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Determination of morphine in human plasma by radioimmunoassay utilising a preliminary liquid–solid extraction

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Abstract: Numerous assays are available for the pharmacokinetic study of morphine. Two of these methods are compared with a new assay procedure, which involves a solid-phase extraction to clean up human plasma before radioimmunoassay allowing the determination of morphine levels to a sensitivity of 1 $\mu g\, l^{-1}$. Data are presented to show the importance of correct method choice if clinical studies are to be used in pharmacokinetic interpretations.

Keywords: *Morphine; radioimmunoassay; solid-phase extraction; reversed-phase chromatography; pharmacokinetics.*

Introduction

In the last 15 years many analytical techniques have been developed to describe the disposition of morphine in a variety of clinical situations. A summary of some of these studies and the conflicting morphine levels related to analytical methodology is given in Table 1 [1–6]. Some doubt has been cast on plasma analgesic concentrations [7]. It was suggested that the use of improved assay techniques for analgesics such as morphine had enabled the anaesthetist to administer such agents on a rational basis by providing pharmacokinetic parameters to calculate an appropriate dose. Pharmacokinetic parameters are often reported with little regard for the possible errors associated with the drug assay [8]. Furthermore, computer programmes, themselves potential sources of inaccuracy, may have led to serious underestimates of the dosage requirement assessed by physicians.

Until recently, many published studies in which plasma analgesic concentrations had been measured gave very little information on the assay method used, particularly its specificity and sensitivity. This is especially important in the administration of morphine, as active metabolites of morphine may interfere with the assay. It is well established that

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Table 1
Plasma concentrations of morphine following an intravenous dose of either morphine sulphate or morphine hydrochloride (10 mg base drug/70 kg)

Reference	Method	Concentration ($\mu g\ l^{-1}$) 0.5 h	1.0 h	2.0 h	4.0 h
1	Solvent extraction + RIA	24	17	9	7
2	Solvent extraction + GLC	37	16	11	6
3	Solvent extraction + ^{14}C-counting	32	20	15	—
4	RIA	27	23	18	11
5	RIA	82	53	29	—
6	RIA	77	78	57	38
15	Solvent extraction + HPLC	37	29	22	—

identical samples analysed by different methods or by different laboratories yield different results [7]. For these reasons, this laboratory decided to investigate the specificity of morphine assays in order to generate a more accurate, rapid method allowing the assay of large numbers of clinical samples per day. The range of procedures available to assay morphine is both large and diverse. Gas chromatography–mass spectrometry (GC–MS) [9] is sensitive although slow and operator dependent, while high-performance liquid chromatography (HPLC) methods are convenient and widely available [10–13]. Both of these techniques normally utilise liquid–liquid extractions which may create emulsion problems in many cases. A Kieselguhr-adsorbent type of extraction before radioimmunoassay (RIA) was used by Sandouk *et al.* [14] and although quite specific, it is slow with low productivity. The new technique summarised in Fig. 1 was compared with two established methods of morphine assay, both of which claim to be specific and sensitive; one uses RIA [6] and the other is based on HPLC [15].

Experimental

Materials and reagents

All chemicals used in this work were of analytical grade (BDH, Poole) with the following exceptions: Triton X-100 and sodium metabisulphite were of technical grade (BDH, Poole); methanol, water and acetonitrile were of HPLC grade (Rathburn, Walkerburn, UK); sodium iodide^{125} was obtained from Amersham International (99.5% purity). Morphine sulphate pentahydrate BP (Evans) was dissolved in 30% v/v methanol to produce a stock standard of 1 mg ml^{-1}. This was serially diluted in working assay buffer to provide standard solutions over the range 1–100 $\mu g\ l^{-1}$ morphine base.

Stock buffer consisted of 0.5 mol l^{-1} disodium potassium phosphate at pH 7.5 This was diluted 1 : 10 with distilled water to provide a working buffer, after the addition of 0.5% v/v Triton X-100 and 0.1% w/v sodium metabisulphite.

Drug free human plasma. This was obtained from a volunteer panel under standard fasting conditions within the Clinical Pharmacology Department of the Napp Research Centre. Blood was collected into 10 ml lithium heparin tubes. All reference plasma was processed within 1 h of collection.

