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Determination of remifentanil in human heparinised whole blood by tandem mass spectrometry with short-column separation

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Abstract

For the use in pharmacokinetic studies, a fast and sensitive assay method was developed for the determination of remifentanil in human heparinised whole blood samples of 0.5 ml. The assay method is based on tandem mass spectrometry detection (LC–MS/MS). The limit of quantification is 0.1 ng/ml and linear up to 50 ng/ml. The precision, accuracy, recovery and applicability were found to be adequate for pharmacokinetic studies.

Keywords: Remifentanil; Liquid chromatography; Mass spectrometry; LC–MS/MS; Human blood

1. Introduction

Remifentanil, methyl-3-[4-methoxycarbonyl-4-[(1-oxopropyl)-phenylamino]-1-piperidine]-propanoate (see Fig. 1), is an ultra-short-acting opioid anaesthetic agent. It is used extensively for short-term anaesthesia, often in combination with sedatives. Remifentanil is rapidly metabolised by esterases in blood, plasma and tissue. Hence its non-organ dependent clearance, it has advantages in clinical situations for anaesthesia of patients with renal or hepatic diseases and ambulatory surgery. For pharmacokinetic studies, sensitive assay methods are required with limited sample preparation and which have a lower limit of quantification of at least 1 ng/ml because of the therapeutic window of 1–40 ng/ml after an infusion rate of 0.04–2 μg/kg bodyweight per min [1–3].

Remifentanil concentrations are frequently assessed. A few assay methods have been published [4–12], but only a limited number do have well-documented lower limits of quantification in a

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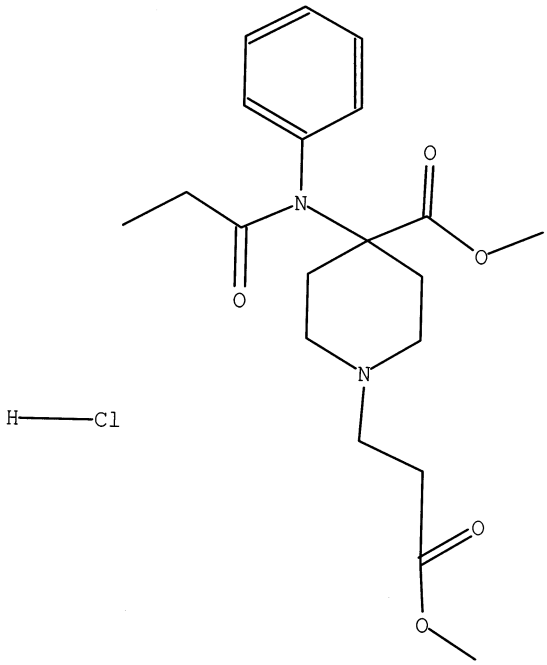

Fig. 1. Structure of remifentanil HCl.

range below 1.0 ng/ml, required for such studies [9,10]. The most sensitive assay methods published so far are based on GC–MS analysis having a limit of quantification of 0.1 ng/ml but requiring time consuming sample preparation and analytical procedures. Beside GC–MS methods, another HPLC method has been published having a limit of quantification of 2.5 ng/ml [4].

The combination of sensitivity, specificity and high sample throughput were the main reasons why it was decided to explore the possibilities of HPLC with tandem mass spectrometry detection. Specificity of the analysis and limited sample preparation are particularly required in case pharmacokinetic profiles of remifentanil in patients, co-administered with other compounds which are also hydrolysed by esterases like esmolol (β-adrenergic blocking agent) and mivacurium chloride (muscle relaxant), are investigated [13,14]. Because of the rapid excretion rate of remifentanil, assessment of pharmacokinetic profiles in patients with continuous infusion of remifentanil often lead to large numbers of samples per subject.

Up to now LC–MS/MS methods have not been published for the assessment of remifentanil in blood. Performance characteristics of analytical methods to determine remifentanil in different matrices have been summarized in Table 1.

