Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 3, pp. 353–360, 1994
Copyright © 1994 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0731–7085/94 $6.00 + 0.00

Evaluation of a differential radioimmunoassay technique for the determination of morphine and morphine-6-glucuronide in human plasma

D.J. CHAPMAN,† S.P. JOEL‡ and G.W. AHERNE*§

† *Biomedical Research Division, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK*
‡ *Imperial Cancer Research Fund, Department of Medical Oncology, St Bartholomew's and Homerton Hospitals, London EC1A 7BG, UK*
§ *Drug Development Section, Institute of Cancer Research, Block E, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5NG, UK*

Abstract: A modified differential radioimmunoassay (RIA) technique for the measurement of morphine and its active metabolite, morphine-6-glucuronide (M6G), in plasma is described. Plasma samples were assayed following appropriate dilution, using a morphine specific antiserum and the results subtracted from those obtained with an antiserum which cross-reacts with both morphine and M6G. The sensitivity of measurement for morphine and M6G was 0.88 and 0.27 nmol l^{-1}, respectively and inter-assay variation ranged from 3.4 to 11.0%. Recovery of morphine and M6G was quantitative over a range of concentrations (1–5000 nmol l^{-1}). The presence of either M6G or morphine-3-glucuronide (M3G) did not affect the recovery of morphine. M6G was quantitatively recovered in the presence of morphine but high concentrations (>1:20) of M3G caused some overestimation of M6G. Results obtained by differential RIA for both morphine and M6G correlated well with the results of HPLC analysis. The assay has been applied to the measurement of M6G in plasma following its administration to human volunteers.

Keywords: *Pharmacokinetics; morphine; metabolites; comparison with HPLC; RIA validation.*

Introduction

Due to its widespread clinical use, and the need for a sensitive analytical technique for its quantitation, morphine was one of the first drug molecules for which radioimmunoassays (RIA) were developed. The major advantages of RIA over classical physico-chemical methods are its sensitivity, speed of throughput and simplicity of operation. The major draw-back with all immunoassays is the potential for interference from structurally related compounds. This is a particular problem with drug RIAs if, like morphine, the parent compound is subjected to extensive metabolism, and the success of these assays is largely dependent upon the specificity of the antiserum used.

Since the first RIA for morphine was described in 1970 [1], a number of anti-morphine antisera have been raised to a range of different immunogens. Predictably, these antisera were found to cross-react by varying degrees with the metabolites of morphine, notably morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) [2]. In an effort to minimize interference from M3G, the major metabolite, antisera were raised against 6-substituted morphine immunogens [3, 4]. As expected, these antisera exhibited low cross-reactivity with M3G, but cross-reacted with M6G by 100% or more. At the time, it was widely believed that M6G was present in negligible quantities [5], but the findings of more recent studies have shown it to be an important metabolite of morphine, especially after oral morphine administration [6, 7]. When these results are considered in the light of information concerning the analgesic and sedative potency of M6G [8, 9], it is apparent that there is a need for the pharmacokinetics to be re-investigated, with particular reference to the formation of M6G and its contribution to the analgesia experienced following treatment with morphine. To assist with these investigations, a specific and robust analytical method capable of analysing low levels of morphine and M6G in plasma is required. Currently, the method of choice is HPLC [6, 10], but this

* Author to whom correspondence should be addressed.

procedure is not without problems being highly labour intensive and having limited sensitivity. A differential RIA for the determination of morphine, M3G and M6G has been described previously [11]. Results from the analysis of spiked samples provided validation for the potential of this technique but the recovery of M6G from spiked samples in the presence of morphine and M3G was not reported.

This report describes the development and validation of a modified differential RIA technique for the analysis of morphine and M6G in human plasma, using two morphine antisera available in our laboratory, and its subsequent application to the investigation of the pharmacokinetics of systematically administered M6G in healthy volunteers.

