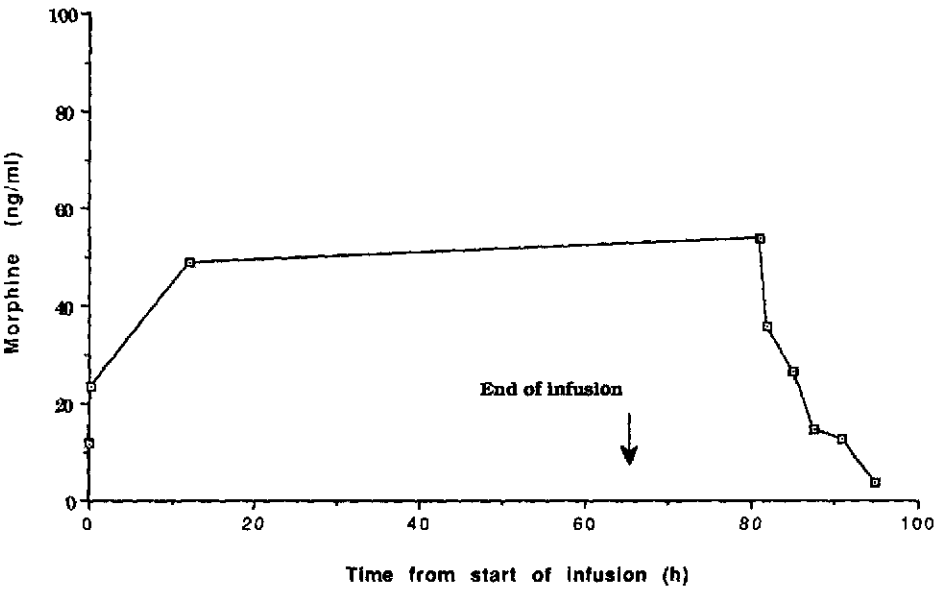

Fig. 7. Morphine plasma concentration *versus* time profile after diamorphine infusion to a premature newborn baby.

CONCLUSION

The method described provides a sensitive and specific assay for morphine and 6-acetylmorphine in small volumes of human plasma. It has been successfully applied to the assay of these compounds in plasma during a study to determine the pharmacokinetics of diamorphine in the human premature neonate.

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REFERENCES

1 A. Elias-Jones, D. A. Barrett, N. Rutter, P. N. Shaw and S. S. Davis. *Arch. Dis. Child.*, in press.
2 D. A. Barrett, A. Elias-Jones, N. Rutter, P. N. Shaw and S. S. Davis, *J. Clin. Pharmacol.*, (1991) in press.
3 F. Tagliaro, D. Franchi, R. Dorizzi and M. Marigo, *J. Chromatogr.*, 488 (1989) 215.
4 J. G. Umans, T. S. K. Chiu, R. A. Lipman, M. F. Schultz, S.-U. Shiu and C. E. Inturrisi, *J. Chromatogr.*, 233 (1982) 213.
5 H. J. G. M. Derks, K. van Twillert and G. Zomer, *Anal. Chim. Acta*, 170 (1985) 13.
6 F. Tagliaro, A. Frigerio, R. Dorizzi, G. Lubli and M. Marigo, *J. Chromatogr.*, 330 (1985) 323.

7 R. W. Frei, R. W. Santi and M. Thomas, *J. Chromatogr.*, 116 (1976) 365.
8 F. Nachtman, H. Spitzy and R. W. Frei, *Anal. Chim. Acta*, 76 (1975) 57.
9 D. A. Smith and W. J. Cole, *Biochem. Pharmacol.*, 25 (1976) 367.
10 E. R. Garrett and T. G. Gurkan, *J. Pharm. Sci.*, 68 (1979) 26.
11 O. Lockridge, N. Mottershaw-Jackson, H. W. Eckerson and B. N. LaDu, *J. Pharmacol. Exp. Ther.*, 215 (1980) 1.
12 C. E. Inturrisi, M. B. Max, K. M. Foley, M. Schultz, S. U. Shin and R. W. Houde, *N. Engl. J. Med.*, 310 (1984) 1213.