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The influence of the sample matrix on LC-MS/MS method development and analytical performance

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Chapter 4

Analysis of remifentanil with LC-MS/MS and an extensive stability investigation in EDTA whole blood and acidified EDTA plasma

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ABSTRACT

Remifentanyl is a mu-opioid receptor agonist and was developed as a synthetic opioid for use in anaesthesia and intensive care medicine. Remifentanyl is rapidly metabolized in both blood and tissues, which results in a very short duration of action. Even after blood sampling remifentanyl is unstable in whole blood and plasma through endogenous esterases and chemical hydrolysis. The instability of remifentanyl in these matrices makes the sample collection and processing a critical phase in the bioanalysis of remifentanyl.

The authors have developed a fast and simple sample preparation method using protein precipitation followed by LC-MS/MS analysis. In order to improve the stability of remifentanyl, citric acid, ascorbic acid and formic acid were investigated for acidification of the EDTA plasma. The stability of remifentanyl was investigated in stock solution, EDTA whole blood, EDTA plasma and acidified EDTA plasma at ambient temperature, 4°C, 0°C and at -20°C.

The analytical method was fully validated based on the FDA guidelines for bio analytical method validation with a large linear range of 0.20–250 ng/mL remifentanyl in EDTA plasma acidified with formic acid. The stability results of remifentanyl in EDTA tubes, containing whole blood placed in ice water showed a decrease of approximately 2% in 2 hours. EDTA plasma acidified with citric acid, formic acid and ascorbic acid showed 0.5%, 4.2% and 7.2% remifentanyl degradation respectively after 19 hours at ambient temperature. Formic acid was chosen because of its volatility and thus LC-MS/MS compatibility. The use of formic acid added to the EDTA plasma improved the stability of remifentanyl, which now showed to be stable for 2 days at ambient temperature, 14 days at 4°C and 103 days at -20°C.

The developed analytical method uses a simple protein precipitation and maximal throughput by a two point calibration curve and short run times of 2.6 minutes. Best sample stability is obtained by placing tubes containing EDTA whole blood in ice water directly after sampling, followed by centrifugation and transfer of the EDTA plasma to tubes with formic acid. The stability of remifentanyl in EDTA plasma was significantly improved by the addition of 1.5 µL formic acid per mL of EDTA plasma. This analytical method and sample pre-treatment is suitable for remifentanyl pharmacokinetic studies.

INTRODUCTION

Remifentanyl is a mu-opioid receptor agonist belonging to the family of phenyl-piperidine derivatives and was developed as a synthetic opioid for use in anaesthesia and intensive care medicine [1, 2]. Remifentanyl has a unique pharmacokinetic profile characterized by its rapid metabolism in both blood and tissues by endogenous esterases. Unlike the other fentanyl congeners, termination of the therapeutic effect of remifentanyl mostly depends on metabolic clearance rather than on redistribution, while pharmacodynamically remifentanyl is similar to the other fentanyl congeners. The half-life of remifentanyl is approximately 3 minutes, independent of the duration of infusion [1]. Even after blood sampling remifentanyl is unstable in whole blood and plasma because of the N-substituted methyl propanoate ester group of remifentanyl, which is highly susceptible to endogenous esterases and chemical hydrolysis.

The therapeutic analgesic concentration of remifentanyl is approximately between 0.5 and 8 ng/mL. In addition to the preferred low sample volume, a sensitive method is mandatory for the pharmacokinetic studies evaluating the time course of remifentanyl concentrations in plasma.

It is well known that degradation of remifentanyl is inhibited by acidification of the blood or plasma with citric acid [3-9]. Most of the previous publications refer to the article of Selinger and co-workers published in 1994 [3]. Here, the acidification of the whole blood with citric acid is described, but the (target) pH of the acidified blood samples was never mentioned. Other publications which described the acidification with citric acid neither mentioned the target pH of the blood or plasma samples [3-9].

Some analytical methods were developed to quantify remifentanyl in whole blood instead of plasma in order to avoid the time necessary for obtaining the plasma [3, 5, 8, 10, 11]. For example, a direct transfer of the whole blood to tubes with acetonitrile in order to overcome the instability of remifentanyl has been described [1, 11].

The applied analytical techniques found in the literature are: High Performance Liquid Chromatography with Ultraviolet detection (HPLC-UV) [3, 7], gas chromatography [8, 11] and HPLC with (tandem) mass spectrometry detection (LC-MS/(MS)) [5, 9, 10]. Most of these methods use time consuming Liquid-Liquid Extraction (LLE) or Solid-Phase Extraction (SPE) procedures to process the whole blood or plasma samples.

