Comparison of Liquid Chromatography–Mass Spectrometry and Radioimmunoassay for Measurement of Fentanyl and Determination of Pharmacokinetics in Equine Plasma

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Abstract

This study evaluated the validity of measuring fentanyl concentrations in equine plasma using radioimmunoassay (RIA) by comparing it to the established technique of liquid chromatography–mass spectrometry (LC–MS). Equine plasma samples were analyzed using a solid-phase Coat-A-Count fentanyl RIA and a validated LC–MS method. The fentanyl concentrations derived by both methods were compared by linear regression and pharmacokinetic analysis. The cross-reactivity of the primary equine fentanyl metabolite, N-[1-(2-phenethyl-4-piperidinyl)]maloanilinic acid (PMA), with the RIA was determined. The binding potency of fentanyl and PMA were compared at three opioid receptor subtypes in equine cerebral cortex using a radioligand binding technique. Fentanyl concentrations determined by RIA and LC–MS correlated, but the RIA overestimated low fentanyl concentrations and underestimated high fentanyl concentrations. The overestimation of low fentanyl concentrations is most likely due to the 29% cross-reactivity of PMA with the RIA. As a result, pharmacokinetic variables determined from an intravenous fentanyl bolus to four anesthetized horses differed depending on the analytical method. Although fentanyl bound with nanomolar potency to the three receptor subtypes, PMA exhibited no binding activity even at micromolar concentrations. In conclusion, when compared with LC–MS, fentanyl concentrations determined by RIA in equine plasma are misleading, especially for the calculation of fentanyl pharmacokinetics.

Introduction

Fentanyl is a synthetic mu-receptor agonist of the 4-anilinopiperidine series that is used commonly as an analgesic and adjunct to inhalant anesthesia in several species (1,2). Significant recent interest has focused on evaluating it as an analgesic in horses and two recent reports characterize the drug concentration profile following intravenous fentanyl in horses (3,4). It is of interest and clinical relevance that the pharmacokinetic parameters derived from measured drug values differ in the two reports; for example, in a study by Thomasy et al. (3), the fentanyl elimination t½, Vd(ss), and MRT were 40–60% of values reported by Maxwell et al. (4). Although the reasons for this are not known, the variability may be partially attributed to the different media from which the drug was measured (serum vs. plasma) or inconsistencies in measurement of values during the terminal distribution phase where fentanyl concentration was close to the limit of the detection system. Notably, two different assay techniques were used by the authors in the prior reports: Maxwell et al. (4) used the radioimmunoassay (RIA), and Thomasy et al. (3) used liquid chromatography–mass spectrometry (LC–MS) for the measurement of fentanyl concentrations. We hypothesize that it was largely this latter difference that contributed to the differences in pharmacokinetic parameters in the two prior reports.

The use of RIA to determine serum or plasma fentanyl concentrations is well-established in humans and non-human primates. In these species, fentanyl is metabolized by N-dealkylation to norfentanyl, a compound that has been reported to have less than 10% cross-reactivity with the RIA (5). In horses, fentanyl is primarily metabolized by oxidation of the propionyl side chain to N-[1-(2-phenethyl-4-piperidinyl)]maloanilinic acid (PMA) (5). Previous reports have shown that the RIA can be used to estimate PMA in horse urine suggesting some cross-reactivity of the assay for this metabolite (6). Others have suggested that this cross-reactivity is in the same range as for the human metabolite, approximately 10% (4,7). Although this might represent the average interference, the degree of cross-reactivity between the fentanyl and PMA and the subsequent
impact of this on pharmacokinetic derivations have not been assessed over the broad range of concentrations of both compounds after IV fentanyl administration to horses. Hence, the first purpose of the current study was to compare RIA to LC–MS for the measurement of fentanyl concentration in horse plasma and to determine if observed differences in pharmacokinetic variables derived from values obtained by different analytical methods are clinically relevant. The second purpose was to determine if PMA cross-reacted with the RIA over a range of concentrations of this metabolite in horse plasma.

Whereas norfentanyl and other fentanyl metabolites possess no significant pharmacological activity in rats, the binding activity and functionality of PMA at the opioid receptor has not been investigated (8). By utilizing a radioligand binding technique with the highly selective ligands [3H]-DAMGO, [3H]-U69593, and [3H]-DPDPE to identify the mu-, kappa-, and delta-receptors, respectively, the binding potency of fentanyl and PMA can be compared in horse cerebral cortex. The final purpose of this study was therefore to assess the binding activity of fentanyl and PMA at opioid receptor subtypes in horse cerebral cortex.

