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Rapid and sensitive determination of fentanyl in dog plasma by on-line solid-phase extraction integrated with a hydrophilic column coupled to tandem mass spectrometry

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Abstract

We have developed and validated a method for the quantification of fentanyl, a synthetic opioid, in dog plasma by on-line SPE with a hydrophilic column coupled to tandem mass spectrometry in positive electrospray mode. A column-switching instrument with 10-port valve and two HPLC pumping systems were employed. Deuterated fentanyl served as the internal standard. A Waters Oasis HLB extraction column and a Waters Atlantis HILIC Silica analytical column in a column-switching set-up with gradient elution were utilized. Both fentanyl (analyte) and the internal standard (fentanyl-d5) were determined via multiple reaction monitoring (MRM) and the MS/MS ion transitions monitored were *m/z* 337.0/188.0 and 342.0/188.0, respectively. Each plasma sample was chromatographed within 5 min. The calibration curves were linear over a widely range of 0.01–50 ng/mL using weighted linear regression analysis ($1/x$). The low limit of quantitation was 0.01 ng/mL. The intra- and inter-day accuracy ranged from 102 to 112% and the overall precision was less than 3%. The recoveries ranged from 90 to 105% in plasma at the concentrations of 0.04, 0.4, 4 and 40 ng/mL. No influence of freeze/thaw and long-term stability were observed. This validated method has been successfully applied to analyze the dog plasma samples of a pharmacokinetics study.

Keywords: Fentanyl; On-line SPE; HILIC; LC/MS/MS; Bioanalysis; Pharmacokinetics

1. Introduction

Fentanyl, *N*-(1-phenethyl-4-piperidyl)propionanilide, is an opiate agonist used in medical procedures as narcotic analgesics. Due to the low plasma concentration of fentanyl after analgesic dose, the development of a sensitive assay, which enables the quantitation in the lower pg/mL range is required [1]. There were many analytical methods of fentanyl had been reported, such as LC/UV [2,3], GC/MS [4–8], immunoassay [9–11], LC/MS [12,13] and LC/MS/MS [14–17]. Among these methods, LC/UV and immunoassay methods show lack of sensitivity, low precision and tend to suffer from cross-interference. GC/MS methods offer enough sensitivity of the existing methods, but require long run times and derivative formation. LC/MS and LC/MS/MS methods offer high specificity and sensitivity. However, as a direct result of the short analysis time offered by LC/MS or LC/MS/MS, sample preparation has become the rate-limiting step for the overall analytical cycle. Several extraction methods had been applied prior to sample analysis, such as liquid–liquid extraction (LLE), protein precipitation (PP), and traditional solid-phase extraction (off-line SPE), which the samples are processed manually in a serial fashion. The drawbacks of these sample clean-up methods are time-consuming and labor intensive. Recently, Msrier et al. reported the automatic on-line SPE sample clean-up method, which offers more sensitive, time-efficient and easier to handle for the analysis of human plasma samples. However, this method needs a special set-up for high throughput and a narrow calibration curves range, which is not practical for general bioanalytical laboratories [18].

Reversed-phase chromatography coupled to LC/MS/MS is the common technique to perform drug analysis. However, due to the poor chromatographic retention, the quantitative determination of highly basic polar compounds in biological samples by LC/MS/MS using reversed-phase chromatography

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is often a challenge and problematic. Fortunately, the recent reported normal-phase high-performance liquid chromatography (HPLC) with silica-based columns and aqueous organic mobile phases becomes one of alternative method that can be applied to analyze polar compounds. There were many reports described the current advances in applying the silica columns with aqueous-polar organic mobile phases to improve LC conditions for the analysis of the polar compounds in biological fluids by LC/MS/MS [19–21]. Shou et al. proposed by using a hydrophilic interaction chromatography (HILIC) as the analytical separation mode following on-line extraction with a hydrophobic extraction column coupled to LC/MS/MS to illustrate good peak shapes of chromatographs and better sensitivity than using the traditional reversed-phase C_{18} column [22]. Naidong and Eerkes also reported using the same technique to improve the LC conditions [23].

The objective of this study was to develop a fast and sensitive on-line SPE hydrophilic chromatography LC/MS/MS assay (on-line SPE HILIC LC/MS/MS) for bioanalysis of fentanyl in biological fluids. To our knowledge, there are no published methods for the determination of fentanyl in dog plasma by using this technique. The method has been applied to a pharmacokinetic study by comparing two fentanyl transdermal patches in dogs.

