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Quantification of Fentanyl in Human Serum by Column-Switching Liquid Chromatography and Tandem Mass Spectrometry

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Abstract

Analytical methods for pharmacokinetic studies with quantification of the highly potent anaesthetic drug fentanyl in serum must be rugged, sensitive, precise and accurate. To address these analytical demands and facilitate an automated approach to sample clean-up, a new method was developed for human serum using back-flush column-switching and high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). Prior to injection, protein precipitation in trichloroacetic acid was performed in each sample. Samples were diluted with internal standard (D_5-fentanyl) in 10% (w/v) trichloroacetic acid, kept at 4°C for 20 min and centrifuged followed by injection of 40µL supernatant onto a BioTrap 500 MS extraction column. Using a time programmed six-port valve switch, the extracted drug was back-flushed onto a Zorbax SB-Aq analytical column, gradient eluted and finally detected after electrospray ionisation with multiple reaction monitoring (MRM) of the transitions *m/z* 337 → *m/z* 188 and *m/z* 342 → *m/z* 188 for fentanyl and D_5-fentanyl, respectively. Mean inter-assay accuracy (*n*=5) ranged from 93 to 101 % and inter-assay precision (*n*=5) ranged from 3.7 to 9.4% (C.V.). The method was used in a preliminary study of 4 patients after application of transdermal patches and found fully applicable for future pharmacokinetic studies. This is the first on-line, column-switching LC-MS/MS method validated and demonstrated suitable for quantification of fentanyl in human serum.

Introduction

Fentanyl, (N-(1-(2-phenylethyl)-4-piperidinyl)-*N*-phenylpropanamide) is a potent, synthetic opioid used as an anaesthetic, peri-operatively and for management of chronic pain. The delivery through transdermal patches (Durogesic®) is increasingly popular, with slow release of the drug into the bloodstream over 2-3 days using patches manufactured for different desorption rates (12-100 µg/h) [1-3]. For transdermal delivery, application of e.g. heat on the patch will increase plasma concentration [4,5]. Analytical methods for pharmacokinetic studies of heating effects must be very sensitive, precise and accurate, as typical, steady-state plasma concentrations are below 1 ng/mL. Early research was based upon radioimmunoassay (RIA) [6], however, these methods are no longer available and their performance suffered from cross-reactivity with metabolites causing overestimation at low concentrations [7]. Hitherto, chromatographic methods have been based upon LC-UV, GC-MS [8-11], LC-MS or LC-MS/MS [12-16]. Among these methods, some were designed for forensic toxicology or involved elaborate and time-consuming pre-treatment such as off-line solid-phase extraction (SPE) or derivatization, and some methods lacked sensitivity. Takashina et al. [16] argued that large inter-individual variations in fentanyl pharmacokinetics justified therapeutic drug monitoring in cancer patients. Their off-line SPE and LC-MS/MS method used papaverine as internal standard, although a deuterium-labelled internal standard (D_5-fentanyl) was available.

Only two papers have demonstrated the use of on-line extraction. Marier et al. [17] used on-line SPE and Chang et al. [18] described two-dimensional chromatography (column-switching) in a study of fentanyl in dog plasma. In the latter case, the authors used reversed-phased chromatography for trapping fentanyl after protein precipitation (PPT) in acetonitrile and hydrophilic interaction chromatography (HILIC) for analytical separation.

The present study demonstrates an alternative approach using a polymer-based, protein-coated, restricted access media (RAM) type of pre-column (BioTrap®) coupled to reversed-phased chromatography to develop a two-dimensional, on-line extraction assay for fast, precise and accurate quantification of low levels of fentanyl in serum. This is to the authors' knowledge the first on-line, column-switching LC-MS/MS method validated and demonstrated applicable for analysis of fentanyl in human serum. In this paper, method validation data are presented including results from a preliminary study of baseline levels of four patients after application of Durogesic transdermal fentanyl patch (25 µg/h).

Experimental

Chemicals and reagents

Acetonitrile was HPLC grade from Merck. All other organic solvents and reagents were of analytical grade. Purified water (18.2 MΩ) was prepared on an Elga Centra RDS system (Buckinghamshire, UK). Certified reference standards of fentanyl and D_5-fentanyl were purchased from Cerilliant (Texas, USA).

