Remifentanil in the Horse: Identification and Detection of its Major Urinary Metabolite*

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Abstract

Remifentanil (4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid methyl ester) is a μ-opioid receptor agonist with considerable abuse potential in racing horses. The identification of its major equine urinary metabolite, 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid, an ester hydrolysis product of remifentanil is reported. Administration of remifentanil HCl (5 mg, intravenous) produced clear-cut locomotor responses, establishing the clinical efficacy of this dose. ELISA analysis of postadministration urine samples readily detected fentanyl equivalents in these samples. Mass spectrometric analysis, using solid-phase extraction and trimethylsilyl (TMS) derivatization, showed the urine samples contained parent remifentanil in low concentrations, peaking at 1 h. More significantly, a major peak was identified as representing 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid, arising from ester hydrolysis of remifentanil. This metabolite reached its maximal urinary concentrations at 1 h and was present at up to 10-fold greater concentrations than parent remifentanil. Base hydrolysis of remifentanil yielded a carboxylic acid with the same mass spectral characteristics as those of the equine metabolite. In summary, these data indicate that remifentanil administration results in the appearance of readily detectable amounts of 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid in urine. On this basis, screening and confirmation tests for this equine urinary metabolite should be optimized for forensic control of remifentanil.

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Introduction

Remifentanil, a new synthetic opioid, is the methyl ester of 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid (Figure 1) and is marketed as a hydrochloride salt (1). Remifentanil exhibits μ-opioid receptor-mediated effects, analogous to those of structurally-related phenylpiperidine derivatives such as fentanyl and sufentanil (2). Remifentanil produces physiological changes in humans consistent with potent μ-receptor agonist activity, including analgesia and sedation. Its adverse effects include ventilatory depression, nausea, vomiting, muscular rigidity, bradycardia, and pruritus.

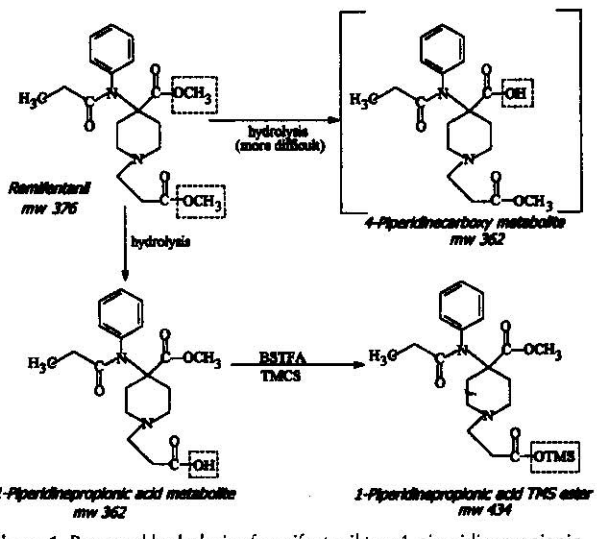

Figure 1. Proposed hydrolysis of remifentanil to a 1-piperidinepropionic acid metabolite, and its derivatization with BSTFA + 1% TMCS to a 1-piperidinepropionic acid TMS ester for GC–MS chromatographic visualization. The 4-piperidinecarboxy hydrolysis product is a formal possibility discussed further in the text, since it is indistinguishable from the 1-piperidinepropionic acid on the basis of molecular weight alone.

Because remifentanil does not release histamine upon injection, it has fewer adverse hemodynamic effects than morphine (3). The reduced heart rate caused by remifentanil can be completely antagonized by naloxone (2,4). Remifentanil is synergistic with hypnotic drugs thereby yielding a decrease of minimum alveolar anesthetic concentration (2).

The presence of a methyl ester on the propionic acid side chain of the piperidine ring increases the susceptibility of remifentanil to hydrolytic cleavage by blood and tissue nonspecific esterases, leading to relatively rapid clearance in humans and measured as a plasma half-life of 8–10 min (1,3). Remifentanil can thus be classified as an ultra-short-acting phenylpiperidine opioid analgesic agent (5). The main remifentanil metabolite in human pharmacology is the carboxylic acid product of hydrolysis [Glaxo Designation # GR90291; cf. (1) and Glaxo Wellcome, 1996]. This same metabolite has also been identified in canine urine samples (2) and is a pure μ-agonist with a potency of $\frac{1}{2000}$ to $\frac{1}{4000}$ that of remifentanil. Because the metabolite is such a weak μ-receptor agonist, it fails to provide any significant additive effect to the pharmacological actions of remifentanil (2). Figure 1 illustrates the known metabolism for remifentanil and the likely metabolic pathway in the horse.

Generally, μ-opioid receptor agonists produce sedation, depressed breathing, and relief of pain in humans. In the horse, however, they increase locomotor activity and stimulate movement at a pace somewhere between a brisk trot and a gallop (6). As a locomotor stimulus and potent analgesic, remifentanil has the potential to increase performance in racehorses. As such, remifentanil is classified by the Association of Racing Commissioners International as a class 1 agent, an agent with the highest potential to affect the performance of a racing horse. In light of its significant potential for abuse on the racetrack, this research is focused on the generation of methods for the detection and confirmation of remifentanil and its metabolites in equine urine.