2. Experimental

2.1. Chemicals and reagents

Remifentanil HCl ([132579-07-2], $C_{20}H_{29}N_2O_5Cl$, batch no. 8) as compound to be measured and D4-remifentanil (D4-$C_{20}H_{25}N_2O_5$, HTS no. 2933.90.55.90) as internal standard were kindly provided by Glaxo Wellcome Operations, Great Britain and Glaxo Wellcome Inc., USA, respectively.

Table 1
Performance characteristics analytical methods[a]

Author	Matrix	Method	Extraction	Range (ng/ml)	PC
Selinger et al [9]	Human and dog blood	HPLC–UV at 210 nm (run time[b]: 13 min)	Liquid–liquid extraction followed by back-extraction	1.0–200	L,P1,P2,A,FT,S2,D and R
Haidar et al [4]	Rat blood	HPLC–UV at 210 nm (run time[b]: 10 min)	Liquid–liquid extraction followed by back-extraction	2.5–250	L,P1,P2,A, LLOQ,S2 and R
Grosse et al [10]	Human blood	GC–MS (run time: 18.6 min)	Precipitation proteins followed by liquid–liquid extraction	0.1–250	L,P1,P2,A,S1,S2,and R

[a] PC, performance characteristics investigated; L, linearity; P1, repeatability; P2, reproducibility; A, accuracy; LLOQ, validation of lower limit of quantification; FT, freeze/thaw experiments; S1, stability sample-extracts; S2, stability QC samples; D, dilution QC samples out of range; R, extraction recovery.

[b] Based on retention time of last peak.

Acetonitrile (FarUV quality) was obtained from LabScan (Boom, Meppel, The Netherlands); chloroform, ammonium acetate, citric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate (all pro analyse quality) were obtained from Merck (Merck, Darmstadt, Germany); hydrochloric acid (pro analyse quality) was obtained from Riedel-de Haën (Riedel-de Haën, Seelze, Germany) and dichloromethane (Ultra Resi Analysed quality) was obtained from Baker (Baker, Deventer, the Netherlands).

2.2. Instrumentation

Experiments were performed on a SCIEX API-III^{plus} triple quadrupole mass spectrometer with the turbo ion spray interface operated in the positive ion mode and the temperature of the turbo set at 200°C. The curtain gas flow was 1.2 l/min, the orifice-60 V, colligion-gas was set at 280×10^{12} molecules/cm^2 and the turbo ionspray was applied with a nitrogen auxiliary flow at 5000 ml/min. The Sciex was equipped with a Perkin Elmer Series 200 pump and a Perkin Elmer ISS 200 autosampler. The mobile phase consisted of a mixture of acetonitrile/chloroform (1:1 v/v), containing ammonium acetate in concentration of 2 mmol/l. The flow, injection volume and runtime were 0.3 ml/min, 20 μl and 3 min respectively. The scanning dwell time in the MRM mode was 100 ms and a pause time of 99 ms was used.

Data analysis was performed using the Macquan software (version 1.5) running on an Apple Macintosh Quadra 800 (operating system: Macintosh system 7.5). The following ions are selected for measurement (precursor/product transitions): remifentanil: precursor-ion 377, product-ion 228; D4-remifentanil: precursor-ion 381, product-ion 232. For the short-column separation of endogenous compounds and remifentanil, a short C_{18} column, Pecophere, Perkin–Elmer, part.no. 0258–0164 3.3 cm, id 4.6 mm was used.

2.3. Extraction procedure

Polypropylene tubes, used for the extraction, contained 20 μl 50% w/w citric acid solution per ml heparinised whole blood to prevent the hydrolysis of remifentanil via pH control [9]. To the polypropylene tubes, 0.5 ml heparinised whole blood and 25 μl internal standard solution 500 ng/ml in 0.001 mol/l HCl were added and mixed. By adding 0.5 ml 0.1 mol/l phosphate buffer pH 7.4, the sample pH was readjusted back to pH 7 enhancing the extraction efficiency. This mixture was vortex-mixed until a homogeneous sample was obtained. Subsequently, 2.0 ml of dichloromethane was added and extraction was conducted by shaking mechanically for 10 min.

