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High sensitive determination of sufentanil in human plasma of parturients and neonates following patient-controlled epidural analgesia (PCEA)

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Abstract

A validated method for the determination of sufentanil in human plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS) is described. Sufentanil was extracted from human plasma with solid-phase-extraction using deuterated sufentanil, [$^2H_5$]-sufentanil, as internal standard. Sufentanil and the internal standard were determined with an API 4000 tandem mass spectrometer equipped with a Turbo-V-Source operating in positive ESI mode on an Alltima HP HILIC straight phase column. The method showed a lower limit of quantification of 0.25 pg/ml (12.5 fg on column). The applicability of the method is shown in a clinical study, in which levels of sufentanil in plasma of parturients and arterial umbilical plasma of their neonates following patient-controlled epidural analgesia (PCEA) under several regimen treatments was analyzed.
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Keywords: Sufentanil; PCEA; Chromatography; Tandem mass spectrometry; ESI; LC–MS/MS

1. Introduction

Patient-controlled epidural analgesia (PCEA) has risen to an important technique in the treatment of labor pain [1,2]. Local anaesthetics, e.g. bupivacaine or *S*-ropivacaine, are used in combination with fentanyl or sufentanil. A typical epidural basal infusion rate averages 4 ml/h of 1.6 mg/ml *S*-ropivacaine plus 0.5 μg/ml sufentanil, but the parturients have the ability to self-administer a bolus according to different pain intensities. Based on the sufentanil infusion rate of 2 μg/h and an half-life of approximately 2 h [3,4], the expecting plasma concentrations in parturients and neonates are rather low.

Several GC–MS, GC–MS/MS, LC–MS and LC–MS/MS-methods for the determination of sufentanil in human plasma are described so far [5–10], but non of them has the potency to monitor plasma levels of sufentanil in parturients and their neonates following PCEA. The most sensitive LC–MS/MS method described so far has a lower limit of quantification (LLOQ) of 10 pg/ml sufentanil in human plasma and employs a rather high volume of 1 ml human plasma [9].

We here describe a precise assay using LC–MS/MS to monitor sufentanil concentrations in small volumes of human plasma of parturients and arterial umbilical plasma of their neonates following PCEA. The LLOQ of sufentanil in the described LC–MS/MS-assay is 0.25 pg/ml human plasma.

2. Experimental

2.1. Materials

Acetonitrile (HPLC grade), water (HPLC grade), formic acid (p.a.), ethyl acetate (p.a.), ammonium hydroxide (25%, p.a) and ammonium acetate (p.a.) were purchased from Merck KgaA (Darmstadt, Germany). Methanol and water used for solid phase extraction were also of HPLC grade, but obtained from Mallinckrodt Baker (Griesheim, Germany). Sufentanil citrate, [$^2H_5$]-sufentanil citrate, alfentanil citrate and fentanyl citrate were obtained from Janssen-Cilag (Neuss, Germany). Isotopic purity of [$^2H_5$]-sufentanil citrate was 99.994%. Blank human plasma was a gift from the Blutspendedienst Hessen (Deutsches Rotes Kreuz, Frankfurt am Main, Germany).

An ammonium acetate stock solution (1 M) was prepared by dilution of 77.08 g ammonium acetate to 1000 ml with HPLC

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water. Mobile phase A was water/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v, pH 2.8) and mobile phase B was acetonitrile/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v).

2.2. Instrumentation

Sample analysis was performed by using liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS/MS). The LC–MS/MS system consisted of an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo-V-source operating in positive ESI mode, an Agilent 1100 binary HPLC pump and degasser (Agilent, Böblingen, Germany) and an HTC Pal autosampler (Chromtech, Idstein) fitted with a 25 μl LEAP syringe (Axel Semrau GmbH, Sprockhövel, Germany). A cooling stack was used to store the samples at 4 °C in the autosampler. An inlet valve was used to truncate non-relevant signals (10-port, VICI Valco Instruments, Houston, USA). High purity nitrogen for the mass spectrometer was produced by a NGM 22-LC/MS nitrogen generator (cmc Instruments, Eschborn, Germany).

2.3. LC–MS/MS conditions

For the chromatographic separation, a straight phase Alltima HP HILIC column with precolumn was used (50 mm × 2.1 mm I.D., 3 μm particle size from Alltech, Unterhaching, Germany). A linear gradient was employed at a flow rate of 0.7 ml/min. Directly after injection of the sample (0 min) the gradient started from 0% mobile phase A to 30% mobile phase A within 2.2 min. Then within 0.3 min mobile phase A was increased to 50% (total 2.5 min) and was held for another 0.4 min (total 2.9 min). To equilibrate the column the gradient composition shifted back to 0% mobile phase A (total 3.6 min) and was held for 3.9 min (total 7.5 min). Total run time was 7.5 min. Injection volume of samples was 20 μl. Retention time of sufentanil and [$^2H_5$]-sufentanil was 2.3 ± 0.01 min (mean ± S.D., $n = 72$). Carry over after an injection of the highest calibration standard was 0.05 ± 0.01% ($n = 6$).

The mass spectrometer was operated in the positive ion mode with an electrospray voltage of +1500 V at 700 °C. Nebulizer gas (GS1) was set to 35 psi, heater gas (GS2) to 80 psi and curtain gas to 30 psi. Collision gas thickness was 2.58×10^{15} molecules/cm^2. Position of the electrospray needle was 5 mm (horizontal) and 5 mm (vertical).

Multiple reaction monitoring (MRM) was used for quantification. The mass transitions used were m/z 387 → m/z 238 for sufentanil and m/z 392 → m/z 238 for the internal standard, [$^2H_5$]-sufentanil, all with a dwell time of 60 ms. Control transitions were m/z 387 → m/z 355 for sufentanil and m/z 392 → m/z 360 for the internal standard, [$^2H_5$]-sufentanil, all with a dwell time of 10 ms. All quadrupoles were working at unit resolution. Quantitation was performed with Analyst Software V1.4 (Applied Biosystems, Darmstadt, Germany) using the internal standard method (isotope-dilution mass spectrometry). Ratios of analyte peak area and internal standard peak area (y-axis) were plotted against concentration (x-axis) and calibration curves were calculated by least square regression with 1/concentration^2 weighting.