2. Experimental

2.1. Chemicals and reagents

Primary standard solutions with a concentration of 1 mg/mL of fentanyl and the internal standard (IS), fentanyl-d5, were obtained from Janssen-Cilag (Neuss, Germany). Ammonium acetate, formic acid, HPLC grade acetonitrile and methanol were purchased from Sigma (St. Louis, MO, USA). The deionized water was purified by the Milli-Q water system (Millipore, Bedford, MS, USA).

2.2. Preparation of standard and quality control samples

The primary stock solution (1 mg/mL) of fentanyl and fentanyl-d5 were stored at −80 °C until used. The secondary working stock solutions were prepared by diluting with acetonitrile from primary stock solution. Calibration standards were prepared by serial dilution with blank dog plasma yielding final concentrations of 0.01, 0.025, 0.05, 0.1, 0.5, 1, 5, 10, 25 and 50 ng/mL. Because of wide linear range of calibration curve, the quality control (QC) samples were prepared by spiking blank dog plasma with independently prepared fentanyl standard solution to give concentrations of 0.04, 0.4, 4 and 40 ng/mL. The working internal standard solutions were prepared by diluting the primary IS stock solution with acetonitrile giving a concentration of 15 ng/mL.

2.3. On-line solid-phase extraction (SPE) and chromatography

A simple protein-precipitation step for sample preparation prior to on-line SPE HILIC LC/MS/MS allows to extend the life of the SPE extraction column and to ensure quantitative suspension of protein binding. Aliquots of 100 μL of dog plasma samples (blank plasma, calibration standards, QC samples and pharmacokinetic plasma samples) were mixed with 100 μL acetonitrile containing 15 ng/mL of fentanyl-d5 (IS). The mixture was vortexed for 30 s and then centrifuged at 21,000 × *g* for 20 min in an Eppendorff Model 5417c centrifuge at room temperature. The supernatant was transferred to a clean tube and then a volume of 100 μL of the supernatant was injected onto on-line SPE HILIC LC/MS/MS. In the first step, The washing solvent, 5% methanol, was delivered by an Agilent Binary pump (Palo Alto, CA, USA) through the sample loop onto the Oasis HLB extraction column (25 μm, 1.0 mm × 50 mm; Waters, Milford, USA) for 1.5 min at a flow rate of 4.0 mL/min. Endogenous components were washed to waste after loading of the samples onto the extraction column. At 1.6 min, the valve was switched; the retained analytes from the extraction columns was flushed to the analytical chromatography with the eluting mobile phase (acetonitrile) for separation and quantification. The following three sequential isocratic steps are applied for the analytical column (Waters Atlantis HILIC Silica, 5 μm, 4.6 mm × 150 mm): 0–1.5 min: 100% solvent B (acetonitrile); 1.6–4.0 min: 30% A (10 mM NH_4OAc containing 0.1% formic acid) to 70% B; 4.1–5.0 min: 100% solvent B. The flow rate was 0.6 mL/min. During analytical chromatography, the extraction column was washed with acetonitrile at a low rate of 4.0 mL/min for 2.0 min and subsequently was re-equilibrated with 5% methanol. The total run time was 5.0 min.

2.4. Mass spectrometric conditions

AB Sciex API 3000 tandem mass spectrometer equipped with an ESI in the positive ion mode (Applied Biosystems, Foster City, CA, USA) was applied. The ion source parameters were as below: the electrospray needle was maintained at 2.0 kV and heated-capillary temperature was set at 450 °C. The auxiliary gas flow was 8.0 L/min. The nebulizing gas, curtain gas and collision gas flows were at instrument settings of 14, 6 and 12, respectively. The declustering potential (DP), entrance potential (EP) and focusing potential (FP) were at 54, 11 and 270 V, respectively. Data acquisition was via multiple reaction monitoring (MRM). Ions representing the $[M + H]^+$ species for both the analyte and IS were selected in MS1 and collisionally dissociated with nitrogen gas to form specific product ions which were subsequently monitored by MS2. The collision energy was 32 eV for the analyte and IS. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity at unit resolution. The ions monitored for fentanyl and fentanyl-d5 (IS) were *m/z* 337.0/188.0 and *m/z* 342.0/188.0, respectively.

