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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 1095–1100
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Determination of fentanyl in human plasma and fentanyl and norfentanyl in human urine using LC–MS/MS

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Received 16 May 2004; received in revised form 28 August 2004; accepted 30 August 2004
Available online 14 November 2004

Abstract

Fentanyl, a potent analgesic drug, has traditionally been used intravenously in surgical or diagnostic operations. Formulations with fentanyl in oral transmucosal delivery system and in transdermal depot-patch have also been developed against breakthrough pain in cancer patients. In this report, LC–MS/MS methods to determine fentanyl in human plasma as well as fentanyl and its main metabolite, norfentanyl, in human urine are presented together with validation data. The validation ranges were 0.020–10.0 and 0.100–50.0 ng/ml for fentanyl in plasma and urine, respectively, and 0.102–153 ng/ml for norfentanyl in urine.

Liquid–liquid extraction of the compounds fentanyl, norfentanyl and the deuterated internal standards, fentanyl-d_5 and norfentanyl-d_5 from the matrixes was applied and separation was performed on a reversed phase YMC Pro C_{18}-column followed by MS/MS detection with electrospray in positive mode. The inter-assay precision (CV%) was better than 4.8% for fentanyl in plasma and 6.2% and 4.7% for fentanyl and norfentanyl, respectively, in urine.

The ruggedness of the methods, selectivity, recovery, effect of dilution and long-term stability of the analytes in plasma and urine were investigated. Effect of haemolysis and stability of fentanyl in blood samples were also studied.

The methods have been applied for the determination of fentanyl in plasma samples and fentanyl/norfentanyl in urine samples taken for pharmacokinetic evaluation after a single intra-venous (i.v.) dose of 75 μg fentanyl.

Keywords: Fentanyl; Norfentanyl; LC–MS/MS; Plasma; Urine; Validation

1. Introduction

Fentanyl is a potent, short-acting narcotic analgesic used as a surgical anaesthetic and for the treatment of pain in tumour patients. Therapeutic levels of fentanyl are as low as 1 ng/ml in plasma and methods with high sensitivity are required for the determination of fentanyl in biological fluids for pharmacokinetic studies. Fentanyl and/or its main metabolite, norfentanyl (Fig. 1) have been determined using GC [1], HPLC/UV [2–5], GC/MS [6–8], LC–MS/MS [9–12] and immunoassays [13,14]. HPLC and immunoassays did not offer the high sensitivity required for low dose studies of fentanyl, GC/MS give good sensitivity but requires long run times. LC–MS/MS offers often rapid and sensitive analysis with simple mobile phase compositions. Fentanyl in human plasma samples has earlier been determined by LC–MS/MS after sample preparation with mixed mode SPE [9,10] with a lower limit of quantification (LLOQ) of 0.05 ng/ml. An LC–MS/MS method for fentanyl and norfentanyl in primate plasma [11], with LLOQ at 0.025 and 0.05 ng/ml, respectively, has also recently been reported using repeated liquid–liquid extraction (LLE) for sample purification. Similarly, fentanyl and norfentanyl in human plasma [12] have been determined at a LLOQ of 0.05 ng/ml using LLE followed by LC–MS/MS. In this report, a rapid and robust LC–MS/MS method with atmospheric pressure ionization (API) technique, e.g. electrospray ionization in positive mode is reported. Sample preparation was performed with a one step LLE and the method, originally developed to analyse fentanyl in plasma samples, has been used with minor modifications to analyse fentanyl and norfentanyl in

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doi:10.1016/j.jpba.2004.09.024

urine. The method has been validated to determine fentanyl in plasma in the range 0.020–10.0 ng/ml and to determine fentanyl and norfentanyl in urine in the range 0.100–50.0 and 0.102–153 ng/ml, respectively.

