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Short Communication

Simple method for the determination of morphine and its active glucuronide metabolite in human plasma by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A simple method for the simultaneous determination of morphine and its pharmacologically active metabolite morphine-6-glucuronide in 0.5 ml human plasma is described. It is based on the method of Svensson [*J. Chromatogr.*, 230 (1982) 427 and 375 (1986) 174], but uses only one solid-phase extraction cartridge prior to chromatography and only a 20-μl injection volume. Mean recoveries of 90 and 85% for morphine and morphine-6-glucuronide, respectively, were obtained, the limit of detection being 2 nmol/l (at a signal-to-noise ratio of 3.0).

INTRODUCTION

First isolated in 1803 [1] morphine (M) has been used pharmacologically as a strong analgesic agent for many years. The metabolism of M in man is primarily through glucuronidation by microsomal UDP-glucuronyltransferase to morphine-3-glucuronide (M3G) and, to a lesser extent, to M6G [2]. The latter is pharmacologically active and is thus of greater clinical significance [3].

The application of high-performance liquid chromatography (HPLC), with electrochemical detection, for the measurement of M in biological samples is a recent development. The advantages over the more established radioimmunoassay [4,5], fluorimetric [6] and ultraviolet spectrophotometric techniques [7–9] are ones of specificity and sensitivity.

Many biological substances co-chromatograph with M; it is therefore necessary to incorporate a clean-up stage prior to analysis. One such technique is alcohol extraction [10], although this tends to be relatively inefficient and time-consuming. Another commonly used technique is solid-phase cartridge extrac-

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tion, using two cartridges [8,11,12], but this can also be time-consuming. However, the sample purification technique reported here is rapid, straightforward and cost-effective, using only one Sep-Pak C_{18} cartridge per extraction.

The described procedure utilises two pH ranges; an alkaline extraction phase followed by chromatography at a high pH. Thus the ability to remove potentially interfering substances is increased, resulting in a clean chromatogram.

Some previous HPLC techniques have used amperometric detection [13–16]. However, coulometric detection has been shown to improve sensitivity [12], the compound under investigation being completely oxidized (or reduced) at a given potential. The electrochemical detector, as reported here, is used in the oxidative screen mode.

EXPERIMENTAL

Materials

Morphine sulphate and morphine-6-glucuronide were purchased from Sigma (Poole, UK); sodium dodecylsulphate (SDS) (AR grade), sodium dihydrogenphosphate (AR grade), acetonitrile (HPLC grade), water (HPLC grade) and methanol (HPLC grade) were purchased from FSA Laboratory Supplies (Loughborough, UK); heptane-1-sulphonic acid and pentane-1-sulphonic acid were purchased from Aldrich (Gillingham, UK); Sep-Pak C_{18} cartridges were purchased from Millipore (Watford, UK).

Apparatus

The HPLC system consisted of an SP8800 pump, SP8780 autosampler, Spectrachrom 100 UV–VIS detector and SP4400 integrator (Spectra Physics, Hemel Hempstead, UK), a 5100 A Coulochem detector with a 5011 analytical cell and a 5020 guard cell (Severn Analytical, Shefford, UK) and a C_{18} Nucleosil, 25 cm × 4.6 mm I.D., 5 μm particle size, reversed-phase column (FSA).

Chromatographic conditions

The mobile phase consisted of 10 m*M* sodium dihydrogenphosphate, 1 m*M* SDS and 26% (v/v) acetonitrile, adjusted to pH 2.1 with orthophosphoric acid (SG 1.69). The flow-rate was 1.5 ml/min and the temperature ambient (range 25–30°C). The Coulochem detector potentials were initially set at +0.35 V, +0.45 V and +0.55 V for detector 1, detector 2 and the guard cell, respectively. However, periodical recalibration was necessary to maintain optimum settings. The injection volume of the autosampler was set at 20 μl.

Standard curve

All standards were made from aqueous stock solutions of M and M6G (both 1 mmol/l), which were stored at −70°C for a maximum of three months. Freshly made aqueous standards were prepared on a daily basis, ranging between 0 and 100 nmol/l.

