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Determination of remifentanil in human blood by liquid–liquid extraction and capillary GC–HRMS–SIM using a deuterated internal standard*

C.M. GROSSE,†‡ I.M. DAVIS,‡ R.F. ARRENDALE,§ J. JERSEY‖ and J. AMIN‖

‡*Laboratory Services Section, Department of Clinical Pharmacology, Glaxo Inc. Research Institute, Five Moore Drive, Research Triangle Park, NC 27709, USA*
§*Solvay Pharmaceuticals, 901 Sawyer Road, Marietta, GA 30062, USA*
‖*Triangle Laboratories Inc., 1703 Webb Drive, Norcross, GA 30093, USA*

Abstract: Remifentanil (GI87084) is a phenylaminopiperidine derivative of the fentanyl type with potent analgesic activity. The compound has an N-substituted labile methyl ester which is highly susceptible to chemical and enzymatic hydrolysis resulting in a short half-life for the drug. A sensitive capillary GC–HRMS–SIM method for the determination of remifentanil in blood has been developed and progressively revalidated in response to pharmacokinetic needs. The method relies on immediate precipitation of blood proteins with acetonitrile to stabilize the drug, followed by liquid–liquid extraction with methylene chloride. Collection tubes are pre-spiked with tetra-deuterated remifentanil as an internal standard to correct for variations in recovery and stability between samples. An initial method was developed on a Hewlett–Packard GC–MSD and had a validated range of 1–100 ng ml^{-1}. Due to sensitivity requirements established during the first clinical study, the analysis was transferred to a VG 70S high resolution mass spectrometer and revalidated. The quantitation range of the current assay is 0.1–250 ng ml^{-1}. To date, the method has been used to analyse several thousand blood samples from remifentanil clinical studies.

Keywords: *Remifentanil; GI87084; gas chromatography; mass spectrometry; human blood.*

Introduction

Bioanalytical methods for fentanyl and its congeners include radioimmunoassay [1–3], enzyme immunoassay [4], high-performance liquid chromatography [5–7] and gas chromatography (GC) using either flame ionization, nitrogen-selective (NPD) or mass spectrometric (MS) detection [8–13]. Sample preparation for analysis of these compounds is primarily limited to selection of plasma, serum, or urine followed by immunoassay or extensive solvent extraction. Radioimmunoassay and GC–NPD techniques provide picogram sensitivity, but can have specificity problems or susceptibility to matrix interferences, respectively [14, 15].

Remifentanil (GI87084), methyl-3-[4-methoxycarbonyl-4-[(1-oxopropyl)-phenylamino]-1-piperidine]-propanoate, a novel ultra-short acting μ-opioid of the fentanyl class with potent analgesic activity [16, 17], presents analytical challenges unusual to the fentanyl congeners. Its labile methyl propanoate ester linkage is sensitive to enzymatic and chemical hydrolysis and rapidly forms the corresponding carboxylic acid (GR90291). Separation of plasma or serum from whole blood prolongs exposure of the ester to conditions favouring hydrolysis, thus hindering preparation for immunoassay. Increasing the pH of the matrix also causes rapid chemical hydrolysis of the ester which limits sample extraction options.

A blood sample preparation method for remifentanil was initially validated using trideuterated remifentanil as an internal standard, liquid–liquid extraction and a Hewlett–Packard GC–MSD operated in selected ion monitoring mode (GC–MS–SIM). Fentanyl, a relatively stable opioid, was also included in the assay as a second internal standard to measure absolute recovery of remifentanil. The method provided a linear range from 1 to 100 ng ml^{-1}, but had inadequate sensitivity for clinical sample analyses.

Transfer of the method to a high resolution

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†Author to whom correspondence should be addressed.

mass spectrometer and use of tetra-deuterated remifentanil, a higher purity internal standard, provided an improved linear range from 0.1 to 25 ng ml^{-1} which was later expanded to 250 ng ml^{-1}. Method validation procedures were employed to determine the sensitivity, specificity, precision, accuracy, and ruggedness of the method.