In addition to the use of citric acid, ascorbic acid and formic acid were investigated for acidification of the EDTA plasma and thus the stabilization of remifentanil. During LC-MS/MS analysis the HPLC solvent flow containing the injected sample is vaporized in the heated electrospray ionization source. This requires the use of volatile additives to prevent deterioration of system performance. Since formic acid is volatile, it is considered a more compatible acid for LC-MS/MS analysis than ascorbic acid or citric acid.

The aim of this study was to develop a fast and simple LC-MS/MS method for the analysis of remifentanil in EDTA plasma and to extensively investigate the stability of remifentanil in untreated EDTA whole blood and in EDTA plasma.

MATERIALS AND METHODS

Chemicals and reagents

Remifentanil was purchased from BDG Synthesis Limited (Wellington, New Zealand). The isotopically labeled internal standard (IS) [$^{13}\text{C}_6$]-remifentanil was purchased from Alsachim (Illkirch Graffenstaden, France). Analytical grade methanol, formic acid, citric acid monohydrate and ascorbic acid were purchased from Merck (Darmstadt, Germany). Purified water was prepared by a Milli-Q Integral system (Billerica, Massachusetts, USA). Ammonium formate was purchased from Acros (Geel, Belgium). Human EDTA whole blood and plasma was made available according to the guidelines of the University Medical Center Groningen.

Equipment and conditions

All experiments were performed on an Agilent 6460A (Santa Clara, Ca, USA) triple quadrupole LC-MS/MS system, with a combined Agilent 1200 series LC system. The Agilent 6460A mass selective detector operated in heated electrospray positive ionisation mode and performed multiple reaction monitoring (MRM) with unit mass resolution. High purity nitrogen was used for both the source and collision gas flows. In the first quadrupole single charged ions were selected for remifentanil and [$^{13}\text{C}_6$]-remifentanil. All precursor ions, product ions, optimum fragmentor voltages and collision energy values were tuned and optimized in the authors' laboratory. For remifentanil the precursor ion was set at a mass-to-charge ratio (m/z) of 377.2 and the product ion at a m/z of 317.2. For [$^{13}\text{C}_6$]-remifentanil the precursor ion was set at a m/z of 383.2 and the product ion at a m/z of 323.2. The fragmentor voltage was 115 V and

the collision energy was 11 V for both substances. The capillary voltage was set at 4,000 V, gas temperature at 300°C, gas flow at 13 L/min, nebulizer gas at 18 psi, sheath gas temperature at 300°C, sheath gas flow at 12 L/min and the nozzle voltage at 0 V. The Agilent 1290 auto sampler was set at 10°C and the 1260 TCC column oven was set at a temperature of 60°C. The mobile phase consisted of methanol and a 20 mM ammonium formate buffer pH 3.5. Analyses were performed with a 50 x 2.1 mm 3- μ m Hypurity® C18 analytical column from ThermoFisher Scientific (Waltham, MA, USA) equipped with a separate 0.5 μ m Varian frit filter (Palo Alto, CA, USA). Chromatographic separation was performed by means of a gradient with a flow of 0.5 mL/min and a run time of 2.6 min with the use of an Agilent 1290 Infinity Binary LC system. The gradient started at 15% methanol and 85% 20 mM ammonium formate buffer pH 3.5 and changed to 30% methanol at 0.11 min and increased slowly to 35% methanol in 1.49 min. At 1.61 min the methanol increased to 95% and was maintained until 2.10 min. From 2.11 to 2.60 min the gradient was kept at 15% methanol to stabilize the column for the next injection. Peak height ratios of the substance and its internal standard were used to calculate concentrations. Agilent Masshunter software for quantitative analysis (version B.04.00) was used for quantification of the analytical results. Regression analysis was performed by applying the software tool Analyze-it, version 2.20 (Analyze-it Software, Ltd.) in Microsoft Excel.

Method development

Early method development showed that acidification of the blood caused coagulation over a period of a few hours, resulting in the rejection of this matrix for further method development. In order to assess the efficacy of varying acids for the acidification of the EDTA plasma, citric acid, ascorbic acid and formic acid were added to the EDTA plasma to lower the pH of approximately 7.6 to approximately 5.3. When EDTA plasma was acidified to a pH below 4.7, coagulation of the EDTA plasma was also observed after 24 hours. Acidification of the EDTA plasma to a pH of 5.3 showed minimal coagulation, while still stabilizing remifentanyl.

