Highly sensitive gas chromatographic–mass spectrometric method for morphine determination in plasma that is suitable for pharmacokinetic studies

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Abstract

A sensitive method was devised to determine morphine plasma concentrations by gas chromatography–mass spectrometry (GC–MS) using selected ion monitoring (SIM) with nalorphine as the internal standard. This method was rugged, reliable, selective and sensitive and was used to determine the morphine content of over 2000 samples. Sample preparation involved extraction of basified sample using n-butyl chloride–chloroform (5:1) and evaporation of the extract to dryness. The residue was derivatised with pentafluoropropionic anhydride, evaporated to dryness, reconstituted in 40 µl toluene and injected onto the GC–MS. For a sample size of 1 ml, the limit of quantitation was 0.75 ng/ml (S/N ratio 10:1) and the estimated limit of detection was calculated to be 0.2 ng/ml (S/N ratio 3:1), expressed as morphine base. Precision ($n=5$) was 4.9% at 0.75 ng/ml, 6.8% at 1.5 ng/ml, 3.0% at 37.5 ng/ml and 2.3% at 150 ng/ml. Standard curves for the range of 0–750 ng/ml morphine in plasma were linear with all r^2 values greater than 0.99. No interfering peaks were seen for either morphine or internal standard in the blank samples. The method is suitable for pharmacokinetic studies after subclinical doses of morphine where it has been used to study morphine plasma concentrations for 6 h after a dose of only 2 mg.

Keywords: Morphine

1. Introduction

Morphine (7,8-dihydro-4,5-epoxy-17-methylmorphinan-3,6-diol) is the prototype opioid analgesic agent (Fig. 1): it has been in constant clinical use from pre-recorded history. During the 1960s analytical methods were developed to measure con-

centrations of morphine in plasma: these introduced the possibility of pharmacokinetic studies of morphine [1].

High-performance liquid chromatography (HPLC) is now commonly preferred for pharmacokinetic studies of morphine as there is no need for derivatisation as is the case with gas chromatography (GC) methods and many of the methods provide for the concurrent measurement of several metabolites of morphine [2–8]. However, many HPLC methods lack the sensitivity required for pharmacokinetic
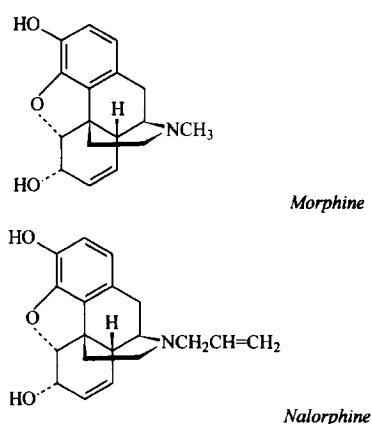
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Fig. 1. Two-dimensional structure of morphine and nalorphine.

studies which involve long durations of blood sampling and/or small doses of morphine.

Radioimmunoassay (RIA) has also been used in morphine pharmacokinetic studies [9], although due to a history of antibody cross-reactivity with morphine metabolites, this method developed a bad reputation.

Gas chromatography with nitrogen–phosphorous detection [10–12], flame ionisation detection [11,13] or electron capture detection [14–16] has been used to determine morphine concentrations for pharmacokinetic studies. However the procedure with unsurpassed sensitivity and selectivity is gas chromatography–mass spectrometry (GC–MS). Many reported methods have used GC–MS as means of analysis [10,11,17–21]. In detection as a drug of abuse, GC–MS is the preferred method for determining morphine because the mass spectrum of the peak provides confirmation which is important for legal purposes.

Krogh et al. [10] used an on-line dialysis system for ease of sample preparation followed by derivatisation and analysis by GC–MS. Although the fully automated sample preparation was advantageous, the limit of detection however was approximately 100-fold higher than presently reported. Wasels et al. [11] and Schuberth and Schuberth [17] both used solid-phase extraction for sample preparation, and Watson et al. [18] and Cone et al. [19] used liquid–liquid extraction as the sample preparation procedure. However none of these methods achieved the subnanogram sensitivity required for phar-

macokinetic studies for low doses of morphine. Jones et al. [20] analysed morphine in urine and body parts by GC–MS. This method stated a similar limit of detection to the previously reported method, however the sample preparation was laborious in comparison.

In our studies, large numbers of plasma samples were needed to be assayed to provide data for gaining insight into the pharmacokinetics of morphine after use of a novel transpulmonary dosing strategy [22]. Under these circumstances, sensitivity was of paramount importance as the doses used were smaller than in common clinical practice and plasma samples were taken for many hours after morphine administration. The method of morphine analysis reported here is generally more sensitive and less complicated than other published assays and allows large sample throughput.