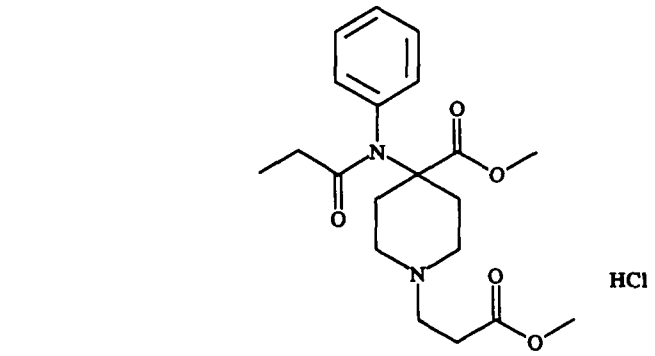
Experimental

Chemicals and reagents

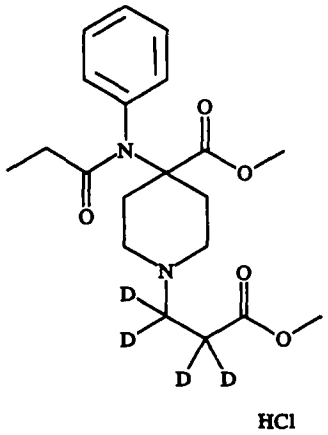
The hydrochloride salts of the reference standards remifentanil (GI87084B) and tetra-deuterated remifentanil (GI87084H) were synthesized at Glaxo Inc. Research Institute (Research Triangle Park, NC). Fentanyl citrate was obtained from Sigma (St Louis, MO). The structures of remifentanil and the two internal standards are shown in Fig. 1. Acetonitrile (UV grade), methylene chloride and ethyl acetate (Capillary GC/GC–MS grade) were obtained from Burdick and Jackson Laboratories (Muskegon, MI). Drug-free heparinized human blood was obtained through the Glaxo Blood Donor program.

Gas chromatography–mass spectrometry

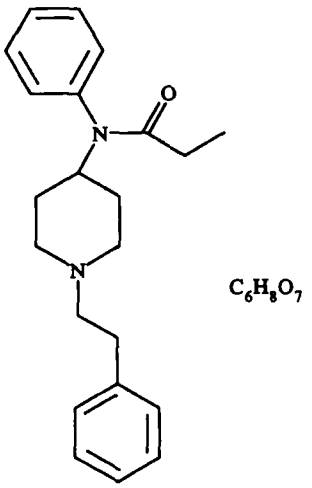
All analyses were performed on an HP Model 5890 gas chromatograph (Hewlett–Packard, Palo Alto, CA) interfaced with a VG Analytical 70S high resolution mass spectrometer (VG/Fisons Instruments, Danvers, MA) operated in electron impact mode. Samples were injected using an HP 7673A autosampler in the splitless mode with a purge-off time of



GI 87084B



GI 87084H



Fentanyl citrate

Figure 1
Structures of remifentanil hydrochloride (GI87084B) and the internal standards $^2H_4$-remifentanil hydrochloride (GI87084H) and fentanyl citrate.

2.5 min. The GC and MS parameters and data integration were controlled by a VAX 3100 work station.

The chromatographic column employed was an Rt_x-1 (100% dimethylpolysiloxane) fused-silica capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Restek, Bellefonte, PA). The carrier gas was ultra-pure grade helium (Air Products, Research Triangle Park, NC) with a column inlet pressure of 15 psi. Residual oxygen and water were scavenged from the carrier gas prior to the GC using a heated High-Capacity Gas Purifier (Supelco, Bellefonte, PA).

Autosampler conditions

The HP 7673A autosampler was programmed to rinse 10 times prior to injection with ethyl acetate, inject a 2 μl sample aliquot, and rinse 10 times after injection with ethyl acetate.

GC conditions

The GC oven was equilibrated at 130°C immediately prior to injection and for 2.5 min post-injection. The oven temperature was initially increased from 130 to 210°C at 50°C min^{-1}, and then from 210 to 280°C at 6°C min^{-1} with no hold time after either increase. The oven temperature was then ramped from 280 to 320°C at 50°C min^{-1} with a 2 min hold at 320°C. The injection port was heated at 250°C. The transfer line was heated at 300°C. The flow rate of helium gas was 1 ml min^{-1}.