2.4. Standard and sample preparation

Stock solutions with 500,000 ng/ml of sufentanil (free base) and 500,000 ng/ml [$^2H_5$]-sufentanil (free base) were prepared in methanol. The stock solution of sufentanil was further diluted with acetonitrile to obtain a calibration standard A of 100 ng/ml. Calibration standard A was further diluted with acetonitrile/water (1:1, v/v) to get working standards with a concentration range of 1.25–3500 pg/ml sufentanil. The stock solution of the internal standard, [$^2H_5$]-sufentanil, was further diluted with acetonitrile to obtain a second stock solution with 100 ng/ml. The second stock solution of [$^2H_5$]-sufentanil was mixed with acetonitrile/water (1:1, v/v) to obtain an internal standard working solution of 1000 pg/ml. All solutions were stored at −20 °C except the working standards of sufentanil and [$^2H_5$]-sufentanil which were kept at 5 °C.

Samples for standard curves and quality controls were prepared prior to extraction by mixing 250 μl blank human plasma with 50 μl of working standard (1.25–3500 pg/ml), 50 μl internal standard (1000 pg/ml [$^2H_5$]-sufentanil) and 700 μl water to obtain calibration standards from 0.25 to 750 pg/ml and with a final concentration of [$^2H_5$]-sufentanil after extraction of 500 pg/ml. Human plasma of parturients and neonates were prepared similarly. Instead of 50 μl working standards (1.25–3500 pg/ml) 50 μl acetonitrile/water (1:1, v/v) was added. Internal standard was added to the study samples just before extraction.

2.5. Sample extraction

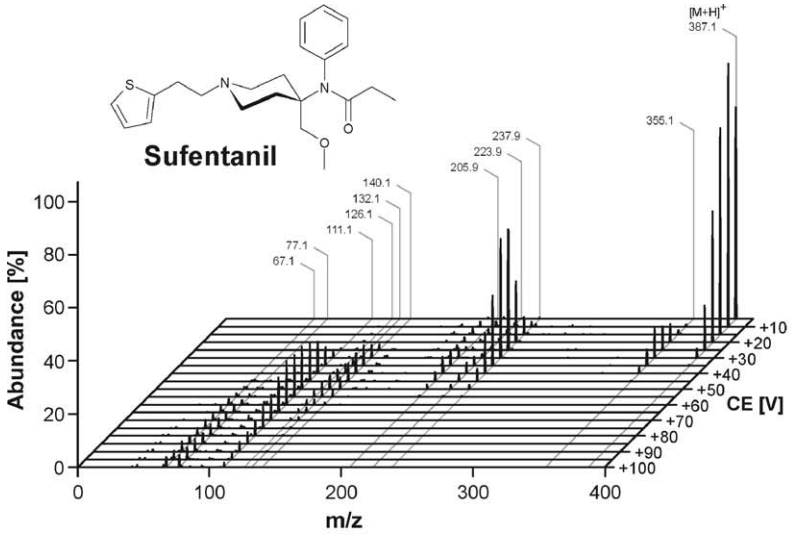
Sufentanil was extracted with solid-phase-extraction. Therefore, 1 ml Strata Screen-C cartridges (phenomenex, Aschaffenburg, Germany) were conditioned with 1 ml of methanol and 1 ml of water. One thousand and fifty microliters of the prepared plasma (250 μl human plasma, 50 μl internal standard, 700 μl water and 50 μl acetonitrile/water (1:1) or 50 μl working standard) was loaded onto the column and washed with 1 ml of water. The cartridges were then dried for 10 min and eluted with methanol/ethyl acetate/ammonium hydroxide 25% (10:90:2, v/v/v). The organic phase was removed at a temperature of 55 °C under a gentle stream of nitrogen. The residues were reconstituted with 100 μl of mobile phase B, centrifuged for 2 min at 10,000 × g and then transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC–MS/MS system.

2.6. Clinical applicability

The applicability of the LC–MS/MS assay method was shown in a prospective, randomized and double-blinded study, which was approved by the local Ethics Committee of the Frankfurt Medical Faculty. Parturients were given detailed infor-

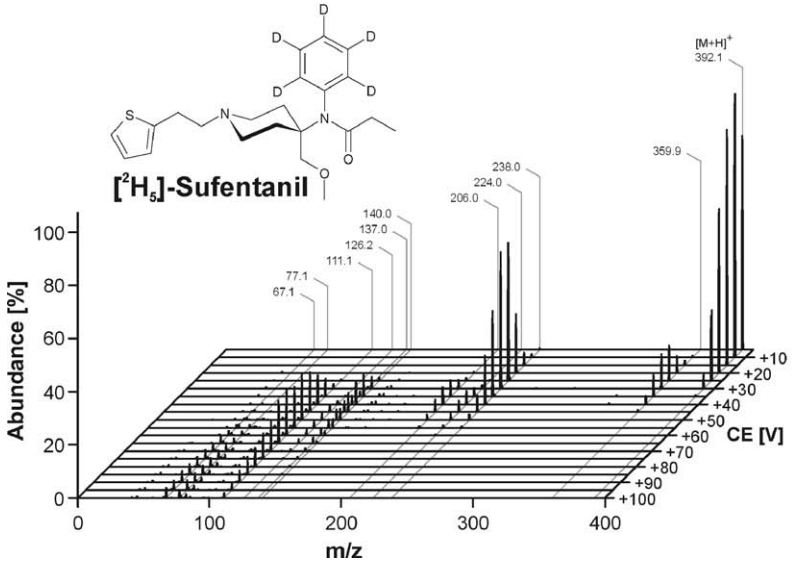
mation about the PCEA procedure and written informed consent was obtained. Only parturients with a completed vaginal birth (no cesarean section) and >36 week of gestation were included in the study. Group I received an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.25 μg/ml sufentanil (29 parturients), group II an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.5 μg/ml sufentanil (23 parturients) and group III an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.75 μg/ml sufentanil (22 parturients).