After the extraction, the polypropylene tubes were centrifuged at 13 000 rpm for 10 min; the upper aqueous layer was discarded. The remaining dichloromethane was evaporated to dryness with the aid of dry nitrogen at ambient temperature. For reconstitution, 125 μl of mobile phase was added and vigorously shaken.

2.4. Calibration

Calibration standards were prepared at 6 different concentrations in the range of 0.1 to 50 ng/ml (0.1, 0.5, 2.0, 5.0, 20 and 50 ng/ml). Appropriate amounts of remifentanil stock solutions prepared in 0.001 mol/l HCl were added to polypropylene tubes containing 0.5 ml heparinised whole blood and 10 μl 50% w/w citric acid solution in deionised water. Calibrators were extracted the same way as the quality control samples. Calibration, based on area ratios, was performed before and after each analytical run; both calibration lines were used for calculations. Weighted linear regression ($1/Y^2$) was employed for calculations.

3. Results

3.1. Spectra

Product spectra of remifentanil $[M+H]^+$ $m/z = 377$ and the internal standard D4-remifentanil $[M-H]^-$ $m/z = 381$ are shown in Fig. 2.

3.2. Calibration

The back-calculated concentrations differed less than ten percent through the range of 0.1–50

ng/ml and within 15% at a level of 0.1 ng/ml, the equation of the calibration curve being $Y = 0.063X + 0.001$ (coefficient of correlation was 0.999, relative standard deviation of slope and intercept 1.4%, respectively 20%).

3.3. Precision and accuracy

The repeatability and reproducibility data were determined using spiked samples containing 0.1, 0.2, 30 and 50 ng/ml. The results are presented in Table 2. Within a concentration range from 0.09 to 47.6 ng/ml the RSD(%) of the repeatability varied between 10 and 2.7%.

Good agreement was observed between the nominal concentrations and the measured concentrations, expressed in percent accuracy of the nominal concentration.

In Fig. 3 chromatograms of remifentanil in blank blood and a sample are shown after a short-column separation. The chromatogram of blank does not show any interference.

3.4. Selectivity

Selectivity was defined as the lack of interfering peaks at the retention times of remifentanil and D-4 remifentanil in the chromatograms. This was confirmed by a six-fold injection of the extracts of six different blank matrix samples. Six blank matrices were obtained from the Leiden University Medical Center. No interfering peaks could be