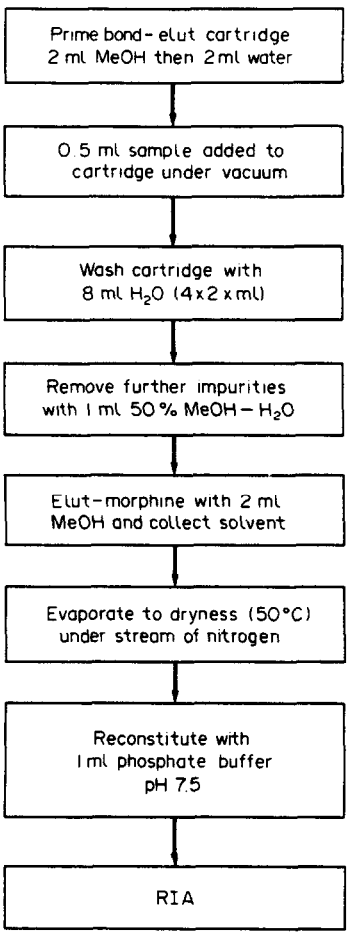

Figure 1

Antisera. These were all commercially available. Goat antimorphine antiserum was supplied as undiluted serum (Bioanalysis, Cardiff). It was stored in a refrigerator at 4°C.

Solid-phase second antibody. Donkey anti-sheep/goat antisera coupled to cellulose beads (Sac-Cel, Wellcome Diagnostics, Dartford) was used undiluted and stored under standard conditions at 4°C in accordance with the manufacturers' recommendations.

Iodination technique

A 1 mg ml^{-1} solution of Iodogen and [1,3,4,6-tetrachloro-3,6-diphenyl-glycouril, Pierce and Warriner (UK) Ltd., Chester] in dichloromethane was diluted 1 : 30 with dichloromethane. A 30 μl volume was pipetted into a small glass vial and the solvent evaporated under a stream of nitrogen. To the reaction vessel was added 10 μl stock buffer with $Na^{125}I$ (1 mCi, Amersham International) and approximately 2 μg morphine sulphate pentahydrate. The contents were mixed with gentle swirling and the reaction allowed to continue at room temperature for 11 min, with intermittent gentle mixing; 0.2 ml of working buffer was then added. An octadecylsilica Bond-Elut Cartridge (Jones Chromatography, Glamorgan) was primed with 2 ml methanol followed by 4 ml distilled water. The contents of the reaction vial were then transferred to the top of the cartridge,

together with four successive reaction vial washings with 0.2 ml working buffer. This volume of approximately 1 ml was forced through the cartridge under positive pressure (Fraction 1). Eight washes each of 2 ml of distilled water were similarly forced through and collected as separate fractions. A small aliquot (10 μl) of each fraction was diluted to 1 ml with buffer and the radioactivity counted. The methanol fractions with the highest count rates were combined and stored at 4°C. For use in the radioimmunoassay, tracer was diluted in working buffer to produce about 30,000 cpm/100 μl (30,000 cpm/assay tube).

Plasma standard and sample purification

Plasma (0.5 ml) was passed through a Bond-Elut C_{18} cartridge (Vac-Elut System, Jones Chromatography). The cartridge was washed with 8 ml of distilled water in 2 ml aliquots and finally 1 ml of 50% *w/w* methanol–water. Morphine was eluted with 2 ml methanol into a previously silanised glass vial. The extract was reduced to dryness at 50°C under a stream of oxygen-free nitrogen. Normally the extract was reconstituted in 1 ml of 50 mM phosphate buffer (pH 7.5).

Radioimmunoassay

Standards, samples or controls (20 μl) were pipetted into previously silanised, round-bottom plastic tubes (LP3, Luckhams, Burgess Hill, Sussex) followed by 100 μl, of both label and antiserum diluted in working buffer.

The antiserum dilution was chosen to produce approximately 50% binding of label at zero dose, typically 1 : 1200. Tubes were vortexed and incubated at room temperature for 15 min; then 100 μl of Sac-Cel was added using an automatic dispenser (Syva). The tubes were mixed and once more incubated at room temperature for 15 min. Finally, 2 ml of distilled water was added and the tubes centrifuged at 2000 g for 5 min.

