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Morphine Pharmacokinetics: GLC Assay *versus* Radioimmunoassay

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Abstract □ The validity of a radioimmunoassay (RIA) for research on the pharmacokinetics of morphine has been questioned because of the possible measurement of cross-reactive metabolites. An RIA using antiserum derived from the 3-*O*-carboxymethylmorphine hapten was compared with a specific GLC assay in the measurement of plasma morphine concentrations in humans. The ratio of values for morphine concentrations measured using RIA and those measured using GLC was determined. The RIA values resulted in a 27% overestimation of this ratio. This overestimation did not significantly affect the values for terminal elimination half-life, volume of distribution at steady state, or total body clearance that were derived using results from each assay and model-independent pharmacokinetic techniques.

Keyphrases □ Morphine—pharmacokinetic determination from radioimmunoassay and GLC assay compared □ Pharmacokinetics—morphine, determination from radioimmunoassay and GLC assay compared □ Radioimmunoassay—morphine, comparison with GLC assay, pharmacokinetics □ GLC—morphine, comparison with radioimmunoassay, pharmacokinetics

The radioimmunoassay (RIA) for morphine, first described by Spector *et al.* (1, 2), has been used to characterize the pharmacokinetic profile of morphine (3, 4). A major concern in using any immunoassay for pharmacokinetic research is its accuracy in measuring the true drug concentration. For antibody generated in one laboratory (at morphine concentrations of 20 ng/ml), at least eight times more morphine-3-glucuronide than morphine was required to produce equivalent displacement of the labeled dihydromorphine (5). At 40 ng/ml, more than 32 times more morphine-3-glucuronide was required for an equivalent displacement. Similar results were obtained when relatively high morphine concentrations (1.8–3.1 μg/ml) were measured in rats using the RIA and a specific fluorometric assay (6). This concentration range markedly exceeds that occurring after therapeutic doses in humans. Catlin (7) questioned the validity of RIA for pharmacokinetic analysis, demonstrating a variability in the specificity of the antibody and interference from morphine metabolites that can result in discrepant interpretations.

Because of the limited sensitivity of currently available analytical methods for measuring morphine, it previously has not been possible to validate the accuracy of RIA for morphine in humans because of the low plasma concentrations (1–50 ng/ml) of morphine attained following pharmacological doses of morphine (0.15 mg/kg). A new specific and sensitive GLC assay was used to reevaluate plasma samples of morphine obtained in a previous pharmacokinetic study that were originally analyzed by RIA. The morphine concentrations measured by the different assays were then compared.

EXPERIMENTAL

Plasma Analysis—Plasma samples that were analyzed in a previous study on morphine pharmacokinetics using RIA were reevaluated using a specific GLC morphine assay. The analysis was undertaken on five of the six subjects who had received 10 mg of intravenous morphine sulfate and on four of the five subjects who had received 10 mg of intramuscular morphine sulfate. All samples had remained frozen at −30° until the time of assay. The GLC assays were performed ~2 years after the RIA measurements. There was no evidence of sample deterioration during this time. Demographic characteristics of the patient population and the drug administration protocol were described earlier (4).

Morphine concentrations in plasma were quantitated using RIA and rabbit antimorphine antisera[1] as described previously (4). The detection limit of this assay was 1 ng/ml, and the pooled coefficient of variation was 8.3% for a series of two to three identical samples containing known concentrations of morphine ranging from 1.0 to 45 ng/ml. Although the specificity of the antisera was not assessed, previous reports of antisera from the same source described its relative affinity for morphine, morphine metabolites, and other opiate alkaloids (5). Morphine concentrations in plasma were also determined using the GLC method described by Edlund (8). The detection limit of the assay was 1 ng/ml. The coefficient of variation of the assay was 4 and 10% at 62 and 0.8 ng/ml, respectively.

Data Analysis—Three analyses were performed on the data to determine differences between the two assays and the consequences of these differences on the derived pharmacokinetic values for morphine. The first analysis used linear regression through the origin (9) to compare the morphine concentrations measured with the two assays. Only plasma morphine concentrations <60 ng/ml were used, which included 118 of 138 possible data pairs. The distribution of the 20 data points in the 70–350-ng/ml concentration range was not sufficiently uniform for accurate regression analysis. The excluded data points represented the high morphine concentrations that occurred immediately after the 2-min rapid intravenous infusion. To determine the possible influence of metabolites on the RIA at low concentrations, linear regression through the origin was performed on only those concentrations measured 1 hr after drug administration. In both regression analyses, the 95% confidence interval of the slope was computed to determine if the slope differed significantly from 1.