2. Experimental

2.1. Chemical and reagents

Fentanyl citrate (purity >99%) was from Sigma (St. Louis, MO, USA). Norfentanyl oxalate solution (1.0 mg/ml in methanol), fentanyl-d_5 (100 μg/ml in methanol), and norfentanyl-d_5 solution (100 μg/ml in acetonitrile), Fig. 1, were from Cerilliant (Pound Rock, TX, USA). Drug free urine and sodium and lithium heparin plasma were obtained within Quintiles AB (Uppsala, Sweden). Drug free citrate buffered plasma was from Uppsala University Hospital (Uppsala, Sweden). Acetonitrile, ethylacetate, *n*-heptane, 2-butanol, sodium hydroxide and formic acid were purchased from Merck (Damstadt, Germany), all were of either LC grade or analytical purity and used as received. Water was purified by passage through two Milli-Q purification systems from Millipore (Bedford, MA, USA).

2.2. LC–MS/MS method

The liquid chromatographic system consisted of two Shimadzu LC-10AD *VP* pumps (Tokyo, Japan), a Gilson 231 sampling injector equipped with a Gilson 832 temperature regulator (CEDEX, France) kept at 12 °C and a column oven, model 7990, from Jones Chromatography (Mid Glamorgan, UK) or a Hot Pocket from Keystone Scientific, Inc. (Bellefonte, PA, USA).

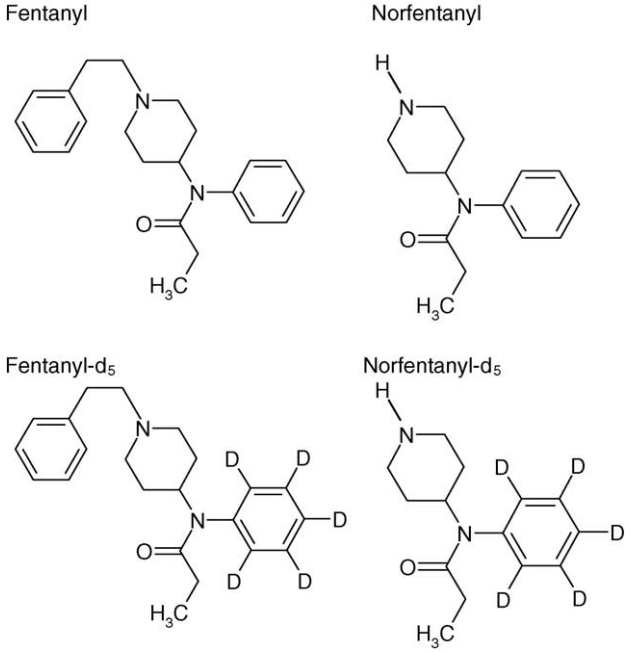

Fig. 1. Fentanyl, fentanyl-d_5, norfentanyl and norfentanyl-d_5.

Gradient elution was used for the separation of fentanyl and norfentanyl in urine samples with a linear gradient from 2 to 30% acetonitrile in 5 mM formic acid for 4 min and maintained at 30% for 1 min. For the determination of fentanyl in plasma, isocratic elution using 18% acetonitrile in 5 mM formic acid aqueous solution was applied. The column used in both methods was a YMC Pro C_{18}-column 50 mm × 2 mm i.d. (Weselerwald, Germany) protected by either an OptiGuard C_{18}, 1 mm pre-column from Optimize Technologies (Oregon City, OR, USA) or a SecurityGuard C_{18}, 4 mm × 2 mm, from Phenomenex® (Torrance, CA, USA).

A triple quadrupole mass spectrometer, Quattro II, with z-spray interface from Micromass (Manchester, UK) was operated in positive-ion mode. The capillary voltage was maintained at 3.5 kV. The cone voltage was 34 and 25 V for fentanyl and norfentanyl, respectively. The source and desolvation temperature was 80 and 250 °C, respectively. The cone gas flow was kept at approximately 80–90 l/h and the desolvation gas flow at 450–500 l/h. The collision gas pressure was (1.0–1.5) × 10^{-3} mbar and the collision energies (in-house frame of reference) were 23 and 18 V for fentanyl and norfentanyl, respectively. Multiple reaction monitoring (MRM), with one channel for plasma analysis and two channels for urine analysis was applied. The transitions were *m/z* 337 → 188 for fentanyl, 342 → 188 for fentanyl-d_5, 232 → 84 for norfentanyl and 237 → 84 for norfentanyl-d_5 with the dwell time 0.3 s for each pair. The software MassLynx, Ver. 3.4 (Micromass, Manchester, UK) was used for data acquisition and processing. A weighted (1/*x*) linear least squares regression was used to establish the calibration curve from the calibration samples and the concentration of the quality control samples was calculated using the calibration curve generated in each analytical run.