Mass spectrometry conditions

The selected ion monitoring (SIM) mode was used for the determination of remifentanil to obtain the desired sensitivity. The ions selected were *m/z* 168.1025 and 227.1158 for remifentanil, *m/z* 172.1276 and 231.1409 for tetra-deuterated remifentanil and *m/z* 245.1655 for fentanyl. An 80 ms dwell time was used for each ion with a 5 ms delay time. In addition, *m/z* 201.8725 and 236.8413 (generated by a molecular leak of hexachlorocyclopentadiene) were monitored as instrument stability and lock mass ions, respectively. Resolution was set at ≥5000 (5% valley definition) which produced peak widths of approximately 200 ppm.

Preparation of solutions

Stock solutions of remifentanil and the internal standards, tetra-deuterated remifentanil and fentanyl, were prepared at 10 μg ml^{-1} as free bases in acetontrile. The solutions were sonicated for 5 min to ensure complete dissolution. The remifentanil stock solution was diluted further with acetonitrile to prepare 1 and 0.1 μg ml^{-1} solutions. The drug and internal standard solutions were combined to prepare spiking solutions to deliver varying amounts of remifentanil, 10 ng tetra-deuterated remifentanil and 50 ng fentanyl per 1 ml whole blood. Separate stock solutions of remifentanil were used for calibration standard and quality control (QC) spiking solution preparation. The stability of refrigerated stock solutions of remifentanil and tetra-deuterated remifentanil was evaluated over several months.

Preparation of standard and quality control (QC) samples

Aliquots (0.2 ml) of standard and QC spiking solutions containing remifentanil and internal standards in acetonitrile were pipetted into screw cap culture tubes. Acetonitrile (2 ml) was then added to each tube, followed by 1 ml of heparinized human whole blood. The tube contents were mixed by vortexing and equilibrated at room temperature for at least 30 min. Methylene chloride (4 ml) was then added to each tube with vortexing and the layers were allowed to separate. The lower organic layer was transferred into a clean tube and evaporated to dryness in a 37°C water bath under a gentle stream of nitrogen. The residue was reconstituted in 50 μl ethyl acetate, placed into GC vials and refrigerated if not analysed immediately.

Standards were prepared in duplicate on the day of analysis using fresh whole blood. QC samples were spiked and extracted as 1 ml aliquots in large batches prior to analysis, and the organic layers were stored at −70°C and processed to completion as needed. The stability of remifentanil in blood and at each processing stage was evaluated under various storage conditions.

Validation procedure

Four analytical runs generated on four separate days were included in the validation of the 0.1 to 25 ng ml^{-1} assay. Analytical runs consisted of calibration standards (0.1, 0.25, 1, 5 and 25 ng ml^{-1}) in duplicate and two sets of QC samples (0.2, 0.8, 4 and 20 ng ml^{-1}) in duplicate. One set of calibration standards was

analysed at the beginning of a run and a second set at the end of a run. QC samples were interspersed throughout the run to monitor method performance. Drug-free blood samples with and without internal standards were included in each run to evaluate background noise. Analytical runs consisted of 52–80 samples per batch.

A cross-validation was completed to extend the upper limit of the assay from 25 to 250 ng ml^{-1}. This involved the preparation and analysis of two analytical batches injected on separate days. Duplicate whole blood calibration standards (0.1, 0.2, 1, 5, 25, 100, 175 and 250 ng ml^{-1}) and two sets of QC samples (0.25, 0.5, 10, 50, 150 and 200 ng ml^{-1}) in duplicate were included in each run.

Quantitation

Individual peak areas and ratios of selected ions from remifentanil and the tetra-deuterated internal standard were received in spreadsheet format from Triangle Labs. The area ratios chosen for quantitation were loaded into an in-house software program (PRANBAS Ver. 2.03.00, Glaxo Inc. Research Institute) using a Lotus-to-PRANBAS transfer program. A linear regression analysis using a least-squares fit was performed with the reciprocal of the squared drug concentration ($1/x^2$) as weight to derive a standard curve. If the percentage error of a standard calibrator exceeded 20% for low, 15% for middle or 10% for high theoretical concentrations, respectively, data for that standard was not used in the regression analysis. The concentrations of analytes in QC and study samples were interpolated from the daily standard curve.