Materials and Methods

Chemicals

Morphine alkaloid and other opioid standards were purchased from MacFarlan Smith Ltd (Edinburgh). Morphine-3,β-D-glucuronide was purchased from Sigma Chemical Co. Ltd (Poole, Dorset) and morphine-6,β-D-glucuronide from Salford Ultrafine Chemicals Ltd (Manchester). Activated charcoal (Norit A) and Iodogen were both supplied by Sigma, and Dextran T-20 was from Pharmacia/LKB (Milton Keynes). All other RIA reagents were of analytical grade and were purchased from BDH Chemicals Ltd (Poole, Dorset).

[^{3}H]-Morphine (27 Ci mmol^{-1}) was obtained from Amersham International (Amersham, Bucks); Na[^{125}I] was supplied by ICN Flow Laboratories (High Wycombe, Bucks). Optiphase 'Safe' liquid scintillation fluid was purchased from Pharmacia/LKB.

For the RIA, the assay buffer was a 0.05 M phosphate buffer (pH 7.4) containing 6.0 g NaCl, 1.0 g gelatin and 13.0 mg thiomersal per litre of distilled water.

Stock standards of morphine alkaloid and M6G were prepared as 3.30 mmol l^{-1} in absolute ethanol and 2.01 mmol l^{-1} in distilled water, respectively. These were then diluted 1:1000 in assay buffer and stored in 100 μl aliquots at −20°C for up to 2 months.

The charcoal suspension contained 10 g of Norit A and 1 g Dextran T-70 per 400 ml 0.05 M phosphate buffer (pH 7.4).

Synthesis of [^{125}I]-morphine and [^{125}I]-M6G

These radiolabels were prepared according to the method used by Moore and colleagues to synthesize [^{125}I]-morphine [12]. In both cases, the three methanolic fractions with the highest counts were combined and stored for up to 2 months at 4°C. Before use, these fractions were diluted in assay buffer to give 25,000 cpm nominal; i.e. the dilution corresponding to 25,000 cpm/assay tube on the day of preparation of the label.

Production of anti-morphine antisera

Antiserum A (code: HP/S/844 11a) was raised in a sheep in response to an *N*-succinylnormorphine–bovine serum albumin (BSA) conjugate [13], and stored at 4°C. The initial antiserum dilution (that which gave rise to 50% binding of [^{3}H]-morphine at zero dose) was 1:1000 in assay buffer.

Antiserum B (code: GG1 VIIa) was raised in a goat in response to a 6-succinylmorphine–BSA conjugate [4], and stored at 4°C. The initial dilutions of this antiserum were 1:2700 and 1:11000, giving rise to 50% binding of [^{125}]-morphine and [^{125}I]-M6G, respectively. Both antisera can be obtained from Guildhay Antisera Ltd (Guildford).

RIA procedure

The previously published RIA protocol [4] was modified slightly. For use with antiserum A, [^{3}H]-morphine was diluted in assay buffer so as to give 2500 cpm/100 μl (equivalent to 0.15 pmol/tube). Following incubation for 1 h at 4°C and phase separation using dextran-coated charcoal suspension, a 500 μl aliquot of supernatant (bound fraction) was added to 4.5 ml of scintillation fluid. Each vial was counted for 4 min in an LKB 1216 Rackbeta liquid scintillation counter. When iodinated radiolabels were in use with antiserum B, the supernatant was aspirated and the charcoal pellet (free fraction) counted for 100 s in a 12-channel LKB Multigamma II counter. The percentage binding was calculated for the standard samples and data reduction was carried out using a smoothed spline plot and the results calculated by interpolation. M6G concentrations were determined using the following equation:

$$[\text{M6G}]\ \text{nmol l}^{-1} = (\text{result using antiserum B}) - (\text{result using antiserum A}).$$

The cross-reactivity profile for each antiserum was assessed by comparing the ability of

a range of structurally related analogues to displace 50% antibody-bound radiolabel [14]. Theoretical assay sensitivity was defined as being 2 SDs from the mean of 15 replicate tests of the zero standard. Inter-assay variation was assessed by the repeat analysis of quality control samples prepared by pooling patient plasma containing a range of concentrations of morphine, M3G and M6G.

