Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 2, pp. 243–248, 1994
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0731–7085/94 $6.00 + 0.00

Determination of remifentanil in human and dog blood by HPLC with UV detection*

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Abstract: Remifentanil (GI87084) is a phenylaminopiperidine derivative of the fentanyl type with a potent analgesic activity and ultra-short half-life. The N-substituted labile methyl propanoate ester group is highly susceptible to cleavage by endogenous esterases and by chemical hydrolysis. The hydrolysis is stopped by addition of 20 μl of 50% citric acid per 1 ml of blood. The method involves a liquid extraction of chilled blood at pH 7.4 with butyl chloride and back-extraction into 0.01 M HCl. The chromatographic conditions are: column — Zorbax SB-CN 4.6 × 250 mm; mobile phase — 7% acetonitrile–14% methanol in phosphate buffer (0.03 M; pH 3.0); detection — UV at 210 nm. The internal standard used was GI97559 — an ethyl analogue of the drug. The method has been validated in human blood over the range of 1–200 ng ml^{-1} and in dog over the range 10–60, 135 ng ml^{-1} with the latter assay being used in a toxicological support study. Additionally, it was used to characterize the hydrolysis of the drug, the enzymes involved in the process, and *ex vivo* drug interactions.

Keywords: *GI87084; remifentanil; HPLC; human blood; dog blood.*

Introduction

Remifentanil (GI87084) is a potent, ultra-short acting anaesthetic, which belongs to the family of 4-anilinopiperidine derivatives (Fig. 1). It has an elimination half-life *in vivo* of 9 min [1], which is much shorter than those of fentanyl, sufentanil and alfentanil — 219, 164 and 94 min, respectively [2]. Remifentanil contains the N-substituted labile methyl propanoate ester group, which is highly susceptible to cleavage by endogenous esterases and by chemical hydrolysis.

Bioanalytical literature on fentanyl and congeners is quite extensive. They have been analysed in biological matrices by gas chromatography (GC) [3–9], gas chromatography–mass spectrometry (GC–MS) [9, 10], high-performance liquid chromatography (HPLC) [11, 12], radioimmunoassay (RIA) [13, 14],

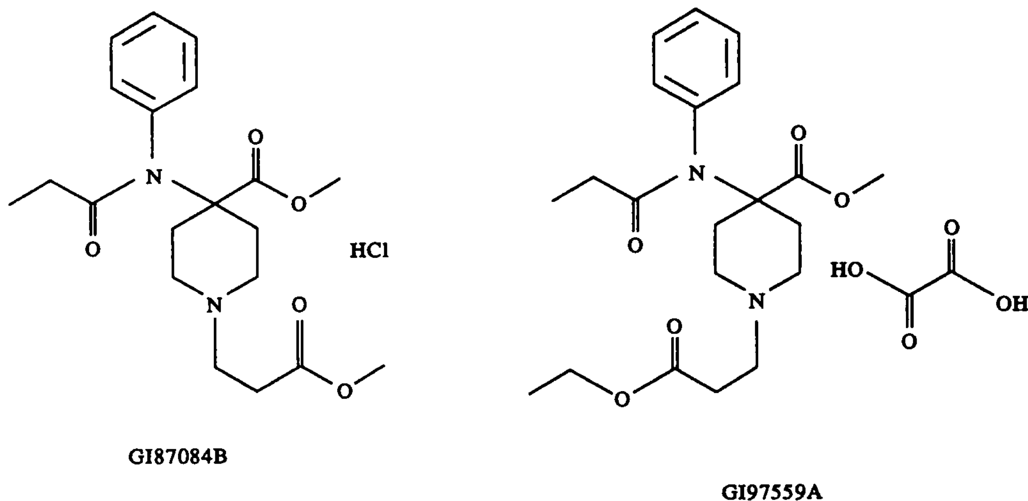

Figure 1
Chemical structures of remifentanil (GI87084) and the internal standard.

*Presented at the 'Fourth International Symposium on Pharmaceutical and Biomedical Analysis', April 1993, Baltimore, MD, USA.