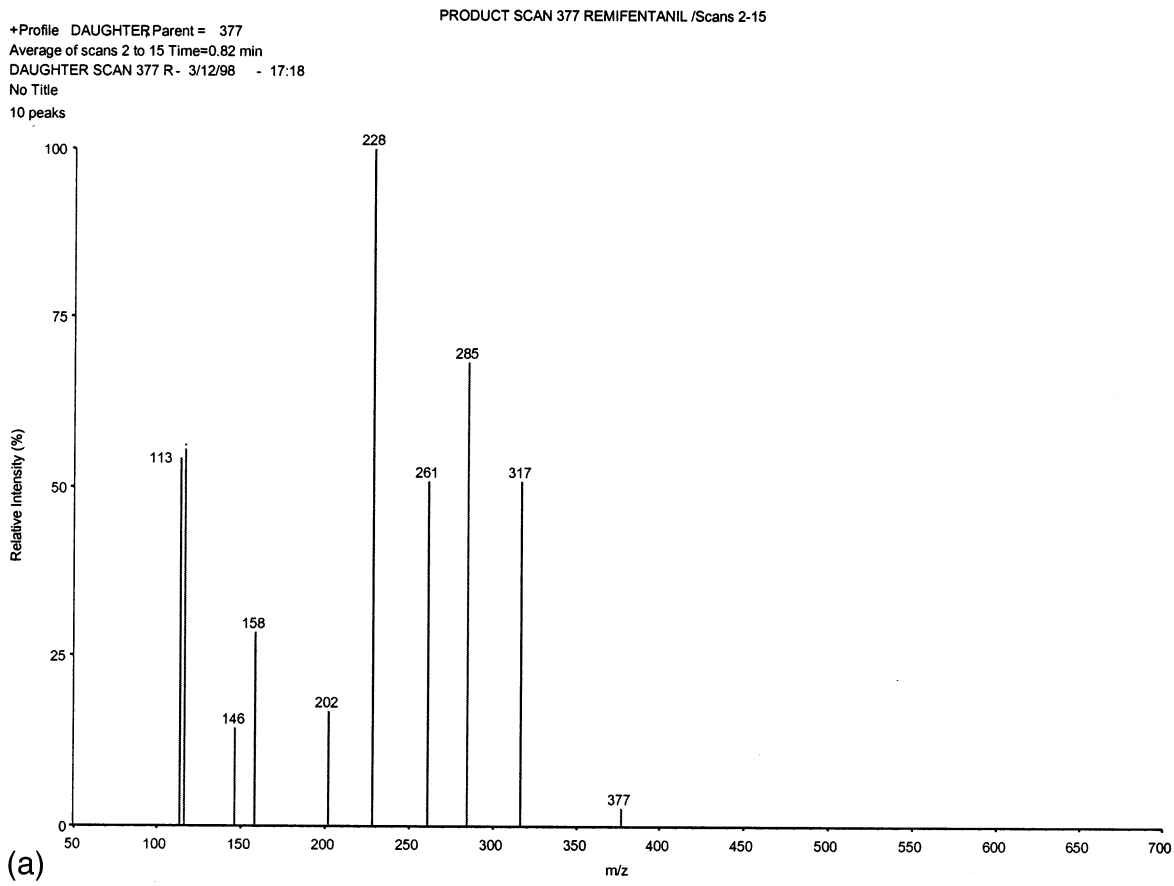

Fig. 2. Product spectra of the precursor parent ion $[M-H]^-$, m/z 377 of remifentanil (a) and the m/z 381 of the internal standard D4-remifentanil (b).

