decreased $^{45}Ca^{2+}$ entry via Na^+/Ca^{2+} exchange (Frelin et al 1984). The contribution to the negative inotropy of DEA in a working preparation without digitalis treatment, mediated by an indirect inhibition of the Na^+/Ca^{2+} exchange system deserves further study.

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Improved method for morphine determination in biological fluids and tissues: rapid, sensitive and selective

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Abstract—Morphine was assayed using a simple two step solvent extraction—acid back extraction sample preparation method, coupled with normal phase high-performance liquid chromatography (HPLC) and dual electrode coulometric detection. HPLC is performed with a 1·0 M Tris–methanol (5:95) mobile phase with subtle pH adjustments to separate morphine and internal standard from any interfering compounds. The use of normal phase HPLC (silica column) substantially reduces problems from interfering lipophilic substances sometimes encountered with reverse phase HPLC following solvent extraction and which would otherwise require more time-consuming sample preparation. Dual electrode detection further improves the selectivity for morphine and gives excellent sensitivity (0·5 ng mL^{-1}), reproducibility and stability for automated sample injection. This method has proven suitable for pharmacokinetic studies of morphine.

The conclusions of many studies of morphine concentration in biological samples have been unsound because of the unintentional co-measurement of morphine conjugated metabolites. Thus a critical issue in the design of new analyses is selectivity. Comprehensive investigations of the pharmacokinetics and the pharmacodynamics of morphine are only possible if the selectivity towards morphine is assured.

Numerous methods have been published for quantitating morphine in biological fluids and tissues. These have been based upon gas-liquid chromatography following derivatization (Dahlström et al 1977; Felby 1979; Jones et al 1984), reverse phase HPLC following solid phase or solvent extraction with electrochemical (Bolander et al 1983; Moore et al 1984; Derendorf & Kaltenbach 1986; Svensson 1986), UV-VIS (Svensson et al 1982; Säwe et al 1985) or fluorescence detection (Nelson et al 1982; Tagliaro et al 1985), or radioimmunoassay (RIA) methodology (Morris 1975; Hahn et al 1979; Stanski et al 1982; Edwards et al 1986). Most of these methods require several time-consuming sample preparation steps before quantitation. These normally include at least two extractions followed by solvent evaporation and reconstitution to clean up and concentrate the sample extract or, in the case of derivatization, to remove excess reagent and isolate the derivative for measurement. Detection by UV (Svensson et al 1982; Säwe et al 1985) is preferred when both the 3- and 6-glucuronide metabolites of morphine are required to be determined, but many recent methods for morphine alone have utilized amperometric detection with good sensitivity. The use of a dual electrode coulometric detector has, in our experience, given much better long term baseline stability at high sensitivities than the glassy carbon sandwich electrochemical cells. This has allowed overnight automated sample runs, increasing laboratory output without compromising sensitivity or reproducibility.

The initial application of this method was to the determination of the regional clearances and tissue solubility of morphine at steady-state in sheep (Sloan et al 1990). The method is now used routinely for monitoring morphine disposition in a pain management setting.

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Methods and materials

Solvent extraction technique. Morphine and hydromorphone (internal standard) were extracted from blood using the following procedure. Blood or diluted urine (1·0 mL) was placed in a glass centrifuge tube (10 mL, Johns Products, Melbourne, Australia) containing heparin (25 units, if blood was being assayed) and hydromorphone (200 ng as HCl, 25 μL for blood, usually 1000 ng as HCl for urine). Following the addition of sodium carbonate—bicarbonate buffer solution (pH 9·06, 1·0 M, 1·0 mL) and acid washed solvent (diethyl ether: n-butanol 85: 15, 5·0 mL), the mixture was vortex-mixed for 1 min, then centrifuged at 3000 rev min^{-1} for 5 min to separate the phases. The organic phase was transferred to a polypropylene tube (10 mL, Johns Products, Melbourne, Australia) containing HCl (0·01 M, 100 μL) and then mixed and centrifuged as before. The organic phase was aspirated to waste and the aqueous phase in the tube was heated in a 60°C water bath for 10 min to evaporate the residual ether. The aqueous phase was cooled to room temperature and 10–40 μL was injected into the liquid chromatograph.