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radioreceptor assay [RRA] [15], and ELISA [16]. The most sensitive GC method achieved a quantitation limit of 20 pg ml^{-1} for fentanyl [8], 30–50 pg ml^{-1} for sufentanil [17] and 1 ng ml^{-1} for alfentanil [11, 18]. In contrast to the older members of the 4-anilinopiperidine derivatives family, virtually no analytical data has been published so far on remifentanil. Grosse *et al.* [19] described a capillary gas chromatographic method coupled with a high resolution mass spectrometer in the selected ion monitoring mode (GC–HRMS–SIM). The method is very sensitive (limit of quantitation of 100 pg ml^{-1}), and robust — so far it has been used to assay *ca* 8000 samples. The hydrolysis problem (instability) was overcome by an immediate inactivating of enzymes by acetonitrile precipitation followed by extraction with methylene chloride.

This approach, although effective, created a number of problems: high costs of assay, availability of GC–HRMS–SIM equipment (productivity, lack of back-up systems), and difficulty of sample preparation adversely affecting recruitment of clinical sites. Hence, the objective of the study was to develop a simpler and less expensive HPLC method with sensitivity of 1 ng ml^{-1}, as well as to develop an alternative and possibly simpler blood collection procedure preserving sample integrity. Additionally, the secondary objective was to validate the method in dog blood over the concentration range 10–60, 135 ng ml^{-1} for use in a sub-chronic toxicity study.

Experimental

Materials

Remifentanil hydrochloride and GI97559 oxalate were synthesized at Glaxo Inc. Research Institute. All the other reagents and solvents were of HPLC grade, obtained from local suppliers and used without further purification.

Chromatographic conditions

Chromatography was performed on a system consisting of a model LC-6A pump, an auto injector SIL-6A, system controller SCL-6A (all from Shimadzu, Columbia, MD), and UV absorbance detector Spectroflow 783 (Kratos Analytical) set at 210 nm. The column was Zorbax SB-CN, 5 μm particle size, 250 × 4.6 mm i.d. (obtained from MAC-MOD Analytical, Chadds Ford, PA), equipped with a CN guard-column, 15 × 3.2 mm i.d. (Brownlee, ABI Applied Biosystems, San Jose, CA). The column was kept at 30°C in a column heater. The flow rate of the mobile phase was 1.5 ml min^{-1} with a resulting back pressure of *ca* 200 kg cm^{-2}. The data were collected by a Hewlett–Packard based Laboratory Automation System (LAS). Linear regression calculations were made by in-house developed software (PRANBAS, ver. 2.03.00).

Mobile phase

Mobile phase was prepared by adding 140 ml of acetontrile, 280 ml of methanol and 60 ml of 1 M phosphate buffer pH 3.0 into a 2 l volumetric flask. The flask was then brought to volume with water, the solution stirred and filtered through a 0.45 μm Nylon filter.

Calibration standards preparation

The calibration standards in human blood were prepared by adding appropriate volumes of remifentanil spiking solutions in 0.001 M HCl to freshly collected heparinized human blood. Within seconds after spiking, 20 μl of 50% citric acid per 1 ml of blood was added, samples were mixed and kept on ice until extraction. The standards were prepared fresh daily. The method validation in human blood was carried out first, when some stability issues still remained unresolved, which necessitated daily calibration curve preparation. The calibration range for human blood was established on the base of earlier results obtained by the GC–MS method [19]. The quality control samples (QCs) were spiked in bulk, and after aliquoting 1 ml were stored at −30°C (±2°C) and thawed on the day of analysis.

The standards and QCs in dog blood were prepared in the same way with the following exceptions: the standards were also spiked in bulk, and 200 μl aliquots in EDTA blood were used.

Extraction procedure

To a standard, quality control blood or clinical sample (1.0 ml for the human blood assay, 200 μl for the dog blood assay) stored on ice, the internal standard was added (100 μl of GI97559, 1000 ng ml^{-1} in 0.001 M HCl), followed by 1 ml of 1 M phosphate buffer pH 7.4. After mixing, 5 ml of *n*-butyl chloride were added and the tubes were mixed for 15 min on a roto-mixer at *ca* 60 rpm min^{-1}.