2.3. Sample preparation

2.3.1. Preparation of standard and quality control samples

2.3.1.1. Urine. Calibration and quality control (QC) samples were made from two separate stock solutions of fentanyl (5.00 μg/ml in methanol:5 mM formic acid aqueous solution, 5:95, v/v) and norfentanyl (10.0 μg/ml in methanol:5 mM formic acid aqueous solution, 1:99, v/v). Calibration samples 0.1–50 and 0.1–153 ng/ml for fentanyl and norfentanyl, respectively, were prepared in blank urine. QC samples at levels of 0.250, 5.00 and 40.0 ng/ml for fentanyl and 0.255, 20.4 and 122 ng/ml for norfentanyl were prepared as well as the samples for determination of lower and upper limits quantification (0.100 and 50.0 ng/ml for fentanyl and 0.100 and 153 ng/ml for norfentanyl, respectively). All spiked urine samples were aliquoted into polypropylene vials and stored frozen at −20 °C.

2.3.1.2. Plasma. Calibration and quality control (QC) samples of fentanyl in plasma were prepared in the same way. Quality control samples (QC) were prepared in blank plasma with either citrate or lithium heparin as anticoagulant.

2.3.2. Liquid–liquid extraction

2.3.2.1. Urine. Urine samples were vortex-mixed and centrifuged at a relative centrifugal force of 1500 × *g* for 5 min on a Sigma 4K10 centrifuge (Osterode am Harz, Germany). Urine, 0.5 ml, was transferred to a glass tube, 50 μl of internal standard solution (5 ng/ml of fentanyl-d_5 and 25 ng/ml of norfentanyl-d_5, 0.5 ml of 1.0 M sodium hydroxide and 3.5 ml of the extraction solution was added. The extraction solution was a mixture of *n*-heptane, ethylacetate and acetonitrile (48:48:2, v/v/v). The samples were extracted by shaking for 30 min on a Stuart Scientific shake board (Stuart Scientific Co. Ltd., Surrey, UK) followed by centrifugation at 1500 × *g* for 5 min and freezing the lower aqueous phase by keeping the tubes at −70 °C for 15 min. The organic phase was then transferred to a new glass tube and the solvent was evaporated using a Liebizch heating block (Bielefeld, Germany) kept at 45 °C with a nitrogen evaporator. The residue was reconstituted in 250 μl of 5 mM formic acid in water and 50 μl was injected onto the LC–MS/MS system.

2.3.2.2. Plasma. Plasma samples were vortex-mixed and the centrifuged plasma, 1.0 ml, was transferred to a conical glass tube, 50 μl of the internal standard solution (10 ng/ml of fentanyl-d_5), 300 μl of 1.0 M sodium hydroxide, 1.0 ml of water and 7.0 ml of extraction solution, *n*-heptane with 3% 2-butanol, were added. The extraction and evaporation of the organic phase as well as reconstitution of the residue was carried out in the same way as for urine samples. A smaller sample volume (0.5 ml) was also used. All other solutions needed for extraction was reduced in the same extension as the plasma volume. The analytical procedures were otherwise unchanged.

2.4. Method validation

The methods were validated according to Guidance for Industry, Bioanalytical Method Validation from FDA [15] with respect to recovery, accuracy, intra- and inter-assay precision, selectivity, ruggedness, effect of dilution, effect of different anticoagulants, effects of haemolysis and stability.

2.5. Authentic samples

Plasma samples from a volunteer administered a single i.v. dose of 75 μg fentanyl (Leptanal®, 100 μg/ml, Cilag Janssen) were collected 3 min before dose and 5, 15, 30, 45, 60120, 180, 240, 360 and 1440 min post-dose. The plasma was isolated by centrifugation at 1300 × *g* for 15 min and transferred to cryo-tubes prior to freezing at −20 °C. The authentic plasma samples were analysed to determine concentration of fentanyl using the method described. Urine samples were also collected during 6 h after dosing, pooled and analysed for determination of urinary fentanyl and norfentanyl.