Table I. Possible Metabolites of Remifentanil and the Mass Spectrometric Visualization of their Molecular Ions

Proposed compound as derived from remifentanil	MW	ESI+ [M+H+]	ESI- [M-H+]	GC–MS mono-TMS	GC–MS bis-TMS
Remifentanil	376	377			
Hydrolysis of a single methyl ester to a COOH	362	363	361	434	
Conversion of COOH to a glucuronide	538	539	537	610	682
Conversion of COOH to a sulfate	442	443	441	514	
Hydrolysis of both methyl esters to a di-COOH	348	349	347	420	492
Conversion of di-COOH to a glucuronide	524	525	523	596	668
Conversion of di-COOH to a sulfate	428	429	427	500	572
Phenyl ring hydroxyl	392	393		464	
Conversion of phenyl ring hydroxyl to a glucuronide	568	569	567	640	712
Diol formation	410	411		482	554
Conversion of diol to a glucuronide	586	587	585	658	730
Conversion of diol to a sulfate	490	491	489	562	634
N-Dealkylation	290	291			
N-Dealkylation + hydrolysis of a single methyl ester	276	277	275	348	
Methylation of a phenyl ring hydroxyl	406	407			

Materials and Methods

Horses, drugs, and clinical signs

Mature Thoroughbred mares weighing 422–503 kg were used for this study. They were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and alfalfa-based protein pellets. They were fed twice daily, vaccinated annually for tetanus, and dewormed quarterly with ivermectin. A clinical examination was performed before each experiment. During experimentation, the horses were provided water and hay ad libitum. The animals used in these experiments were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care Use Committee, which also approved the experimental protocol.

Remifentanil (Ultiva, Glaxo Wellcome, Inc., Research Triangle Park, NC) was administered intravenously (i.v.) in doses of 1 and 10 mg (two 5-mg doses given 10 min apart). Urine samples were collected at 0, 1, 2, 4, 6, 8, 24, 48, and 72 h after administration and were stored at –20°C.

ELISA quantitation of apparent remifentanil in urine

The one-step ELISA assay used for this analysis has been described previously (7). Standard curves for remifentanil and alfentanil in the Fentanil Group ELISA test kit (Neogen Corp., Lexington, KY) were constructed using assay buffer. The assays were started by adding 20 μL of the standard, test, or control samples to each well of the kit. During the test, the presence of remifentanil eqivalents in the sample competitively prevented the binding of the hapten-HRP (horseradish peroxidase) complex to the antibody. Because the HRP enzyme is responsible for the color-producing reaction in the ELISA, the log of the concentration of remifentanil and its metabolites in the sample is inversely related to the percent of maximal optical density of the test well, which was determined at a wavelength of 650 nm with an automated microplate reader (Bio-Tek Instruments, Winooski, VT) approximately 30 min after addition of substrate. All assay reactions were run at room temperature (22°C). Also, urine samples collected from a horse dosed with remifentanil (1 mg, i.v.) were screened with this test kit to determine the response of the ELISA kit to remifentanil and its metabolite(s).

Urine sample preparation

To facilitate passage of equine urine through solid-phase extraction (SPE) columns, urine samples were first sonicated with a Fisher Scientific Sonic Dismembrator Type 60 equipped

with an Ultrasonic Convertor horn type CML-3 (Fisher Scientific, Pittsburgh, PA). Three pulses 30 s in duration were sequentially applied with increasing intensity for each pulse (30, 60, and 90 watts). Samples were kept on ice throughout the procedure to minimize heating.

Urine extraction procedure

Clean-screen (#ZSDAU-020, Worldwide Monitoring, Philadelphia, PA) SPE columns were conditioned by sequential addition of 2 mL of methanol, 5 mL of deionized water, 2 mL of 100mM sodium phosphate buffer (pH 6.0), after which 5 mL of sonicated urine samples buffered with 2 mL of 100mM sodium phosphate buffer (pH 6.0) were loaded. Each column was then washed sequentially with 3 mL of deionized water, 1 mL of 0.1M hydrochloric acid, and 3 mL of methanol. The drug metabolites were eluted with 2 mL of dichloromethane/isopropanol/NH_4OH (78/20/2, v/v/v). Dimethylformamide (15 µL) was added as a "keeper solvent". The eluent was evaporated to dryness at ~ 40°C under a stream of nitrogen gas. The sample was resuspended in 200 µL of dichloromethane, transferred to an autosampler vial equipped with a 200 µL spring-loaded insert, and again evaporated to dryness at ~ 40°C under a stream of nitrogen gas. For derivatization, each dried sample was dissolved in 40 µL of *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) (Pierce Chemicals, Rockford, IL), vortex mixed briefly, and incubated at 70°C for 30 min to produce the trimethylsilyl (TMS) derivative of remifentanil carboxylic acid metabolites.