In the second data analysis, the relative precision and bias of the RIA, as compared with the GLC morphine assay, were determined using approaches suggested previously (10). Relative precision measures the deviation or prediction error of the morphine concentration determined using the RIA compared to the value measured by GLC. This prediction error may have a systematic component called relative bias. The relative bias is the degree to which the typical RIA prediction is either too high

[1] Obtained from Dr. Sidney Spector, Roche Institute of Molecular Biology, Nutley, N.J.

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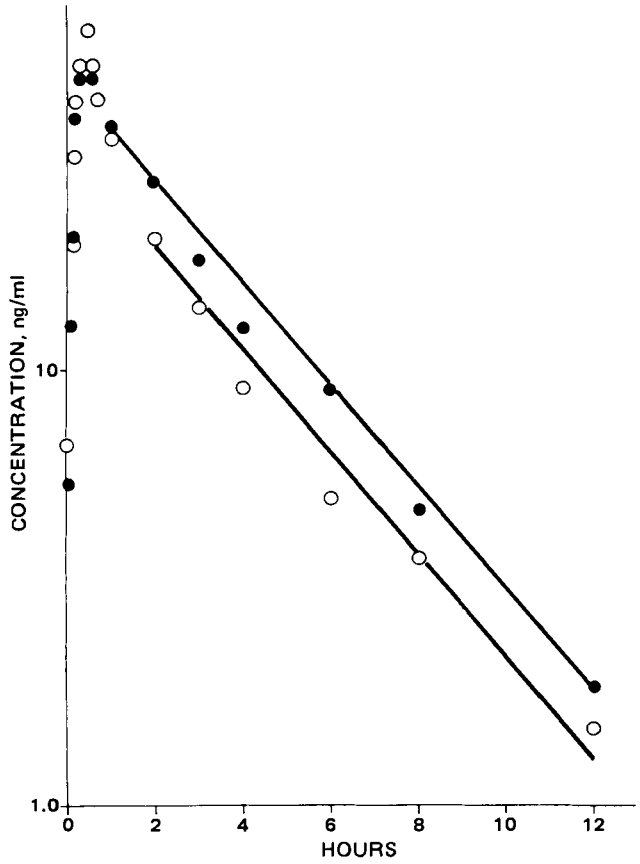

Figure 1—*Plasma concentration data determined by RIA (●) and GLC (○) versus time, for one subject receiving morphine sulfate 10 mg intramuscularly. Lines represent the terminal elimination phases characterized by linear regression.*

or too low. A measure of relative precision is the mean squared prediction error (*MSE*):

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(\text{RIA} - \text{GLC})^2 \qquad \text{(Eq. 1)}$$

where n is the number of data points.

A measure of relative bias is the sample mean prediction error (*ME*):

$$ME = \frac{1}{n}\sum_{i=1}^{n}(\text{RIA} - \text{GLC}) \qquad \text{(Eq. 2)}$$

In the following relationship, the *MSE* is composed of bias (*ME*) and random, nonsystematic error:

$$MSE = ME^2 + \frac{1}{n}\sum_{i=1}^{n}[(\text{RIA} - \text{GLC}) - ME]^2 \qquad \text{(Eq. 3)}$$

in which the last term of the equation is an estimate of the variance of the prediction error and thus a measure of the random error. The relative precision (*MSE*), bias (*ME*), and their 95% confidence interval, along with the proportion of the precision that was bias and random error, were determined using all the values for morphine plasma concentrations that were <60 ng/ml and all the values for 1 hr after drug administration.

In the third data analysis, pharmacokinetic parameters for each individual patient were computed. Model-independent approaches were

Table I—Comparison of Relative Assay Precession and Bias

	All Data <60 ng/ml	Data after 1 hr
Relative precession (*MSE*), ng/ml	27.8	20.4
95% confidence bounds	18.1–37.5	13.6–27.2
Relative bias (*ME*), ng/ml	1.97	3.21
95% confidence bounds	1.02–2.92	2.41–4.01
Components of the *MSE*		
Relative bias, %	14	50
Random error, %	86	50