Supernatant fluid was aspirated and the white precipitate counted for 1 min on a multi-head gamma counter (Multigamma 1260, LKB Ltd., Selsdon, Surrey). The counts per minute versus log concentration for standards were fitted to a spline function, from which results of unknowns were calculated automatically.

Pharmacokinetic study

Following Ethical Committee approval, five healthy volunteers (four female, one male), mean age 23.2 years (range 20–25 years) and mean weight 63.8 kg (range 53–76 kg) took part in the study. The volunteers reported to the Trials Unit at 08.00 hours, having fasted overnight, but being allowed small quantities of water.

A baseline blood sample was taken (10 ml) prior to administration of one 30 mg morphine sulphate tablet by mouth. Blood samples (10 ml) were withdrawn from an in dwelling venous cannula at the following times after dosing: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0 and 24 h. Blood samples were transferred to heparinised tubes and gently mixed. Following centrifugation the supernatant plasma was split into three aliquots and stored at −20°C prior to analysis.

Results

Recovery

Recovery of spiked morphine using liquid–solid extraction followed by RIA averaged 88% in the range 0.4–100 ng ^{14}C-morphine base/ml plasma (Table 2). This was

Table 2
Recovery of [^{14}C]-morphine base from spiked human plasma

Concentration of morphine base (μg l^{-1})		% Mean recovery*
0		80
0.4		112
0.8		79
1.6		122
3.1		89
6.3		81
12.5		79
25		80
50		82
100		78
	Mean	88.2
	SEM	4.95
	RSD (%)	17.8

* Calculated from d.p.m. in total recovered methanol.

measured by diluting fractions from the solid-phase extraction with 5 ml PCS scintillation cocktail (Amersham International).

Counting efficiency was determined by overspiking each fraction with 10,000 dpm C^{14}-hexadecane and recounting each sample.

Reproducibility and recovery experiments

Both the within-batch (Tables 3 and 4) and between-batch (Table 5) reproducibility were studied together with recovery by adding known amounts of morphine base to 1-ml volumes of drug-free plasma. Due to the sigmoidal response of competitive binding experiments it was found necessary to prepare two calibration curves over the ranges 1–6 μg l^{-1} morphine base and 5–20 μg l^{-1} morphine base. Recovery in the lower range averaged 92% and in the upper range 104%. The mean relative standard deviation in the lower range was 15% and in the upper range 13%.

Since at the 1 μg l^{-1} level the RSD was 21.4%, this was set as the limit of detection. The between-batch RSD (Table 5) was assessed on eight separate occasions. The average recovery in the range 5–50 μg l^{-1} was 106%, the average RSD being 21.1%.

The new procedure was evaluated in comparison with two established methods [6, 15]. The levels obtained by RIA [6] were significantly different from those by HPLC [15] and liquid–solid extraction-RIA (LSE-RIA) ($P > 0.001$). LSE-RIA and HPLC [15] were found to be not significantly different from each other ($0.10 > P > 0.05$). Figure 2 illustrates plasma concentrations of morphine with time using the RIA [6] and LSE-RIA methods.

Data analysis

Individual plasma morphine concentrations were fitted to a tri-exponential function as shown in equation (1):

$$C = x \exp(-\alpha t) + y \exp(-\beta t) + z \exp(-\gamma t) \quad (1)$$

where C is the morphine concentration at time t; x, y and z are concentration terms; and α, β and γ are first order rate constants. Parameter values were obtained from the

Table 3
Within-batch reproducibility and recovery

No. of determination	Concentration increment after addition of morphine base ($\mu g\,l^{-1}$) 5	10	20
1	4.1	10.3	19.5
2	4.7	10.3	25.6
3	4.8	10.6	22.5
4	4.7	11.2	17.9
5	4.3	11.3	17.3
6	4.0	10.1	23.1
7	5.1	10.5	20.8
8	6.7	10.2	22.6
9	6.7	9.4	23.4
10	5.3	11.7	18.9
x	5.04	10.6	21.2
SD	0.97	0.67	2.71
RSD (%)	19.1	6.4	12.8
Recovery (%)	100	106	106