Liquid chromatography

The HPLC system modules were all from Agilent Technologies (Palo Alto, CA, USA) including a 1200 binary pump, 1200 SL autosampler and 1200 column department unit with a six-port switching valve embedded. The damper and mixer were bypassed in

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order to optimize the pumping system to low dead volume as described in the Agilent User Manual. Autosampler injection volume was 40 µL. The analytical column was an Agilent Zorbax SB-Aq, 50 x 2.1 mm, i.d., packed with 3.5 µm particles. Flow rate was 600 µL/min. Column temperature was 40°C. Mobile phase A consisted of 4% 2-propanol in 10 mM ammonium acetate adjusted to pH 6.5 with diluted formic acid. Mobile phase B was acetonitrile. The binary pump gradient started at 20% phase B for 1 min and then went up to 90% phase B in 4 min. It was maintained at 90 % phase B for 0.5 min and then brought back to initial conditions for 1.5 min of equilibration. Total run time was 6 min. The six-port switching valve brought the BioTrap extraction column in series with the analytical column for back-flush elution after 1 min and was switched back at 4 min thus allowing for 2 min equilibration before next sample injection. Extraction was controlled using an Agilent 1100 isocratic pump directly connected to the autosampler. The extraction column was a BioTrap 500 MS, 20 x 2.0 mm, i.d. (Dalco Chromtech, Sweden). Flow rate was 1 mL/min using 4% 2-propanol in 10 mM ammonium acetate adjusted to pH 10.0 with 25% (w/w) ammonium hydroxide solution.

Tandem mass spectrometry

The MS system consisted of an Agilent 6460 triple quadrupole mass spectrometer (Palo Alto, CA,USA) equipped with a jet stream electrospray ion source operated in positive mode. Capillary voltage was 2000 V, nebuliser pressure 40 p.s.i., gas temperature 350°C, gas flow 8 L/min, sheath gas temperature 400°C, sheath gas flow 8.5 L/min, nozzle voltage 0 V, depolarisation potential 120 V. Multiple Reaction Monitoring (MRM) parameters for fentanyl were: *m/z* 337 → *m/z* 188 (collision energy: 23 - signal used for calibration), *m/z* 337 → *m/z* 105 (collision energy: 39 - qualifying ion). For internal standard D_5-fentanyl: *m/z* 342 → *m/z* 188 m/z (collision energy: 21 –signal used for calibration), *m/z* 342 → *m/z* 105 (collision energy: 39 - qualifying ion). Dwell time was 100 ms at unit resolution. All calculations were performed used the MassHunter® software (Agilent Technologies) and Excel 2007 (Microsoft).

Preparation of calibrators and quality control samples

A stock serum pool (10 mL) containing fentanyl at 100 ng/mL was prepared by diluting 1000 µL fentanyl solution (1 µg/mL in 25 mM formic acid) and 1000 µL NaCl solution (9 mg/mL) with blank serum. Serum calibrators (*n*=8) were prepared by spiking appropriate volumes of spiked serum (fentanyl: 10 ng/mL) into blank serum. The calibrator concentrations were 0.05, 0.10, 0.25,0.50, 1.0, 2.0, 3.0 and 5.0 ng/mL. An eight-point calibration curve was constructed for each batch of samples, with serum calibrators and serum control samples undergoing similar sample preparation. Standard curves were based on peak area with a linear curve fit, not forced through zero, with no weighting. Quality control samples (QCs) in five levels (0.05, 0.10, 0.25, 1.0 and 2.5 ng/mL) used for validation were prepared like serum calibrators, but from separate serum pools and stock solutions. Pooled blank serum free from fentanyl was centrifuged at 3000 x *g* for 10 min and used for preparation of spiked samples. All calibrators and QCs were aliquoted and kept frozen (below -18°C) until used.

Sample preparation

The reagent used for protein precipitation was prepared as follows: 20 ml 10% (w/v) trichloroacetic acid was mixed with 30 µL D_5-fentanyl aqueous working solution (1 µg/mL). Sample preparation was equivalent for calibrators, QCs and patient samples. Serum (200 µL) was mixed for 1 min with 100 µL protein precipitation reagent (4°C) in 1.5 mL micro-centrifuge tubes (Sarstedt, Germany), left at 4°C for 20 min and then centrifuged at 13.000 x *g*. An aliquot of 100 µL of the clear supernatant was transferred to autosampler vials.