Collection of clinical samples

Subjects were administered remifentanil by intravenous infusion. Blood samples were collected from the opposite arm prior to administration of drug, during and after infusion at the sampling times indicated in the clinical protocols. A recent protocol included a 20 min infusion of remifentanil with sampling times at baseline; every 30 s from 0.5 to 5 min, every minute from 6 to 10 min, and every 2 min from 12 to 20 min during and post-infusion; and then at 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210 and 240 min after the end of the infusion. Blood samples were collected in heparinized Vacutainer tubes and inverted several times to ensure sample homogeneity. A 1 ml aliquot was withdrawn from the Vacutainer within 1 min of collection and pipetted into a glass tube that contained 2.2 ml acetonitrile, 10 ng tetra-deuterated remifentanil, and 50 ng fentanyl. The samples were immediately processed as described previously through transfer of the organic layer to clean tubes. The tubes containing the organic extracts were then capped and stored at −70°C until analysis.

Results and Discussion

Precision and accuracy

The mass spectra of remifentanil and the stable isotope internal standard are shown in Fig. 2. The area ratios of the base peak ions, *m/z* 168.1025 for remifentanil and *m/z* 172.1276 for the tetra-deuterated internal standard were selected for quantitation. A linear regression analysis using a least-squares fit was performed with the reciprocal of the squared drug concentration ($1/x^2$) as weight to minimize error at the low end of the calibration curve.

Daily calibration data during the 4-day validation demonstrated acceptable assay precision with relative standard deviation (%RSD) values for the calibrator concentration groups ranging from 3.5 to 6.3% after rejection of outliers. Individual back-calculated concentrations for the calibration standards were within 12.4% of nominal. Daily correlation coefficients (r) were ≥ 0.9966 indicating acceptable goodness of fit over the concentration range of 0.1–25 ng ml^{-1}.

The within- and between-day assay precision and accuracy of the QC sample results from the validation are summarized in Tables 1 and 2, respectively. The RSD values for the within-batch precision results ranged from 3.3 to 12.0%. The RSD values for the between-batch precision results ranged from 6.4 to 15.5%. The interpolated QC concentration averages were within 10% of nominal for all four QC levels.

Results from the 2-day cross-validation of the 0.1 to 250 ng ml^{-1} assay also demonstrated acceptable assay precision and accuracy with %RSD values for the QC samples ranging from 7.0 to 15.9%, and interpolated QC concentration averages within 3.2% of nominal for all levels. Daily correlation coefficients were ≥ 0.9965.