A lumbar epidural catheter was placed in the L2–L3 interspace. Following insertion of the epidural catheter, a test dose of 5 ml of 2 mg/ml *S*-ropivacaine (10 mg) was administered to exclude intrathecal or intravascular malpositioning. After a priming dose of 8 ml 1.6 mg/ml *S*-ropivacaine (12.8 mg) plus 10 μg sufentanil a PCEA device was connected (Deltec Grasby, St. Paul, MN, USA) and started 30 min later. Epidural background infusion rate was 4 ml/h of 1.6 mg/ml *S*-ropivacaine plus 0.25 (group I), 0.5 (group II) or 0.75 μg/ml sufentanil (group III). The parturients had the ability to self-administer a maximum of four 3 ml boli (lack-out time 15 min) according to different pain intensities. At time of birth, 5 ml maternal and 5 ml arterial umbilical blood samples were collected in EDTA vacutainer (Sarstedt, Nümbrecht, Germany). After 10 min centrifugation with 5000 rpm at room temperature, plasma was transferred into 5 ml plastic vials (Sarstedt, Nümbrecht, Germany) and stored at −40 °C.



Sufentanil
$C_{22}H_{30}N_2O_2S$
Molecular mass 386.6 amu
Monoisotopic mass 386.2 amu

[M+H]+		Fragment	DP	EP	CE	CXP
387.1	→	67.1	64	8	93	4
387.1	→	77.1	64	8	93	6
387.1	→	111.1	64	8	53	10
387.1	→	126.1	64	8	49	10
387.1	→	132.1	64	8	47	8
387.1	→	140.1	64	8	39	10
387.1	→	205.9	64	8	37	14
387.1	→	223.9	64	8	39	16
387.1	→	237.9	64	8	28	16
387.1	→	355.1	64	8	30	12



[$^2H_5$]-Sufentanil
$C_{22}H_{25}N_2O_2SD_5$
Molecular mass 391.6 amu
Monoisotopic mass 391.2 amu

[M+H]+		Fragment	DP	EP	CE	CXP
392.1	→	67.1	64	8	93	6
392.1	→	77.1	64	8	81	4
392.1	→	111.1	64	8	53	8
392.1	→	126.2	64	8	51	10
392.1	→	137.0	64	8	43	10
392.1	→	140.0	64	8	39	10
392.1	→	206.0	64	8	39	16
392.1	→	224.0	64	8	41	22
392.1	→	238.0	64	8	28	16
392.1	→	359.9	64	8	25	26

Fig. 1. Mass spectra of sufentanil and [$^2H_5$]-sufentanil by using product ion scans in the positive ionization mode and several collision energies. The inserted tables present the optimized multiple reaction monitoring (MRM) conditions for the mass transitions (DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exit potential). Product ion scans were obtained by infusion of 10 ng/ml methanolic solutions of sufentanil and [$^2H_5$]-sufentanil (10 μl/min). Twenty product ion scans at each collision energy (5–100 V, step 5 V) were summed up.

3. Results and discussion

3.1. Optimization of the LC–MS/MS conditions

To assess the optimal parameters of the mass spectrometer methanolic tuning solutions of 10 ng/ml of both compounds were infused into the mass spectrometer with an infusion rate of 10 μl/min. Intense molecular peaks and significant fragments were found in the positive ionization mode (Fig. 1). Substance specific voltages for declustering (DP), cell entrance (CE) and cell exit potential (CXP) were roughly optimized with the ramping function of the Analyst software.

Best sensitivity was obtained by adding formic acid to the mobile phase leading to positively charged sufentanil, but these conditions cause weak retention and broad and tailing peaks on 150 mm × 2 mm C_8 or C_{18}-columns (phenomenex, Aschaffenburg, Germany). Additionally, larger amounts of water in the mobile phase, which is necessary to retain sufentanil on C_8 or C_{18}-columns, decrease the signal intensity. Straight phase columns resulted in more symmetric peaks of sufentanil, when high percentage of acetonitrile as mobile phase and formic acid was used. Sufentanil and [$^2H_5$]-sufentanil were only measured between 2.0 and 2.8 min. Thus, unwanted flow was truncated before 2.0 min and after 2.8 by an inlet valve. Gradient elution was found to be 50% more sensitive (based on signal-to-noise ratios) than isocratic elution with mobile phase A/mobile phase B (10:90) (Fig. 2). Although the total run time with isocratic elution can be reduced to 3.5 min, gradient elution was chosen because of better sensitivity for low sufentanil concentrations in neonate plasma samples.

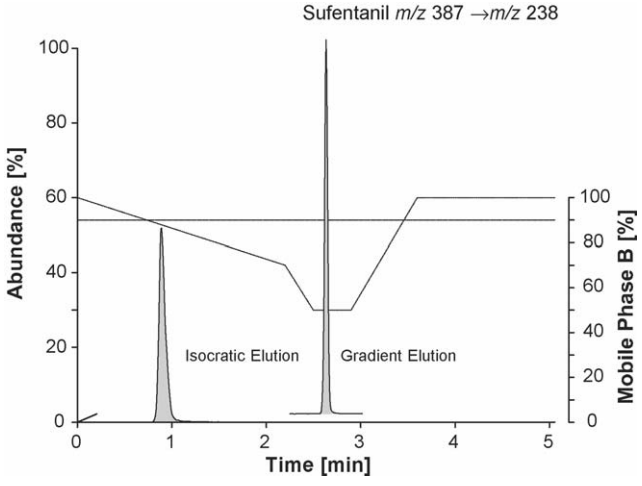


Fig. 2. Comparison of isocratic and gradient run for sufentanil on an Alltima HP HILIC (50 mm × 2.1 mm I.D., 3 μm particle size). Mobile phase A was water/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v, pH 2.8) and mobile phase B was acetonitrile/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v). Concentration of sufentanil was 100 pg/ml in mobile phase B. Flowrate was 700 μl/min and injection volume 20 μl in both runs. Isocratic run has been performed with mobile phase A/mobile phase B (10:90, v/v). Retention times of sufentanil was 0.88 min. Retention times under gradient elution (0 min: A 0%, B 100%; 2.2 min: A 30%, B 70%; 2.5 min: A 50%, B 50%; 2.9 min: A 50%, B 50%; 3.6 min: A 0%, B 100%; 7.5 min: A 0%, B 100%) of sufentanil was 2.43 min. Positive ionization mode was used for detection.

After development of the chromatographic conditions declustering potential, cell entrance, cell exit potential, nebulizing gas (GS1), heater gas (GS2), curtain gas (CUR), collision-activated dissociation (CAD) gas, electrospray voltage (IS), temperature, and orientation of the electrospray needle were further optimized by repeated injections of 100 pg/ml solutions of sufentanil and [$^2H_5$]-sufentanil in mobile phase B under the final chromatographic conditions.