The accuracy of the assays was assessed by determining the recovery of morphine and M6G from artificial mixtures containing morphine, M6G and/or M3G spiked in at levels that might be expected in clinical samples. Percentage recoveries for morphine and M6G were calculated by dividing the actual result by the expected result, i.e. the result that would be predicted taking into consideration the known specificity characteristics of the antiserum in use.

Comparison of HPLC and RIA results for morphine and M6G in plasma

A random selection of plasma samples obtained from healthy volunteers recruited at the Department of Medical Oncology, Homerton Hospital, London were analysed. These subjects had received a single dose of morphine sulphate, and plasma levels of morphine, M6G and M3G measured using a specific HPLC method [10]. These chromatographic results were compared with those obtained using the RIA procedures described in this section.

M6G pharmacokinetics

Approval for these studies was obtained from the Ethical Committee of St Bartholomew's Hospital, London. Six healthy volunteers were dosed with 1 mg/70 kg M6G as an intravenous bolus and serial plasma samples were collected over a 12-h period. These samples were stored at −20°C prior to analysis for levels of morphine and M6G by differential RIA. The plasma M6G concentration–time profiles were analysed using an interactive curve-stripping program, using weighted nonlinear least-squares regression [15].

Results

Antiserum A

Specificity characteristics. The cross-reactivity of this antiserum with a range of structural analogues is shown in Table 1.

Table 1
Percentage molar cross-reactivities of antiserum A

Opioid	Cross-reactivity (%)
Morphine	100
M6G	<0.1
M3G	<0.1
Normorphine	32.1
Codeine	<0.01
Diamorphine	<0.1
Naxolone	<0.1
Buprenorphine	<0.1
Pethidine	<0.1

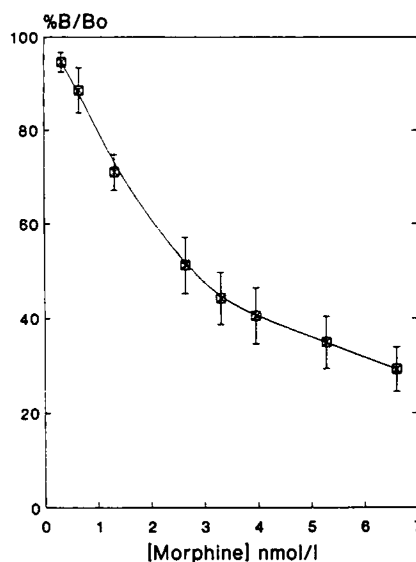

Figure 1
Mean (±SD) of seven separate RIA standard curves using antiserum A and ^{3}H-morphine.

Negligible interference was evident with these compounds, with the exception of normorphine.

RIA performance. The inter-assay variation (mean + SD) for each point (0.33–6.60 nmol l^{-1}) for seven separate standard curves is shown in Fig. 1. The overall mean relative standard deviation (RSD) for this calibration range was 10.4% (2.3–16.1). Non-specific binding was always <6% of the total amount of radioactivity added and the theoretical assay sensitivity was calculated to be 0.88 nmol l^{-1}. Analysis of quality control samples revealed inter-assay RSDs of 3.4 and 5.8% at levels of 37.1 and 347.0 nmol l^{-1}, respectively (n = 15). No matrix effects were observed with the addition of up to 100 μl plasma, and clinical samples diluted in parallel to the standard curve displayed no systematic bias.

Analytical recovery. The recovery of morphine alone and in the presence of increasing concentrations of either M3G or M6G is shown in Tables 2 and 3.

Comparison of HPLC and RIA results for morphine. There was a significant correlation between results obtained using the two methods ($r = 0.9777$; $n = 40$). Linear regression analysis of the data gave the equation $y = 1.15x - 0.85$ (where y = RIA; x = HPLC). These data are presented in Fig. 2.

Antiserum B

Specificity characteristics. The ability of a range of structurally related analogues to displace 50% antibody-bound [^{125}I]-morphine or [^{125}I]-M6G is shown in Table 4. The cross-reactivity of this antiserum with morphine and M6G approached 100% and substantial interference from codeine and diamorphine was also observed. Significantly, cross-reaction with M3G was low (1–2%).