After shaking, the samples were centrifuged

at 1000g for 10 min at 4°C and the upper organic layer was transferred into a conical tube containing 300 μl of 0.01 M HCl. The tubes were shaken for 3 min on a vortex and centrifuged. The upper organic phase was aspirated off and the aqueous phase was put into a limited volume insert. The volume of 50–120 μl (depending on the range of the calibration curve) was injected into the chromatographic system.

Results and Discussion

Stability

The issue of remifentanil stability was of key importance in the design of the assay as well as in the sample handling procedure. Initial attempts aimed at finding a specific esterase inhibitor were doomed to failure, as the chemical hydrolysis is another factor to deal with. The remifentanil half-life in an *ex vivo* experiment in fresh blood was *ca* 60 min at 37°C, and *ca* 3 h at room temperature of 22°C. Its half-life in buffer pH 7.4 is *ca* 100 min. This was the rationale behind choosing whole blood as the assay matrix, so the time needed to harvest plasma or serum could be saved. The addition of citric acid (10 mg ml^{-1}) lowers the blood pH sufficiently to preserve integrity of samples. The concentration of citric acid is critical; at lower concentrations (2–5 mg ml^{-1}) citric acid does not ensure stability; at higher (20 mg ml^{-1}) the blood proteins could be precipitated. In fact, dog blood preserved in this way has a tendency to thicken and coagulate, especially at the drug concentrations above 10 μg ml^{-1} and after a prolonged storage. Hence, it is advisable to aliquot dog blood immediately after collection. Also, during the extraction the sample pH is readjusted back to 7.4; sample extraction should then be done as expediently as possible, as only the low temperature stops the hydrolysis at this stage.

GI97559, the ethyl-analogue of remifentanil used as the internal standard in this assay is susceptible to hydrolysis also. The rates of chemical and enzymatic hydrolysis of both substances are almost identical.

The human blood samples preserved with citric acid can be safely stored for 1 year at −30°C, kept on ice for 20 h, and passed through three freeze–thaw cycles. The stability results are presented in Tables 1, 2 and 3. In all these experiments the internal standard dissolved in diluted HCl and prepared fresh daily

Table 1
Stability of remifentanil. Fresh human blood was spiked with remifentanil at 20 ng ml^{-1}. The samples were preserved with citric acid (10 mg ml^{-1}) and stored on ice at room temperature of 22°C ± 2°C. The results are presented as the peak height ratio of the drug to the internal standard with relative standard deviation in brackets

Lapsed time (h)	On ice	Room temperature
0	—	0.225 (1.5%)
1	0.227 (1.7%)	0.223 (5.5%)
5	0.222 (1.9%)	0.222 (2.2%)
20	0.231 (2.0%)	0.223 (4.2%)

Table 2
Stability of remifentanil in human blood. Quality controls at 3.0, 60.0 and 160.0 ng ml^{-1} were analysed after the exposure; results obtained are in ng ml^{-1}

Long-term storage*	2.60	58.92	159.20
Processing stability†	3.30	55.90	158.80
Freeze–thaw stability‡	3.45	56.15	158.05

*Samples were stored for 1 year at −30°C, n = 4.
†Samples were thawed and kept on ice for 5 h, n = 2.
‡Samples were thawed and frozen three times before analysis, n = 2.

Table 3
Stability of remifentanil in dog blood. Quality controls at 30.0, 400.0, 4000.0 and 40,000.0 ng ml^{-1} were analysed after exposure; results are in ng ml^{-1}

Processing stability*	31.40	378.18	3701.40	37325.5
Freeze–thaw†	31.65	379.98	3725.43	36841.1

*Samples were thawed, and kept on ice for 5 h, n = 4.
†Samples were thawed and frozen three times, n = 4.

was added immediately before the extraction. The concentration differences between the stability samples and control samples are not statistically significant (two-sided t-test, at the 5% significance level) and are of no practical relevance.