The following acidic solutions were added to EDTA plasma to obtain a pH of approximately 5.3. From an almost saturated 1,000 g/L citric acid solution, 6 μ L was added to 1 mL of EDTA plasma. For ascorbic acid, 37.5 μ L of an almost saturated solution of 250 g/L was added to 1 mL of EDTA plasma. And 1.5 μ L of a 100% formic acid solution was added to 1 mL of EDTA plasma. Remifentanyl was spiked to all three acidified EDTA plasma's and to untreated EDTA plasma to obtain concentrations of 50 ng/mL. The spiked EDTA plasma samples were processed in single for each time point according to section 2.4 and analyzed at time zero

or stored at ambient temperature and were, subsequently, processed and analyzed at 1.5, 3.0, 19, 26, 44 and 50.5 hours.

Sample preparation

Formic acid was added to the EDTA plasma to improve the remifentanil stability. To each mL of EDTA plasma 1.5 μ L formic acid was added.

The sample preparation was performed by means of a protein precipitation. An aliquot of 100 μ L EDTA plasma was transferred into a glass 1.5 mL screwneck vial (Fisher Scientific, The Netherlands) and 400 μ L methanol, containing 10 ng/mL [$^{13}\text{C}_6$]-remifentanil was added. After precipitation, the vials were vortex mixed (Multi-tube vortexer, Labtek Corporation Ltd., Christchurch, New Zealand) for 1 minute and stored at -20°C for at least 10 minutes. Afterwards the vials were again vortex mixed for 1 minute, centrifuged at 10,000g for 5 minutes and 5 μ L of the clear upper layer of supernatant was injected into the LC-MS/MS.

Validation

The analytical method validation in acidified EDTA plasma included linearity, accuracy, precision, selectivity, specificity and stability based on international guidelines [12]. Two stock solutions were weighed and dissolved in purified water. One stock solution was used for the preparation of the calibration curve, while another stock solution was used for the preparation of the quality control (QC) concentrations. The volume of the spiked stock solution never exceeded 5% of the total EDTA plasma volume used for the preparation of the EDTA plasma standards. An eight point calibration curve was prepared at 0.20, 0.50, 2.0, 5.0, 25, 100, 200 and 250 ng/mL. The following QC concentrations were used for the validation: 0.20 ng/mL (Lower Limit Of Quantification (LLOQ)) ng/mL, 0.50 ng/mL, 100 ng/mL, 200 ng/mL and the Over the Curve concentration of 500 ng/mL. The Over the Curve concentration was diluted 10 times with blank EDTA plasma prior to sample processing in order to validate the dilution. One calibration curve, consisting of eight calibration points, was analyzed each day to determine linearity on three separate days. All three calibration curves were assessed with the use of Analyse-it to assess the linear regression of the 8-point calibration curve and to assess whether a 2-point calibration would provide the same linear fit. In order to maximize sample throughput for routine analysis, the accuracy and precision were calculated for all 3 days using a two point calibration curve, consisting of the lowest (0.20 ng/mL) and highest (250 ng/mL) concentrations of the calibration curve. In this way,

the use of a two point calibration curve was embedded in the validation procedure. The validation was performed with a maximum tolerated bias and Coefficient of Variation (CV) of 20% for the LLOQ and 15% for all other Calibration and QC samples including the stability validation. For the determination of the accuracy, precision and Over the Curve, all QC concentrations were processed and measured five times and the run of five was repeated on each of three separate days. For each accuracy and precision concentration bias and CV were calculated per run. Within-run, between-run and overall CV's were calculated with the use of one-way ANOVA.

In order to test the Limit Of Detection (LOD) a concentration of 0.015 ng/mL was prepared in acidified EDTA plasma and was processed five times and analyzed to assess the CV, with a maximum tolerated CV of 20%.

To assess the variation of the acidification procedure between different EDTA plasma lots of different healthy volunteers, 9 lots of EDTA plasma were acidified with 1.5 μ L formic acid / ml EDTA plasma and followed by pH measurement. These 9 acidified EDTA plasma lots were also used for the selectivity and specificity testing, since the FDA guideline states the testing of at least 6 sources of the used biological matrix. From every batch a LLOQ was spiked and processed together with a blank sample from each batch. Peaks found in the blank samples should not exceed 20% of the peak height of the LLOQ.