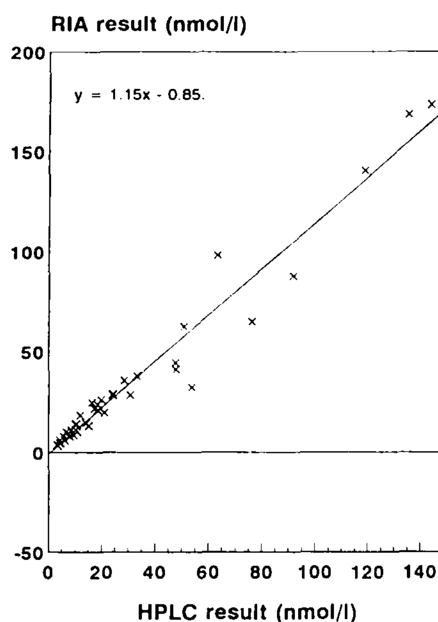

Figure 2
Correlation of morphine concentrations in plasma (n = 40) obtained by HPLC and by RIA using antiserum A and ^{3}H-morphine.

Table 2
Recovery of morphine added to control drug-free plasma using RIA with antiserum A

[Morphine](nmol l^{-1})	Mean % recovery
1	80.7 ± 5.5
5	101.0 ± 2.0
10	105.8 ± 4.2
50	105.9 ± 10.0
100	119.9 ± 7.5
500	104.3 ± 3.3

Mean ± SD; (n = 4).

RIA performance. The inter-assay variation for each point (0.20–4.02 nmol l^{-1}) for seven separate standard curves using M6G as the standard and radiolabel is shown in Fig. 3. Similar curves were obtained using morphine as the standard and radiolabel. The overall mean RSD was 6.1% (2.2–9.8). Theoretical assay sensitivity was calculated to be 0.27 nmol l^{-1}. Analysis of quality control samples revealed inter-assay RSDs of 11.0, 7.5 and 5.4% at levels of 19.1, 215.4 and 303.4 nmol l^{-1}, respectively (n = 10). No matrix effects were

Table 3
Recovery of morphine in the presence of M6G or M3G added to control drug-free plasma using RIA with antiserum A

Molar ratio morphine–metabolite (1 = 100 nmol l^{-1})	% Recovery + M6G	% Recovery + M3G
0:1	ND	ND
1:0	101.2 ± 4.3	111.3 ± 9.9
1:0.1	100.5 ± 3.7	—
1:0.2	97.5 ± 4.0	—
1:0.5	93.2 ± 2.5	—
1:1	99.9 ± 1.4	94.1 ± 7.7
1:2	96.5 ± 4.0	—
1:5	97.5 ± 6.1	108.3 ± 15.0
1:10	98.6 ± 5.2	112.3 ± 13.0
1:25	96.6 ± 2.3	—
1:50	107.0 ± 9.3	104.7 ± 8.8
1:100	107.5 ± 8.2	134.6 ± 19.0

*Mean ± SD; (n = 4).
ND = none detected.

Table 4
Percentage molar cross-reactivities of antiserum B using morphine and M6G radiolabels

Opioid	[^{125}I]-morphine (%)	[^{125}I]-M6G (%)
Morphine	100	79.2
M6G	314	100
M3G	1.0	2.2
Normorphine	0.4	1.0
Codeine	40.0	43.5
Diamorphine	75.2	67.3
Naloxone	<0.01	<0.01
Buprenorphine	<0.1	<0.01
Pethidine	<0.1	<0.01