Materials and Methods

Study samples

Two sample sets were examined. One sample set consisted of plasma samples from horses participating in a study investigating the effects of anesthesia with isoflurane on the pharmacokinetics of fentanyl and PMA (3). The second sample set consisted of plasma samples from horses participating in a study investigating the effects of intravenous fentanyl administration on the minimum alveolar concentration of isoflurane (9). In both studies, blood samples were transferred to a tube containing sodium heparin, centrifuged for 10 min, and the plasma collected and frozen at −20°C (3,9). From these two sample sets, 122 plasma samples were chosen.

HPLC–MS of fentanyl and PMA

Analytical reference standards of fentanyl and fentanyl-d₅ were commercially obtained (Cerilliant, Round Rock, TX). PMA was synthesized using a previously described procedure and determined to have 95% purity by LC–MS (10). All liquid chromatography solvents were HPLC grade (Burdick and Jackson, Muskegon, MI), and formic acid was spectroscopy grade (Aldrich, St. Louis, MO). Standard solutions of fen-.

radioimmunoassay of fentanyl

The solid-phase Coat-A-Count fentanyl RIA (Diagnostic Products, Los Angeles, CA) was performed according to manufacturer’s instructions. Briefly, all calibrators, quality control samples, and test samples were run in duplicate. Samples with a high concentration of fentanyl by LC–MS were diluted 1:10 or 1:20 in drug-free horse plasma. Four blank polypropylene tubes (not antibody coated) were used for the total and non-specific binding counts. Fentanyl antibody-coated RIA tubes were used for the preparation of fentanyl calibrators, controls, and samples. A 50-µL aliquot of fentanyl calibrators, controls, and samples were added to the coated tubes. The fentanyl calibrators were at concentrations of 0.1, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 ng/mL and prepared in drug-free horse plasma. Two fentanyl quality control samples were prepared at con-
centrations of 0.313 and 2.5 ng/mL in drug-free horse plasma. One milliliter of I\textsuperscript{125} fentanyl tracer was added to each of the prepared tubes, and the tubes were vortex mixed and allowed to stand for 1 h. All except for the total count tubes were then decanted and struck sharply against the countertop to remove remaining tracer solution. A single-well gamma counter (Beckman gamma 5500, Beckman Instruments, Fullerton, CA) was operated on the I\textsuperscript{125} channel, and each sample was counted for 60 s.

The calibration curve was constructed using least squares linear regression of the logit transformed counts versus the log of fentanyl concentration, with good linearity of the resulting curve for all runs ($R^2 > 0.996$). The limit of quantitation (LOQ) for the assay was 0.156 ng/mL. To determine the cross-reactivity of PMA with the RIA, spiked PMA samples were prepared in drug-free horse plasma at concentrations of 0.5, 1, 5, 15, and 30 ng/mL. These samples were assayed on two different days.

**Pharmacokinetic and statistical analysis**

Weighted linear regression was used to determine the relationship between fentanyl concentrations in equine samples determined by LC–MS (independent variable) and those determined by RIA (dependent variable) using Graphpad Prism software (version 3.02, San Diego, CA). From the regression analysis, 95% confidence intervals of the slope and intercept were calculated. If 1 and 0 were not included in the 95% confidence intervals for the slope and intercept, respectively, then they were considered significantly different.

Nonlinear least-squares regression was performed on plasma fentanyl concentration versus time curves for four anesthetized horses from the first sample set using WinNonlin 5.0.1 (Pharsight, Mountain View, CA). The RIA and LC–MS fentanyl data were weighted by the reciprocal of the plasma fentanyl concentration squared, respectively, and the data were fit to compartmental models (11). The appropriate model was selected using Akaike’s information criterion and standard compartmental equations were used to estimate pharmacokinetic variables (12).