It should be noted, that the other members of the fentanyl family are generally far more stable. Although fentanyl concentration degrades by 50% while boiled in 3 M hydrochloric acid for 4 h at 90°C [20], yet solutions of fentanyl and alfentanil in diluted hydrochloric acid, that had been kept in the refrigerator are stable for 4 months [3]. Solutions of fentanyl, alfentanil and sufentanil in isopentanol stored at room temperature for 2 weeks showed minor degradation of approximately 3% [3]. These observations agree with other stability studies evaluating the fentanyl citrate stability in parenteral solutions [21, 22]. Sufentanil on the other hand, while chemically stable, shows

a tendency to be absorbed by PVC containers [23].

Precision and accuracy

Human blood assay. A set of seven calibration standards, a zero, a drug-free blood sample (all in duplicate), quality control samples at three concentration levels (in duplicate) prepared by a primary analyst, and a set of quality control samples formulated by a secondary analyst were analysed on each of four validation days. The calibration curve covered the range from 1.0 to 200.0 ng ml^{-1}. Peak height ratio was used for quantitation. A linear regression analysis using a least-squares fit was performed with the reciprocal of the drug concentration as weight. The correlation coefficient as a measure of goodness of fit was ≥0.9986. Precision of the method, expressed as relative standard deviation (RSD) was 1.8–12.0%, while accuracy, expressed as a percentage of nominal concentration, was 94.2–106.7%. The complete data are shown in Table 4.

Dog blood assay. A set of nine calibration standards, a zero, a drug-free blood sample (all in duplicate), quality control samples at four concentration levels (in duplicate) prepared by a primary analyst, and a set of quality control samples formulated by a secondary analyst were analysed on each of four validation days. The calibration curve covererd the range from 10.0 to 60,135.0 ng ml^{-1}. Peak area ratio was used for quantitation. A linear regression analysis using a least-squares fit was performed with the reciprocal of the squared drug concentration as weight. This was necessary due to the 3.5 orders of magnitude of the calibration curve. The correlation coefficient as a measure of goodness of fit was ≥0.9938. Precision of the method, expressed as RSD was 3.4–10.7%, while accuracy, expressed as a percentage of nominal concentration, was 95.4–112.0%. The data are shown in Table 5.

Recovery

Recovery was calculated by comparing extracted quality control samples with an unextracted additionally prepared calibration curve in 0.01 M HCl which represented 100% recovery and correcting for all the losses in volume due to sample transfer. The mean recovery of remifentanil extracted from human blood was 91.53% at 3.0 ng ml^{-1} (RSD = 14.6%, n = 8) and 101.56% at 160.0 ng ml^{-1} (RSD = 6.2, n = 8), while from the dog blood it was 115.07, 100.93, 90.14 and 93.46% at 30.0, 400.0, 4000.0 and 40,000.0 ng ml^{-1}, respectively (RSD = 1.8–14.4, n = 7 and 8).

Table 4
Remifentanil quality control concentrations in human blood (between-run precision and accuracy)

	Nominal conentration (ng ml^{-1})					
	3.0a	3.0b	60.0a	60.0b	160.0a	160.0b
Mean	3.20	2.98	57.34	56.51	154.27	151.68
SD	0.107	0.358	1.131	1.825	2.836	4.941
RSD (%)	3.3	12.0	2.0	3.2	1.8	3.3
% Nominal	106.7	99.3	95.6	94.2	96.4	94.8
n	8	8	8	8	7	8

a, first analyst.
b, second analyst.

Table 5
Remifentanil quality control concentrations in dog blood (between-run precision and accuracy) (ng ml^{-1})

	QC concentrations (ng ml^{-1})							
	30.0a	30.0b	400.0a	400.0b	4000.0a	4000.0b	40000.0a	40000.0b
Mean	33.61	32.10	390.19	382.88	4272.89	3977.88	43611.81	38167.09
SD	3.393	3.420	17.527	31.491	216.024	133.938	4318.515	3237.742
RSD (%)	10.1	10.7	4.5	8.2	5.1	3.4	9.9	8.5
% Nominal	112.0	107.0	97.5	95.7	106.8	99.4	109.0	95.4
n	8	7	7	8	8	8	8	7

a, first analyst.
b, second analyst.