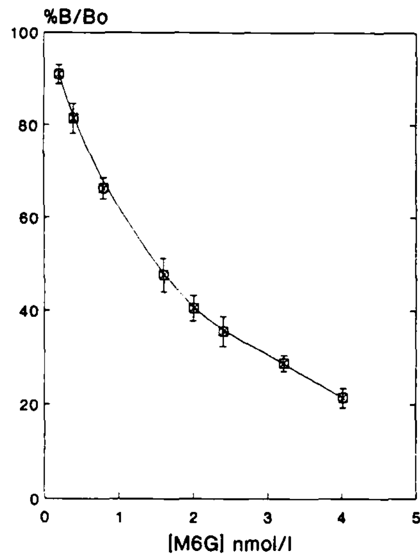

Figure 3
Mean (± SD) of seven separate RIA standard curves using antiserum B and ^{125}I-M6G.

observed with the addition of up to 100-μl of human plasma and clinical samples diluted in parallel with either the morphine or M6G standard curves and displayed no systematic bias.

Analytical recovery. The recovery of morphine, M6G or M3G, and M6G in the presence of increasing quantities of M3G, is shown in Tables 5 and 6. The recoveries of morphine, M6G or M3G alone were similar regardless of

Table 5
Recovery of morphine, M6G or M3G from control drug-free plasma using RIA with antiserum B and M6G as standard and radiolabel

Conc. added (nmol l^{-1})	Mean % recovery of morphine	Mean % recovery of M6G	Mean % recovery of M3G
1	120.7 ± 18.0	122.0 ± 8.5	—
5	92.7 ± 8.2	119.0 ± 10.0	—
10	105.3 ± 7.4	110.0 ± 4.0	—
50	97.7 ± 8.3	104.3 ± 12.0	
100	87.3 ± 5.1	97.3 ± 4.0	1.7
500	99.3 ± 6.1	104.7 ± 9.1	1.2
1000	—	—	1.2
2500	—	—	1.0
5000	—	—	0.8
10000	—	—	1.0

Mean ± SD; (n = 4). Mean of three results.

Table 6
Recovery of M6G in the presence of increasing concentrations of M3G added to control drug-free plasma using RIA with antiserum B and M6G as standard and radiolabel

M6G:M3G ratio (1 = 100 nmol l^{-1})	Result (nmol l^{-1})	Mean % recovery using M6G
1:0	96.9 ± 13	100 (100)
1:1	101.1 ± 9.7	104 (103)
1:5	114.1 ± 4.4	118 (135)
1:10	118.3 ± 6.0	122 (138)
1:20	146.2 ± 8.7	151 (174)
1:40	164.3 ± 9.1	169 (221)
1:50	184.0 ± 7.5	190 (237)
1:100	240 ± 6.9	248 (379)

Mean of four results ± SD.
Results in parentheses are those obtained for the same spiked samples when RIA with antiserum B with morphine as standard and radiolabel.

whether morphine or M6G was used as the standard and radiolabel but, for the sake of brevity, only the data obtained using M6G as the standard and radiolabel are included in Table 5. For the purposes of comparison, data obtained using both morphine and M6G as the standard and radiolabel are shown in Table 6.

Comparison of HPLC and RIA results for M6G. There was a significant correlation between the M6G results obtained on 35 plasma samples using the differential RIA technique described here and those measured by HPLC. Correlation coefficients of 0.9733 and 0.9012, and linear regression equations $y = 1.43x + 3.27$ and $y = 1.08x + 5.94$ (where y = RIA; x = HPLC), were obtained when morphine and M6G were used as the standards and radiolabel respectively. These results are illustrated in Fig. 4.

M6G pharmacokinetics. The mean (±SEM) M6G plasma concentration–time profile for the six volunteers who received 1 mg/70 kg M6G i.v. is shown in Fig. 5 and are similar to those obtained by HPLC analysis [19]. M6G exhibited a bi-exponential decline comprising a rapid distribution phase followed by a more prolonged elimination phase. The mean half-life in the elimination phase was 1.7 ± 0.07 h (mean ± SEM). Low levels of M6G were still detectable in all subjects 12 h after dosing (results not shown). Mean pharmacokinetic parameters calculated following this treatment are included in Table 7. Morphine was undetectable in all the samples taken from these subjects.