Several extraction protocols (SPE, liquid–liquid-extraction and protein-precipitation) were tested, but only solid phase extraction with an ion-exchange material produced the most reproducible recovery.

3.2. Method validation

Method validation was performed according FDA recommendations [11]. Assay accuracy was calculated with six different standard series in the range from 0.25 to 750 pg/ml. Each calibration curve was prepared with different drug free human plasma. For calculation, ratios between peak areas of sufentanil and internal standard, [$^2H_5$]-sufentanil, were used. Best values were obtained with weighted least square regression (weighting factor 1/concentration^2). Mean accuracy of the assay was found to be 100.1 ± 5.8% over the calibration range of 0.25–750 pg/ml. Detailed data are given in Table 1.

Intraday precision of the assay was determined using eight concentrations (0.25, 0.5, 0.75, 1, 2.5, 7.5, 75 and 750 pg/ml), blank (with internal standard spiked matrix) and double blank samples (only matrix). Due to the injection volume of 20 μl, only four injections per sample were possible and two extracts of the same concentration had to be combined. Each sample was then analyzed six times in a row. Intraday precision was

Table 1
Accuracy of sufentanil

	Accuracy ± S.D. (n = 6)		R.S.D. (%)
	pg/ml	%	
Nominal concentration (pg/ml)			
DblBlank	No peaks	–	–
Blank	No peaks	–	–
0.25	0.249 ± 0.025	99.5 ± 10.0	10.0
0.5	0.514 ± 0.03	102.9 ± 6.3	6.1
0.75	0.751 ± 0.06	100.2 ± 8.7	8.7
1	0.975 ± 0.05	97.5 ± 4.6	4.7
2.5	2.46 ± 0.17	98.4 ± 6.7	6.8
7.5	7.42 ± 0.4	98.9 ± 5.4	5.4
10	10.1 ± 0.5	101.1 ± 4.6	4.6
25	25.0 ± 0.8	99.8 ± 3.1	3.1
75	79.3 ± 5.9	105.7 ± 7.9	7.4
100	99.2 ± 7.3	99.2 ± 7.3	7.4
250	241.5 ± 12.3	96.6 ± 4.9	5.1
750	754.3 ± 34.3	100.6 ± 4.6	4.5
Mean (%) ± S.D.		100.1 ± 5.8	5.8

Accuracy was determined using six different standard curves prepared in six blank human plasmas. No peaks of sufentanil were visible in unspiked drug free plasma samples. DblBlank, extracted blank plasma; blank, extracted blank plasma + 500 pg/ml [$^2H_5$]-sufentanil.

Table 2
Intraday and interday precision for sufentanil of extracted plasma samples

	Intraday		Interday	
	Mean (%) ± S.D. (n = 6)	Precision, R.S.D. (%)	Mean (%) ± S.D. (n = 4 days)	Precision, R.S.D. (%)
Nominal concentration (pg/ml)				
DblBlank	No peaks	–	No peaks	–
Blank	No peaks	–	No peaks	–
0.25	102.4 ± 8.6	8.4	103.8 ± 7.8	7.6
0.5	97.4 ± 10.1	10.3	94.3 ± 7.0	7.4
0.75	98.0 ± 2.2	2.2	98.7 ± 3.8	3.9
1	97.5 ± 3.4	3.5	98.2 ± 5.6	5.7
2.5	99.8 ± 2.6	2.6	99.4 ± 2.8	2.8
7.5	100.5 ± 1.2	1.2	103.4 ± 1.0	1.0
75	102.5 ± 1.0	1.0	102.7 ± 1.0	0.9
750	99.7 ± 1.2	1.2	99.7 ± 1.0	1.0

No peaks of sufentanil were visible in unspiked drug free plasma samples. DblBlank, extracted blank plasma; blank, extracted blank plasma + 500 pg/ml [$^2H_5$]-sufentanil.

repeated on four different days and values were used to calculate the interday precision (Table 2). Every sample of intra- and interday-precision was prepared with the same drug free human plasma. LLOQ was defined as the concentration where standard deviation of accuracy did not exceed 15% and relative standard deviation (R.S.D.) of intra- and interday-precision was less than 15%. LLOQ for sufentanil was found to be 0.25 pg/ml (12.5 fg on column)(Fig. 3).

Extraction efficacy of sufentanil and [$^2H_5$]-sufentanil was determined at three different concentrations (7.5, 75 and 750 pg/ml). Relative recovery was calculated by comparing the mean peak areas of six extracted standards of one concentration with the mean peak areas of six extracted blank matrix samples which were reconstituted after evaporation of the organic phase with standards prepared in mobile phase B. Relative recovery was constant over the calibration range. Mean relative recovery was 89.9 ± 6.1% for sufentanil and 89.9 ± 5.8% for [$^2H_5$]-sufentanil (Table 3). Absolute recovery was ascertained by comparing mean peak areas of extracted samples with matrix free solvent-standards in mobile phase B. Absolute recovery was constant over the calibration range. Mean absolute recovery was 39.0 ± 3.1% for sufentanil and 39.0 ± 3.3% for [$^2H_5$]-sufentanil (Table 3), which was caused by a high matrix effect. Each replicate of absolute and relative recovery was prepared with a different drug free human plasma.

Matrix and suppression effects were assessed after the method of Matuszewski et al. [12] with six extracted blank matrix samples of six different drug free human plasmas, which were reconstituted with a 75 pg/ml standard in mobile phase B. The mean peak areas of all samples were compared with the mean peak areas of the 36 matrix free 75 pg/ml standards in mobile phase B. An ion suppression effect of 45.6 ± 1.7% for sufentanil and 46.1 ± 2% for [$^2H_5$]-sufentanil was observed. Since ion suppression effects sufentanil and [$^2H_5$]-sufentanil signal in the same way quantification can be performed without any problems.

To visualize the matrix effect the post-column infusion technique of Bonfiglio et al. [13] was used. The LC–MS/MS method was the same as described under Section 2.3 but no switching valve was used.