2.5. Method validation

2.5.1. Linearity of calibration curves

Calibration curves were constructed from working standard solutions of fentanyl at concentrations range 0.01–50 ng/mL by plotting peak area ratio (*y*) of fentanyl to the internal standard, fentanyl-d5, versus fentanyl concentration (*x*). The samples were

prepared in five replicates. The regression parameters of slope, intercept and correlation coefficient were calculated by weight ($1/x$) linear regression in Analyst 1.3 software used in Applied Biosystems API 3000.

2.5.2. Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of this analytical method were evaluated using QC samples. The solution of a certain concentration was injected onto on-line SPE HILIC LC/MS/MS for quantitative determination five times a day to evaluate intra-day precision. The same procedure was performed once a day for five consecutive days to determine inter-day precision. Accuracy was determined by comparing the calculated concentration using calibration curves to nominal concentration.

2.5.3. Recovery test and matrix effect

The concentration-dependent matrix effect of fentanyl was examined by evaluation of the recovery which by comparing the peak areas obtained from five extracted samples spiked with known amounts of standards with those obtained from the pure compounds of the same concentrations in the solvent. The concentration of fentanyl-d5 was 15 ng/mL. The recovery studies were assessed at least five replicates at four concentration levels (0.04, 0.4, 4 and 40 ng/mL).

2.5.4. Stability

To evaluate sample stability after three freeze/thaw cycles and room temperature, five replicates of QC samples at concentrations of 0.04, 0.4, 4 and 40 ng/mL were subjected to three freeze/thaw cycles or were stored at room temperature for 6 h before sample processing. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

2.6. Pharmacokinetic study

The validated method was applied to evaluate the pharmacokinetics of Durogesic® and a novel National Bureau of Controlled Drugs (NBCD) fentanyl transdermal patches to beagle dogs. The animal study including dose administration and blood sample collection was performed by the Development Center for Biotechnology (DCB), Taipei, Taiwan. Three male adult beagle dogs weighing 10–12 kg were used in this study. Animals were surgically implanted of catheters before dosing. An angiocatheter was inserted in the cephalic vein for administration of anesthetics, and a second angiocatheter was placed in the right jugular vein for blood sampling. Dogs were fed a commercial dry dog food during the study. Water was available *ad libitum*.

A patch with 2.5 mg fentanyl per patch (25 μg/h) was dermal applied to each dog on the clipped site for 72 h. After 72 h, the patch was removed. Serial blood samples were collected from each dog according to the schedule described below: 0 (prior to patch application), 2, 4, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h after patch application and at 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h after patch removal. A blood sample (2 mL) was collected from each animal via the jugular vein catheter of each animal and stored in ice (0–4 °C). Heparin was used as the anticoagulant. Immediately after collecting the blood sample of each time point, the jugular-vein catheter was flushed with 2 mL of physiological saline (containing 30 units of heparin per mL). After a 2-week washout period, the same dogs were each applied with a NBCD fentanyl transdermal patch and the blood samples were collected according to the same schedule of Durogesic® patch treatment. Plasma was separated from the blood by centrifugation (3000 rpm for 15 min at 4 °C in a Beckman Model Allegra™ 6R centrifuge) and stored in a freezer (−70 °C). Control plasma was obtained from separate dogs receiving no drug. All plasma samples were stored in a freezer prior to transferring to analysis of the parent drug by on-line SPE HILIC LC/MS/MS.

Plasma concentrations after treatment with the fentanyl transdermal patch were analyzed by non-compartmental model. The partial area under the plasma fentanyl concentration versus time curve (AUC) (from the first measured time point to the last, 0 h to 96 h), the total AUC (from the first measured time to infinity, 0 h to infinity), the mean plasma concentration at steady-state (calculated by averaging the mean plasma concentration for each dog during the 24–72-h period), the apparent elimination rate constant (Kel) (determined by performing linear regression on the terminal portion of the natural log concentration versus time curve after patch removal), the half-life for elimination of fentanyl after removal of the patch will be estimated from the apparent elimination rate were calculated using WinNonLin software program (version 3.1, Pharsight, CA, USA).