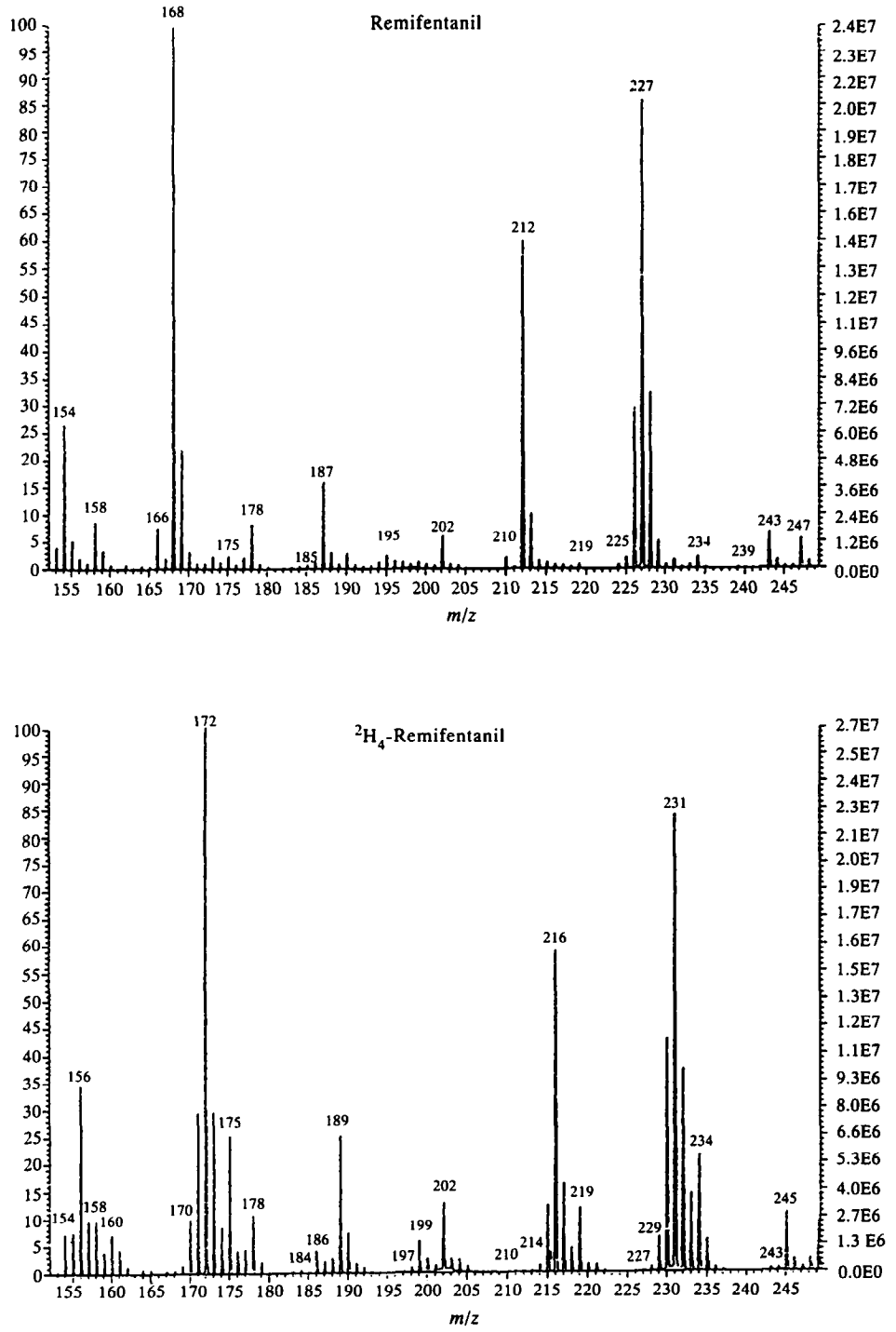


Figure 2
Mass spectra of remifentanil and $^2H_4$-remifentanil.

Recovery

Recovery was determined by comparing the peak areas of remifentanil in extracted QCs at 0.25, 20 and 200 ng ml^{-1}, to those in non-extracted QCs at equivalent concentrations. It was necessary to spike the non-extracted QCs into drug-free blood extracts to eliminate matrix-related chromatographic differences. As presented in Table 3, the mean recovery of remifentanil from human blood was 72.7% at 0.25 ng ml^{-1} (%RSD = 16.5%, n = 6), 75.6% at 20 ng ml^{-1} (%RSD = 9.6%, n = 6) and 83.0% at 200 ng ml^{-1} (%RSD = 7.1%, n = 6).

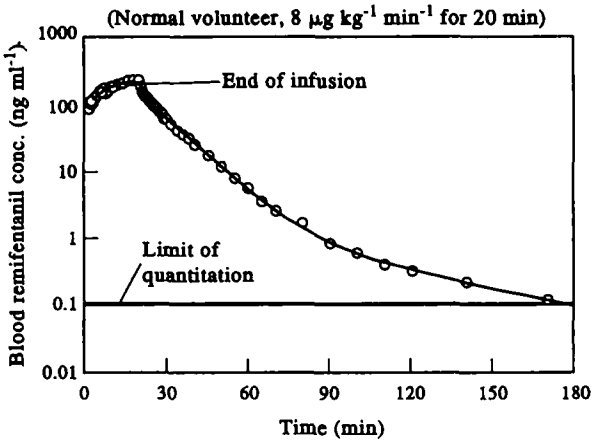


Figure 3
Blood remifentanil concentration–time profile.