Gas chromatographic–mass spectrometric (GC–MS) analysis

Injection of TMS derivatives was made through the 250°C injector port of a Hewlett-Packard 6890/5972 GC–MSD (Atlanta, GA), with sample deposition onto a DB-5MS 5% phenyl-95% methylpolysiloxane column (30-m length, 0.25-mm i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA) with oven programming beginning at 70°C (held for 2 min), then ramping at 20°C/min to 280°C (held for 12 min). Under these conditions, remifentanil eluted at 13.6 min retention time, and the carboxylic acid metabolite-TMS derivative eluted at 14.1 min. The MS was set to acquire from *m/z* 50 to 550 at 1.53 scans/s with a threshold of 150. In quantitative experiments, selected ion monitoring (SIM) was performed for ions *m/z* 319, 303, 227, 212, and 168 for remifentanil and *m/z* 377, 303, 285, 270, and 226 for remifentanil carboxylic acid-TMS. Mass Spec Calculator Pro (Version 4.03, Quadtech Associates, Inc., 1998) assisted in the interpretation of full scan mass spectra where necessary. Table I presents calculated molecular ions for various predicted end-products of phase I and phase II metabolism of remifentanil and their visualization by GC–MS with TMS derivatization, as well as their appearances to electrospray ionization-MS (ESI-MS) as protonated or deprotonated species. These calculations assisted with screens for metabolites.

Hydrolysis of remifentanil to 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidine-propionic acid

Remifentanil HCl (20 mg) was dissolved in 1 mL of methanol/water (1:1), and a 0.1N sodium hydroxide solution was added drop-by-drop until pH ~12 was achieved. The mixture was stirred overnight at room temperature (22°C), acidified by addition of 0.1N hydrochloric acid to pH ~4, and evaporated under reduced pressure to dryness. Chloroform (5 mL) was added, and the mixture was stirred for 1 h and filtered. Chloroform was evaporated to obtain a colorless oil (~10 mg).

MS–MS (general)

Full scan ESI mass spectra were obtained on analytical standards at 10 µg/mL in 50:50 acetonitrile/0.05% formic acid (aq), approximate pH 3, by infusion at 0.6 ml/h via a Harvard syringe pump into the electrospray probe of a Micromass Quattro II MS–MS (Micromass, Beverly, MA) set in positive ion mode. Negative mode spectra were obtained similarly but with dissolution in 50:50 acetonitrile/0.5% (v/v) NH_4OH (aqueous, from conc.), approximate pH 10. All spectra were optimized by combination of 1–2 min of uniformly acquired data, background subtraction, and peak smoothing.

MS–MS tuning

The Quattro II MS was tuned for positive ion spectra by direct infusion of 10 ng/µL of remifentanil in 50:50 acetonitrile/0.05% formic acid (aqueous). The peak shape and intensity of the monoprotonated remifentanil *m/z* 377 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5V. Negative ion mode was optimized similarly with focus placed on the monodeprotonated remifentanil carboxylic acid *m/z* 361 ion as visualized in 50:50 acetonitrile/0.5% (v/v) NH_4OH (aqueous, from conc.). Collision gas (argon) and collision energy were adjusted for collisionally induced dissociation (CID) in the central hexapole by optimizing settings as needed for MS–MS. Generally the collision gas was set to 1×10^{-3} mbar. In general, for positive mode the source cone voltage was set at +35 V, the collision energy was set at –25 V, the capillary of the ESI probe was set at +3.5 kV, skimmer was set at 1.0 volts and the HV lens was set at +0.7 kV. For negative mode the source cone voltage was set at 25 volts, the collision energy was set at –24 V, the capillary of the ESI probe was set at +2.9 kV, skimmer was set at 1.9 V and the HV lens was set at +0.65 kV. Source temperature was set at 100°C.

Direct examination of urine for glucuronide metabolites by MS–MS

Urine from horses taken 0, 2, 4, 6, or 8 h after treatment with 1 mg of remifentanil was passed through a ~30Q0 molecular weight cutoff Centrifree filter (Amicon, Inc., Beverly, MA) to remove high molecular weight materials. Specifically, 900 µL of urine was centrifuged for 90 min at $1200 \times g$ in a swinging bucket rotor (Type AH-4) in an AccuSpinFR centrifuge (Beckman, Palo Alto, CA). The filtrate was diluted 1:10 with 50:50 acetonitrile/0.5% (v/v) NH_4OH (aqueous, from conc.) for negative mode. The mixture was infused at 0.9 mL/h via a Harvard syringe pump equipped with a 500-µL Hamilton gas-tight syringe. Infusion was direct into the electrospray probe of the Quattro II MS–MS for ESI-MS with the source tuned as described.