2. Experimental

2.1. Materials and reagents

Morphine sulphate pentahydrate and nalorphine hydrobromide were BP grade. HiPerSolv grade n-butyl chloride was obtained from BDH Chemicals (Melbourne, Australia). All other chemicals used were either pharmacopoeia or AR grade. Water was purified by a Milli-Q system from Millipore (Sydney, Australia).

All glassware was supplied by Alltech (Sydney, Australia). A Searle vortex mixer was used as a heating module for the derivatisation process as well as vortex mixing (Buchler Instruments, NJ, USA). A Medos JAK vacuum evaporator (Dynavac Engineering, Sydney, Australia) was used for evaporation after the extraction procedure. The vacuum was achieved using a water aspirator (40°C).

2.2. Samples and standards

Blood samples, obtained from healthy human subjects before (10 ml for blank and standard curve preparation) and after receiving morphine, were placed in heparinized blood collecting tubes and centrifuged at 1500 g for 15 min. The plasma was separated and stored at -80°C until analysis. A stock

solution (0.33 mg/ml) of morphine sulphate, prepared each day, was serially diluted in water then added to each subjects drug-free plasma to give standard curves covering the concentration range of 1 to 750 ng/ml. Internal standard solutions of 5 µg/ml were prepared from a stock solution of 10 mg/ml nalorphine hydrobromide.

Reproducibility of the method was assessed by assay of 5 replicate aliquots of pooled plasma samples containing 0.75, 1.5, 37.5 and 150 ng/ml morphine.

2.3. Preparation of glassware

Glassware was siliconised by immersion in a 5% solution of SurfaSil in n-hexane for 10 min, rinsed with acetone, then n-hexane and oven dried at 100°C.

2.4. Extraction and derivatisation

One-ml aliquots of the plasma samples and the plasma morphine standard solutions were pipetted into 4 ml vials containing 10 µl (=50 ng) nalorphine hydrobromide as the internal standard (Fig. 1) and made alkaline to pH 9 with 200 µl carbonate buffer; 2 ml n-butyl chloride–chloroform (5:1) was added and the mixture vortex mixed for 10 min followed by centrifugation at 2000 g for 5 min. The carbonate buffer was made by preparing 4 M sodium hydrogen carbonate solution and adjusting to pH 9 with 4 M sodium carbonate solution. The samples were frozen in a dry ice bath for 15 s, the organic layer decanted into a 2 ml vial and evaporated to dryness under vacuum at 40°C. Extraction residues were derivatised by the addition of 50 µl acetonitrile and 50 µl pentafluoropropionic anhydride (PFPA) with the samples capped and heated at 70°C for 1 h. The samples were then cooled to room temperature, centrifuged and evaporated to dryness under vacuum at 40°C. The samples were reconstituted in 40 µl toluene, vortex mixed for 30 s, centrifuged for 1 min and then transferred into 200 µl siliconised glass inserts. Of this final extract, 2 µl was injected into the GC–MS via splitless mode. Autosampler sequences allowed a sample throughput of 48 per day including standards and quality control samples.

A study of the derivatisation reaction was made.

First, the completeness of the derivatisation reaction was assessed by performing the reaction at a morphine concentration of 375 ng/ml. Samples were taken out of the heating block at 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min then treated as described in Section 2.4. Second, the effect of unevaporated derivatisation residues on the assay was assessed. Five samples each containing 7.5 µg morphine and nalorphine were prepared and assayed as described in Section 2.2 but the samples were evaporated for only 5, 10, 15, 20 and 30 min and the GC–MS method was used in scan mode.

2.5. Instrumentation

Analyses were performed on a Hewlett-Packard 5890 SERIES II Plus gas chromatograph equipped with a Model 7673 autosampler and a 5972 SERIES mass selective detector (MSD) in EI mode (70 eV). High purity helium was used as the carrier gas at a constant flow-rate of 1.5 ml/min. The column was a 30 m 5% phenyl methyl siloxane capillary column (HP-5MS, Hewlett-Packard) with an internal diameter of 0.25 mm and a film thickness of 0.25 µm. The injector and detector temperatures were maintained at 250°C and 280°C, respectively. The oven temperature was programmed as follows: 150°C for 1 min after injection, increasing to 250°C at 40°C/min, finally holding at 250°C for 21.5 min (overall run time 25 min). The MSD was operated in selected-ion monitoring mode (SIM) using $m/z = 414.3$ and $m/z = 440.3$ for the morphine and nalorphine (internal standard) derivatives, respectively. Each ion had a dwell time of 100 ms.