Table 1
Within-batch precision and accuracy for the quantitation of remifentanil in human blood

Nominal QC conc. (ng ml^{-1})	Calc. QC conc. (ng ml^{-1})	SD	RSD (%)	Accuracy (%)	*n*
0.2	0.190	0.0228	12.0	95.0	7
0.8	0.802	0.0490	6.1	100.3	7
4.0	4.13	0.1361	3.3	103.3	8
20.0	20.5	1.1337	5.5	102.5	8

Table 2
Between batch precision and accuracy for the quantitation of remifentanil in human blood

Nominal QC conc. (ng ml^{-1})	Calc. QC conc. (ng ml^{-1})	SD	RSD (%)	Accuracy (%)	*n*
0.2	0.180	0.0279	15.5	90.0	15
0.8	0.788	0.0503	6.4	98.5	16
4.0	4.09	0.3199	7.8	102.3	16
20.0	20.1	1.3909	6.9	100.5	16

Table 3
Recovery of remifentanil from human blood

Nominal QC conc. (ng ml^{-1})	% of nominal	*n*
0.25	72.7	6
20.0	75.6	6
200.0	83.0	6

Specificity

The original method for the detection of remifentanil in human blood had a validated range of 1–100 ng ml^{-1} and used trideuterated remifentanil as an internal standard. The LOQ of the assay was limited by the presence of an internal standard fragment ion which produced a response in the mass channels selected for the analyte. Since this response was a constant, initial revalidations of the assay involved successive lowering of the internal standard concentration. This approach was facilitated by the concurrent transfer of the analysis to a contract mass spectrometry laboratory (Triangle Labs, RTP, NC) with high resolution instruments, and eventually produced a method with an LOQ of 0.1 ng ml^{-1} at an internal standard level of 1 ng ml^{-1}.

Following the completion of the first clinical study, a tetra-deuterated internal standard was synthesized by the Drug Metabolism department (Glaxo). The higher isotopic purity of the tetra-deuterated remifentanil made it possible to improve the reliability of the assay by increasing the concentration of the internal standard from 1 to 10 ng ml^{-1} while still maintaining the LOQ at 0.1 ng ml^{-1}. The peak

area ratio for background noise in drug-free blood samples containing tetra-deuterated remifentanil was generally one-third the average peak area ratio for the lowest standard as specified in departmental validation guidelines. No interferences were observed in drug-free blood samples without internal standard.

Stability

Early *in vitro* testing of remifentanil in human blood at 37°C indicated that the half-life of the drug was approximately 10 min. Although the actual *in vitro* half life was later determined to be closer to 60 min, the earlier results preceded the clinical studies, and had a significant impact on the development of the method and on the subsequent design of the stability experiments.

Due to the susceptibility of remifentanil to chemical and enzymatic hydrolysis, whole blood was chosen as the matrix to minimize processing time. However, since the blood proteins are denatured and the drug is extracted immediately after collection, the medium stored is an acetonitrile–methylene chloride mixture. This extract does not freeze at temperatures as extreme as −70°C, so no evaluation of freeze–thaw cycling stability was necessary. In addition, since remifentanil is immediately paired in blood samples with the stable isotope internal standard, variations in stability and recovery between samples are corrected relative to a species with nearly identical physicochemical properties. Consequently, the stability testing of remifentanil in blood included evaluation of processing stability prior to the extract stage, long-term storage stability of acetonitrile–methylene chloride extracts, and stock solution and final extract stability.

Addition of acetonitrile preserved the integrity of blood samples stored at room temperature for at least 4 h. Stability studies of acetonitrile–methylene chloride extracts separated from whole blood and stored at various conditions indicated that peak area ratios of the drug and internal standard were stable for at leat 210 days at −20°C and 90 days at −70°C (Table 4). A dramatic increase in peak area ratios was observed in extracts stored at −70°C for 210 days and an experimental error was suspected. The stability of remifentanil and tetra-deuterated remifentanil in acetonitrile stock solutions stored at 4°C was at least 18 months, and their relative response remained constant in final extracts for up to 1 week.

Clinical pharmacokinetics

The described analytical method was used to determine subject blood concentrations of remifentanil during and after a 20 min intravenous infusion. A representative blood concentration versus time profile obtained from one of these subjects is presented in Fig. 3. Ion chromatograms from extracts of a drug-free blood sample with internal standard, a 0.1 ng ml^{-1} standard, and a subject sample are shown in Fig. 4.

Conclusions

A sensitive and specific assay for the determination of remifentanil in human whole blood using a stable isotope internal standard and capillary GC–HRMS–SIM has been developed. The method has a validated range of 0.1–250 ng ml^{-1} and has been used to analyse several thousand clinical blood samples to date.