To assess whether the method of analysis had a significant effect on the fentanyl pharmacokinetic variables, a paired difference t test with a Hochberg correction for multiple t tests was used to compare extrapolated time zero plasma drug concentration ($C_{(t=0)}$), apparent volume of the central compartment ($V_{(C)}$), apparent volume of distribution at steady state ($V_{(dss)}$), total body clearance (CL), area under the plasma drug concentration–time curve (AUC), and mean residence time (MRT) using either LC–MS or RIA to determine plasma fentanyl concentrations. A Wilcoxon signed rank test was used to compare rapid distributional half-life ($t_{1/2a}$), slow distributional half-life ($t_{1/2s}$), and elimination half-life ($t_{1/2e}$) of fentanyl using either LC–MS or RIA to determine plasma fentanyl concentrations. Significance was set at $p < 0.05$. Data are reported as mean ± SD or harmonic mean ± pseudo-standard deviation.

**Radioligand binding assay**

Following euthanasia, brain tissue was obtained as a secondary use of tissue. Whole brains were obtained from three adult horses that were euthanized by an overdose of sodium pentobarbital at the UC Davis Veterinary Medical Teaching Hospital. Following euthanasia, the brains were removed and frozen at −80°C. Brain homogenates were prepared by a previously described method (13).

Binding experiments were based on the methods of Kosterlitz et al. (14). The binding assay was carried out with 1.3 nM [$\text{H}$]–DAMGO, [$\text{H}$]–DPDPE, or [$\text{H}$]–U69593 (Perkin Elmer Life Sciences, Boston, MA) assayed in the absence (control) or presence of varying concentrations of fentanyl or PMA. The incubation times for [$\text{H}$]–DAMGO, [$\text{H}$]–DPDPE, and [$\text{H}$]–U69593 were 3.5, 6.5, and 1 h, respectively. Non-specific and total binding of [$\text{H}$]–DAMGO, [$\text{H}$]–DPDPE, and [$\text{H}$]–U69593 were determined in the presence and absence, respectively, of 1, 10, and 1 µM unlabelled naloxone, respectively. Each experiment was performed in duplicate and incubated at 25°C in polypropylene tubes. The experiment was terminated by rapid vacuum filtration over Whatman GF/B glass filters soaked in a 0.1% polyethyleneimine solution. Each filter was washed three times with 3 mL of ice-cold 10 mM Tris-HCl buffer (pH 7.4). The filter-bound radioactivity was counted by liquid scintillation spectrometry (Beckman LS 6000IC, Beckman Instruments, Fullerton, CA) with 40% efficiency for tritium. Specific binding was defined as the amount of binding obtained by subtracting non-specific binding from total binding. The IC\textsubscript{50} was determined using Graphpad Prism software for each assay. Data are presented as mean ± SD of three experiments.

**Results**

The LC–MS calibration curves for fentanyl and PMA were linear ($R^2 > 0.993$) in the ranges 0.05–60 and 0.05–40 ng/mL, respectively. By diluting samples in drug-free horse plasma, fentanyl and PMA concentrations were measured from the detection limit (0.1) up to 110 and 50 ng/mL, respectively. The overall accuracy and precision for fentanyl in equine plasma as measured by quality control samples at two concentrations (0.2 and 12.0 ng/mL) was 99% and 1%, respectively, and relative standard deviation was 0.7%. The overall accuracy and precision for PMA in equine plasma as measured by quality control samples at two concentrations (0.2 and 12.0 ng/mL) was 93% and 8%, respectively, and relative standard deviation was 2.3%. The RIA calibration curves were linear ($R^2 > 0.996$) in the range 0.16–5 ng/mL. By diluting samples in drug-free horse plasma, fentanyl concentrations could be measured from the detection limit (0.16) to 90 ng/mL. The overall accuracy and precision for fentanyl in equine plasma as measured by quality control samples at two concentrations (0.2 and 12.0 ng/mL) was 93% and 8%, respectively, and relative standard deviation was 2.3%. The RIA calibration curves were linear ($R^2 > 0.996$) in the range 0.16–5 ng/mL. By diluting samples in drug-free horse plasma, fentanyl concentrations could be measured from the detection limit (0.16) to 90 ng/mL. The overall accuracy and precision for fentanyl in equine plasma as measured by quality control samples at two concentrations (0.2 and 12.0 ng/mL) was 93% and 8%, respectively, and relative standard deviation was 2.3%.