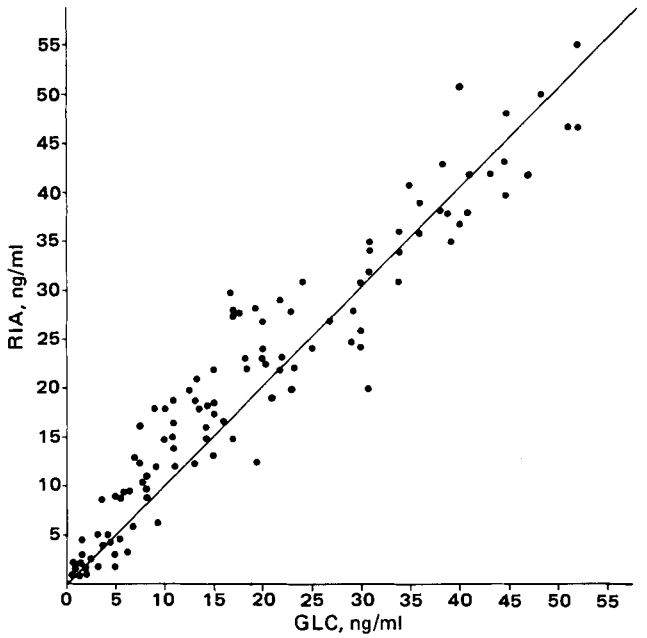

Figure 2—*Regression through the origin using all the morphine concentrations below 60 ng/ml as determined by RIA and GLC. The line represents linear regression.*

applied to the morphine concentration data derived from the two assays. Linear regression of the log plasma concentration *versus* time data was used to determine the apparent terminal elimination half-life. The plasma concentration values that occurred 1 hr after drug administration (*i.e.*, when distribution or absorption was complete) were used. Total body clearance was determined by dividing the dose by the area under the plasma concentration *versus* time curve, determined by the linear trapezoid rule. Complete bioavailability was assumed for intramuscular administration of morphine on the basis of a previous study (4) that demonstrated comparable area under the curves from intravenous and intramuscular administration in the same subject. A model-independent estimate of the volume of distribution at steady state was determined

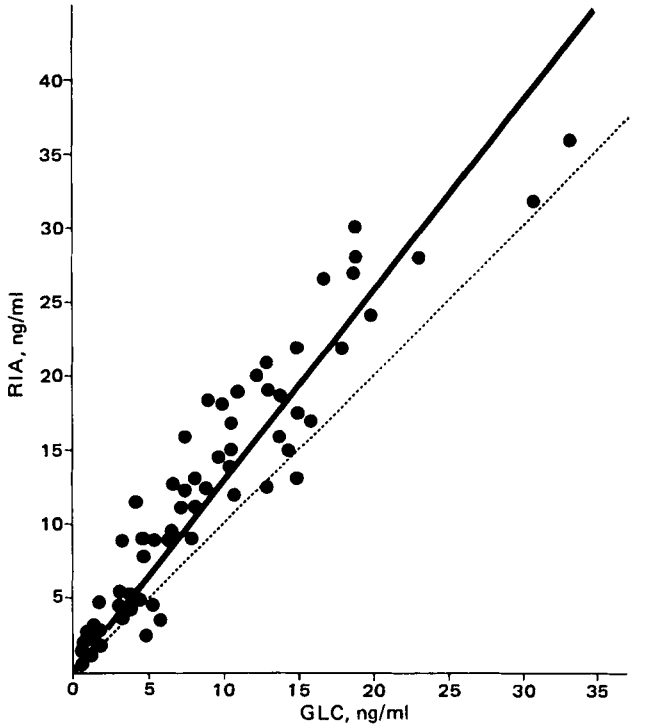

Figure 3—*Regression through the origin of the morphine concentrations as determined by RIA and GLC. Only the values obtained 1 hr after drug administration were used. Linear regression (—) and identity (- - - -) are also shown.*

Table II—Pharmacokinetic Parameters Derived from RIA and GLC Assays

Subject	Age, yr	Weight, kg	Dose, mg	Elimination Half-Life, min			Total Body Clearance, ml/kg/min			Volume of Distribution at Steady-State, liters/kg		
				RIA	GLC	Difference	RIA	GLC	Difference	RIA	GLC	Difference
1	25	64	10 iv	169	190	−21	11.4	15.8	−4.4	2.48	3.35	−0.87
2	28	84	10 iv	159	159	0	14.4	13.8	+0.6	2.72	2.51	+0.21
3	29	80	10 iv	198	233	−35	14.7	17.4	−2.7	3.73	4.48	−0.75
4	28	64	10 iv	184	157	+27	17.1	23.9	−6.7	3.57	3.44	+0.13
4	28	64	10 im	158	153	+5	12.3	14.7	−2.4	2.56	2.11	+0.45
5	34	75	10 iv	183	288	−105	12.3	13.2	−0.9	2.07	4.89	−2.82
5	34	75	10 im	166	234	−68	11.3	12.1	−0.8	2.46	3.28	−0.81
6	29	100	10 im	161	195	−34	12.0	15.0	−3.0	2.97	2.81	+0.16
7	32	76	10	158	161	−3	11.1	11.5	−0.4	2.30	2.44	−0.14
Mean	29.7	75.7		170	197	−26	13.0	15.2	−2.3	2.76	3.25	−0.49
±*SD*	3.0	11.6		14	47	41	2.0	3.7	2.3	0.56	0.89	1.0

