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Note

Rapid, high-sensitivity method for measurement of morphine in guinea-pig serum

C.A. TURNER and R. MURPHY*

Department of Anatomy and Histology, School of Medicine, Flinders University, Bedford Park, S.A 5042 (Australia)

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In general, methods for screening blood samples for morphine have used either solid-phase [1–4] or liquid-phase [5–9] extraction regimes to recover the drug from plasma or serum, followed by high-performance liquid chromatographic (HPLC) analysis with either electrochemical or spectrometric quantification of drug content. The solid-phase methods generally utilise commercially available, pre-packed mini-columns or cartridges and are expensive to run routinely, whilst the liquid-phase methods are often cumbersome and/or use relatively toxic solvents to extract the drug. In addition, these methods have been optimised for extraction of morphine from human blood and may not be suitable for measurement of blood levels in experimental animal models. In fact, in our hands, these methods have not given consistent results when we have attempted to measure morphine levels in the blood of guinea-pigs chronically treated with the drug. It is, however, important that a reliable method should be available for measuring morphine in guinea-pig serum, as this animal is a widely used model for the study of opiate dependence and tolerance.

The guinea-pig small intestine, after chronic exposure to morphine, has been used as a model for opiate dependence and tolerance for many years [10–12]. The route of chronic administration of the opiate to experimental animals varies from repeated subcutaneous injection (e.g. ref. 13) to implantation of compounded morphine pellets [14], and tissue taken from these animals is routinely maintained in physiological buffers containing morphine after dissection to prevent premature withdrawal phenomena. The concentration of morphine in such buffers should reflect the levels of the drug achieved in the blood, but the lack of a validated method for measuring these levels in guinea-pigs makes this difficult

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to accomplish. Thus it may be impossible to interpret the results of biological experiments when it is not known if these results arise from the chronic treatment regime or from acute exposure of tissue to inappropriate concentrations of morphine in the buffers.

Consequently, we have devised a rapid liquid–liquid extraction regime for recovery of morphine from guine-pig serum and a high-sensitivity analytical method, using HPLC with electrochemical detection, to quantify the drug in these extracts.

EXPERIMENTAL

Guinea-pigs (200–300 g of either sex) were implanted subcutaneously with a compounded pellet [10] containing both morphine sulphate (15 mg) and free morphine base (60 mg), and after six to eight days they were killed by a blow to the head and exsanguinated by cardiac puncture. Collected blood was allowed to clot at room temperature and was then centrifuged at 4°C for 20 min at 2000 *g*, and the serum collected into a fresh tube. Control animals (i.e. morphine naive) were similarly bled and their serum was harvested. All sera were stored at −20°C until used in the assay. Known amounts of morphine and naloxone (the internal standard; see below) were added to control (drug-free) serum samples for estimation of recovery of both drugs.

Extraction

Aliquots (200 μl) of serum samples from morphine-treated guinea-pigs or morphine standards in control serum were added to 1.5-ml polypropylene micro test tubes and were diluted with aqueous sodium bicarbonate (1.0 *M*; pH adjusted to 9.0 with sodium hydroxide; 200 μl). An internal standard, naloxone (50 μl, either 10^{-3} or 10^{-4} *M*), was added and the mixture was mixed thoroughly by vortexing. The mixture was extracted with chloroform (2×1.0 ml) and the combined chloroform extracts were evaporated to dryness in glass tubes. The residue was resuspended in methanol (50 μl) and then diluted with hydrochloric acid (0.1 *M*, 150 μl). The sample was filtered through a disposable filter (0.45 μm porosity, 4 mm diameter) and an aliquot (25–50 μl) analysed as described below.

Chromatographic analysis

Chromatographic analyses were carried out at 25°C on an Ultrasphere ODS column (150 mm×4.6 mm; Altex/Beckmann) fitted with a hand-packed guard column (30 mm×2 mm) of LiChroprep RP-18. Chromatograms were developed isocratically with a mobile phase consisting of 0.1 *M* sodium dihydrogenphosphate, 0.002 *M* octanesulphonic acid, 0.001 *M* disodium ethylenediaminetetraacetic acid and 25% (v/v) HPLC-grade methanol. Flow-rate was maintained at 1.0 ml/min with a Kortec K25M HPLC pump (Kortec/ICI Australia) and samples were loaded onto the column with a Valco C6U injection valve (Valco Instruments). Output from the column was monitored with a glassy carbon electrochemical flow cell (Bioanalytical Systems) maintained at a potential of +0.9 V against an Ag/AgCl reference by a BME 1162 dual-channel electrochemical controller (Biomedical Engineering Department, Flinders Medical Centre).