Stability of remifentanil in acidified EDTA plasma was assessed at 0.5 ng/mL and 200 ng/mL and was processed five times and analyzed after 2 days at ambient temperature, 14 days at 4°C and 14 days as processed sample in the auto sampler at 10°C. Long term stability of remifentanil in EDTA plasma at -20°C was assessed at 103 days, with the use of freshly prepared at 0.5 ng/mL and 200 ng/mL. Freeze thaw stability was assessed after one and three times freezing and thawing and was processed five times and analyzed.

Stock stability at 100 μ g/mL in purified water was assessed after a 1,000 times dilution to 100 ng/mL and followed by triplicate injections after 6 months storage at -20°C and at +4°C.

The stability of remifentanil in EDTA whole blood without acidification was investigated at ambient temperature, 4°C and at 0°C (ice water). EDTA whole blood was spiked at 0.5 ng/mL and at 200 ng/mL and divided over multiple tubes which were labelled and sealed. At multiple time points during 5 hours, one tube of each concentration was centrifuged and the EDTA plasma was stored at -20°C. The stored samples were prepared in triplicate and analyzed the following day.

Ion-suppression, extraction recovery, matrix effect and process efficiency

The presence of ion-suppression during an analytical run was tested by infusion of stock solution of remifentanil and [¹³C₆]-remifentanil with the use of a t-piece to combine the flows of the syringe pump with stock solution and the LC pump. The 9 acidified EDTA plasma lots used for selectivity and specificity, were processed as described in section 2.4, without [¹³C₆]-remifentanil. Ion-suppression chromatograms were recorded for all 9 lots of processed EDTA plasma.

The extraction recovery, matrix effect and process efficiency were assessed at 2 concentrations in triplicate. Remifentanil was spiked at 0.5 ng/mL and 200 ng/mL in EDTA plasma acidified with formic acid (solutions A). For the extraction recovery, acidified blank EDTA plasma samples were spiked at 0.5 ng/mL and 200 ng/mL after processing (solutions B). For the matrix effect and process efficiency, methanol was spiked at 0.5 ng/mL and 200 ng/mL (solutions C). The average peak height responses were used to calculate extraction recovery, matrix effect and process efficiency. The calculations of the extraction recovery, matrix effect and process efficiency were as follows: extraction recovery = $A/B \times 100$, matrix effect = $100 \times (B/C - 1)$, process efficiency = $A/C \times 100$. Where A, B and C refer to the prepared solutions mentioned above.

RESULTS

Method development

The stability test with un-acidified EDTA plasma and EDTA plasma acidified with citric acid, ascorbic acid and formic acid was performed by means of single sample analysis for all 7 time points and each matrix. The results of the stability test in EDTA plasma showed that untreated EDTA plasma was very unstable with a decrease in remifentanil concentration of 13% within 3 hours. Citric acid and formic acid showed 0.5% and 4.2% remifentanil degradation respectively after 19 hours at ambient temperature, while ascorbic acid showed 7.2% degradation, see figure 1. Formic acid was chosen to acidify the EDTA plasma for the validation, because of the volatility (LC-MS/MS compatibility) and the fact that this acid was also used in the mobile phase.

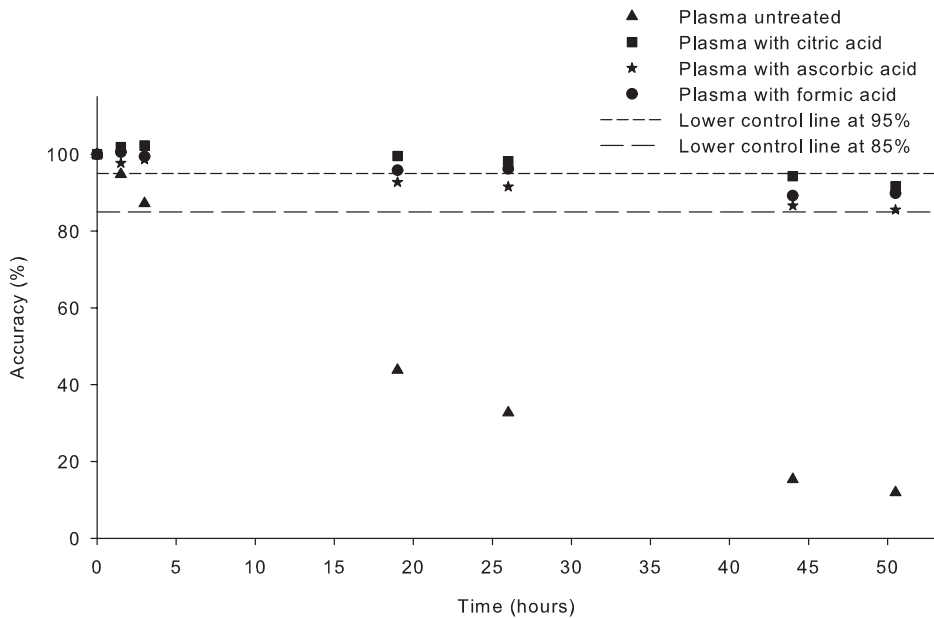


Figure 1 Results of the stability test for remifentanyl at ambient temperature in untreated and acidified EDTA Plasma.