Method validation

Acceptance criteria for the linearity of calibration curves were correlation coefficients (*r*) better than 0.999. Intra-assay precision and accuracy was assessed by replicate analysis of quality control samples (*n*=5) at five levels (0.05, 0.10, 0.25, 1.0 and 2.5 ng/mL). Precision was reported as coefficient of variation (C.V.%) and accuracy [(measured concentration / spiked concentration) x 100%]. Inter-assay precision was assessed by analysis of quality control samples at four levels (0.10, 0.25, 1.0 and 2.5 ng/mL) in different batch runs (*n*=5). Specificity was assessed by analysis of different, blank patient serum samples (*n*=6) and by determination of the retention times (RT) of three commonly used fentanyl analogues (alfentanil, remifentanil and sufentanil). The acceptance criteria for accuracy (recovery) and precision (C.V.) was ± 15% of the theoretical values, except at the limit of quantification (LOQ), where it was ±20%. These criteria are stated in current guidelines for bioanalytical method validation [19]. Limit of detection (LOD) was calculated as "mean + 3 x standard deviation (S.D.)" in blank samples (*n*=3). Matrix effects from ion suppression were calculated from the response ratio (%) of serum calibrators compared to aqueous calibrators. Relative qualifying ion intensities were expressed as the percentage of the intensity of the target MRM transitions for fentanyl and D_5-fentanyl, respectively. The acceptance criterion for variation of relative qualifying ion intensities was ± 20%. Fentanyl has been reported to be stable in serum after freezing for at least 6 months at -20°C [15], at ambient temperature for at least 6 hours before processing [18], in plasma at 4°C for at least 3 days [10], in both serum and whole blood after three freeze-thraw cycles [9,14,18] and in serum at -18°C for six freeze-thraw cycles up to 84 days [20]. The authors found it unnecessary to repeat these stability experiments in this paper.

Acceptance criteria for routine method application

The retention time of fentanyl should be within 3.2 ± 0.2 min. The relative variation of qualifying ions for the standard curve should be within ± 20%. Results of QCs at three levels (0.1, 0.25 and 2.5ng/mL) should be within ± 15% of the theoretical (spiked) values.

Pharmacokinetic study samples

Patient samples were obtained 12 hours after application of a Durogesic® patch (25 µg/h). The study was approved by the regional ethics committee (Ref. N-2011007). In order to find typical baseline concentrations for patients (*n*=4) fentanyl serum concentrations were determined in series of samples (*n*=13) during a time period of 5:45 h.

Results and Discussion

Choice of method parameters

The column-switching technique with specific reference to BioTrap column and a detailed discussion of general issues concerning optimization and principles has been publishe previously [21]. This extraction column can handle direct injection of serum, plasma, urine, drain blood and other biomatrices, however, for sub-ng/mL concentrations of fentanyl, it was obligatory to couple the technique with protein precipitation (PPT) due to matrix interferences causing distorted peak shapes and baseline noise when serum diluted with

extraction mobile phase was injected directly. The classic organic solvents for PPT (acetonitrile, methanol) cause low retention on the BioTrap column and the use of trichloroacetic acid was found to be an optimal alternative for LC-MS/MS [22].

Specificity

Chromatograms were free from interferences for all calibrators and samples making auto integration and data handling very easy (Figure 1). Separation of fentanyl homologues and analogues can be challenging as shown earlier by Lurie and Reiko [23]. The gradient run of the present method allows for separation of fentanyl from the analogues alfentanil (RT: 2.71 min), remifentanil (RT: 2.80 min) and sufentanil (RT: 3.50 min) that could otherwise interfere by ion suppression.The response in serum calibrators was below 30% of similar treated aqueous calibrators showing ion suppression effects, but the effect on quantification was minimised due to the co-eluting deuterium-labelled internal standard, that compensates for alternating matrix effects during quantification of each sample. Fluctuations in the response of D_5-fentanyl were typically below 8% (C.V.), which indicates robust and stable extraction efficiency.