The current measured by the controller was monitored with a DP 600 dual-pen chart recorder (ICI Instruments).

RESULTS AND DISCUSSION

The chromatographic system described above gave a single sharp peak for morphine, with a retention time of 4.7 min ($k'=2.33$). A typical analysis for morphine standard is shown in Fig. 1A. This chromatogram represents 50 pg of morphine base injected on-column, and extrapolation from this chromatogram gives an estimated limit of detection for standards of 1 pg morphine base (at a signal-to-noise ratio of 2:1). Fig. 1B shows a typical chromatogram for the analysis of an extract of guinea pig-serum spiked with morphine standard and prepared as described above. The morphine peak, representing 0.65 ng injected on-column, is clearly visible and is not obscured by any components of the serum that co-extract with the opiate. The recovery of exogenous morphine was $70.1 \pm 8.0\%$ ($n=6$). Similarly, the internal standard, naloxone (10^{-3} M was used here), gave a sharp peak with a retention time of 8.5 min ($k'=5.33$) and did not co-elute with any electrochemically active serum component. Recovery of naloxone was $98.1 \pm 0.3\%$ ($n=2$). Fig. 1C shows a typical chromatogram obtained from serum of a morphine-treated guinea-pig. The measured amount of morphine here was 17 pg injected on-column in a volume of 25 μl, corresponding to a serum concentration of 0.67 ng/ml. Extrapolation from this chromatogram gives an estimated limit of detection corresponding to a serum concentration of 20 pg/ml morphine base (signal-to-noise ratio of 2:1).

Naloxone was chosen as the internal standard for this assay because of its

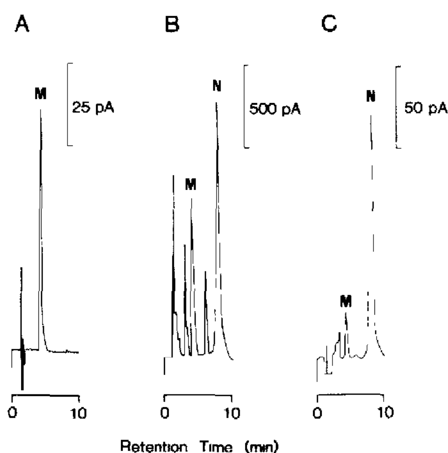

Fig 1 HPLC analysis of (A) 50 pg morphine base (M), (B) guinea pig serum spiked with morphine (M) and naloxone (N) and (C) serum from morphine-treated guinea-pig, showing measured morphine (M) and naloxone (N), internal standard. The amount of morphine measured in C was 17 pg, corresponding to a serum concentration of 0.67 ng/ml. Chromatographic details are reported in the text Scale bars indicate measured current in pA. Injections were made at time zero, and the deflections at 1.4 min are injection artifacts.

structural similarity to morphine (see Fig. 2), giving it similar solubility and extraction properties to morphine and having the same electrochemically active group (the phenol moiety). Standard curves for the assay were constructed by spiking control serum with naloxone standard and with increasing amounts of morphine standard. After extraction and analysis by the above procedures, the ratio of peak heights for the morphine and naloxone peaks was plotted as a function of the original concentration of morphine in the serum to generate a standard curve as shown in Fig. 3. This curve was linear for morphine concentrations ranging up to 250 ng/ml (Fig. 3A, correlation coefficient 0.9989; using 10^{-3} *M* naloxone as internal standard), but for assays of guinea-pig serum it was typically constructed over the range 0.25–1.0 ng/ml (Fig. 3B, correlation coefficient 0.9996; using 10^{-4} *M* naloxone as internal standard). Linear regression analysis indicated that both curves pass through the origin. Using this assay system, the serum concentration of morphine in guinea-pigs six to eight days after implantation of morphine pellets was measured at 0.39 ± 0.06 ng/ml ($n=13$).