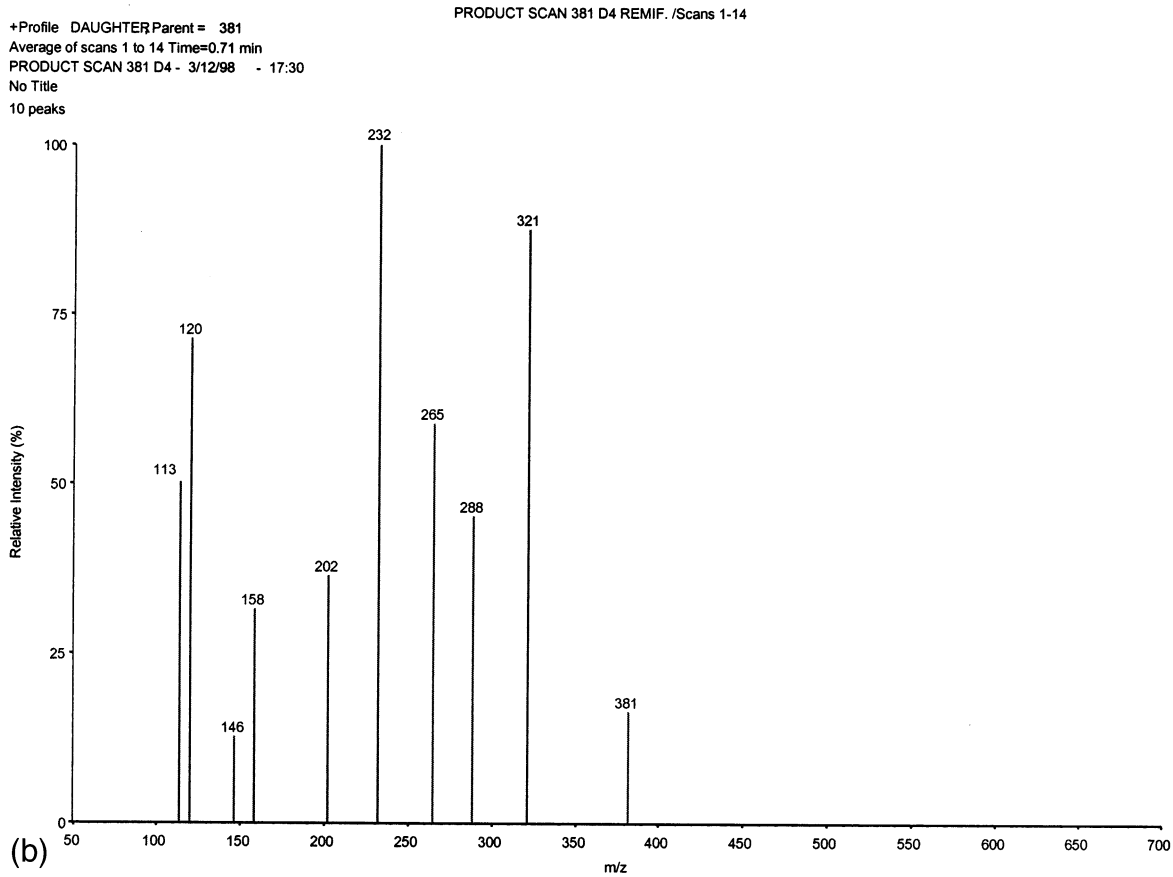

Fig. 2. (*Continued*)

detected; the calculated amount of remifentanil was in all six blank matrices below the lower limit of quantification of 0.1 ng/ml.

3.5. Stability

The influence of frequent freezing and thawing of quality control samples at 0.2, 30 and 50 ng/ml was assessed after three freeze–thaw cycles, each separated by at least 1 day. Freezing occurred at −20°C; thawing at ambient temperature. The calculated concentrations were all within ±15% of the nominal concentration: after three freeze–thaw cycles, the decrease in concentration was 2–6%, being within the precision of the method.

Table 2
Repeatability and reproducibility data of the assay ($n = 6$)

Nominal concentration (ng/ml)	Repeatability (rsd%)	Reproducibility (rsd%)	Accuracy (%)
0.1	10	2.1	93
0.2	7.8	9.0	97
30	2.5	3.8	98
50	2.7	3.7	95