Tissues were assayed as follows: Weighed tissue (approx 5–10 g) was homogenized (Omni-Mixer, Sorvall, Newton, CT, USA) with a double weight of potassium dihydrogen orthophosphate solution (pH 4·5, 0·1 M). Samples of homogenate (1·0 g) were extracted in a glass centrifuge tube (Johns Products) fitted with a PTFE-lined screw cap using solvent (5·0 mL) in the presence of internal standard (usually 500 ng, but varied in accordance with expected morphine concentration) and carbonate bicarbonate buffer (1·0 mL) in the same manner as for the blood samples. Morphine concentrations in fat were determined as above after initial homogenization of the samples (0·5 g) in methylene chloride (2·0 mL) before extraction into phosphate buffer solution.

A calibration standard curve was constructed for each study over the expected concentration range using morphine-free blood from the same animal or morphine-free frozen-stored (−20°C) tissues or body fluids to which had been added known amounts of morphine sulphate pentahydrate and internal standard. These standards were extracted in the same manner as the unknown samples in the same batch. Results were calculated by internal standardization on the basis of peak height ratios from the calibration curves for that particular experiment.

A high-performance liquid chromatograph was equipped with an electrochemical detector (Coulochem dual cell; ESA Bedford, MA, USA; detector 1: +0·30 V, detector 2: +0·65 V, guard cell +0·85 V and a 15 cm × 5 μm Spherisorb silica column (Phase Separation, Queensferry, UK). Chromatographic analysis was carried out using a mobile phase consisting of 95:5 methanol–tris (1·0 M) buffer (pH 9·10) which was filtered (0·2 μm) and degassed under vacuum before use. A flow rate of 2·0 mL min^{-1} was used which gave retention times of 3·4 and 6·0 min for morphine and hydromorphone, respectively. Detector 2 of the ESA Model 5010 analytical cell was monitored and the chromatograms recorded on a dual pen recorder.

Sensitivity, reproducibility and selectivity of assay. Limits of sensitivity for biological fluids and tissues assays were calculated on the basis of a signal to noise ratio of 3:1 on the maximum gain used in the assay. Replicate 1·0 mL samples of pooled blood with 20 (n = 15) or 200 ng mL^{-1} (n = 15), and with muscle tissue with 150 ng g^{-1} (n = 12) of added morphine (as sulphate pentahydrate) were assayed as described. Selectivity was assessed by comparison of chromatograms obtained in the presence and absence of morphine, and in the presence and absence of other substances, including the morphine 3- and 6- glucuronide metabolites. (see Results).

Results and discussion

Fig. 1 shows chromatograms of morphine-free blood and blood with 20 ng mL^{-1} morphine (peak 1, 3·3 min) and 200 ng hydromorphone (peak 2, 6·4 min) internal standard. Similar chromatograms were obtained from extracted tissue homogenate (Fig. 2), (lung, liver, kidney, brain, muscle, gut and fat) and diluted (10:1) urine samples. The retention time of an endogenous substance (peak 3, 5·1 min) could be altered by small changes (0·02–0·2 units) in pH of the mobile phase to adjust for changes of column condition with age. The mobile phase pH range of 8·9–9·2 was sufficient to resolve morphine and hydromorphone from any interfering peaks encountered in the extracted samples. No interfering peaks were observed when sodium bromosulphophthalein, sodium *p*-aminohippurate, sodium iothalamate, pethidine, methadone, paracetamol, aspirin, morphine 3- and 6-glucuronides, various benzodiazepines or various tricyclic antidepressants were also present in the samples.

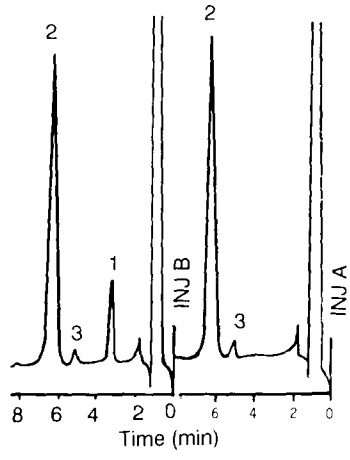

FIG. 1. HPLC traces of (A) blank blood to which internal standard (hydromorphone, 200 ng mL^{-1} as HCl) was added and (B) blood with 20 ng mL^{-1} morphine (as sulphate pentahydrate) and internal standard added. Peak 1 = morphine, Peak 2 = hydromorphone, Peak 3 = endogenous substance.