using the first statistical moment described by Benet and Galeazzi (11). An appropriate correction for the intramuscular absorption phase was made in the calculation of the volume of distribution at steady state. A paired Student *t* test was used to determine if there was a significant ($p \leq 0.05$) difference in the pharmacokinetic parameters derived from the RIA and GLC assays.

RESULTS AND DISCUSSION

Examining the individual curves of plasma morphine concentration *versus* time in four of five subjects receiving intravenous morphine, and in three of the four receiving intramuscular morphine, the RIA gave consistently higher plasma concentrations than the GLC assay for time periods 1 hr after drug administration. Figure 1 represents the plasma morphine concentrations determined by the two assays in a subject who received intramuscular morphine.

In the first data analysis, when all plasma concentrations <60 ng/ml were compared (118 data pairs) using regression through the origin, the slope of the regression line was 1.03 and the 95% confidence interval was 0.993–1.064 (Fig. 2). This indicates that the ratio of RIA/GLC morphine concentrations did not differ significantly from 1 when measured with the two assays. When only the data after 1 hr were evaluated (66 data pairs), the slope of the regression through the origin was 1.27 and the 95% confidence interval was 1.23–1.30, indicating that the ratio of RIA/GLC plasma concentrations differs significantly from 1 (Fig. 3). This results in a 27% overestimation of the true morphine concentration by RIA.

In the second data analysis (Table I), the measures of RIA *versus* GLC assay relative precision (*MSE*) and bias (*ME*) are given. The relative biases differ significantly from zero for all plasma morphine concentrations <60 ng/ml and for plasma morphine concentrations 1 hr after drug administration. The plasma concentration data 1 hr after drug administration represents a greater portion of the *MSE* than can be attributed to bias relative to all the plasma morphine concentration data <60 ng/ml.

From the third data analysis, Table II shows the individual pharmacokinetic parameters derived by model-independent techniques for each patient and assay. The GLC assay resulted in a longer mean apparent elimination half-life, higher mean total plasma clearance, and a larger mean apparent volume of distribution at steady state. These differences were not statistically significant when evaluated with a paired *t* test.

Comparison of the morphine concentration determined by RIA and GLC demonstrated a 27% overestimation of the true morphine concentration by RIA at time intervals 1 hr after drug administration. The RIA was significantly less precise than the GLC assay, and had a greater systematic bias for morphine concentrations obtained 1 hr after drug administration. While the morphine antiserum samples have less affinity for morphine metabolites (specifically the main metabolite, morphine-3-glucuronide), they can affect the accurate measurement of morphine with the RIA in several ways. The standard curves for morphine and morphine-3-glucuronide are not parallel, resulting in dose-dependent inhibition ratios of morphine to morphine-3-glucuronide (7). In addition, there is also evidence that the elimination half-life of morphine-3-glucuronide is longer than that of the parent drug in rabbits (7) and humans (3). A previous report (12) showed that morphine metabolites present at concentrations 8–12 times higher than the true morphine concentration 1 hr after drug administration. Overestimation of the true morphine concentration 1 hr after drug administration probably results from cross-reaction of morphine-3-glucuronide with the antiserum samples when true morphine concentrations are lower than the metabolite concentrations.

Overestimation of plasma morphine concentrations by the RIA did not affect derived pharmacokinetic values. The elimination half-life calculated using the values from the GLC assay did not differ markedly from that determined using RIA values. The higher clearance and larger volume of distribution of morphine calculated using the results of the GLC assay reflect the lower plasma concentrations measured by GLC. While statistical differences were not present for the morphine elimination half-lives, clearances, and volumes of distribution derived from the two assays, the variability (standard deviation) of the mean of differences between individual pharmacokinetic values derived from each assay was large and the sample size was small. Both variability and sample size contribute to the probability of detecting a difference in the statistical analysis.