For all samples at or above 0.05 ng/mL, the relative qualifying ions ratios fentanyl and D_5-fentanyl were within ± 20% of the averaged values for calibrators in the same batch, showing no co-eluting, isobaric matrix interferences on the MRM transitions and confirming high detection specificity. This approach is rarely used in clinical biochemistry, but has been proposed by Vogeser and Seger [24].

Linearity

Linearity was assessed by linear regression analysis of calibrators in different batches (n=5). The C.V. of the calibration curve slope was 3.6%, indicating reproducible response ratios with correlation coefficients (r) better than 0.999 (Table 1).

Precision and accuracy

Method validation data (Table 1) showed acceptable precision and accuracy data for the concentrations 0.05 to 2.5 ng/mL, which was chosen as the final working range for the assay. These validation data were fully adequate for pharmacokinetic studies and are well in line with international acceptance criteria for biomedical analysis. Unfortunately, no certified reference material, external quality control samples or reference laboratory was available for method comparison.

Method performance data are comparable to results by Chang et al. [18] although these authors reported a C.V. of 1% for LOQ, which was not obtained here, as well as a range of linearity up to 50 ng/mL. However, the recommended serum concentration for analgesia is 1–2 ng/mL and for anaesthesia it is 10–20 ng/mL. Thus, 0.05 to 5 ng/mL covers a normal (non-toxic) range after transdermal application.

Limits of quantification (LOQ) and detection (LOD)

The LOD was calculated to 0.003 ng/mL and the LOQ was 0.05

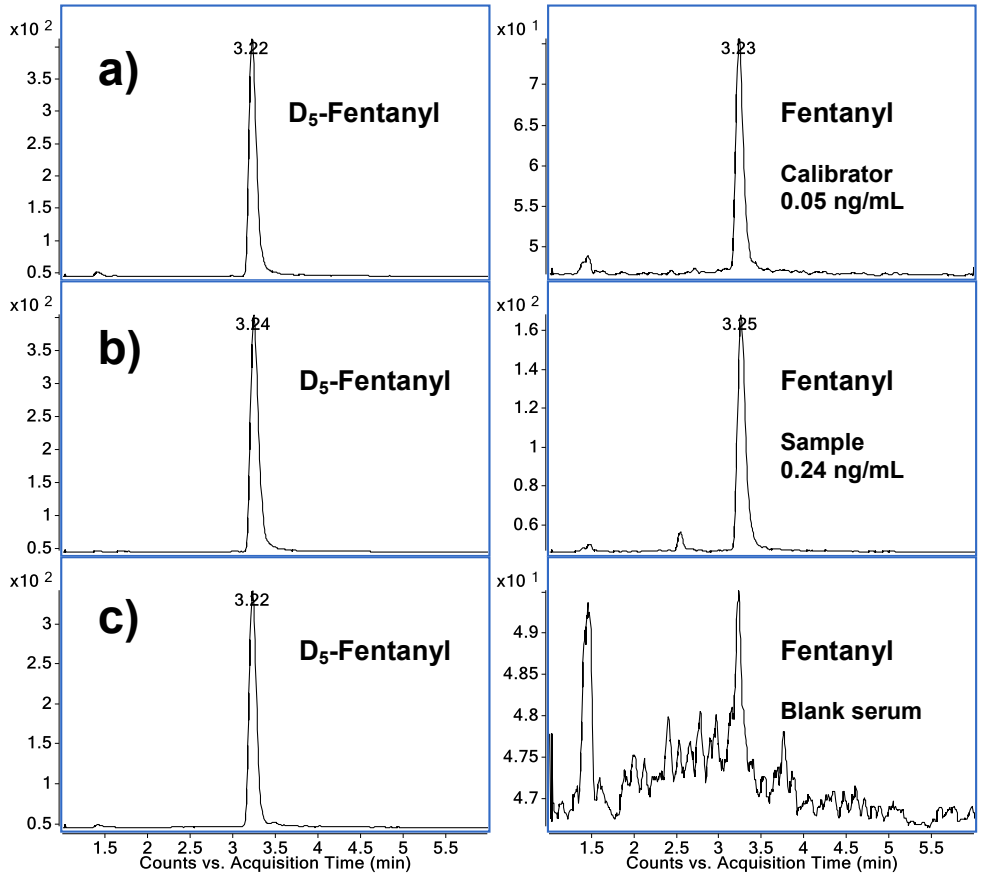

Figure 1: Multiple Reation Monitoring (MRM) transitions for internal standard, D_5-fentanyl (m/z 342 → m/z 188) (*left column*) and fentanyl (m/z 337 → m/z 188) (*right column*) in a) lowest serum calibrator 0.05 ng/mL; b) patient sample (0.24 ng/mL); c) blank serum.