Results

Clinical responses

In a preliminary experiment, 1 mg of remifentanil caused no discernible effect on locomotor or behavioral activity. In a subsequent experiment, remifentanil was administered in two 5-mg doses. Within 1 min of administration of the first 5-mg dose, the horse began rapid pacing around the stall, which lasted about 5 min. When pacing ceased, a second 5-mg injection was administered, and the locomotor effects were similar in intensity and duration to those seen following the first injection. Mild sweating was observed about 5 min after the second injection, and it persisted for about 10 min.

ELISA detection

Using a Fentanil Group ELISA test kit, the standard curve for remifentanil was equivalent to the standard curve for alfentanil (I_{50} ~ 0.4 ng/mL) showing that the test kit readily detects remifentanil (Figure 2). Consistent with the in vivo data, Figure 3 shows the percent of ELISA activity in urine samples from a horse treated with intravenous remifentanil at a dose of 1 mg, a subclinical dose in terms of an observable clinical response.

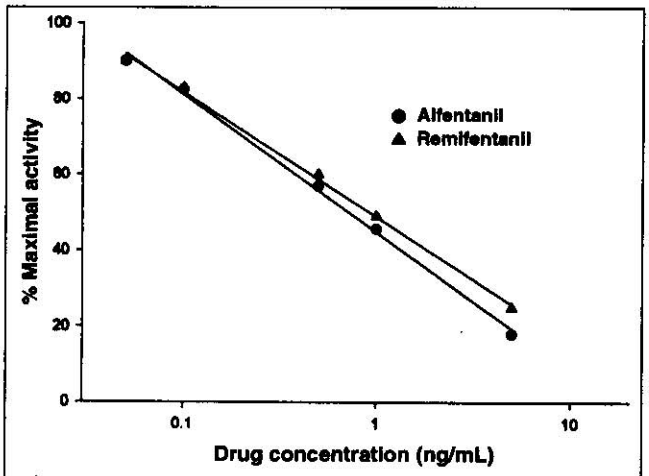

Figure 2. ELISA standard curves for remifentanil and alfentanil in assay buffer solution. Maximal activity is percentage of optical density at 650 nm of standard compared to the optical density of buffer zero.

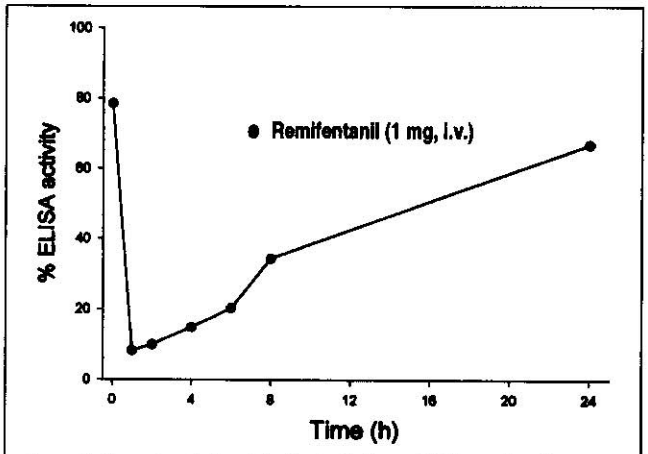

Figure 3. Percent activity of the Fentanil Group ELISA test in urine samples from a horse treated with intravenous remifentanil (1 mg).

The inhibitory effect of apparent remifentanil in the urine samples was maximal at 1 h after dosing and thereafter declined to where it was indistinguishable from control at 24 h.

MS analysis of remifentanil

Figure 4A shows that the remifentanil standard from Glaxo-Wellcome gave a sharp peak without derivatization and was > 95% pure based on TIC area. Figure 4B and Table II present the full scan mass spectrum of this peak and the interpretation of this spectrum, respectively. Urine from a horse treated with remifentanil yielded a peak with the same retention time, and the peak had the same mass spectrum (data not shown), which suggested parent remifentanil was in the urine sample.

The concentrations of parent remifentanil in the urine sam-

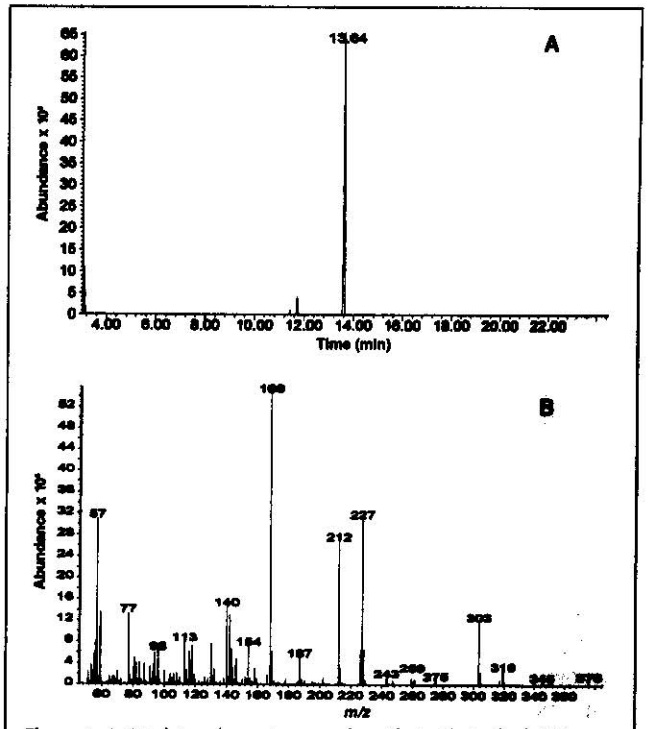

Figure 4. A, Total ion chromatogram of remifentanil standard. TIC area analysis indicated the remifentanil standard was > 95% pure. B, Electron impact mass spectrum of remifentanil peak at 13.6 min.