The RIA results from the equine samples correlated well with the LC–MS data over the entire fentanyl concentration range of 5–110 ng/mL and at low fentanyl concentrations of 0–5 ng/mL with $R^2$ values of 0.89 and 0.88, respectively, as shown in Figure 1. However, the RIA consistently overestimated low fentanyl concentrations as indicated by the signifi-
significantly higher slope of 1.77 and intercept of 0.76 as seen in Table I for the 0–5 ng/mL data. In contrast, the RIA consistently underestimated high fentanyl concentrations as indicated by the significantly lower slope of 0.75 for the 5–110 ng/mL data.

For both analytical methods, a three-compartment model best described the decrease in plasma fentanyl concentrations over time in the anesthetized horses (Figure 2). Significant differences between the LC–MS and RIA methods were found for Cl and AUC, while there were no significant differences for the other pharmacokinetic variables as seen in Table II. Plasma PMA concentrations as measured by LC–MS increased rapidly in the anesthetized horses with the $C_{\text{max}}$ of 10.0 ± 1.9 ng/mL occurring at 17 ± 3 min as shown in Figure 2.

Increasing concentrations of PMA in equine plasma displaced the RIA I\(^{125}\) fentanyl tracer from binding to the antibody coated tubes but was less effective than fentanyl as shown in Figure 3. The mean (± SD) cross-reactivity of PMA determined from the spiked equine plasma samples was 29 ± 14%.

Fentanyl completely displaced [\(^{3}\text{H}\)-DAMGO, [\(^{3}\text{H}\)-U69593, and [\(^{3}\text{H}\]-DPDPE from mu-, kappa-, and delta-receptors in horse cerebral cortex, with inhibitory constants of 2.97 ± 1.18, 288 ± 5, and 210 ± 84 nM, respectively. However, PMA was not effective at displacing [\(^{3}\text{H}\]-DAMGO, [\(^{3}\text{H}\)-U69593, and [\(^{3}\text{H}\]-DPDPE from mu-, kappa-, and delta-receptors, respectively,

![Figure 1](https://academic.oup.com/jat/article-abstract/32/9/754/868032)

**Figure 1.** Correlation between fentanyl plasma concentrations at 5–110 ng/mL (A) and 0–5 ng/mL (B) as measured by RIA and LC–MS. The solid line represents the slope of the regression line (Table I).

![Figure 2](https://academic.oup.com/jat/article-abstract/32/9/754/868032)

**Figure 2.** Plasma fentanyl and PMA concentrations (mean ± s.d.) versus time following a single 4 µg/kg dose administered intravenously to four horses anesthetized with isoflurane using LC–MS or RIA to measure fentanyl and LC–MS to measure PMA.

### Table I. Comparison of RIA versus LC–MS for Fentanyl Concentrations Using Linear Regression Analysis

<table>
<thead>
<tr>
<th>Data Set</th>
<th>Slope (95% confidence limits)</th>
<th>Intercept (ng/mL) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay (5–110 ng/mL) (n = 65)</td>
<td>0.75* (0.68–0.82)</td>
<td>4.94† (2.12–7.76)</td>
</tr>
<tr>
<td>Radioimmunoassay (0–5 ng/mL) (n = 57)</td>
<td>1.77* (1.63–1.92)</td>
<td>0.76† (0.49–1.02)</td>
</tr>
</tbody>
</table>

* Significantly different from 1.
† Significantly different from 0.

* Significantly different from 1.
† Significantly different from 0.

### Table II. Pharmacokinetic Variables Determined Using LC–MS or RIA to Measure Fentanyl Following a Single i.v. 4 µg/kg Dose of Fentanyl to Four Horses Anesthetized with Isoflurane

<table>
<thead>
<tr>
<th>Variable</th>
<th>LC–MS</th>
<th>RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{ph}}$ (ng/mL)</td>
<td>99 ± 27</td>
<td>140 ± 30</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>1.3 ± 0.08</td>
<td>0.66 ± 0.42</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>20 ± 3.7</td>
<td>29 ± 11</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>70 ± 20</td>
<td>180 ± 110</td>
</tr>
<tr>
<td>$V_c$ (L/kg)</td>
<td>0.042 ± 0.010</td>
<td>0.031 ± 0.008</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>0.31 ± 0.12</td>
<td>0.54 ± 0.24</td>
</tr>
<tr>
<td>$CL$ (mL/min/kg)</td>
<td>6.9 ± 1.1</td>
<td>3.2 ± 0.18</td>
</tr>
<tr>
<td>AUC (ng·min/mL)</td>
<td>600 ± 120</td>
<td>1300 ± 67</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>44 ± 14</td>
<td>170 ± 68</td>
</tr>
</tbody>
</table>