Chromatography

Figure 2(a) shows a chromatogram of a drug-free human blood sample, which is followed by the standard 2.0 ng ml^{-1} (Fig. 2b), and by the standard 10.0 ng ml^{-1}. Figure 3(a) presents a chromatogram of a drug-free dog blood sample, followed by a calibration standard 100.0 ng ml^{-1} (Fig. 3b), and by sample obtained from the dog 20 min after intravenous bolus injection of remifentanil at 10 mg kg^{-1} day^{-1} (Fig. 3c). The signal-to-noise ratio at the limit of quantitation of 1 ng ml^{-1} is 3:1, which is sufficient to obtain precision and accuracy necessary in studies of this type.

The chromatographic run time is *ca* 15 min. The retention times of remifentanil and the internal standard are 8.5 and 13 min, respectively. The retention time of GI90291, the main metabolite and hydrolysis product of remifentanil, is 4.5 min. Additionally, GI90291 is not extractable under conditions described above. The analytical system throughput is approximately 100 samples every 24 h.

The methods utilizes non-specific UV

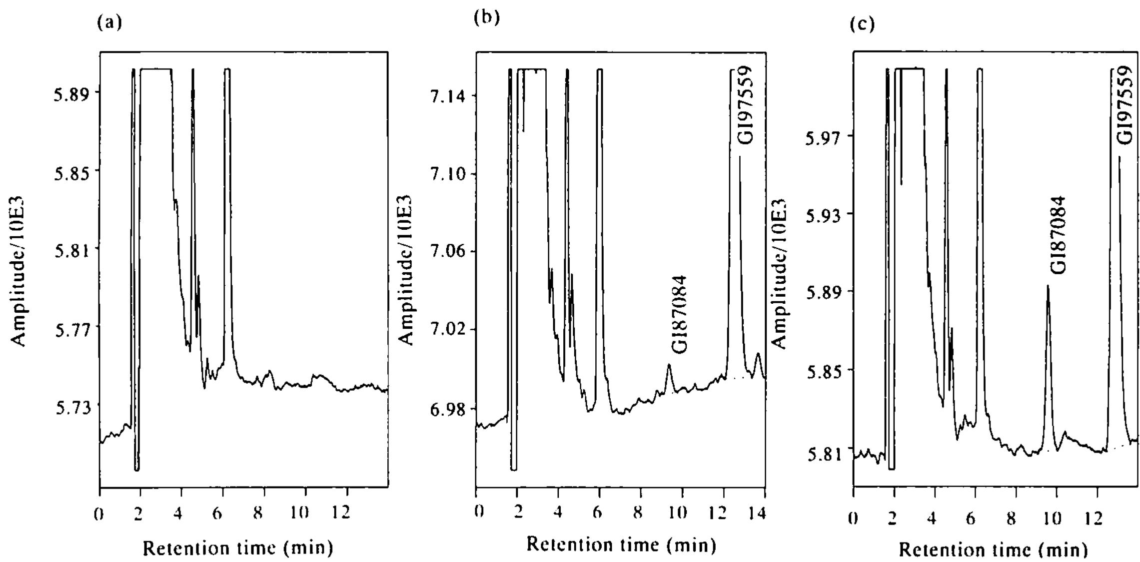

Figure 2
Representative chromatograms of human blood extracts. (a) Drug-free human blood, (b) calibration standard 2.0 ng ml^{-1}, (c) calibration standard 10.0 ng ml^{-1}.