The major mechanism by which morphine is removed from the body is hepatic metabolism. Morphine's high hepatic extraction ratio (13, 14) indicates that clearance is very dependent on hepatic blood flow. The difference in mean total morphine clearance between the two assays (12.9 *versus* 15.3 ml/kg/min) was relatively small given the variability in hepatic perfusion that can exist in humans. The difference in mean volume of distribution between the two assays (2.76 *versus* 3.25 liters/kg) was also small. The differences in derived values for pharmacokinetics between the two assays was minimal and does not affect the interpretation of the fate of morphine in the human body.

Overestimation of the morphine concentration by RIA can be minimized by use of antiserum samples that exhibit minimal cross-reactivity with morphine-3-glucuronide (15, 16).

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Peak Homogeneity Determination for the Validation of High-Performance Liquid Chromatographic Assay Methods

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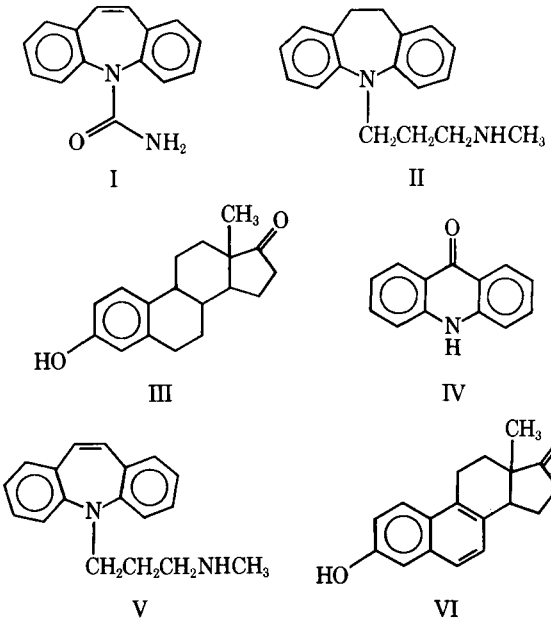
Abstract □ To validate high-performance liquid chromatographic assay procedures with regard to specificity, methods were developed to determine the homogeneity of the chromatographic peaks. These methods employed a rapid-scanning UV-visible spectrophotometer to monitor the chromatographic effluent. The absorption data were processed to nullify the signal due to the drug substance specifically, while allowing the detection of coincident impurities. Results from three model systems indicated the ability of these methods to detect as little as 0.1% of a coincident impurity.

Keyphrases □ High-performance liquid chromatography—validity, determination of peak homogeneity □ Impurities—high-performance liquid chromatography, determination by measurement of peak homogeneity □ UV spectrometry—use in determination of peak homogeneity of high-performance liquid chromatographic assays

The use of high-performance liquid chromatography (HPLC) for quantitative analyses of pharmaceuticals has been increasing rapidly (1). HPLC offers excellent sensitivity, accuracy, and precision, as well as convenience. Perhaps the most significant advantage of HPLC is the specificity obtained, since the drug substance is assayed following separation from any impurities. It is this specificity which has led to the acceptance of HPLC methods for stability-indicating assays. Naturally, the validity of such procedures is dependent on the homogeneity of the chromatographic peak of interest. In ordinary practice, a procedure is considered sound in this regard if the chromatographic peak representing the drug-substance is resolved from all known or theoretical synthetic impurities as well as decomposition products (2). Such indirect methods do not actually examine the homogeneity of the peak and are limited in scope to compounds previously identified as potential impurities. However, homogeneity, within specified limits, can be shown for any chromatographic technique, if it can be demonstrated that a critical physical property of the peak in question does not change with time. For example, GC peak homogeneity can be shown by using rapid-scanning mass spectrometers as specific detectors to demonstrate the constancy of the mass spectrum of the eluting peak with time (3, 4).

This report presents a similar method which evaluates the homogeneity of HPLC peaks directly, by monitoring the constancy of the UV–visible (UV/VIS) absorption spectrum of the moving eluting substance without the use of a stopped-flow apparatus, which examines only a small portion of the peak of interest. Specifically, it is the ratio between absorbances at specified wavelengths in the absorption spectrum of the eluted peak which is examined. Homogeneity is demonstrated by the fact that for sufficiently dilute solutions of a pure substance, the ratio of absorbances should remain constant, regardless of concentration throughout the chromatographic peak. To accomplish this, a rapid-scanning, microcomputer controlled UV/VIS spectrophotometer was employed to examine the chromatographic effluent. Several reports have appeared (5–9) concerning the use of rapid-scanning spectrophotometers as detectors for HPLC. This report represents the first application of these detectors for HPLC method validation in pharmaceutical analysis. Results obtained for three model systems are described; each of these systems contains a drug substance: carbamazepine (I), desipramine (II), or estrone (III), plus a representative impurity designed to coelute.



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