3. Results and discussion

3.1. Recovery and lower limit of quantification

Extraction recovery was determined by comparing the peak area obtained after extraction with the peak area obtained after direct injection of the compound dissolved in blank extract. For urinary fentanyl and norfentanyl, the recovery was 96% and 89% for fentanyl at QC levels of 0.250 and 40.0 ng/ml, and 33% for norfentanyl at both 0.255 and 122 ng/ml. Although the extraction recovery for norfentanyl was low, robust extraction procedures and the high sensitivity of the MS-instrument made it possible to reach the lower limit of quantification of 0.10 ng/ml with good precision. The signal-to-noise ratios at the LLOQ were approximately 200 for fentanyl (LLOQ = 0.100 ng/ml, CV = 4.0%, $n = 6$) and 50 for norfentanyl (LLOQ = 0.102 ng/ml, CV = 3.3%, $n = 6$) in the urine assay. The mean accuracy at the LLOQ was −7.6% and −10.0% of the nominal value for fentanyl and norfentanyl, respectively. The recovery of fentanyl in plasma was 96% and 81% for QC levels 0.040 and 7.50 ng/ml, respectively. The signal-to-noise ratio was about 15 for fentanyl in plasma at the LLOQ of 0.020 ng/ml (CV = 12%, $n = 6$) and the mean accuracy, expressed as percentage deviation from the nominal value, was −4.8%.

3.2. Intra-assay and inter-assay precision and accuracy

3.2.1. Urine

Intra- and inter-assay precision were determined for three quality control levels, 0.250, 5.00 and 40.0 ng/ml for fentanyl and 0.255, 20.4 and 122 ng/ml for norfentanyl. Six replicates of each level were analysed on three different days. The coefficients of variation for the intra- and inter-assay precision were 2.0–6.2%, for fentanyl and 2.1–4.7%, for norfentanyl. The mean accuracies, expressed as the percentage deviations from the nominal values, for the three QC levels investigated, were −3.6 to 8.1%.

3.2.2. Plasma

Intra- and inter-assay precision were also determined for three quality control levels, 0.04, 1.00 and 7.50 ng/ml in plasma. The coefficients of variation for the intra- and inter-assay precision were 1.4–5.0%. The mean accuracies were 1.7–2.8%.

Details of accuracy, intra- and inter-assay precision for the plasma and urine quality control samples are presented in Table 1.

Table 1
Validation statistic: accuracy, intra- and inter-assay precision of quality control samples of fentanyl in plasma and fentanyl/norfentanyl in urine

Matrix/compound	Concentration (ng/ml)	Accuracy (% deviation)	Intra-assay precision (% CV)	Inter-assay precision (% CV)	*n*
Plasma fentanyl	0.0400	1.7	5.0	4.8	17
	1.00	2.2	1.4	1.6	18
	7.50	2.8	1.6	2.5	18
Urine fentanyl	0.250	−3.1	2.6	3.3	18
	5.00	8.1	2.0	2.9	18
	40.0	−3.6	5.4	6.2	18
Urine norfentanyl	0.255	−2.4	4.5	4.7	18
	20.4	2.7	2.1	4.1	18
	122	−0.8	4.5	4.4	18

3.3. Selectivity, ruggedness and effect of dilution

Blank urine/plasma samples were analysed with each validation batch and no interference peak with area greater than 20% of the peak area for the LLOQ samples was found. The selectivity was also investigated by analysing blank plasma/urine from six individuals as well as samples containing common drugs such as acetylsalicylic acid, salicylic acid, paracetamol, caffeine, nicotine and ibuprofen (Fig. 2). No interference occurred in the samples tested. The methods were robust and up to 97 plasma samples and 88 urine samples could be analysed in one batch. Fig. 3 shows the reproducibility for norfentanyl of the urine method where a large number of QC samples, 20.4 ng/ml, were analysed in one of the validation batches. Urine samples containing 250 ng/ml of fentanyl and 1020 ng/ml of norfentanyl were diluted 10-fold with blank urine and analysed in six replicate. The coefficients of variation were 6.9% and 6.7%, respectively. Mean deviation from nominal concentration was 1.0% and 0.6% for fentanyl and norfentanyl, respectively. Plasma samples could also be diluted with blank plasma, tested with 50.0 ng/ml of fentanyl before being processed.