Table II. Interpretation of Fragments Observed in Remifentanil Mass Spectrum*

Ion (*m/z*)	Interpretation
376	molecular ion, M^+
361	$[M^+] - CH_3$
345	$[M^+] - OCH_3$
319	$[M^+] - CH_3CH_2C{=}O$
303	$[M^+] - CH_2COOCH_3$
227	303 - $[C_6H_5 - H^+]$ or $[M^+] - [C_6H_5NC{=}OCH_2CH_3 + H]$
212	227 - CH_2
168 (base peak)	227 - $COOCH_3$
77	C_6H_5
57	$CH_3CH_2C{=}O$

* As seen in Figure 4B.

ples were estimated using blank urine to which known concentrations of remifentanil had been added. Extracts were analyzed by a GC–MS SIM protocol using diagnostic ions derived from the major peaks seen in Figure 4B, specifically *m/z* 319, 303, 227, 212, and 168, with the *m/z* 168 peak being used for external standardization. This approach yielded a linear standard curve. By interpolation of data points on this curve, peak urinary concentration was reached 1 h after administration (Figure 5), which is in basic agreement with the ELISA findings (Figure 3). Furthermore, parent remifentanil was detected in urine samples for up to 6 h, also in general agreement with the ELISA data.

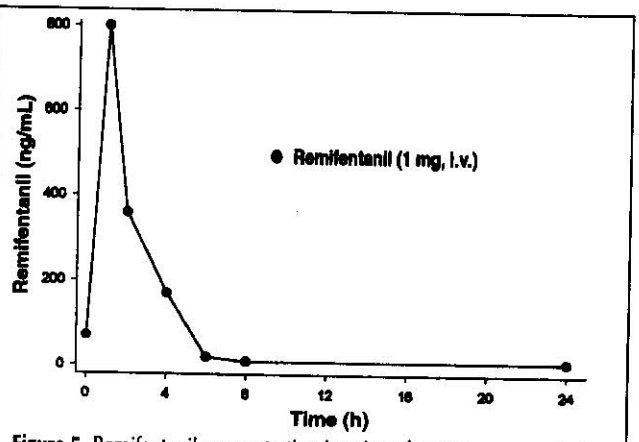

Figure 5. Remifentanil concentration in urine after intravenous administration of 1 mg of remifentanil as determined by GC–MS.

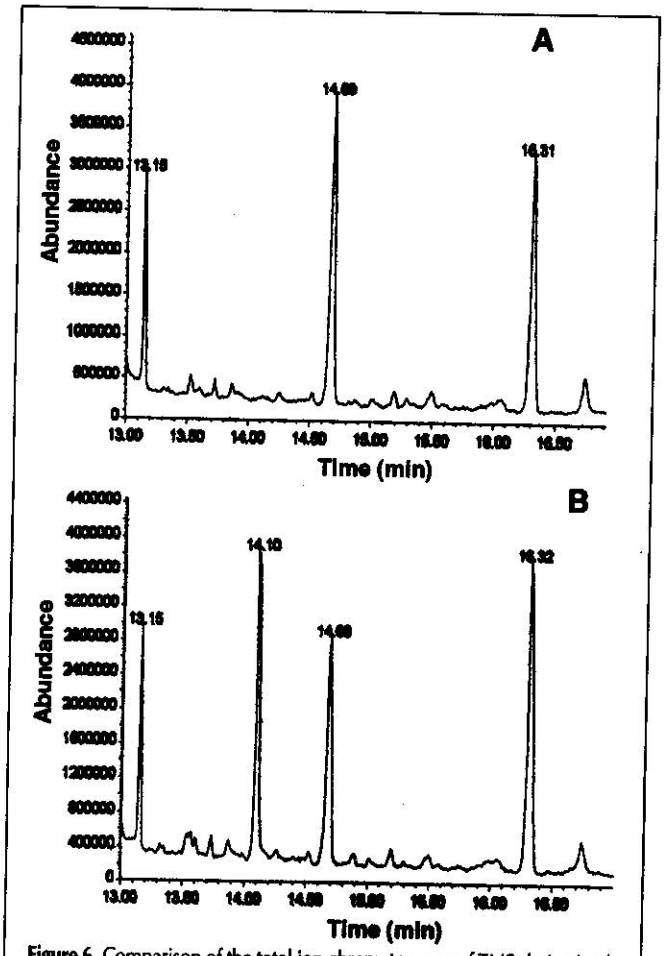

Figure 6. Comparison of the total ion chromatograms of TMS-derivatized, equine urine extracts. A, control sample and B, sample taken 1 h after administration of 1 mg remifentanil (note the appearance of the 14.10 min peak)