The 95% control line is to detect a 5% deterioration and the 85% control line marks the QC rejection point of 15% bias. The stability test was performed by means of single sample analysis for all 7 time points and each matrix.

Validation results

The eight point calibration curve of remifentanyl proved to be linear from 0.20 to 250 ng/ml with a R^2 of 0.9995. The linear regression equation with its 95% confidence intervals (CI) was $y = 0.02495$ (CI 0.02471 to 0.02519) $x - 0.02342$ (CI -0.05192 to 0.00508) for the 8-point calibration curve. While the regression equation was $y = 0.02483$ (CI 0.02400 to 0.02566) $x - 0.001312$ (CI -0.14854 to 0.14592) for the 2-point calibration curve. The 95% CI of the intercepts and the slopes of both calibration curves showed overlapping CIs. Therefore it can be concluded that both calibration curves did not significantly deviate from each other and the use of a 2-point calibration curve was justified. The accuracy and precision results calculated with a two point calibration curve showed the highest overall bias during the validation of -5.0% (CV 4.3%) for 0.5 ng/mL, while the highest overall CV was 5.6% for 0.20 ng/mL. The validation results regarding accuracy, precision and dilution are shown in table 1.

In figure 2 chromatograms are shown for a representative blank, LLOQ (0.20 ng/mL) and highest calibration standard (250 ng/mL) for remifentanyl and the internal standard [$^{13}\text{C}_6$]-remifentanyl. The LOD was tested at 0.015 ng/mL and showed a CV of 7.5%.

Table 1 Accuracy and precision results of remifentanyl

Concentration (ng/mL)	Within-run CV (%) n=15	Between-run CV (%) n=15	Overall CV (%) n=15	Overall bias (%) n=15
0.20 (LLOQ)	5.1	2.3	5.6	0.5
0.50	3.1	2.9	4.3	-5.0
100	3.7	0.0	3.7	3.7
200	3.2	0.0	3.2	5.1
500 (OC)	1.5	1.6	2.1	-1.0

LLOQ is Lower Limit of Quantitation. OC is Over the Curve concentration which is diluted 10 times. N=15 means 5 replicate analyses on each day on each of the 3 validation days. Within-run, between-run and overall coefficient of variation (CV) were calculated with One-Way ANOVA.