3. Results and discussion

3.1. Method sensitivity

In order to increase the sensitivity of this assay several factors needed to be addressed. Precautionary silicone deactivation of all glass surfaces with which the morphine came into contact, including the glass injection port liner of the GC, was carried out to decrease the potential for analyte adsorption. A "blank" plasma sample from the same subject was run with each standard and sample set to verify

possible contamination; none had measurable concentrations of morphine or nalorphine. Precautions such as using high purity solvents and cleaning solvent dispensers regularly aided in avoiding contamination.

In comparison to other methods involving liquid–liquid extraction [19,20], this method did not require back extractions for sample purification. Sample loss was therefore minimised by minimising the number of extraction steps. Even greater sensitivity could have been achieved by injecting a greater volume. The morphine extract was reconstituted in toluene. With the specified GC inlet conditions, it would have been possible to raise the injection volume of a sample reconstituted in toluene to 5 µl without overloading the injection port. The injection volume of this assay was 2 µl as sensitivity was sufficient for this study, however, a higher injection volume would involve more frequent cleaning of the MSD.

3.2. Solvent choice

The extraction solvent, n-butyl chloride–chloroform (5:1) was chosen as it causes little emulsification or protein precipitation and produces extracts that are relatively clean. Moreover it is less dense than the plasma thereby facilitating its removal for further processing by the centrifugation and freezing manoeuvre. After vortex mixing, the sample was placed in a dry ice bath to freeze the aqueous phase, thereby allowing the organic phase to be easily decanted for evaporation. Thus the extraction is rapid, reproducible and cost effective. The evaporator used accommodates over one hundred
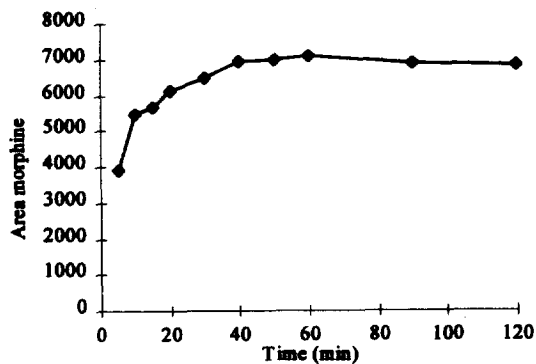
Fig. 3. Completeness of the derivatisation reaction was assessed from the area of morphine peak plotted against time allowed for derivatisation.

samples allowing large volumes of samples to be handled simultaneously. Fig. 2 shows a typical chromatogram for a plasma sample taken from a patient where the concentration was 5.8 ng/ml morphine.

3.3. Derivatisation reaction

PFPA was used as the derivatisation agent as it gave cleaner chromatograms from plasma extracts than trifluoroacetic anhydride used for producing acetyl derivatives. Previously reported methods incorporating PFPA derivatisation [11,17,20], have allowed derivatisation to proceed for 20 or 30 min. In establishing the optimum time to be allowed for derivatisation, the area of morphine was measured and the profile shown in Fig. 3 was obtained by plotting area of the morphine peak versus time.
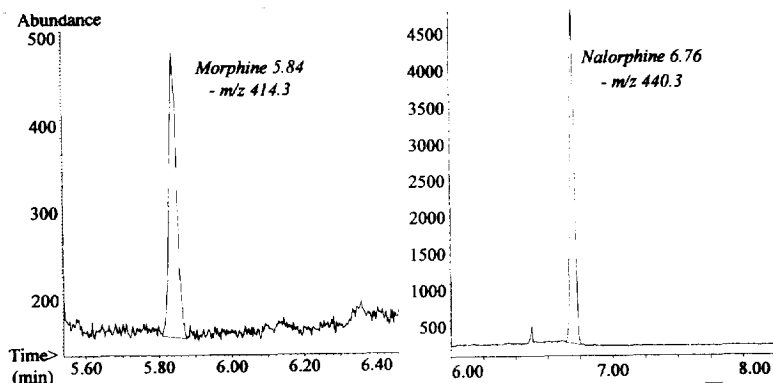
Fig. 2. Chromatogram of plasma sample taken from a subject receiving morphine in which the concentration was 5.8 ng/ml.