3.4. Anticoagulants

Quality control samples in citrate buffered plasma and lithium heparin plasma were prepared at two levels, 0.0500 and 7.50 ng/ml, these samples were analysed in six replicates and evaluated against a calibration curve made by calibration samples prepared in plasma with citrate as an anticoagulant. The results gave no significant differences between citrate and heparin as anticoagulant.

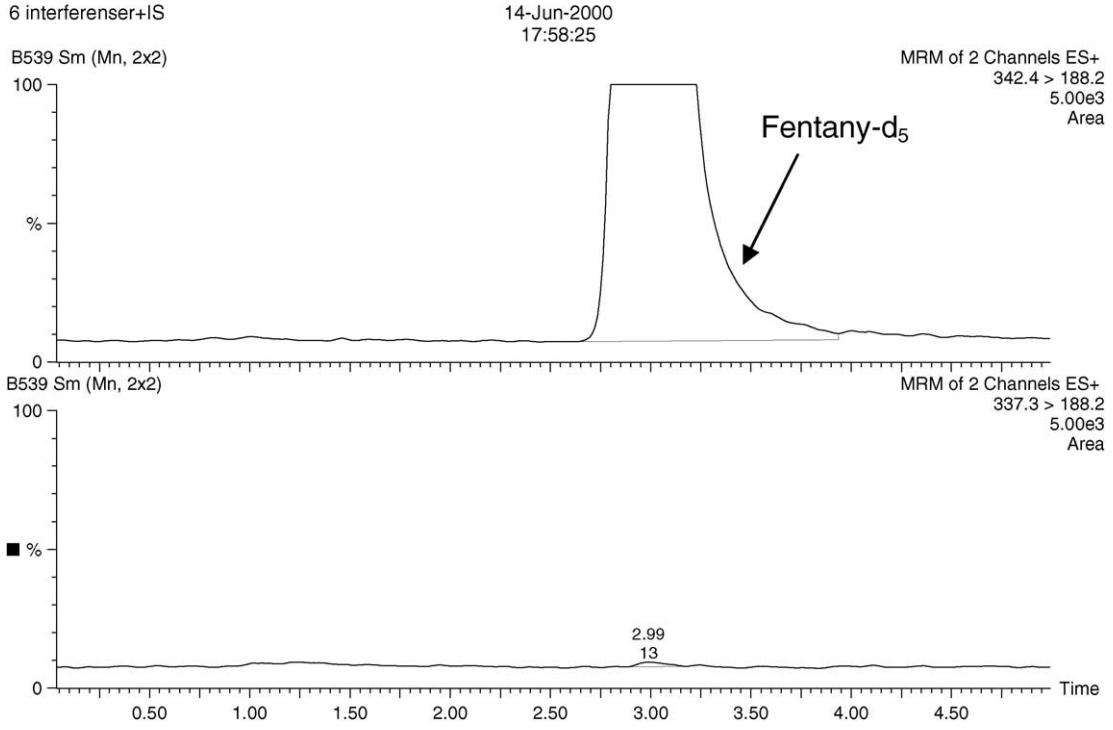

Fig. 2. Blank samples containing 0.5 ng/ml of fentanyl-d_5 and acetylsalicylic acid, salicylic acid, paracetamol, caffeine, nicotine and ibuprofen.