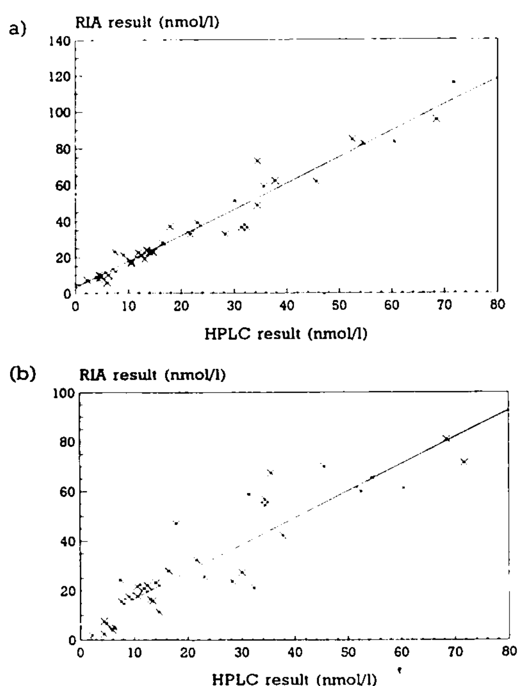

Figure 4
Correlations of M6G plasma concentrations (n = 35) obtained by HPLC and by (a) differential RIA using morphine standardization and ^{125}I-morphine and (b) differential RIA using M6G standardization and ^{125}I-M6G.

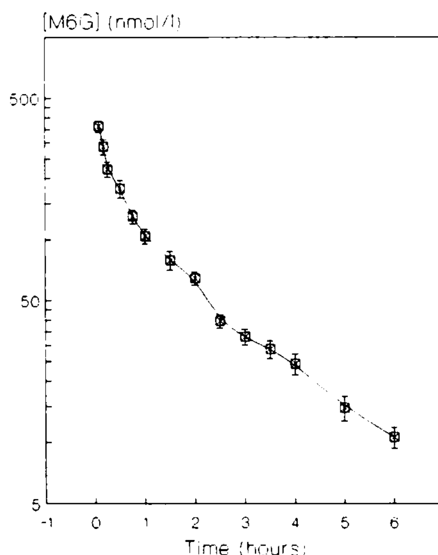

Figure 5
M6G plasma concentrations (mean ± SEM) determined by differential RIA following i.v. administration of 1 mg/70 kg M6G to healthy volunteers (n = 6).

Table 7
Plasma pharmacokinetic data following administration of 1 mg/70 kg i.v. M6G to healthy volunteers

Subject ID	Dose (mg)	C_{max} (nmol l^{-1})	$T_{½}$ (h)	Cl (ml min^{-1})	V_d (l)	AUC (0–∞) (nmol l^{-1} h^{-1})
A	2.43	384.0	1.77	108.9	17.0	453.0
B	1.71	279.2	1.48	64.6	8.4	376.9
C	2.29	402.0	1.71	80.1	11.2	546.7
D	1.73	424.0	1.88	59.3	9.7	414.5
E	2.35	374.0	1.85	128.3	19.7	355.7
F	1.63	308.0	1.51	60.5	8.0	363.1
Mean (±SEM)	2.02 (0.15)	361.9 (23.0)	1.70 (0.07)	83.6 (11.7)	12.3 (2.0)	418.3 (29.7)

Discussion

In order to make confident use of the specificity characteristics of a particular antiserum in a differential RIA technique such as that described here, a thorough investigation of the properties of the antiserum is required. For this reason, in addition to employing the accepted criteria for the assessment of antiserum specificity [14], the recovery of morphine and M6G from a wide range of recovery standards was also determined for both antisera, and morphine and M6G results obtained were compared with those measured using a specific HPLC method.