Sample extraction and preparation

Blood samples were collected in 10-ml lithium heparinized tubes. They were centrifuged at 2000 *g*, 4°C for 10 min. The plasma was then decanted off; at least 0.5 ml was required per sample. The collected plasma was then either used immediately or stored at −70°C for up to twelve months. All subsequent work was carried out using disposable polypropylene test tubes.

Prior to use, 0.5 ml of HPLC-grade water was added to 0.5 ml of either sample or standard. This was followed by the addition of 1 ml of 0.2 mol/l borate buffer pH 9.0 and 0.2 ml of 0.1 mol/l pentane-1-sulphonic acid. Between each stage the sample was shaken using a vortex mixer for 15 s.

Sample purification

Sep-Pak C_{18} cartridges were used to prepare samples for HPLC analysis. Prior to use the cartridges were primed with 2 ml of methanol followed by 2 ml of HPLC-grade water. The samples and standards were then passed through the cartridges and the eluates discarded. This was followed by washing through 5 ml of HPLC-grade water to remove molecules of greater polarity than M and M6G. Finally, 1 ml methanol, used to extract the M and M6G, was washed through the cartridge and the eluate collected for analysis. The methanol was evaporated under a stream of oxygen-free nitrogen at 60°C. The dried residues were then reconstituted in 1 ml of mobile phase, 20 μl of which were injected into the HPLC column.

Extraction efficiency

A series of aqueous standards not taken through the procedure outlined above, representing maximum recovery, were used to investigate the extraction efficiency.

Comparison of ion-pair reagents

The three ion-pair reagents investigated were heptane- and pentane-1-sulphonic acid and SDS, which were compared for their morphine-retaining properties. The extraction procedure was as described, except that 4 ml of methanol were used to elute the morphine. Aliquots of 1 ml were collected and analysed.

Stability

Pooled plasma was used to investigate sample stability at ambient temperature. The autosampler was set to sample at regular intervals for up to 60 h, the M and M6G content then being analysed.

Between-run reproducibility

Between-run reproducibility was investigated using pooled plasma as a quality control. One 2-ml plasma aliquot was used per sample run, being stored at −70°C prior to use.

RESULTS AND DISCUSSION

Comparison of ion-pair reagents

SDS was found to be totally unsuitable due to solubility problems. With pentane-1-sulphonic acid all M and M6G was eluted within the first 2 ml of methanol. However, when using heptane-1-sulphonic acid, the elution profiles of M and M6G within methanol were much wider and there was also some loss in the aqueous wash. As a consequence, pentane-1-sulphonic acid was the ion-pair reagent of choice and used in all subsequent extractions.

Optimum reproducibility was obtained by eluting M and M6G in 1 ml of methanol. This had the effect of concentrating the samples and minimised evaporation time. Typical recoveries for a 100 nmol/l standard of M and M6G were 81 and 91%, respectively.

Measurement of M and M6G

Fig. 1 shows chromatograms of peaks for both M and M6G obtained from (A) 50 nmol/l aqueous standard, (B) plasma blank and (C) plasma sample spiked