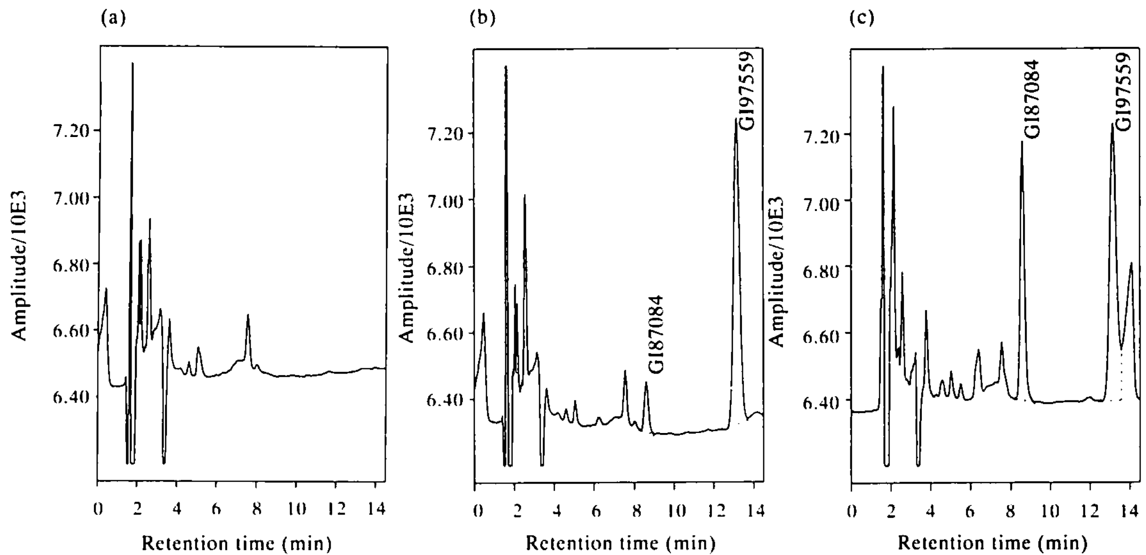

Figure 3
Representative chromatograms of dog blood extracts. (a) Drug-free dog blood, (b) calibration standard 100.0 ng ml^{-1}, (c) dog sample collected 20 min after the drug administration (dose — 10 mg kg^{-1}, i.v. bolus) and containing 424.0 ng ml^{-1} of remifentanil.

absorption of the drug at 210 nm. At this wavelength many interferences could be expected. In order to avoid them two critical steps must be followed: Teflon liners (or aluminium foil) should be used underneath the screw caps as well as glass (not polymer) limited volume inserts to store the back-extracts in the autosampler.

Toxicological study

The dog blood assay was used to analyse toxicity study samples in pure-bred beagle. In the example presented here, dogs received 0.1, 1.0, 10.0 and 40.0 mg kg^{-1} day^{-1} of remifentanil in an intravenous bolus injection, which lasted 60 s. Blood samples were taken at 0.017, 0.087, 0.167, 0.333, 0.5, 1.2 and 3 h after the dose. Plot of the logarithm of plasma concentration versus time was constructed for remifentanil and is presented in Fig. 4.

Conclusions

The described method is simple, quick, selective with regard to the degradation product and sensitive enough to be used in most pharmacokinetic studies in man and dog. The addition of citric acid as a preservative indicates stability of samples while stored, processed and freeze-thawed.

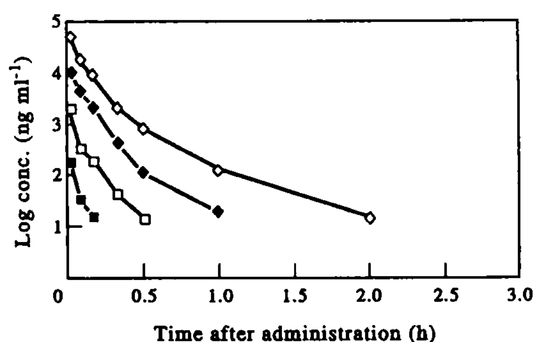

Figure 4
Concentration of remifentanil in dog blood as a function of time after the drug administration. Dose: open diamonds, 40 mg kg^{-1}; full diamonds, 10.0 mg kg^{-1}; open squares, 1.0 mg kg^{-1}; full squares, 0.1 mg kg^{-1}. Remifentanil given as a 60 s bolus i.v. injection.

Acknowledgements — The authors thank Dr Stephen Kutz and Dr Douglas Rickert for the permission to use the dog data in this publication.

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[*Received for review 19 April 1993; revised manuscript received 25 July 1993*]