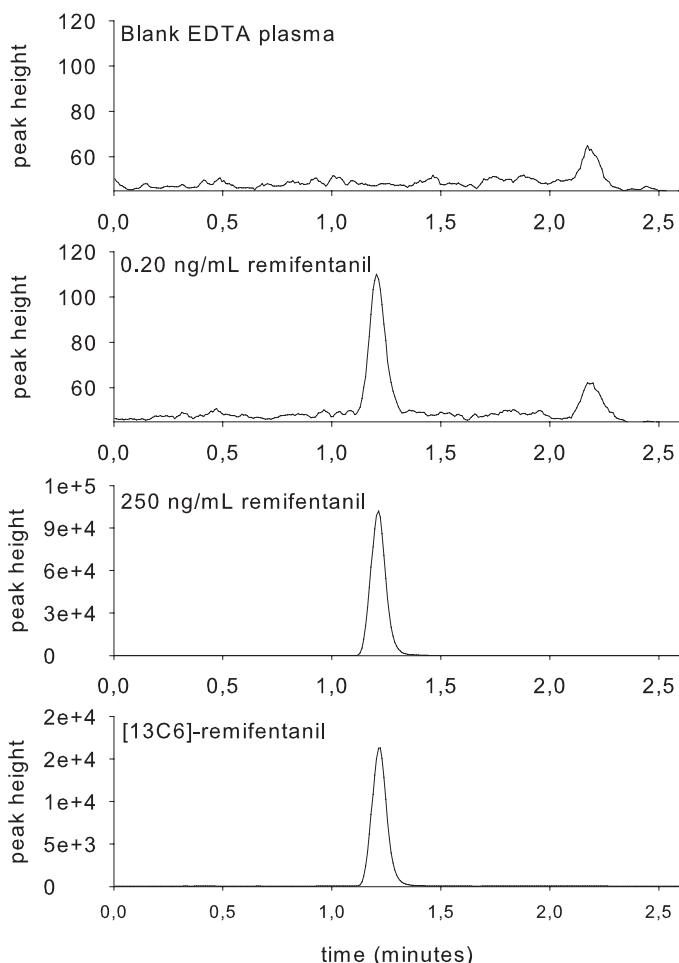


Figure 2 Chromatograms of representative processed samples of a blank, 0.20 ng/mL remifentanyl, 250 ng/mL remifentanyl and the internal standard [$^{13}\text{C}_6$]-remifentanyl.

The 9 lots of EDTA plasma acidified with formic acid showed reproducible pH values ranging from pH 4.7 to pH 5.4, with a mean pH of 5.08. Peaks found in the blank samples of the 9 lots of EDTA plasma acidified with formic acid did not exceed 20% of the peak height of the LLOQ.

The results of the stability validation are shown in table 2.

Table 2 Results of the stability testing of remifentanyl in EDTA plasma acidified with formic acid

Stability	Time (days)	0.50 ng/mL		200 ng/mL	
		Bias (%)	CV (%)	Bias (%)	CV (%)
EDTA plasma F/T 3	n.a.	0.6	2.3	0.9	3.7
EDTA plasma AT	2	-3.3	3.0	-11.6	2.2
EDTA plasma 4°C	14	-6.6	5.5	-11.8	2.7
EDTA plasma -20°C	103	5.5	4.4	-7.2	1.3
AS 10°C	14	1.3	3.5	-3.4	3.6

F/T 3 is the stability of three freeze and thaw cycles. AT is the stability at ambient temperature. AS is autosampler stability in processed sample.

The stock solutions of 100 mg/L proved to be stable at -20°C and at +4°C for 6 months with a maximum bias of 4.3% (CV 1.3%). The stability of remifentanyl in EDTA blood during 2 hours showed an average decrease of approximately 42% at ambient temperature, 12% at +4°C and less than 2% decrease at 0°C (ice water) at 0.50 and 200 ng/mL. The results of the stability tests in untreated EDTA whole blood can be seen in table 3 and figure 3.

At ambient temperature, remifentanyl showed to be even more unstable in untreated EDTA whole blood than in EDTA plasma without acidification. After 3 hours at ambient temperature the remifentanyl concentration decreased with approximately 54% in untreated EDTA whole blood (figure 3) compared to a 13% decrease in untreated EDTA plasma (figure 1).

Ion-suppression, extraction recovery, matrix effect and process efficiency

The developed analysis method showed extraction recoveries of 99% (CV 8.2%) and 107% (CV 1.3%) for 0.5 ng/mL and 200 ng/mL respectively. Matrix effects were -8% (CV 4.1%) for 0.5 ng/mL and -10% (CV 0.3%) for 200 ng/mL. The total process efficiency was 91% (CV 8.2%) for 0.5 ng/mL and 96% (CV 1.3%) for 200 ng/mL. The ion-suppression chromatograms showed no ion-suppression near the retention time of remifentanyl in all 9 lots of acidified EDTA plasma, see figure 4.

Table 3 Results of the stability testing of remifentanil in untreated EDTA whole blood in triplicate, at ambient temperature, 4°C and 0°C at 0.50 and 200 ng/mL