Table 3
Relative (as compared to spiked matrix samples) and absolute recovery (as compared to spiked matrix free samples) for sufentanil and [$^2H_5$]-sufentanil of six different plasma samples

Relative recovery	Sufentanil Mean (%) ± S.D. (n = 6)	[$^2H_5$]-Sufentanil Mean (%) ± S.D. (n = 6)
Nominal concentration (pg/ml)		
7.5	91.2 ± 6.0	90.5 ± 4.5
75	88.9 ± 5.4	89.1 ± 5.5
750	89.7 ± 7.0	90.0 ± 7.2
Mean (%) ± S.D.	89.9 ± 6.1	89.9 ± 5.8
Absolute recovery	**Sufentanil Mean (%) ± S.D. (n = 6)**	**[$^2H_5$]-Sufentanil Mean (%) ± S.D. (n = 6)**
Nominal concentration (pg/ml)		
7.5	39.0 ± 3.3	39.3 ± 3.8
75	35.4 ± 2.6	35.2 ± 2.5
750	42.5 ± 3.4	42.6 ± 3.5
Mean (%) ± S.D.	39.0 ± 3.1	39.0 ± 3.3

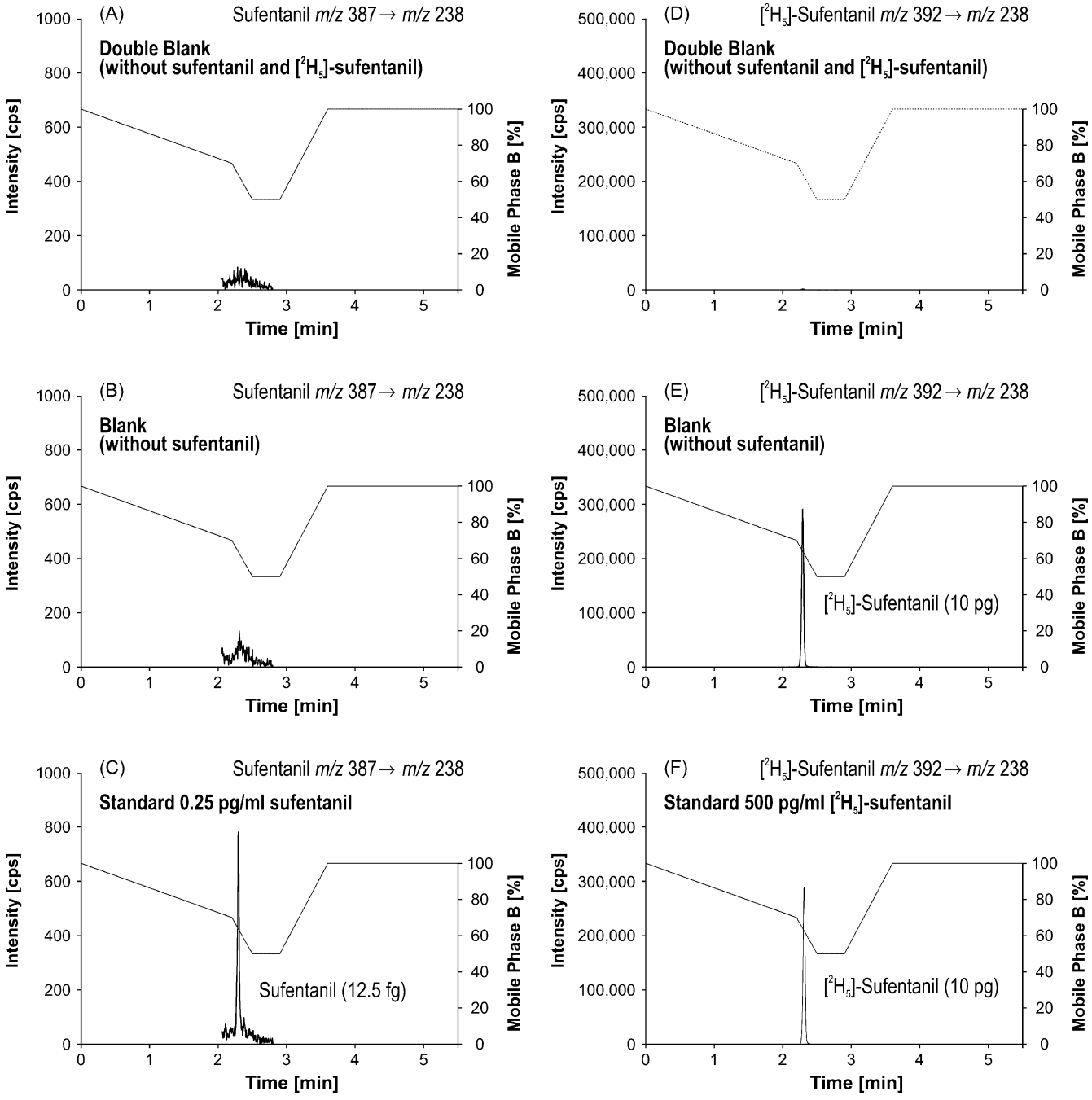


Fig. 3. Representative chromatograms of extracted standard samples. Mobile phase A was water/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v, pH 2.8) and mobile phase B was acetonitrile/formic acid/1 M ammonium acetate stock solution (100:0.25:0.5, v/v/v). Chromatogram (A) represents an extracted unspiked plasma sample (Double Blank) obtained in MRM mode at transition *m/z* 387 → 238 for sufentanil. The corresponding transition at *m/z* 392 → 238 for the internal standard, [$^2H_5$]-sufentanil, is shown in chromatogram (D). Chromatograms (B) and (E) (Blank) represent an extracted plasma sample with added concentrations of 500 pg/ml internal standard [$^2H_5$]-sufentanil (10 pg on column). An extracted plasma sample with added concentrations of 0.25 pg/ml sufentanil (12.5 fg on column) and 500 pg/ml [$^2H_5$]-sufentanil (10 pg on column) is shown in chromatograms (C) and (F) which represents the LLOQ of sufentanil.