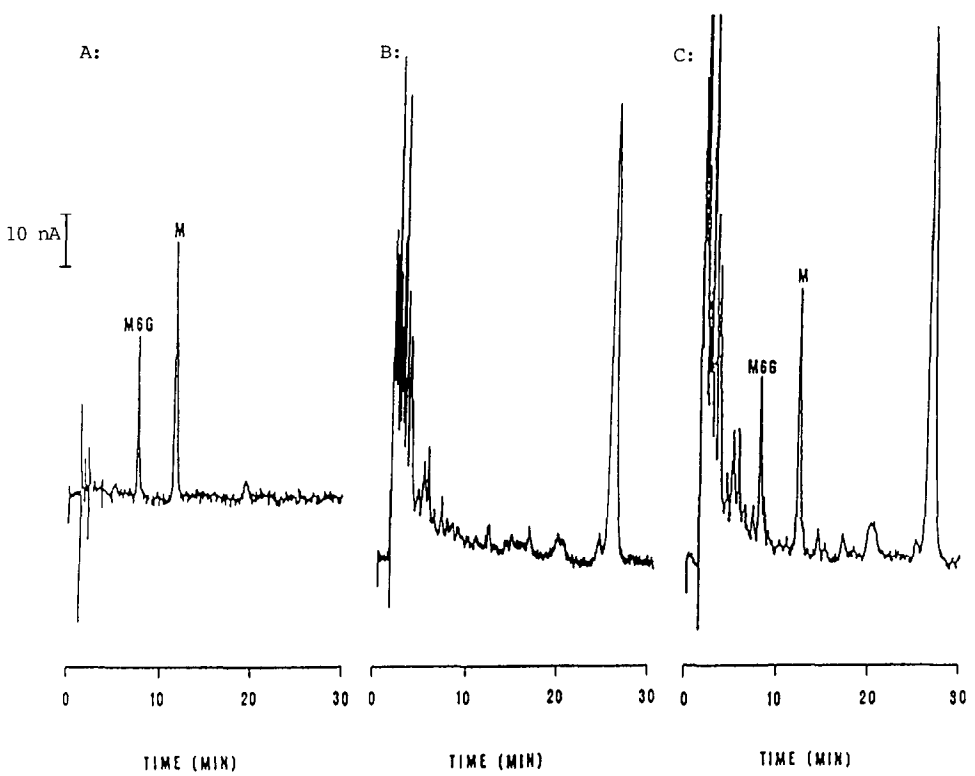

Fig. 1. Chromatogram showing recovery of 50 nmol/l morphine (M) and morphine-6-glucuronide (M6G) standard from plasma. (A) Aqueous standard; (B) plasma blank; (C) plasma spike.

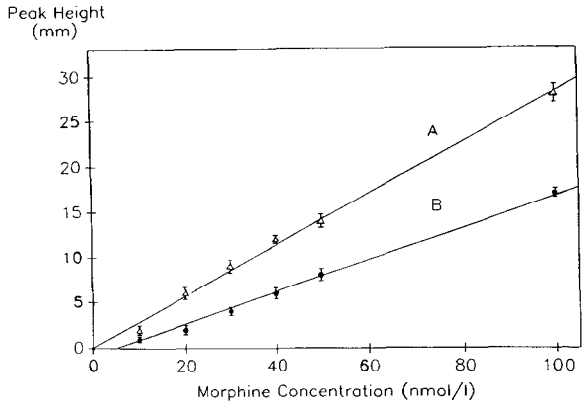

Fig. 2. Standard curve for (A) morphine and (B) morphine-6-glucuronide.

with 50 nmol/l M and M6G. Approximate elution times were 7.5 and 12.0 min for M6G and M, respectively. Detector sensitivity was set at 8 mV.

Standard curve

The standard curve for both M and M6G was linear between 2 and 100 nmol/l (Fig. 2). The linear regression equations for the M and M6G standard curves were $y = 0.28x + 0.07$ ($r = 0.998$) and $y = 0.18x - 0.84$ ($r = 0.996$), respectively. The limit of detection was 2 nmol/l (signal-to-noise ratio = 3.0).