Figure 6 shows the results when control and 1 h urine samples were derivatized with BSTFA + 1%TMCS. The 1 h sample (Figure 6B) shows a significant peak at 14.10 min that is absent in the control sample (Figure 6A). Ion chromatographic screens for potential metabolites listed in Table I indicate that the 14.10 min peak corresponds in molecular weight to that anticipated for a carboxylic acid hydrolysis product of remifentanil. No evidence was found for any other potential metabolite listed in Table I, nor were there any other TIC peaks in the postadministration samples that were not in the control samples (data not shown).

Figure 7A represents selected ion chromatography derived from full scan GC–MS runs of 1-h urine extracts. The ion chromatography illustrates nested symmetrical peaks within the remifentanil (13.55 min) and remifentanil carboxylic acid TMS derivative (14.10 min) peaks. The relative ion intensities suggest that the carboxylic acid metabolite was present at about 10-fold greater concentrations than parent remifentanil. Figure 7B illustrates the full scan mass spectrum of the TMS derivative of the 14.10 min carboxylic acid metabolite. Interpretation of peaks (Table III) supports the suggestion that this is the major equine metabolite of remifentanil. Further support for the structure of the metabolite is provided by the comparability of certain mass-to-charge ratio peak values listed in Tables II and III, particularly the *m/z* 303 peak representing loss of either CH_2COOCH_3 or $CH_2COOTMS$, respectively. Figure 7C shows that the integrated area for a key diagnostic ion (*m/z* 226) for the carboxylic acid of remifentanil, has the same profile as that of parent remifentanil (Figure 5).

A single peak of TMS-derivatized hydrolysis product at 16.2 min (Figure 8A) shows a mass spectrum (Figure 8B) that matches the mass spectrum of the equine metabolite (Figure 7B). Examination of the hydrolysis product on the 6890/5972 GC–MSD verified that the base hydrolysis product retention time matched that of the equine metabolite seen in Figure 7.

Discussion

As a fentanyl-based μ-opiate agonist, remifentanil at a clinically effective dose would be expected to produce a clear-cut locomotor response, as previously reported for other members of this group of agents. Consistent with this expectation, a 5-mg dose of remifentanil produced locomotor and related responses. As such, this dose has the ability to influence the racing performance of horses, and effective screening and confirmation methods are required for forensic control of this agent. The need for a screening method is further emphasized by the fact that there have been no reported identifications of remifentanil in postrace drug testing in North America, despite its commercial availability since 1996.

Remifentanil administration could be readily detected with a Fentanil Group ELISA kit. The presence of apparent remifen-

tanil was demonstrated in equine urine for up to 8 h, indicating its suitability as a screening method.

Remifentanil was also readily detectable in our standard GC injection and oven temperature regimens, which are based on a 5%-phenyl-95%-methylpolysiloxane-lined capillary column programmed from 70°C to 280°C at 20 dpm. Additionally, it was amenable to SPE methods, adapted from Worldwide Monitoring assays for fentanyl-related drugs (United Chemical Technologies, Inc. Catalog: Worldwide Monitoring Bonded Phase Extraction Sorbents, 1996). The SPE method worked well on both remifentanil and its carboxylic acid metabolite. Application of these techniques enabled the detection of parent remifentanil in urine 1–6 h after administration (Figure 4).

Full scan GC–MS acquisitions were analyzed by ion chromatographic screening based on predicted values for metabolites in Table I, and this procedure revealed that the major equine metabolite of remifentanil is essentially the same as that previously described in dogs and humans. No other potential candidate metabolites listed in Table I could be visualized, supporting the contention that the carboxylic acid is the primary equine urinary metabolite.

Remifentanil appears to be rapidly metabolized in the horse. Figure 1 presents two alternatives for the generation of a remifentanil carboxylic acid, one that would result in a 1-piperidinepropionic acid metabolite, the other a 4-piperidinecarboxy metabolite. Either of these potential metabolites would undergo TMS derivatization. Comparison of equine metabolism of remifentanil to analogous metabolism described in the literature for humans and dogs has enabled the selection between the identical molecular weight carboxylic acid metabolites shown in Figure 1. Further support for this contention can be made by mass spectral analysis. Mass Spec Calculator Pro software predicted identical mass spectral peaks for the two candidates, with the exception of McLafferty rearrangement products: *m/z* 88 for the 4-carboxy TMS derivative and *m/z* 285 for the 1-propionic acid TMS derivative. Because both the equine metabolite (Figure 7B) and hydrolysis product (Figure 8B) have significant *m/z* 285 peaks, it is indirect support that the sterically less-hindered 1-propionic acid methyl ester is more prone both to nonspecific esterases in vivo and base hydrolysis in situ. Because there are two potential targets for hydrolysis (Figure 1), it is of interest that chemical hydrolysis produced the same carboxylic acid as did esterases in vivo.