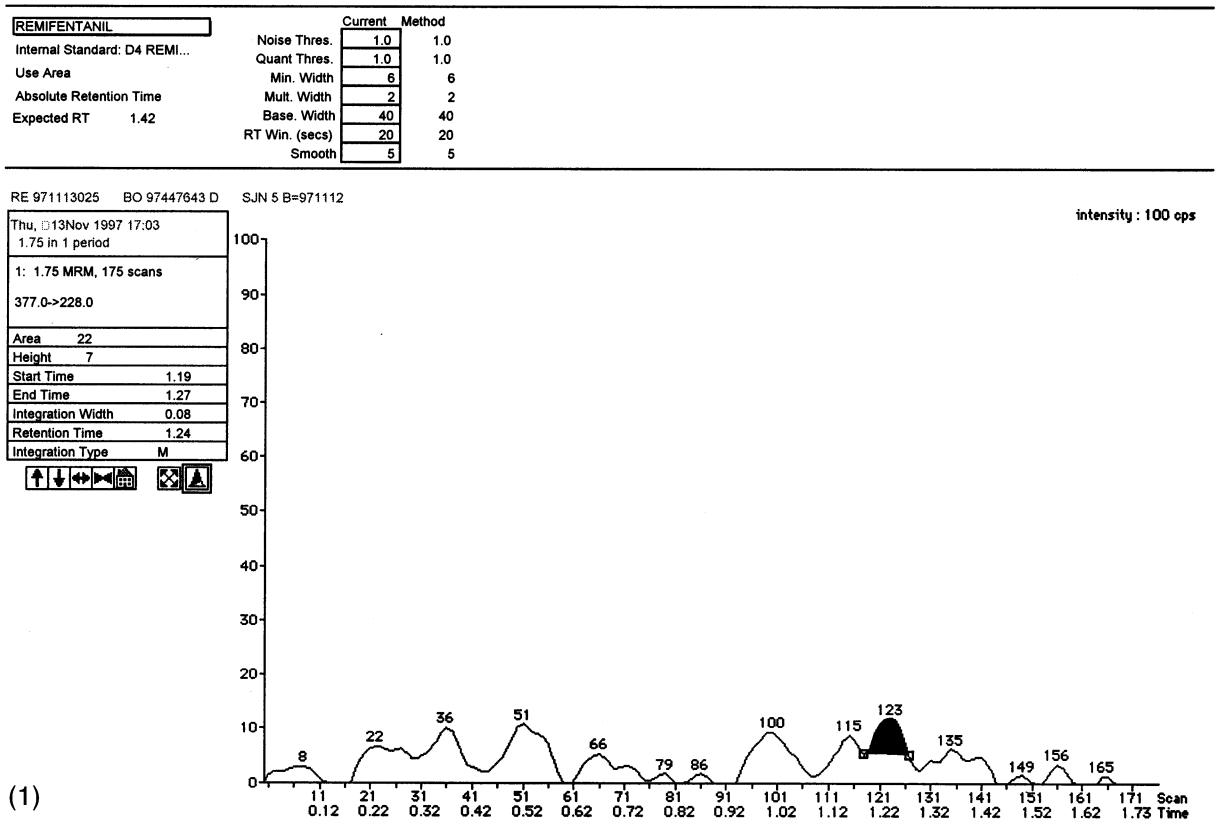

Fig. 3. MRM (multiple reaction mode) chromatograms of remifentanil: (1) blank blood, (2) blood extract (0.2 ng/ml) of remifentanil and (3) internal standard D4-remifentanil.

To determine the extract's stability, three different extracts of a quality control sample, within the analytical range, were assayed in duplicate directly after extraction. Extracts, stored at ambient temperature as well as at 2–8°C, were analysed after 24, 48, 72 and 125 h. Extracts were re-injected within another analytical validation run. At each time-point, the calculated average concentration was within $\pm 15\%$ of the nominal concentration. Sample extracts were stable for 1 month when stored in a monitored freezer at -20°C.

Stability of quality control samples was established in monitored freezers at -20 and -70°C. After 6 months storage at the indicated conditions, concentrations were within $\pm 15\%$ of the nominal concentration of 60 and 100 ng/ml.

In order to assess the long-run stability of the analytical procedure, quality control samples having concentrations of 0.2 and 30 ng/ml were determined at the beginning and at the end of an analytical run (number of injections in between: 144). The obtained values were within $\pm 15\%$ of the nominal concentrations. Therefore, the performance of the analysis is stable through the whole run.

3.6. Carry-over

Carry-over was determined by comparing the signal of a blank sample, directly followed by a determination of the highest calibration standard. The blank signal was approximately 0.008% compared with the signal of the highest calibration standard, being 50 ng/ml.

3.7. Samples out of range

For measuring samples out of range, a quality control sample, at concentration 500 ng/ml, was diluted with whole blood to obtain concentrations falling within the calibration range (1.0 and 20 ng/ml). These dilution controls were analysed six times within one analytical run. The back-calculated concentration was within $\pm 15\%$ of the nominal concentration.

3.8. Recovery

The extraction recovery was determined by the ratio of the slope of the standard/internal standard ratios for an extracted and an unextracted standard curve. Regarding the extracted samples, D4-remifentanil was added after the extraction step, and was used as an external standard. The overall remifentanil recovery through the analytical range was calculated to be 76%.

4. Discussion

The instability of remifentanil in human whole blood and plasma has been investigated in detail [4,7,10,14]. Glaxo Wellcome data indicate that the hydrolysis in blood or plasma follows the Arrhenius equation in the range of 25–45°C. The rate of hydrolysis will therefore be negligible at −20°C, taking into account a Q_{10}-factor of 1.45 and a k_e of 0.15 at 25°C (K. Selinger, personal communication). Based on these findings, appropriate measures were taken to avoid decomposition of the *N*-substituted ester group of remifentanil [9]. These measures included the analysis of remifentanil in whole blood, avoiding hydrolysis, induc-