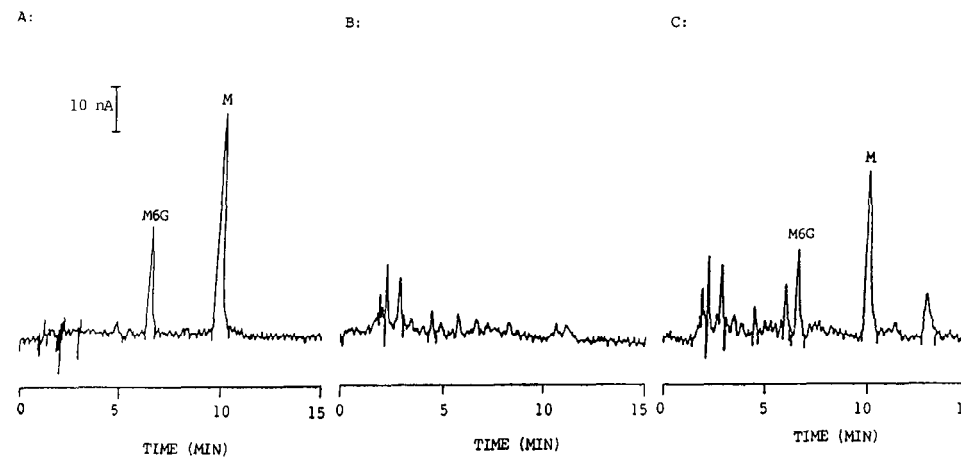

Fig. 3. Typical chromatogram of (A) aqueous standard of 50 nmol/l morphine (M) and morphine-6-glucuronide (M6G), (B) plasma blank and (C) plasma 10 min after an oral dose of a 10-mg bolus of morphine.

TABLE I

MEAN PERCENTAGE RECOVERY FROM SPIKED PLASMA SAMPLES OF MORPHINE AND MORPHINE-6-GLUCURONIDE (n = 9)

Concentration (nmol/l)	Recovery (mean ± S.D.) (%)
Morphine	
2	72.2 ± 21.9
10	96.3 ± 5.8
50[a]	90.0 ± 7.8
Morphine-6-glucuronide	
2	81.5 ± 16.5
10	101.7 ± 6.0
50[a]	85.1 ± 7.7

[a] n = 8.

Stability

For up to 36 h the M and M6G content of the pooled plasma was stable, having a mean ± S.D. of 73.5 ± 1.85 and 8.0 ± 1.20 nmol/l, respectively. If left for longer periods there was a reduction in the measured concentrations of M and M6G. Consequently, all samples were analysed within 36 h of preparation.

Recovery from spiked plasma samples

The following were all passed through the described procedure and analysed for M and M6G: aqueous standards (50 nmol/l); plasma spiked with 50 nmol/l M and M6G; and control "blank" plasma (Fig. 3).

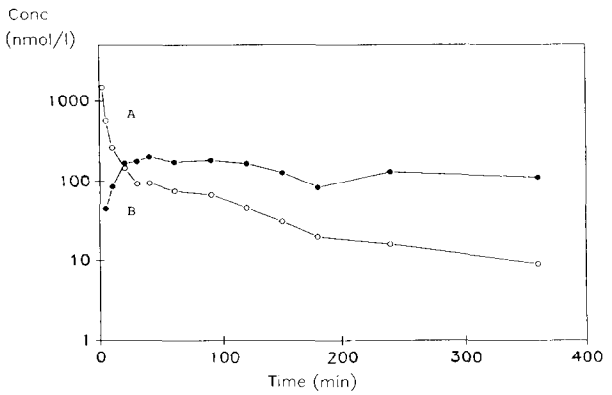

Fig. 4. Time course graph showing plasma levels of (A) morphine and (B) morphine-6-glucuronide after an oral dose of a 10-mg bolus of morphine.

Comparing the spiked plasma samples to the non-extracted standards gave the mean percentage recoveries and standard deviations shown in Table I.

Between-run reproducibility

Over a six-month period, the quality control plasma samples had M and M6G mean values of 61 and 44 nmol/l, the coefficient of variation being 5.3 and 3.4%, respectively.

Determining M and M6G in plasma

The procedure as described was used to determine the concentration of M and M6G in the plasma of patients, after receiving a 10-mg bolus of M (Fig. 4).

Using the procedure as described allowed the measurement of concentrations as low as 2 nmol/l (signal-to-noise ratio = 3.0). At higher concentrations, *e.g.* 50 nmol/l, recovery was 90% for M and 85% for M6G. Even near the limits of detection recovery was 72 and 82% for M and M6G, respectively. Once samples have gone through the extraction procedure they can be left for up to 36 h at ambient temperature before being analysed. This means that a large number samples can be prepared simultaneously and the autosampler programmed to run overnight.

It is possible to analyse sample sizes of less than 0.5 ml or, alternatively, to increase the sensitivity of the assay simply by altering the volume of mobile phase used for reconstitution. Sample sizes as low as 0.2 ml have been used.

The procedure as described is a sensitive, selective and cost-effective means of measuring nanomole concentrations of M and M6G in patients and has been successfully used clinically to quantify these opiates.

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