Derivatisation was incomplete until 40 min; however, 60 min was used in practice to allow for any variability which might slow the rate of derivatisation. In this study GC–MS run time was the rate determining step, so the additional 20 min allowed for derivatisation did not adversely affect the sample throughput.

In determining the effect of unevaporated derivatisation residues on assay, there was visible residue in the 5, 10 and 15 min samples and the chromatograms showed high baselines which obscured the morphine peak. Fig. 4a shows the high baseline obtained when evaporation was stopped at 5 min. Although the morphine peak was visible in extracted ion chromatogram mode, the unseen high baseline quickly contaminated the MS source. After evaporation for 30 min, there was no visible residue and the baseline in the chromatogram was significantly reduced (Fig. 4b).

3.4. GC–MS analysis of morphine in plasma

Fig. 5a shows the mass spectrum and the structure of 3,6-di-PFP-morphine and the fragmentation that produced the base peak. A very intense base peak at m/z 414.3 was observed. Another intense peak was seen at m/z 119 and this peak was due to fragmentation of $CF_3CF_2^+$ and was seen in the 3,6-di-PFP-nalorphine mass spectrum and throughout the chromatographic run. This was therefore unacceptable as a qualifying ion. All other peaks, compared to the base peak, were small (5–10% of the base peak). The ion m/z 430.3 was chosen as a qualifying ion since at high morphine concentrations as this ion was 10% of the base peak. At very low levels, this ion was not seen and no interfering ions at m/z 414.3 were observed in any of the blank samples. Thus the routine monitoring of a qualifying ion was deemed not necessary. The mass spectrum of 3,6-di-PFP-nalorphine (Fig. 5b) shows a base peak at m/z 440.3 which is due to the fragment indicated on the structure shown in Fig. 5b. Again no qualifying ions were deemed to be necessary for the internal standard.

Standard curves covering the concentration range of 0–750 ng/ml morphine in plasma were found to be linear. The slopes of forty four standard curves prepared in individual subjects drug free plasma obtained over a 6 month period, gave r^2 values always >0.99. The average slope of all these standard curves was 0.012, with a C.V. of 16.7%. A typical calibration curve is shown in Fig. 6 and this
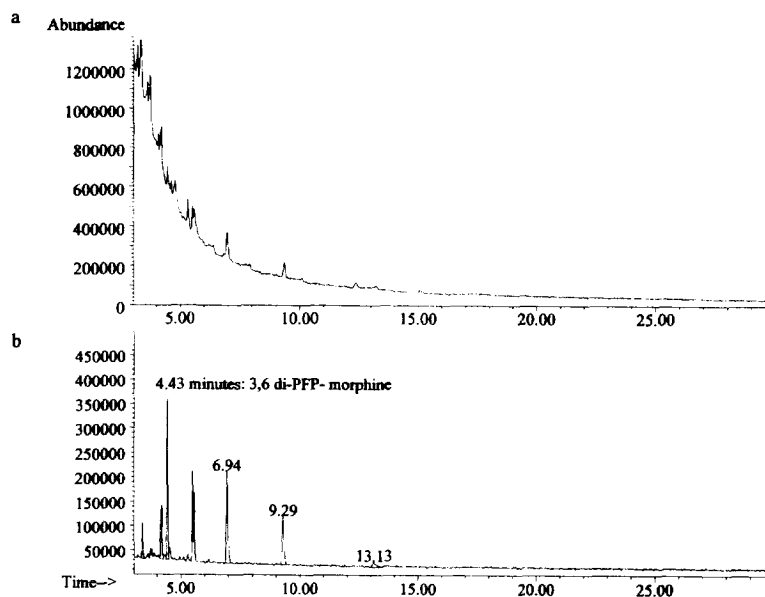