During the course of this study, it was noted that electrode poisoning began to cause a decrease in peak heights for both morphine and naloxone after 30–40 injections. The cause of this poisoning is still under investigation, but may be due

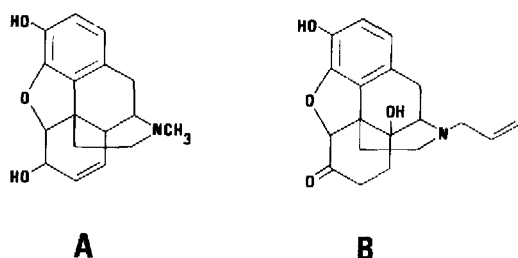

Fig. 2. Structure of morphine (A) and naloxone (B).

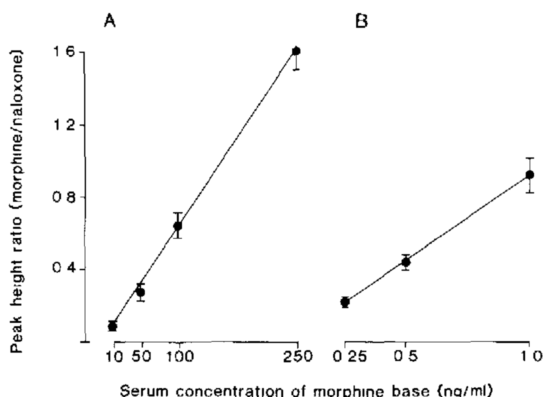

Fig. 3. Standard curves for measurement of morphine in guinea-pig serum. Curves were constructed over two concentration ranges: (A) 10–250 ng morphine base per ml serum ($n=4$), using 10^{-3} *M* naloxone as internal standard; (B) 0 25–1.0 ng morphine base per ml serum ($n=3$), using 10^{-4} *M* naloxone as internal standard.

to polymerization of morphine oxidation products on the electrode at this operating potential [7]. It was found to be reversed if the cell potential was set at +0.2 V and mobile phase was recirculated through the chromatograph overnight. This circumvented the need to dismantle and polish the glassy carbon electrode and avoided the re-equilibration period associated with such an operation.

Thus we have described a rapid, efficient and sensitive method for extracting and measuring morphine in the serum of guinea-pigs chronically treated with this opiate. This method should be suitable for measurement of morphine in sera of other animals (including man). This will enable better correlation of morphine concentration in physiological buffer solutions with concentrations attained in the animal and would enable future studies of the time course of release of morphine from chronic treatment regimes such as pellets or other depot injections.

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REFERENCES

1 G.K. Gourlay, C.F. McLean, G.A. Murphy and N.R. Badcock, J. Pharmacol Methods, 13 (1985) 317
2 R.W. Abott, A. Townsend and R. Gill, Analyst, 112 (1987) 397.
3 J -O. Svensson, A. Rane, J. Säwe and F. Sjöqvist, J. Chromatogr., 230 (1982) 427.
4 J.-O. Svensson, J. Chromatogr., 375 (1986) 174.
5 M.W White, J. Chromatogr., 178 (1979) 229.
6 R.G. Peterson, B H. Rumack, J.B Sullivan, Jr. and A. Makowski, J. Chromatogr., 188 (1980) 420.
7 R.D. Todd, S.M. Muldoon and R.L. Watson, J. Chromatogr., 232 (1982) 101.
8 H. Vandenberghe, S.M. Macleod, H. Chinyanga and S J. Soldin, Ther. Drug Monit., 4 (1982) 307.
9 R.A. Moore, D. Baldwin, H.J. McQuay and R.E.S. Bullingham, Ann. Clin. Biochem., 21 (1984) 125.
10 H.O.J. Collier, Br. J Addict., 67 (1972) 277.
11 H.O.J. Collier and D.L. Francis, in J. Fishman (Editor), The Basis of Addiction, Dahlem Konterenzen, Berlin, 1978, p. 281.
12 H.O.J. Collier, N.J. Cuthbert and D L. Francis, Br. J. Pharmacol., 73 (1981) 921.
13 S.M. Johnson, J.T. Williams, M. Costa and J.B. Furness, Neuroscience, 21 (1987) 595.
14 S.M. Johnson, D.P. Westfall, S.A. Howard and W.W. Flemming, J. Pharmacol. Exp. Ther., 204 (1978) 54.