Time (hours)	Ambient temperature				4°C				0°C			
	0.50 ng/mL		200 ng/mL		0.50 ng/mL		200 ng/mL		0.50 ng/mL		200 ng/mL	
	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)
0.00	100.1	3.5	100.0	4.4	100.1	3.5	100.0	4.4	100.1	3.5	100.0	4.4
0.17	95.9	1.3	94.2	0.6	98.7	4.5	101.1	3.7	103.3	3.1	105.8	2.6
0.33	92.4	0.3	95.5	3.8	96.2	3.6	98.6	3.1	103.2	1.8	106.9	3.6
0.50	86.2	1.3	91.2	3.4	95.0	2.0	98.3	4.5	102.5	2.6	105.8	4.0
0.67	86.3	1.6	86.7	3.3	93.1	3.3	97.0	3.3	100.8	3.4	104.2	2.3
0.83	75.7	1.2	83.8	1.2	91.1	2.1	97.4	1.2	100.4	1.4	103.2	3.3
1.00	75.9	3.1	79.5	0.7	89.7	2.2	94.7	2.9	100.2	4.3	104.9	0.8
1.17	70.8	3.0	75.2	3.6	89.1	3.5	95.4	1.0	98.8	3.0	105.1	0.4
1.33	68.2	1.2	72.2	1.8	88.3	2.9	93.4	1.1	97.7	4.5	103.2	1.2
1.50	65.8	2.9	70.4	0.2	86.8	2.3	91.6	1.8	96.2	3.3	102.8	0.8
2.00	56.1	5.2	60.3	0.8	85.5	3.4	90.6	1.5	95.2	2.1	101.3	1.3
2.50	51.0	4.0	51.8	2.8	84.2	3.6	88.6	0.6	92.9	1.3	98.0	3.2
3.00	44.3	1.4	46.9	0.3	82.8	2.1	86.6	0.6	93.8	2.9	98.2	1.4
3.50	39.6	5.2	41.2	0.6	80.6	3.9	85.3	1.7	91.2	1.8	95.6	0.6
4.00	33.6	4.6	35.7	2.0	79.8	4.1	83.4	1.0	92.7	0.3	93.0	1.0
4.50	29.9	5.1	32.2	0.6	79.0	2.9	83.0	0.6	88.0	4.2	91.6	1.0
5.00	26.2	2.3	28.4	2.4	80.0	4.1	83.8	0.6	83.6	3.7	88.8	0.6

Biases are calculated in relation to time is 0.00 hours.

CONCLUSION

We propose LC-MS/MS analysis combined with the use of formic acid to acidify the EDTA plasma sample as a new analytical method to measure remifentanil concentrations in EDTA plasma. We studied the concentration stability over time, at different temperature conditions and with or without acidification of the EDTA plasma to stabilize esterase activity.

During method development it became clear that acidifying the EDTA whole blood causes coagulation, which impairs the integrity of the sample. For this reason the stability of remifentanil was also investigated in untreated EDTA whole blood at ambient temperature, 4°C and 0°C. At ambient temperature, remifentanil showed to be even more unstable in untreated EDTA whole blood than in EDTA plasma without acidification, which is in agreement

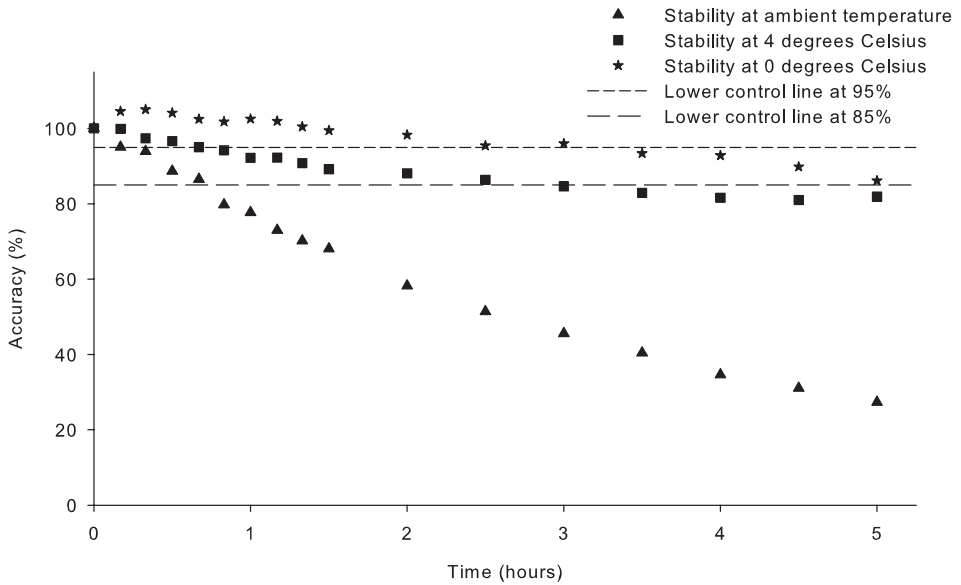


Figure 3 Results of the stability test of remifentanyl in untreated EDTA whole blood at ambient temperature 4°C and at 0°C.

The 95% control line is to detect a 5% deterioration and the 85% control line marks the QC rejection point of 15% bias.