Table 4
Within-batch reproducibility and recovery

No. of determination	Concentration increment after addition of morphine base ($\mu g\,l^{-1}$) 1	4	6
1	0.86	2.97	5.21
2	1.24	3.34	5.66
3	1.22	3.52	5.36
4	0.86	3.04	5.93
5	0.84	3.17	5.53
6	0.75	3.55	5.34
7	0.78	4.10	6.17
8	0.84	3.60	6.38
9	0.88	4.62	5.74
10	0.65	4.56	5.33
x	0.89	3.65	5.67
SD	0.19	0.59	0.39
RSD (%)	21.4	16.2	6.9
Recovery (%)	89.2	91.3	94.5

Table 5
Between-batch reproducibility

Concentration increment after addition of morphine base ($\mu g\,l^{-1}$)	Observed mean value ($\mu g\,l^{-1}$)	SD	N	Relative standard deviation (%)
0	0.2	0.3	8	—
5	5.8	2.0	8	34
20	19	2.8	8	14.1
50	54	7.0	8	13.0

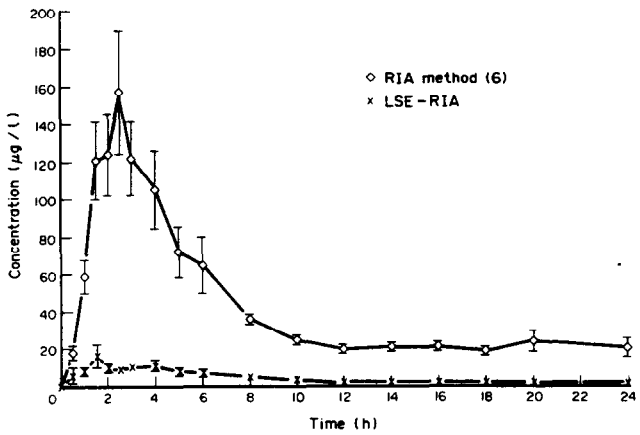

Figure 2

Table 6
Summary of pharmacokinetic data

Parameter	RIA	LSE-RIA	HPLC
C_{max} (μg l^{-1})	157	16	15.4
t_{max} (h)	2.5	1.5	2.0
AUC (μg.h l^{-1})	990	93.5	105
x	29.9	2.7	7.7
y	203	15.1	32.0
z	−233	−17.8	39.8
α (h^{-1})	0.02	0.03	0.09
β (h^{-1})	0.29	0.26	0.47
γ (h^{-1})	1.06	1.48	1.05
Lag time (h)	0.22	0.23	0.17

programme ESTRIP [16]. Key pharmacokinetic parameters obtained from this analysis are shown in Table 6, which illustrates a close agreement between the HPLC and the LSE-RIA methods, whilst highlighting the differences with the RIA method. Using LSE-RIA values, plasma profiles of morphine from 30-mg oral tablet doses were found to be characterised by peak levels of 16 μg l^{-1} occurring at 1.5 h, followed by biphasic elimination with fast- and slow-component half-lives of approximately 0.5 and 2.7 h, respectively.

Discussion

The value of this improved assay for morphine is apparent from Fig. 2. The large discrepancies in levels shown between LSE-RIA, HPLC [15] and RIA [6] pose many problems for valid pharmacokinetic studies.

The RIA technique on its own would appear to have serious limitations in pharmacokinetic studies. In similar studies it was suggested [6] that there may be a lack of specificity of the assay method due to the presence of morphine-6-glucuronide [17]. Recent unpublished observations suggest that morphine-6-glucuronide is not an

important metabolite in single dose morphine studies. However, after multiple doses it is present in amounts sufficient to affect significantly the assayed morphine by the RIA method [6].

In this work the authors have attempted to eliminate some of the problems in correlating blood levels of morphine with analgesic activity, which in earlier publications have caused some confusion [18]. Further work should be undertaken to extract metabolite(s) from biological fluids in clinical studies to facilitate a better understanding of morphine disposition in diseased states.

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