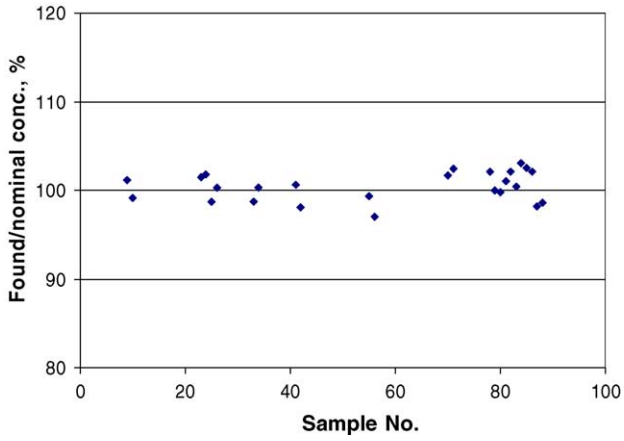

Fig. 3. Ruggedness of the method: found concentrations (%) in relation to the nominal concentration of norfentanyl (20.4 ng/ml) in human urine.

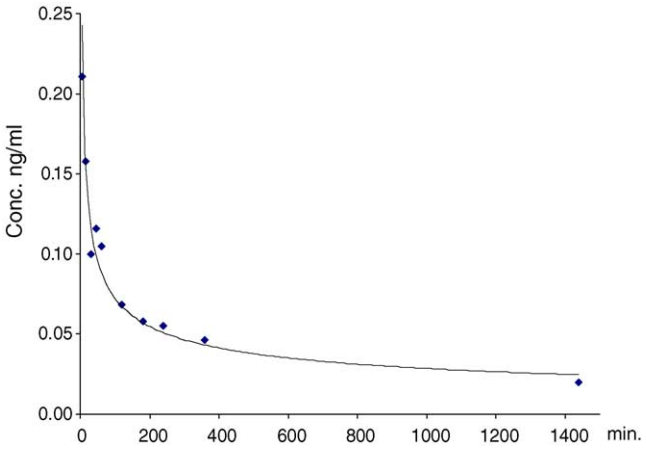

Fig. 4. Concentration of authentic samples from one volunteer administered one single i.v. dose of 75 μg fentanyl.

3.5. Stability

3.5.1. Heparinized blood samples

Sodium heparin blood samples (spiked with fentanyl), left at ambient temperature for 5, 15, 30, 45, 60, 120 min prior to centrifugation for isolation of plasma, were analysed. Two plasma samples for each time point were prepared and each sample were analysed in duplicate. No significant differences in concentration of fentanyl were found for fentanyl blood samples that were left on the bench