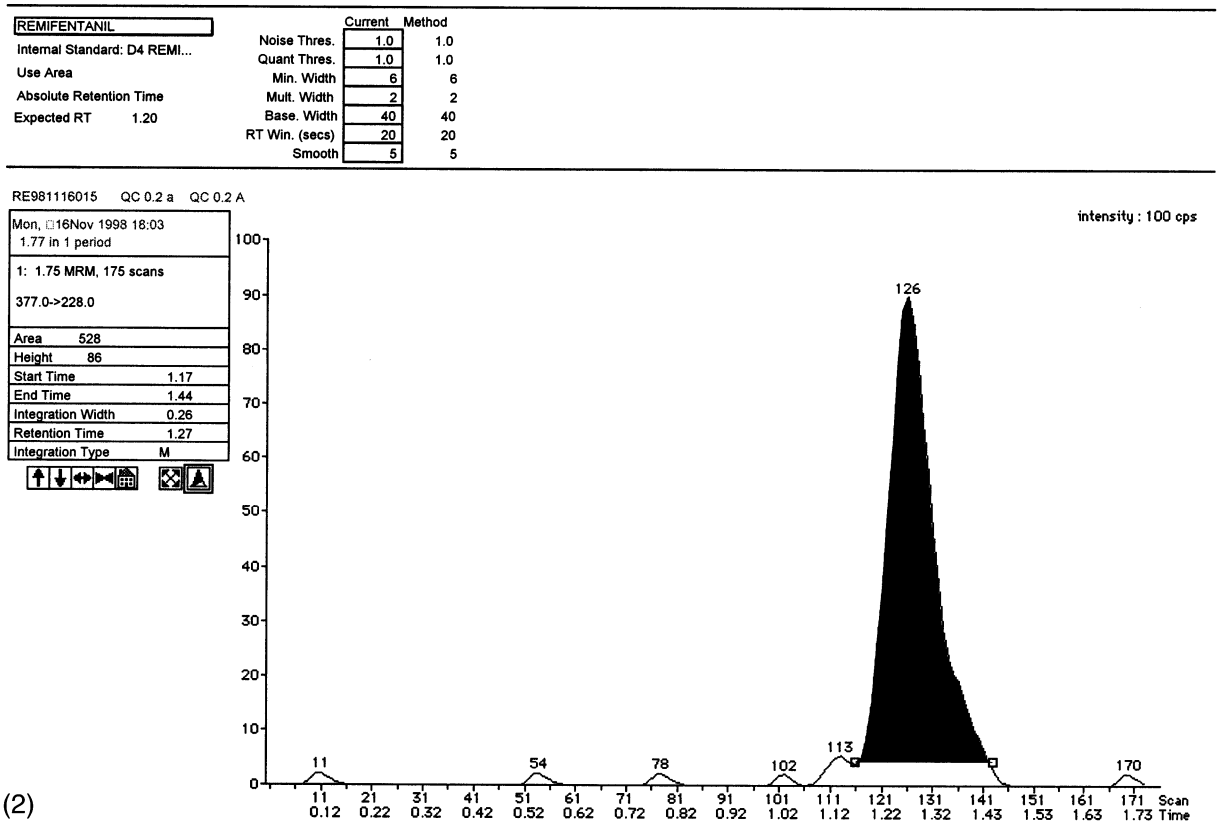

Fig. 3. (*Continued*)