Further support for the presence of the carboxylic acid in equine extracts independent of TMS derivatization was provided by HPLC–ESI$^+$-MS–MS, in which the hydrolysis product and equine metabolite had matching retention times and mass transitions from the calculated *m/z* 363 cation (Table 1) to *m/z* 214, 247, 259, 303, and 331 fragments (Lehner, unpublished results). Other experiments showed that the integrated area for a key diagnostic ion (*m/z* 226) for the carboxylic acid of remifentanil peaks with the same profile as that of parent remifentanil (Figure 7C). Comparison of the 14.1 min *m/z* 226 area and its ratio to the *m/z* 168 13.6 min area over the 0–24 h sampling period suggested an approximately 10-fold preponderance of the carboxylic acid metabolite over parental remifentanil (data not shown). The relatively higher concentrations of the remifentanil 1-piperidinepropionic acid hydrolysis product suggest that screening and

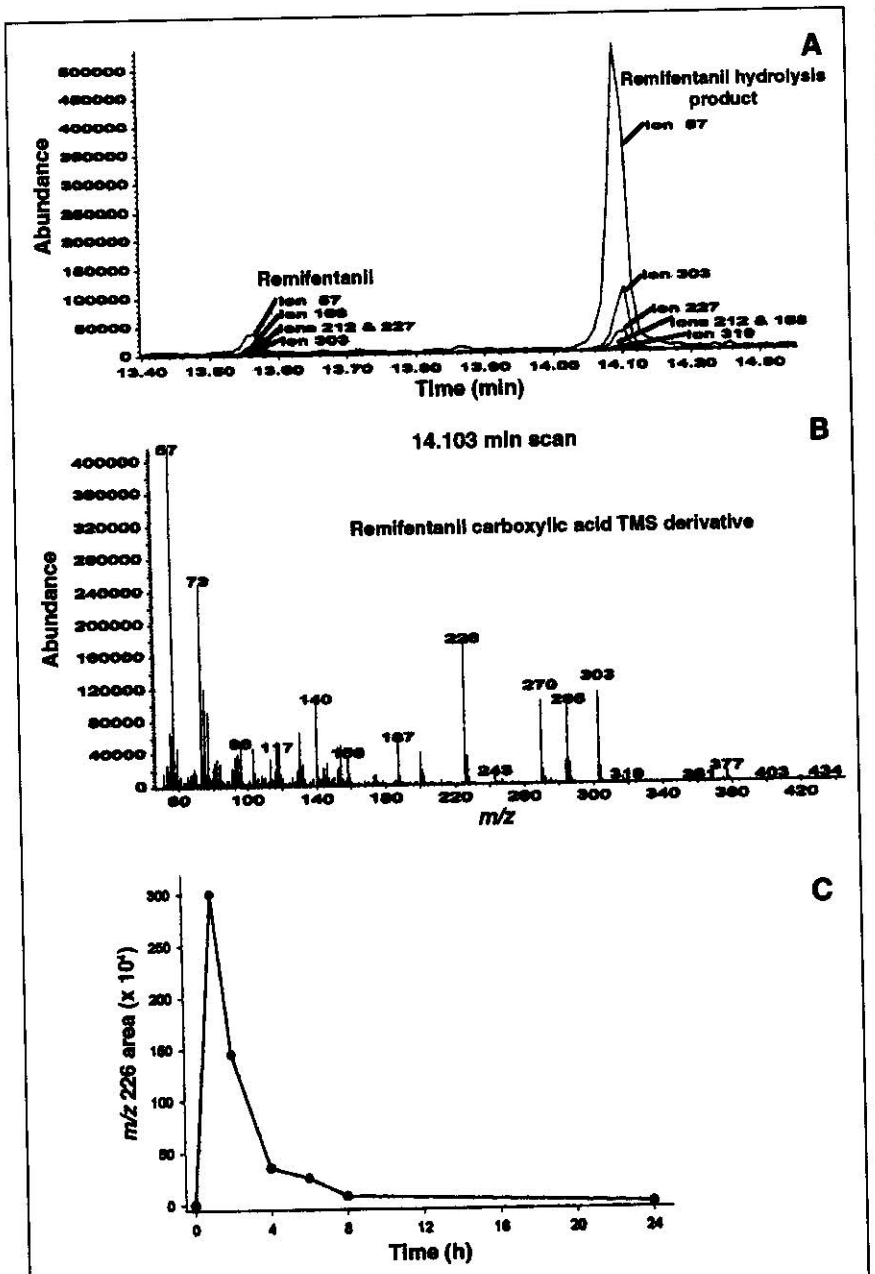

Figure 7. Equine urine extract 1 h after remifentanil administration. A, Ion chromatography of remifentanil and remifentanil carboxylic acid hydrolysis product (as TMS derivative) for ions relevant to each compound; B, mass spectrum of the equine carboxylic acid metabolite as a TMS derivative; and C, mass spectral area count of the proposed remifentanil carboxylic acid metabolite: ion *m/z* 226 area count of the 14.1 min peak as a function of the number of hours after dosing.