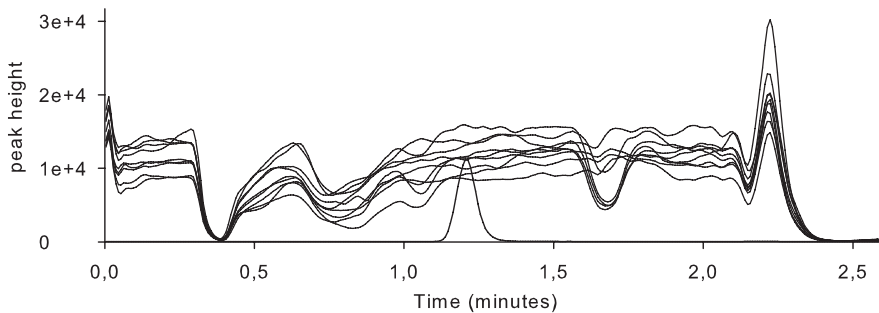


Figure 4 Ion suppression chromatograms of 9 lots of EDTA plasma acidified with formic acid, including a chromatogram with the retention time of the remifentanyl peak at 1.2 minutes.

with the findings of Davis et al. [13]. These results showed that the collected EDTA whole blood should not be stored at ambient temperature. The collected EDTA whole blood tube is best stored temporarily in ice water to cool down and should then be centrifuged as soon as possible (preferably before the first hour but not later than 2 hours after the sampling time). To stabilize the esterase activity in EDTA plasma, the resulting EDTA plasma fraction should be acidified with 1.5 μL formic acid per mL of EDTA plasma. Subsequently the sample can be stored in a -20°C freezer.

Compared to earlier used citric acid [3-9], formic acid is volatile and the use of formic acid was considered a more compatible acid for LC-MS/MS analysis than the non-volatile ascorbic acid and citric acid.

The pH of the acidified EDTA plasma is considered the primary determinant for the stability of remifentanyl. We found the pH values in the 9 lots of acidified EDTA plasma within an acceptable range. This indicates that remifentanyl concentrations are stable in acidified EDTA plasma of different patient samples. The stability of remifentanyl in EDTA plasma acidified with formic acid was extensively investigated and showed good stability results, even at ambient temperature.

The developed analytical method could be limited by the current LLOQ when bolus injections are used. However, the validated Limit Of Quantitation of 0.20 ng/mL assured that the method was robust and not readily vulnerable to a deteriorating sensitivity. The LOD was tested at 0.015 ng/mL and showed a CV of 7.5%. This may indicate that a lower LLOQ could be possible in order to monitor remifentanyl even at the tail end of the pharmacokinetic curve of bolus injections. In addition, the use of a more sensitive mass spectrometer could also improve the LLOQ.

The application of a 2-point calibration curve, which included the LLOQ and the highest concentration of the linear range provided excellent accuracy and precision results. The approach of applying a minimal calibration curve already proved to be very efficient for therapeutic drug monitoring [14]. The 2-point calibration curve could be impaired when the curve may become non-linear, possibly due to changing ionization characteristics or overdue maintenance. In our method, an isotopically labelled internal standard is used, which can compensate for changing ionization characteristics. In addition, with the use of QC samples throughout the linear range, linearity issues would result in unacceptable biases for the QCs, and run rejection. The two point calibration curve was validated during the 3 days validation of the linearity, accuracy and precision, indicating no such linearity problem. The QC samples throughout the linear range proved that the use of the two point calibration curve was valid. During routine analysis, QC samples are also incorporated in the run, ensuring valid results at all times.

Remifentanyl concentrations were frequently reported in whole blood in the past because of the stability issues. Blood was drawn from the patient and directly mixed with acetonitrile in order to stop the endogenous esterases [1, 11]. With our procedure, this instability is no longer a problem and plasma analysis can be easily performed. Due to the instability

of remifentanil and the following time consuming sample processing, the established pharmacokinetic models were based on whole blood. La Colla et al. evaluated the predictive performance of the model of Minto et al for morbidly obese patients [15-17]. It should be noted that their analyses were performed in plasma, while the model of Minto was developed for whole blood analysis. It is unclear if La Colla et al. acknowledged the blood to plasma ratio [15]. As a future perspective, the blood to plasma ratio should be investigated before the current pharmacokinetic models can be used with plasma analysis.

The developed analytical method uses a simple protein precipitation and maximal throughput by a two point calibration curve and short run times of 2.6 minutes. The validation showed excellent accuracy and precision results with a very large linear range of 0.2 ng/mL to 250 ng/mL. The method showed no ion-suppression for all 9 lots of acidified EDTA plasma.

In conclusion, we found that the developed LC-MS/MS method is suitable for measuring remifentanil concentrations in EDTA plasma for pharmacokinetic trials.

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