The characterization of antiserum A (HP/S/844 11a) using [^{3}H]-morphine and its use in an RIA for morphine is described. Cross-reactivity of this antiserum with M3G and M6G was negligible. Interference from normorphine was to be expected, taking into consideration the structure of the immunogen used. However, although *N*-demethylation is a recognized metabolic pathway, significant quantities of normorphine have not been found, even after chronic oral morphine administration [7]. The recovery of morphine over a wide range of concentrations using this assay was excellent, even when relatively high levels of M3G or M6G were also present; and results obtained using the RIA correlated well with those obtained by HPLC. This assay is robust, reliable, reproducible and offers an attractive alternative to other RIAs currently available for the analysis of morphine. In addition, the ability of the method to analyse directly small volumes of plasma, in the absence of any sample pretreatment, and its capacity for a large sample throughput, makes it an excellent overall choice for use in studies investigating the pharmacokinetics of morphine.

The characteristics of antiserum B (GG1 V11a) using [^{125}I]-morphine and [^{125}I]-M6G radiolabels are also described. Cross-reaction with M3G was low (1–2%), but significant interference was observed from codeine and diamorphine. However, concomitant administration of these compounds with morphine is rare and, although codeine is a recognized metabolite of morphine, negligible quantities are produced [16]. Hand *et al.* [11] reported the use of an equivalent antiserum that was found to cross-react with M6G by more than 100%. In an attempt to correct for this, they divided each result by a 'cross-reactivity factor', calculated for each assay. Similar cross-reactivity data were obtained in the present study. It should be remembered that cross-reactivity is conventionally determined in the absence of the analyte, a situation which does not generally occur in the analysis of samples. Our data indicate that both morphine and M6G were accurately recovered over a wide range of concentrations using this assay (regardless of whether morphine or M6G was used as the standard and radiolabel). The results of the recovery of M6G in the presence of morphine and M3G showed that the use of a 'cross-reactivity factor' was not necessary using the present assay. Furthermore, the use of a cross-reactivity factor does not take into consideration the variability in the cross-reactivity of M6G within individual samples, a value that is likely to vary depending on the levels of morphine and M3G present.

In the present study, M6G levels determined by the assay employing morphine as the standard and radiolabel correlated well with those obtained by HPLC, but were consistently higher. Our recovery data show that, although interference from high levels of M3G alone was negligible, the presence of high concentrations of M3G, relative to M6G in the same sample (M3G–M6G > 20:1) can lead to an apparent increase in the recovery of M6G. This possibility was noted in another study employing a similar antiserum [17]. M3G–M6G ratios between 6:1 and 10:1 have been reported following chronic dosing with oral morphine [7]. This potential interference is likely to be less of a problem *in vivo* when morphine is also present. In our modified assay using antiserum B, we used M6G as a standard in place of morphine and for the preparation of the radiolabel. Comparison of the M6G data using these two approaches revealed that this modification alleviated the problem of M3G interference to some extent, and also gave rise to M6G results which showed closer agreement to those obtained by HPLC. We believe that these improvements were due to the standardization of our assay with the metabolite, rather than the less abundant parent drug. Nevertheless, a degree of caution still needs to be introduced when interpreting M6G results obtained using this differential RIA. The findings discussed so far confirm our belief that, in a situation where a mixture of compounds are competing for binding sites on antibodies with

varying degrees of avidity, traditional methods of assessing cross-reactivity alone are not sufficient. In these situations, extensive characterization of the antiserum is required in the form of recovery assessments such as those conducted in this study. This information is of particular importance if the antiserum is being considered as a candidate for use in a differential RIA technique.

The differential RIA technique described in this report was used to follow plasma concentrations of M6G in six healthy volunteers, treated with a potentially therapeutic i.v. dose of M6G, for up to 12 h. The pharmacokinetic data observed for i.v. M6G in this study provide evidence for the high initial plasma concentrations, small volume of distribution, and low clearance of M6G. In addition, the terminal half-life (1.7 h) of i.v. M6G is not significantly different from that measured for morphine and metabolically formed M6G in an earlier study [18]. It was also of interest to note the absence of morphine following the systemic administration of M6G in these subjects. These findings are in agreement with those obtained in two studies investigating the kinetics of M6G in patients with normal renal function, in which HPLC was used as the analytical tool [9, 19].