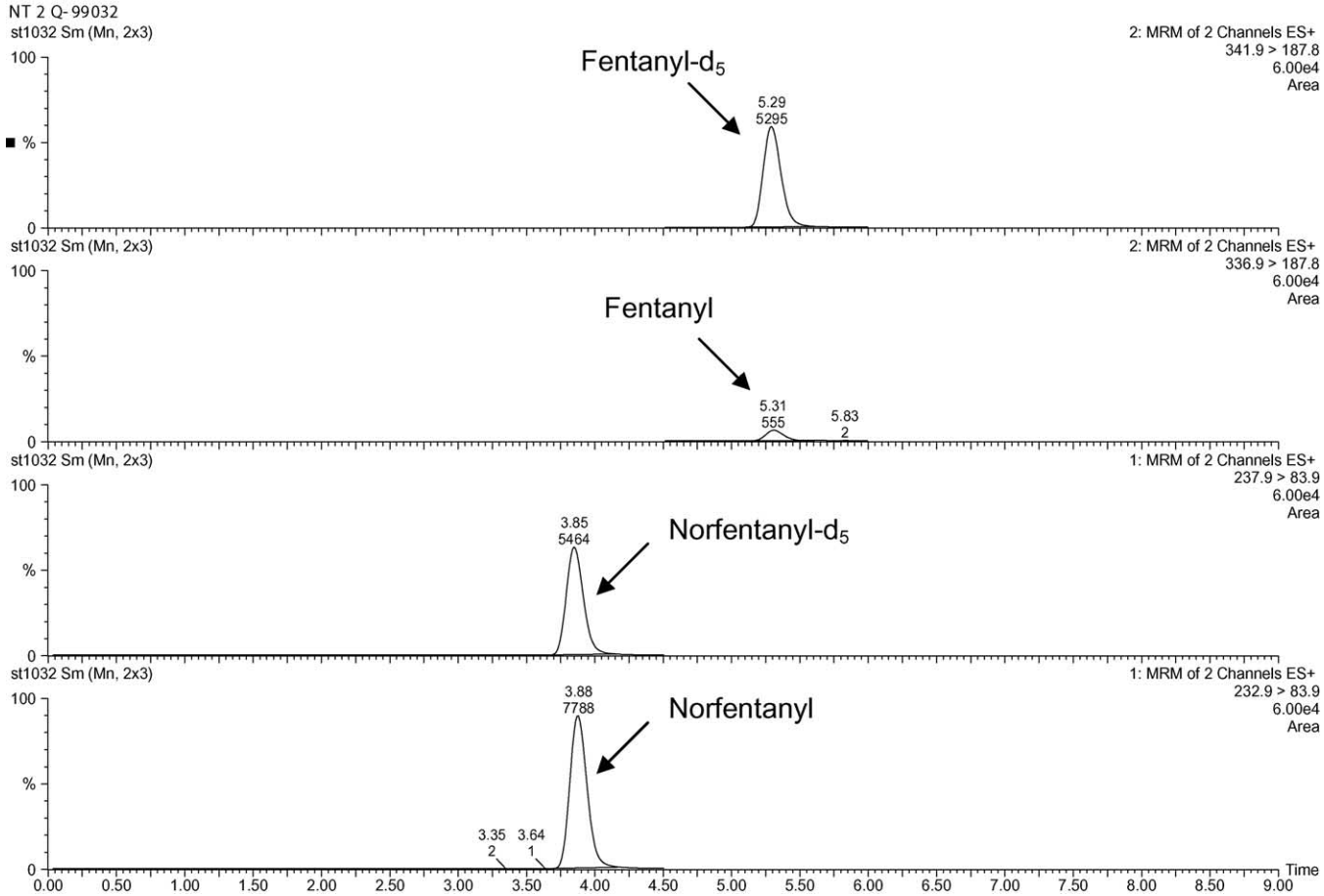

Fig. 5. Authentic urine sample collected 0–6 h post-dose (75 μg, i.v.), containing <0.10 ng/ml of fentanyl and 3.6 ng/ml of norfentanyl.

at ambient temperature for 120 min before being centrifuged.

3.5.2. Long-term stability

Fentanyl and norfentanyl in frozen urine were analysed after 1- and 3-month storage at −20 °C. Both fentanyl and norfentanyl are stable for at least 3 months in frozen urine. Fentanyl in frozen plasma was also found to be stable for at least 6 months at −20 °C.

3.6. Effect of haemolysis

Plasma samples obtained from non-haemolysed and haemolysed blood were analysed and compared. No effect from haemolysis was observed since the accuracy was 3.7% and 5.0% for samples spiked in plasma obtained from non-haemolysed blood and haemolysed blood, respectively.

3.7. Authentic samples

The concentration of fentanyl in the plasma samples from one volunteer administered a single i.v. dose of 75 μg fentanyl was determined. The concentration could be determined for samples taken during 24 h after the dose (Fig. 4). The concentration of fentanyl and norfentanyl in the urine pool during a period of 6 h post-dose was determined, Fentanyl was <0.10 ng/ml in this sample and 3.6 ng/ml of norfentanyl was found (Fig. 5). In the present set up fentanyl could not be measured in urine collected during 6 h post-dose. This can however, be made as it is possible to reduce the quantification limit of fentanyl in urine at least five-fold to 0.02 ng/ml. The present signal-to-noise ratio at LLOQ of 0.10 ng/ml fentanyl in urine is 200.

4. Conclusion

Selective, sensitive and robust LC–MS/MS methods have been validated for determination of fentanyl/norfentanyl in urine samples as well as fentanyl in plasma. Simultaneous determination of fentanyl and its main metabolite, norfentanyl in urine has been performed in series of approximately 100 samples without loss of column performance. Prepared plasma samples could be injected every 4th min to determine concentrations as low as 0.02 ng/ml of fentanyl. The methods are suitable for determination of fentanyl and norfentanyl in samples generated from clinical trials.

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