A solution of 1000 pg/ml sufentanil and [$^2H_5$]-sufentanil in mobile phase B was infused through a splitting tee of the API 4000 using a Harvard Apparatus Pump 11 Plus (Harvard Apparatus, Holliston, MA, USA). Infusion rate was 10 μl/min. Twenty microliters of six extracted blank human plasmas were injected into the LC–MS/MS system and compared to an injection of 20 μl mobile phase B. The resulting chromatograms were normalized to the mean signal between 1.0 and 1.5 min which represented a constant signal in all chromatograms (Fig. 4). The relative intensity of six extracted blank human plasmas at the expected retention time of sufentanil and [$^2H_5$]-sufentanil were compared to the relative intensity of an injection of mobile phase B at the same time point (2.4 min in Fig. 4). Mean ion suppression effect with post-

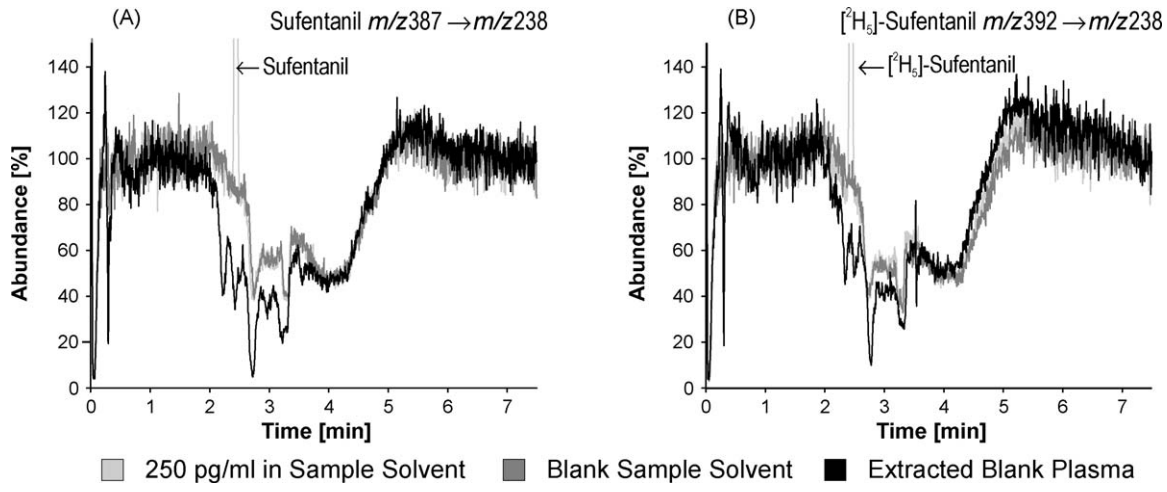


Fig. 4. Post-column infusion of sufentanil and [$^2H_5$]-sufentanil after Bonfiglio. Chromatograms in (A) represent the transition *m/z* 387 → 238 of sufentanil and chromtograms in (B) transition *m/z* 392 → 238 of [$^2H_5$]-sufentanil. A solution of 1000 pg/ml sufentanil and [$^2H_5$]-sufentanil in mobile phase B was infused through a splitting tee. Infusion rate was 10 μl/min and injection volume 20 μl. To determine the region of interest a standard in sample solvent (250 pg/ml sufentanil and [$^2H_5$]-sufentanil) was injected into the system to obtain the expected retention time of sufentanil and [$^2H_5$]-sufentanil (truncated peaks in A and B).

column infusion technique was 42.8 ± 11.1% for sufentanil and 41.1 ± 10.1% for [$^2H_5$]-sufentanil (t = 2.4 min). Mean ion suppression effect in the region ±0.05 min of the expected retention time was 46.9 ± 10.8% for sufentanil and 44.0 ± 9.1% for [$^2H_5$]-sufentanil (t = 2.35–2.45 min).

In contrast to the method of Matuszweski determination of matrix effects by post-column infusion of sufentanil and [$^2H_5$]-sufentanil resulted in imprecise values for signal influencing effects, because all chromatograms had to be normalized and the time point for calculation of matrix effects had to be assumed (no peak of sufentanil or [$^2H_5$]-sufentanil is visible with post-column addition).

Determination of signal influencing effects with the method of Matuzewski produced more accurate values as compared to post-column infusion even if the results were calculated with six instead of 36 injections (sufentanil 47.1 ± 5.3%; [$^2H_5$]-sufentanil 48.0 ± 5.4%). However, determination of matrix effects with both methods leads to comparable values for ion suppression of sufentanil and [$^2H_5$]-sufentanil.

3.3. Stability of sufentanil

Plasma samples of parturients and arterial umbilical plasma of neonates were stored at −40 °C until LC–MS/MS measurement. Each freeze/thaw stability, long-term stability and short-term stability of sufentanil was determined with six standard samples of two different concentrations (7.5 and 750 pg/ml), which were prepared with six different blank human plasmas. The final concentration of the internal standard, [$^2H_5$]-sufentanil, in the stability tests was always 500 pg/ml. Spiked samples for freeze/thaw stability were stored at −40 °C. After 24 h, the samples were allowed to thaw for 60 min at room temperature and refrozen at −40 °C. After the third thawing cycle, samples were extracted and measured against a freshly prepared calibration curve (Table 4). Sufentanil was stable under the freeze/thaw stability conditions. Long-term stability under the storage conditions of −40 °C was ascertained over a period of 6 months. No difference was observed to freshly prepared calibration standards. To assess short-term sta-

Table 4
Recovery of six different plasma samples after performing storage, short-term and freeze/thaw stability for sufentanil and stability of stock solutions of sufentanil and [$^2H_5$]-sufentanil

	Short-term stability Mean (%) ± S.D. (n = 6)	Long-term stability Mean (%) ± S.D. (n = 6)	Freeze/thaw stability Mean (%) ± S.D. (n = 6)
Nominal concentration (pg/ml)			
7.5	97.3 ± 1.4	92.6 ± 1.8	90.3 ± 4.1
750	97.3 ± 0.5	95.7 ± 0.5	95.2 ± 2.5
Mean (%) ± S.D.	97.3 ± 1.0	94.1 ± 1.2	92.7 ± 3.3

Stock-stability	Sufentanil Mean (%) ± S.D. (n = 6)	[$^2H_5$]-Sufentanil Mean (%) ± S.D. (n = 6)
Nominal concentration (ng/ml)		
100	99.4 ± 2.4	99.9 ± 2.9

bility, spiked samples were extracted, reconstituted with 100 μl mobile phase B and stored in the autosampler at 4 °C. After 24 h, the samples were measured against a freshly prepared calibration curve (Table 4). Sufentanil was stable under those conditions.