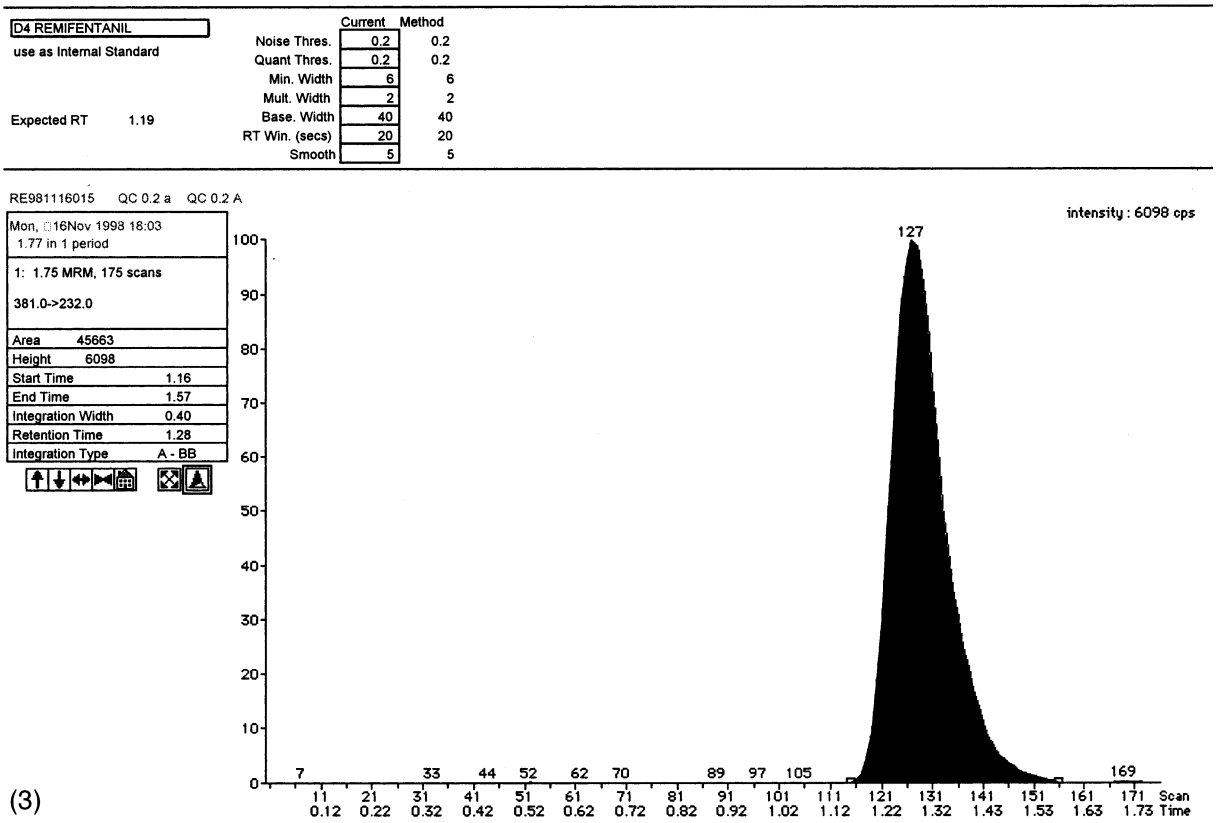

Fig. 3. (*Continued*)

ing preparation of plasma or serum. Additionally, based on Selinger et al. [9], Nalgene® tubes were pre-filled with 20 μl of 50% w/w citric acid solution in deionised water in which, after sampling at the clinic (or for the preparation of a quality control sample), 1.0 ml of blood was transferred into, mixed and flash frozen at −20°C, or lower, within 30 min. It was proven that these measures sufficiently protected remifentanil against decomposition after three freeze–thaw cycles as well as 6 months storage in the freezer at −20°C and subsequent processing at room temperature.

Grosse et al. [10] extracted the samples with methylene chloride using precipitation with acetonitril after which sample treatment was performed by addition of 1 ml of 1 mol/l phosphate buffer pH = 7.4 to the samples, cooling and extraction with *n*-butyl chloride. After freezing out of the water phase, the organic phase was back-extracted with 0.01 mol/l HCl [4,9]. By changing the organic phase of the later method from *n*-butyl chloride to dichloromethane, it proved that back-extraction could be discarded. Short chromatographic separation between remifentanil and the endogenous components on a short C_{18} column proved to be efficient to prevent ion suppression in the ion source.

HPLC–UV methods are very suitable for toxico-kinetic studies of remifentanil. GC–MS methods provide both sensitivity and a wide analytical range. However, both methods are time consuming, either in sample preparation or speed of analysis. Since the newly developed LC–MS/MS method combines sensitivity and high sample throughput in an appropriate analytical range, the use of LC–MS/MS might be considered to be

superior to the GC–MS and HPLC methods for pharmacokinetic studies. Additionally, the principle of LC–MS/MS probably results in an assay that is specific with reference to co-administered compounds.

5. Conclusion

The method for the determination of remifentanil in human heparinised whole blood with LC–MS/MS showed acceptable precision, recovery, robustness and applicability at a concentration range of 0.1–50 ng/ml of remifentanil.

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