Table 4
Long-term storage stability of remifentanil human blood extracts. Results are in % nominal

Elapsed (days)	Stability conc. = 0.2 ng ml^{-1}		Stability conc. = 8 ng ml^{-1}	
	−20°C	−70°C	−20°C	−70°C
3	99	94	102	102
7	116	101	107	103
30	99	99	101	98
90	97	95	100	99
210	97	E*	97	E*

*, Experimental error.

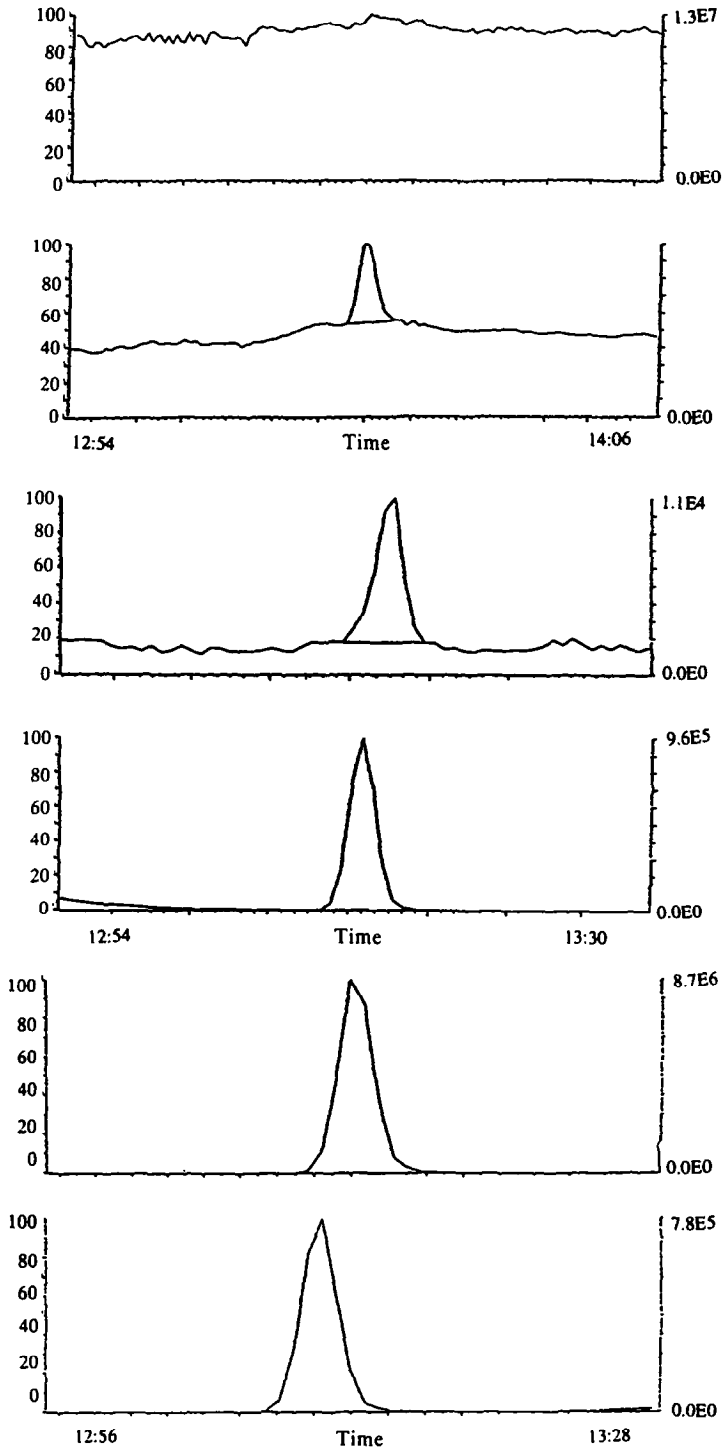


Figure 4a

Figure 4b

Figure 4c

Figure 4
Ion chromatograms of remifentanil (*m/z* 168.1025, upper chromatogram) and the internal standard $^{2}H_{4}$-remifentanil (*m/z* 172.1276, lower chromatogram) from extracted human blood samples of (a) drug-free blood, (b) 0.1 ng ml^{-1} standard and (c) subject no. 10 after 1 min of infusion.

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