Six different stock solutions of 100 ng/ml of sufentanil in acetonitrile and 100 ng/ml of [$^2H_5$]-sufentanil in acetonitrile were freshly prepared and stored for 6 h at room temperature. To assess the stability of the stock solutions they were diluted to a concentration of 100 pg/ml with mobile phase B and measured against a freshly prepared calibration curve (Table 4). Stock solutions were stable at room temperature.

Post-preparative stability was determined with 20 extracted plasma samples of parturients and neonates (1.74–50.6 pg/ml). The extracted plasma samples were stored after measurement at −40 °C and remeasured after 6 weeks against a freshly prepared calibration curve. Mean sufentanil concentration after 6 weeks was 102.9 ± 4.1%. Sufentanil was stable during all stability determinations.

3.4. Interferences in LC–MS/MS

No interferences of several blank human plasmas ($n = 12$) with sufentanil were observed, but with the internal standard, [$^2H_5$]-sufentanil. In four blank human plasmas, a peak with the same retention time and an intensity of 10,000–15,000 cps occurred (Fig. 5), which was not caused by carry over in the extraction procedure or in the HPLC-system. The interference was visible in the control transition (*m/z* 392 → 360), too. The mean intensity of the internal standard in those tests was ~50,000 cps (1.2 pg on column) and the interference of 20–30% of the internal standard peak high resulted in poor assay accuracy at low sufentanil concentrations (data not shown). To overcome those possible interferences, the internal standard was increased to 10 pg on column, what resulted in an acceptable assay accuracy. The complete method validation was performed with the increased internal standard concentration of 10 pg on column.

Another interference occurred in most of the study samples (Fig. 6) and in both ion transitions of sufentanil. The mean

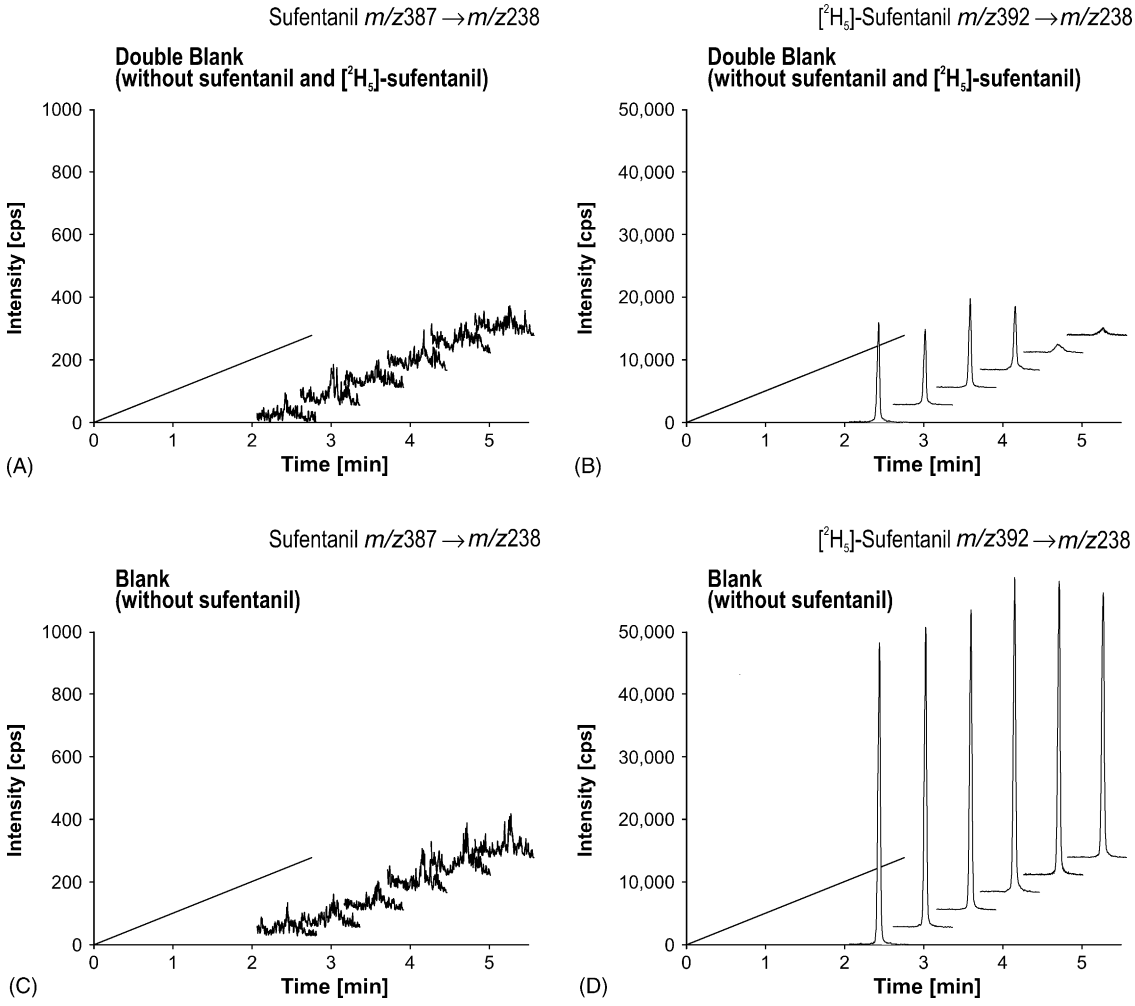


Fig. 5. Interferences with [$^2H_5$]-sufentanil. Chromatograms (A) and (B) represent extracts of six unspiked blank human plasmas and chromatograms (C) and (D) show extracts of the same plasmas, but spiked with 1.2 pg [$^2H_5$]-sufentanil. The two rear peaks in (B) represent a normal interference, which can be seen in most plasmas (8 out of 12), but the four front peaks show ~30% of the peak high as compared to spiked blank human plasmas (1.2 pg [$^2H_5$]-sufentanil on column) in chromatograms (D).

peak area of this interference was 7.2 ± 9.0% as compared to the sufentanil peak in the corresponding chromatogram, but did not complicate integration of sufentanil peaks.

Investigation of the structure of the interferences with precursor ion scans was not possible due to the low concentration of the interferences.