Fig. 4. The chromatograms of derivatised morphine extracts when (a) excess PFPA residues remain in the final solution; (b) when excess PFPA residues are removed from the final solution. The retention time for 3,6-di-PFP-morphine is slightly shorter than shown in Fig. 2 having been chromatographed on a slightly shorter column.
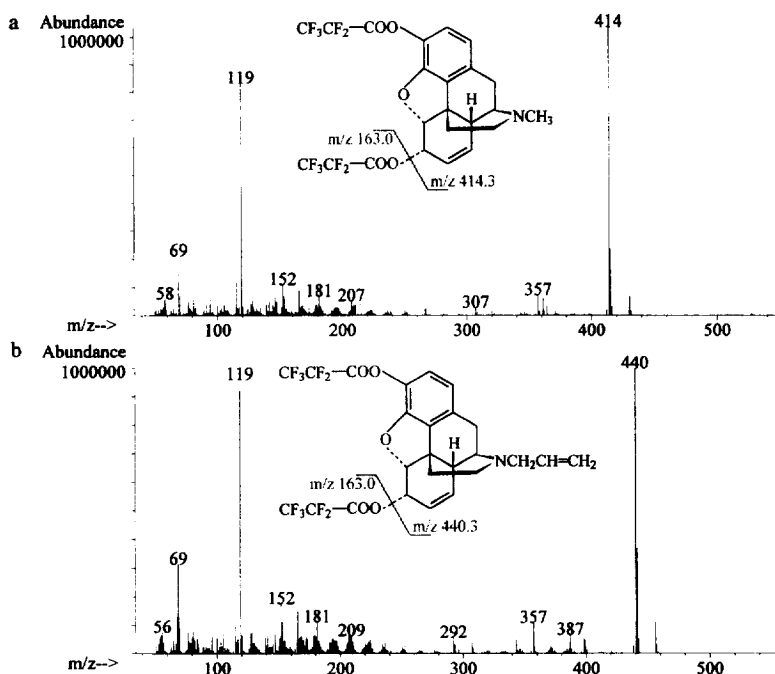
Fig. 5. Mass spectrum and fragmentation patterns for (a) 3,6-di-PFP-morphine and (b) 3,6-di-PFP-nalorphine.

had a slope of 0.012 and correlation coefficient of 0.9994.

Assay of five replicate samples at 0.75, 1.5, 37.5 and 150 ng/ml gave coefficients of variation of 4.9%, 6.8%, 3.0% and 2.3%, respectively.

Blank plasma samples were analysed to check for co-elution and there were no interfering peaks at the elution time of either morphine or the internal standard. The limit of quantitation for morphine was 0.75 ng/ml; this concentration gave a signal to noise

Fig. 6. Standard curve for morphine analysis. Insert: Lower region of the curve 0–15 ng/ml.

ratio of 10 to 1 for m/z 414.3. The limit of detection was estimated to be 0.2 ng/ml ($S/N=3$) expressed as morphine base.

3.5. Application of the method

Clinical studies were carried out where morphine was given either intravenous or by transpulmonary administration, the latter by AERx system (Aradigm Corporation, Hayward, CA, USA) [22]. The assay was established to determine the pharmacokinetic profile of morphine using the AERx device and it was important to maximise the sensitivity of the assay so the most comprehensive profile could be obtained. Blank samples for each subject enrolled in the study were shown not to contain an interfering ion at either 414.3 or 440.3. Thus it was possible to maximise sensitivity by maximising the dwell time on the two ions m/z 414.3 and 440.3. This approach gave a detection limit of 0.2 ng/ml. A typical morphine concentration–time profile (Fig. 7) shows that concentrations of morphine in peripheral arterial
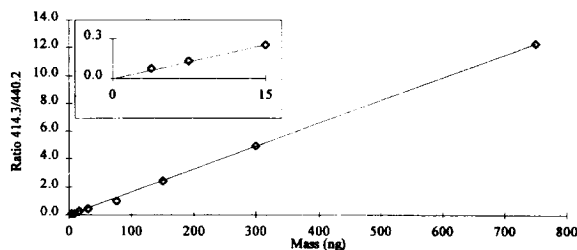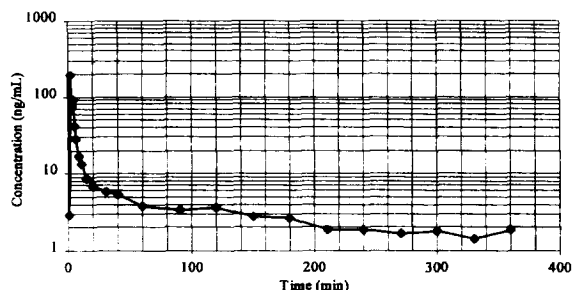
Fig. 7. Application of the method: a morphine plasma concentration–time profile after an intravenous dose of 2 mg morphine.

plasma were measurable after a single intravenous dose of 2 mg for least 360 min.

4. Conclusions

The GC–MS method described here has been used to process more than 2000 blood and plasma samples. All calibration curves had a correlation coefficient greater than 0.99 over the plasma concentration range of 0–750 ng/ml expressed as morphine base. The majority of published GC–MS methods had higher limits of detection than our method and thus were unsuitable for our study. While the method reported by Jones et al. [20] achieved a similar limit of detection, the method reported here requires significantly less sample preparation.

Acknowledgments

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