Table III. Interpretation of Fragments Observed in the Remifentanil Carboxylic Acid TMS Mass Spectra*

Ion (*m/z*)	Interpretation
434	molecular ion, M^+
419	$[M^+] - CH_3$
403	$[M^+] - OCH_3$
377	$[M^+] - CH_3CH_2C{=}O$
317	$[M^+]$ - COOTMS
303	$[M^+] - CH_2OOTMS$
285	$[M^+] - C_6H_5NCOCH_2CH_3 - H$
270	$285 - CH_3$
226	$303 - C_6H_5$
200	$285 - CH_2{=}C{-}COOCH_3$
187	$200 - CH_2$
154	$285 - CH_2COOTMS$
140	$285 - CH_2CH_2COOTMS$
73	TMS
57	$CH_3CH_2C{=}O$

* As seen in Figure 7B and 8B.

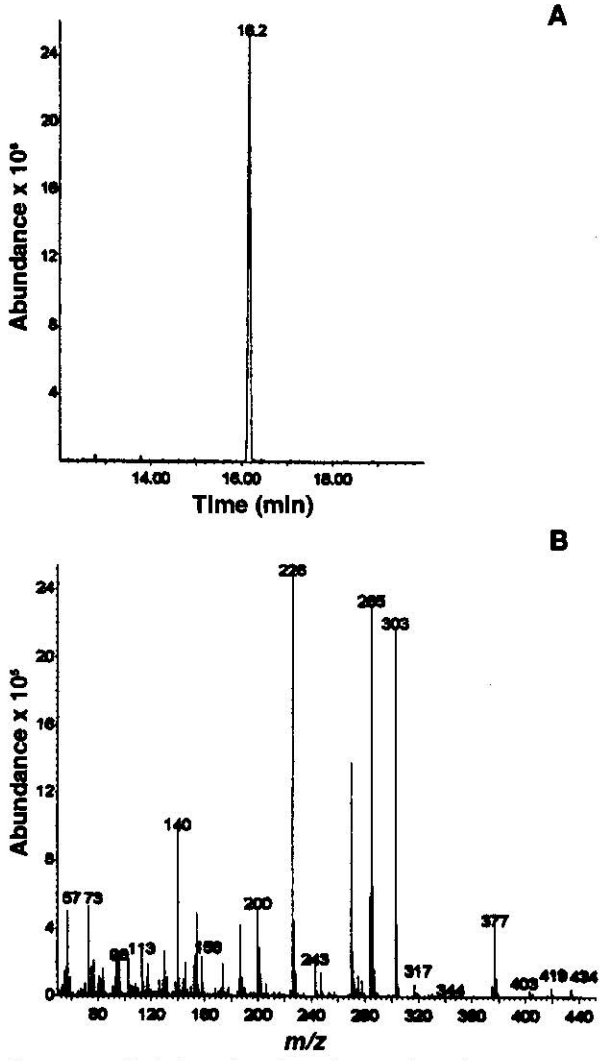

Figure 8. Base hydrolysis of remifentanil. A, Total ion chromatogram of remifentanil producing a single peak following TMS derivatization and B, mass spectrum of the 16.18 min peak seen in A.

confirmation methods should focus on this urinary metabolite of remifentanil, particularly for forensic samples.

In other experiments, conjugates of the carboxylic acid metabolite in urine were screened by electrospray-negative mode MS. Sulfate and glucuronide conjugates were excluded by this method, because neither *m/z* 441 nor *m/z* 537 peaks were observed. Additionally, no other potential candidate metabolite listed in Table I could be visualized, supporting the implication that the 1-piperidinepropionic acid hydrolysis product of remifentanil is the primary urinary metabolite in horses. Since the remifentanil-COOH metabolite could be visualized without prior glucuronidase or sulfatase treatment, and since direct screening for such conjugates was negative, it was tentatively concluded that the carboxylic acid was excreted directly. In particular, the near-identity of negative mode ESI-MS–MS zero hour and 1–8 h samples in the ranges for conjugates dictated by Table I (*m/z* 427–585) implies that such conjugates are unlikely to be detectable after clinically effective doses of this highly potent drug.

In summary, a dose of about 5 mg i.v. of remifentanil in horses produces clear-cut evidence of locomotor stimulation, consistent with the previously described pharmacological characteristics of this group of agents. The evidence presented here suggests that remifentanil is metabolized in the horse in much the same way as in humans and canines, namely by hydrolysis to 4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid. Remifentanil and its metabolites are readily detected as fentanyl equivalents in urine samples by ELISA-based screening. Confirmation methods employing extraction, TMS derivatization, and mass spectrometric verification of the major equine urinary metabolite should allow effective control of the unauthorized use of this substance in racing horses.

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