Calibration			
Linearity range		0.05 - 5 ng/mL	
Slope ± S.D. (*n*=5)		0.97633 ± 0.03495	
C.V.% (slope)		3.6	
r (mean ± S.D)		0.99984 ± 0.00016	
Intercept (mean ± S.D)		0.01256 ± 0.00458	
Limit of quantification (LOQ)		0.05 ng/mL	
Limit of detection (LOD)		0.003 ng/mL	
Intra-assay precision and accuracy (*n*=5)			
Conc. (ng/mL)	Measured mean (ng/mL) ± S.D.	C.V.(%)	Mean accuracy (%)
0.05	0.04902 ± 0.0024	4.9	98
0.10	0.09542 ± 0.0029	3.1	96
0.25	0.22957 ± 0.0052	2.2	92
1.0	0.88797 ± 0.0205	2.1	89
2.5	2.23212 ± 0.0379	1.5	89
Inter-assay precision and accuracy (*n*=5)			
Conc. (ng/mL)	Measured mean (ng/mL) ± S.D.	C.V.(%)	Mean accuracy (%)
0.10	0.10009 ± 0.00948	9.4	101
0.25	0.24932 ± 0.01437	5.8	100
1.0	0.93760 ± 0.04746	5.1	94
2.5	2.32050 ± 0.08518	3.7	93

Table 1: Method validation data.

ng/mL. Although excellent signal to noise ratios (> 65) were found for serum calibrators at 0.01 ng/mL, two factors were in favour of a raised LOQ: (1) decreasing accuracy values below 0.05 ng/mL; (2) content in blanks. Method performance was clearly more limited by autosampler carry-over (worst case 0.6 % peak area) than apparatus sensitivity, but within the validated concentration range, the effect on quantification values is minimal.

Pharmacokinetic study samples

The serum baseline concentrations from patients (*n*=4) after minimum 12 h transdermal application of a 25 µg/h Durogesic® patch are presented in Figure 2. All results were above LOQ. Consequently, the assay is applicable for pharmacokinetic studies of heating to facilitate systemic delivery of fentanyl from transdermal patches. With the maximum effect reported in the literature being a factor 3 increase in serum concentration [5], results are expected to be within the validated concentration range.

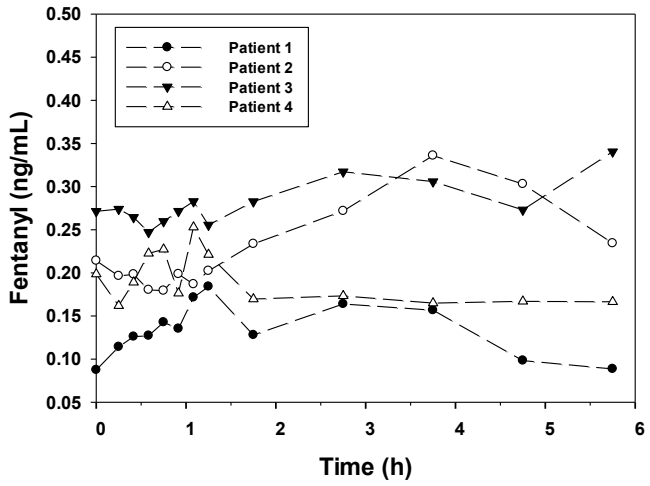

Figure 2: Serum concentrations-time profiles of fentanyl for patients (*n*=4) after minimum 12 h application of Durogesic® transdermal patches (25 µg/h).

Conclusion

The method presented complies with the criteria for biopharmaceutical and pharmacokinetic studies. The performance in terms of specificity, precision, and accuracy is comparable to other LCMS/MS methods. In this case the advantage is an easy sample preparation with no use of organic solvents, low sample volume and short time of analysis. The column-switching technique is a stable and robust technique which is simple to implement in a routine laboratory. In the future, the method will be used for pharmacokinetic studies investigating drug uptake of fentanyl delivered by different routes.

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