In summary, this report describes the validation of a modified differential RIA technique for the determination of morphine and M6G in samples of human plasma, using two morphine antisera available in our laboratory. Potential problems associated with the original method proposed by Hand *et al.* [11] have been highlighted, and the assay improved by the use of M6G in place of morphine as the assay standard and radiolabel. In the absence of a specific antiserum for M6G, differential RIA remains the only immunoassay technique currently available for the determination of M6G, and the advantages of RIA as an analytical tool makes this assay a useful alternative to existing methodologies. A specific M6G antiserum for use in an immunoassay has now been produced in our laboratory [20], and the development and validation of immunoassays suitable for M6G pharmacokinetic studies will be described in a later paper.

Acknowledgements — We would like to thank South West Thames Regional Health Authority and the Royal Marsden Hospital for financial support.

References

[1] S. Spector and C.W. Parke, *Science* **168**, 1347–1348 (1970).
[2] G.W. Aherne, *Int. Congress Symp. Ser. Royal Soc. Med.* **58**, 21–29 (1983).
[3] S. Spector, B. Berkowitz, E.J. Flynn and B. Peskar, *Pharmacol. Rev.* **25**, 281–291 (1973).
[4] G.W. Aherne, E.M. Piall, J.D. Robinson, B.A. Morris and V. Marks, in *Radioimmunoassay for Clinical Biochemistry* (C.A. Pasternak, Ed.), pp. 81–91. Heydon and Son, London (1975).
[5] U. Boerner, S. Abbott and R.L. Roe, *Drug Metab. Rev.* **4**, 39–73 (1975).
[6] J.O. Svensson, A. Rane, J. Sawe and F. Sjoquist, *J. Chromatogr. (Biomed. Appl.)* **230**, 427–432 (1982).
[7] J. Sawe, J.O. Svensson and A. Rane, *Br. J. Clin. Pharmacol.* **16**, 85–93 (1983).
[8] K. Shimomura, O. Kamato, S. Veki, S. Ida, K. Oguri, H. Yoshimura and H. Tslikamato, *Tohoku J. Exp. Med.* **105**, 45–52 (1971).
[9] R. Osborne, S. Joel, D. Trew and M. Slevin, *Lancet* **1**, 828 (1988).
[10] S.P. Joel, R.J. Osborne and M. Slevin, *J. Chromatogr. (Biomed. Appl.)* **430**, 394–399 (1988).
[11] C.W. Hand, R.A. Moore, H.J. McQuay, M.C. Allen and J.W. Sear, *Ann. Clin. Biochem.* **24**, 153–160 (1987).
[12] R.A. Moore, D. Baldwin, M.C. Allen, P.J.Q. Watson, R.E.S. Bullingham and H.J. McQuay, *Ann. Clin. Biochem.* **21**, 318–325 (1984).
[13] B.A. Morris, J.D. Robinson, E. Piall, G.W. Aherne and V. Marks, *J. Endocrinol.* **64**, 6–7 (1974).
[14] G.E. Abraham, *J. Clin. Endocrinol. Metab.* **29**, 866–870 (1969).
[15] A. Johnson and R.C. Woollard, *J. Pharmacol. Meth.* **9**, 193–199 (1983).
[16] S.Y. Yeh, *Experimentia* **30**, 264–266 (1974).
[17] M.P. Shelly, B.L. Taylor, K.G. Quinn and G.R. Park, *Anaesthesia* **43**, 733–737 (1988).
[18] P.J. Hoskin, G.W. Hanks, G.W. Aherne, D. Chapman, P. Littleton and J. Filshie, *Br. J. Clin. Pharmacol.* **27**, 499–505 (1989).
[19] R. Osborne, P. Thompson, J. Joel, D. Trew, N. Patel and M. Slevin, *Br. J. Clin. Pharmacol.* **34**, 130–138 (1992).
[20] G.W. Aherne, M. Cross, D.J. Chapman, A. Hardcastle, S.P. Joel and M.L. Slevin ML, *Proc. Am. Assoc. Cancer Res.* **33**, 558 (1992).

[*Received for review 13 July 1993*]