* Abbreviations: $C_{\text{ph}}$, extrapolated time zero plasma drug concentration; $t_{1/2}$, rapid distributional half-life; $t_{1/2}$, slow distributional half-life; $t_{1/2}$, elimination half-life; $V_c$, apparent volume of the central compartment; $V_d$, apparent volume of distribution at steady state; $CL$, total body clearance; $AUC$, area under the plasma drug concentration-time curve; and MRT, mean residence time.
† Values are expressed as mean ± s.d.
‡ Values are expressed as harmonic mean ± pseudo s.d.
§ Significantly different from analysis of fentanyl concentrations by LC–MS ($p < 0.05$).
Discussion

The RIA method described in this study is misleading in comparison to the established method, LC–MS, for the measurement of fentanyl in equine plasma. While fentanyl concentrations determined by RIA and LC–MS correlated, the RIA consistently overestimated low concentrations of fentanyl and underestimated high concentrations of fentanyl. The overestimation of low fentanyl concentrations by the RIA is most likely due to interference by the primary metabolite of fentanyl in the horse, PMA, or variability near the limit of quantitation for the assay. It was necessary to perform a 1:10 or 1:20 dilution in drug-free horse plasma for samples with a high concentration of fentanyl so that the tested sample was within the narrow RIA calibration range. It is possible that sample dilution may have played a role in the underestimation of high fentanyl concentrations by the RIA. Samples analyzed by LC–MS with a high fentanyl concentration were also diluted, but only a 1:4 dilution played a role in the underestimation of high concentrations of fentanyl. The overestimation of low fentanyl concentrations by the RIA is most likely due to interference by the primary metabolite of fentanyl in the horse, PMA, or variability near the limit of quantitation for the assay. It was necessary to perform a 1:10 or 1:20 dilution in drug-free horse plasma for samples with a high concentration of fentanyl so that the tested sample was within the narrow RIA calibration range. It is possible that sample dilution may have played a role in the underestimation of high fentanyl concentrations by the RIA. Samples analyzed by LC–MS with a high fentanyl concentration were also diluted, but only a 1:4 dilution was necessary due to the broader calibration range.

Pharmacokinetic variables determined following an intravenous fentanyl bolus to four horses anesthetized with isoflurane differed depending on the method of analysis as seen in Table II, and parallel the differences in pharmacokinetic variables noted in previous studies of awake horses (3,4). For example, clearance was significantly different between the LC–MS and RIA analysis at 6.9 ± 1.1 and 3.2 ± 0.18 mL/min/kg, respectively, in the present study of anesthetized horses. In previous studies in awake horses, clearance was 9.2 ± 1.7 and 5.9 ± 1.3 mL/min/kg in samples analyzed by LC–MS and RIA, respectively (3,4). The most notable differences in fentanyl concentrations between the two analytical methods are apparent at the terminal disposition phase as shown in Figure 3, where fentanyl concentrations declined much more slowly using the RIA in comparison to LC–MS. Interestingly, the disparity in fentanyl concentrations between the two methods begins at approximately 20 min when PMA concentrations are near their maximum.

The 29% cross-reactivity of PMA in equine plasma noted in this study is higher than the previously measured cross-reactivity of less than 10% in equine serum (4). These differences may be due to matrix differences (plasma versus serum), purity of the PMA standard, or concentration range of PMA examined. In the present study, PMA was synthesized by a previously described procedure and the purity of 95% was determined by LC–MS (10). Although the interference of PMA with the RIA has not been reported in plasma, the RIA has been used to estimate PMA concentration in horse urine (6).

In comparison to fentanyl, PMA has no binding affinity to the three major opioid receptor subtypes in the horse. Thus, PMA is unlikely to have any important functional activity in the horse, and is consistent with previous reports of norfentanyl and other human fentanyl metabolites possessing no pharmacological activity (8).

In conclusion, the RIA is less accurate than LC–MS for measurement of fentanyl in equine plasma, especially for the determination of fentanyl pharmacokinetics. PMA exhibited 29% cross-reactivity with the RIA, but possessed no binding or presumed functional activity at the three opioid subtype-receptors in the horse.

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References


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