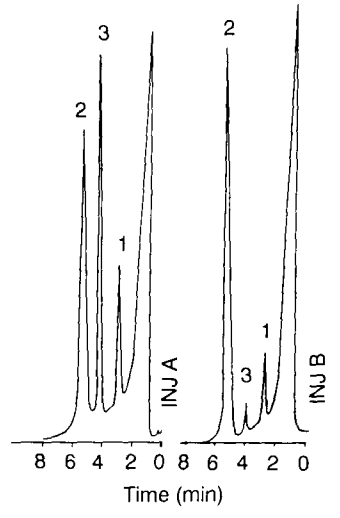

FIG. 2. HPLC traces of extracts of (A) sheep liver containing 189 ng g^{-1} morphine and (B) sheep kidney containing 73 ng g^{-1} morphine following a 2·5 mg h^{-1} infusion for 25 h. Peak 1 = morphine, Peak 2 = hydromorphone, Peak 3 = endogenous substance.

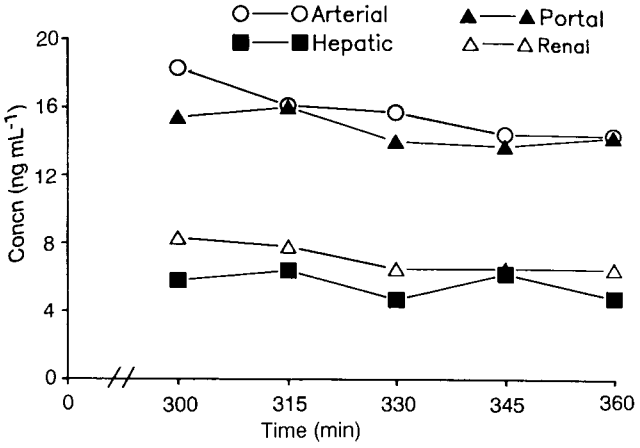

FIG. 3. Regional blood concentrations of morphine during the sixth h of an infusion of morphine (2·5 mg h^{-1}) in a chronically catheterised sheep (Mather & Runciman 1985).

The pK_a of the carboxylate group of morphine 3- and 6-glucuronides is 3·2 (Svensson et al 1982) so that, at the extraction pH of 9·06, these metabolites will be highly ionized and hence will not partition into the organic phase. Furthermore, the addition of morphine 3-glucuronide and morphine 6-glucuronide to previously chromatographed urine extracts resulted in no further peaks in the chromatogram because these substances would be highly bound to the silica stationary phase at the pH of the mobile phase.

The sensitivity of the assay was determined to be 0·5 ng mL^{-1} for blood with a signal to noise ratio of 3:1. Due to a dilution factor of 2:1 in preparing homogenates, the sensitivity for tissues was 2 ng g^{-1}. The range of morphine concentrations encountered in the present studies (Sloan et al 1990) was 1-250 ng mL^{-1} in blood and 2–1100 ng g^{-1} in the various tissues. Following extraction of blood with added morphine 20 ng mL^{-1} (n = 15) and 200 ng mL^{-1} (n = 15), the coefficients of variation (CV) were 2·4 and 2·7%, respectively; for muscle containing 150 ng g^{-1} (n = 12), the CV was 5·4%. For all samples, calibration curves were linear ($r^2 > 0.998$) over the range 5–500 ng mL^{-1} and passed essentially through the origin.

Regional blood morphine concentrations from a sheep receiving 2·5 mg h^{-1} morphine are shown in Fig. 3, demonstrating significant concentration gradients across the liver and kidney.

In developing this method, attention was given to selectivity for morphine by chromatographic separation and this was enhanced by choice of detector conditions to ensure accuracy of results. This was achieved by using a combination of normal phase HPLC and a dual electrode electrochemical detector. Electrode 1 of the analytical cell was run at +0·30 V which was high enough to oxidize a small peak occurring in some samples with a similar retention time to morphine but did not oxidize morphine which was oxidized at +0·65 V at electrode 2. A guard cell, placed between the pump and injector, operating at +0·85 V preoxidized any mobile phase contaminants, thus ensuring a stable base line with low noise at operating gain.

The small number of extraction steps in sample preparation maximizes extraction efficiency (83%) giving the method excellent sensitivity, linearity and lower coefficients of variation than previously reported methods. These factors are important for accurate quantitation in biodisposition studies. A fast analysis time of 7 min has been achieved that allows an automated high sample throughput commensurate with large sample numbers obtained from multi-site sampling studies and for rapid monitoring of patients.

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