3.5. Application of the LC–MS/MS-method

The applicability of the LC–MS/MS assay method was shown in a prospective, randomized and double-blinded study. Group I received an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.25 μg/ml sufentanil (29 parturients), group II an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.5 μg/ml sufentanil (23 parturients) and group III an infusion of 1.6 mg/ml *S*-ropivacaine plus 0.75 μg/ml sufentanil (22 parturients). Mean plasma levels of sufentanil in parturients were 9.4 ± 4.2 pg/ml in group I, 12.2 ± 8.2 pg/ml in group II and 18.7 ± 10.1 pg/ml in group III. Mean arterial umbilical plasma levels of sufentanil in neonates was 3.7 ± 1.6 pg/ml in group I, 4.2 ± 2.6 pg/ml in group II and 7.3 ± 4.2 pg/ml in group III. Representative chromatograms of sufentanil in parturients and neonates are presented in Fig. 6. Mean PCEA

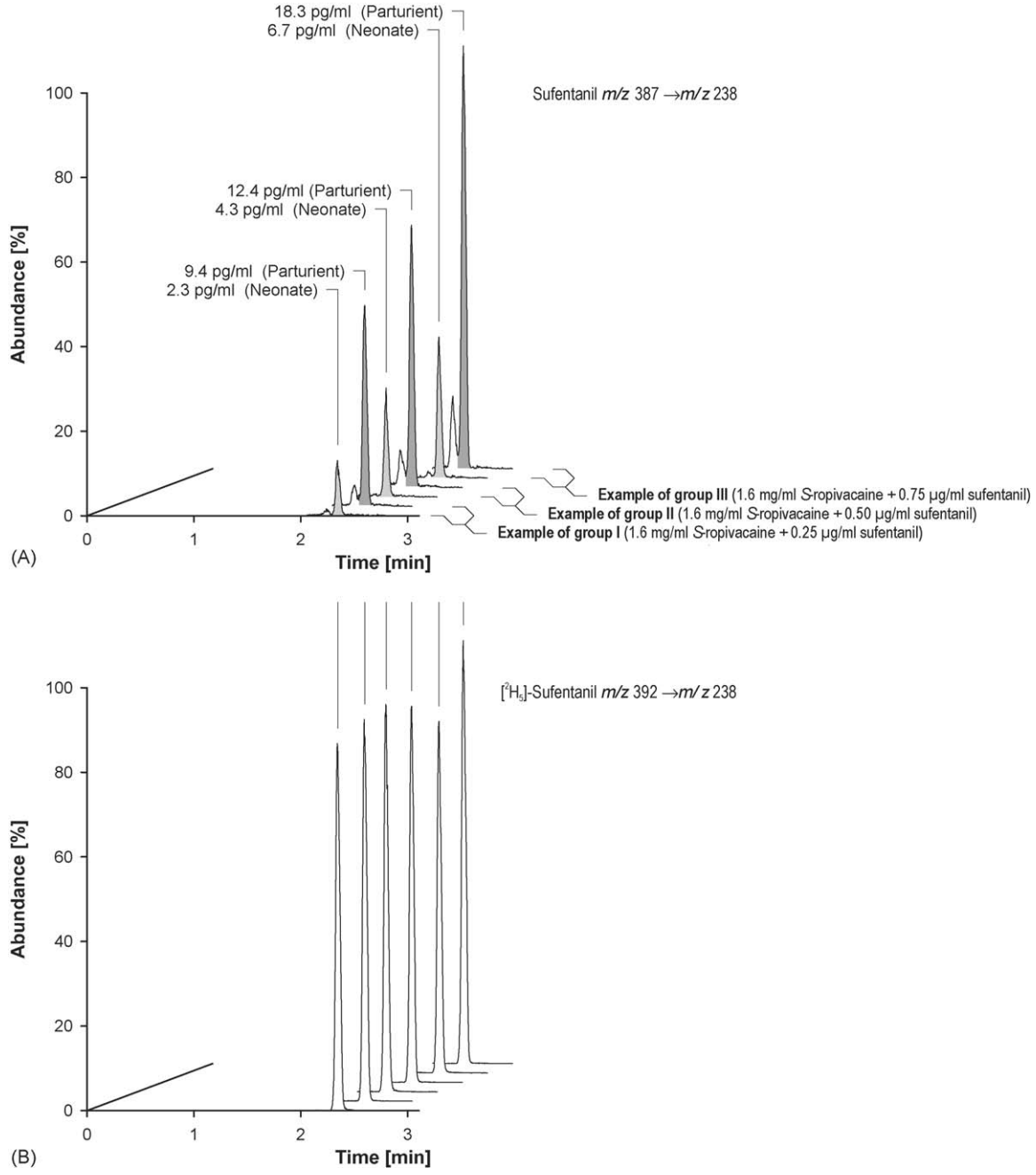


Fig. 6. Typical LC-MS/MS chromatograms from extracted plasma of parturients and neonates after PCEA. Chromatograms in (A) represent the transition *m/z* 387 → 238 of sufentanil and chromtograms in (B) transition *m/z* 392 → 238 of [$^2H_5$]-sufentanil. Parturients of group I received an infusion of 1.6 mg/ml *S*-ropivacaine and 0.25 μg/ml sufentanil, parturients of group II an infusion of 1.6 mg/ml *S*-ropivacaine and 0.50 μg/ml sufentanil and parturients of group III an infusion of 1.6 mg/ml *S*-ropivacaine and 0.75 μg/ml sufentanil.

duration was 4:59 ± 2:49 (h:min) in group I, 4:26 ± 3:55 (h:min) in group II and 5:51 ± 3:35 (h:min) in group III. The whole amount of sufentanil during PCEA was 18.9 ± 5.3 μg in group I, 24.3 ± 12.4 μg in group II and 38.5 ± 16.3 μg in group III. The detailed clinical outcome of the study will be published elsewhere.

4. Conclusion

The developed LC–MS/MS assay enabled to quantify sufentanil in plasma of parturients and arterial umbilical plasma of their neonates. This was shown in a clinical study under different regimen treatments as presented in Fig. 6. The LLOQ of sufentanil was found to be 0.25 pg/ml (12.5 fg on column) and is more than 10 times lower than in other GC- or LC- based mass spectrometrical methods reported so far.

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