3. Results and discussion

3.1. Development of the on-line SPE procedures

Fentanyl is a basic drug (pK_a: 8.4), which makes it particularity suitable for extraction by using cation-exchange cartridges. Unfortunately, the use of these cartridges is not suitable for the on-line SPE HILIC LC/MS/MS because of the damage of silica-based column which caused by elution high pH solvent, e.g. 5% NH_4OH in methanol. The problem was resolved by using the mixed-mode (hydrophilic and lipophilic) solid-phase extraction column (e.g. Oasis HLB). To extend the life of the SPE extraction column, a simple protein precipitation with acetonitrile was applied prior to injection. After the sample was loaded on to the SPE column, a wash by 5% methanol was used to remove water-soluble interferences and to retain the analyte and the internal standard on the extraction column. Then, the compound was eluted by organic solvent (acetonitrile) and flowed onto the analytical column. There was no blockage in the extraction column or significant loss of extraction performance observed, indicating this on-line procedure was acceptable.

3.2. Mass spectrometry

The MS/MS parameters were optimized to the maximum response for the fentanyl and fentanyl-d5 in positive ion mode. Under electrospray ionization (ESI) condition, proto-

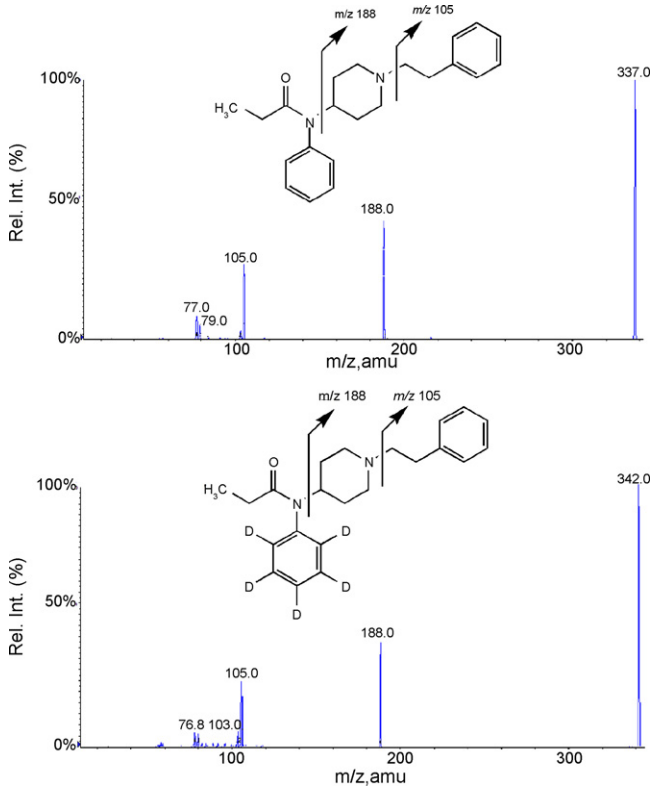

Fig. 1. Product ion mass spectra of $[M+H]^+$. (A) Fentanyl ($[M+H]^+$, *m/z* 337.0) and (B) fentanyl-d5 ($[M+H]^+$, *m/z* 342.0).

nated molecular ions $[M+H]^+$ were the major peaks. Mass spectrums of fentanyl $[M+H]^+$ *m/z* 337.0 and the internal standard fentanyl-d5 $[M+H]^+$ *m/z* 342.0 are shown in Fig. 1. The most intense fragment ion of fentanyl at *m/z* 188.0 is suggested to originate from a charge-driven elimination of *N*-phenyl-propionimidic acid including a transannular hydrogen transfer [16]. Therefore, the MS/MS transition 337.0/188.0 and 342.0/188.0 for fentanyl and fentanyl-d5 were selected since the ion scan product presented a higher abundance.

3.3. Chromatography

One of the advantages by using LC/MS/MS was to quantify the analyte and the internal standard at short retention times even though without complete separation. The gradient program was adjusted to the best condition, which the eluting analyte was observed approximately at 4 min. The total run time was 5 min. Under optimized LC conditions, the chromatographic retention times of fentanyl and fentanyl-d5 were both 3.6 min and showed symmetrical good peak shape (Fig. 2). There was no significant interference from endogenous compounds observed at the retention times of the analytes.

3.4. Method validation

3.4.1. Linearity of calibration curves

Table 1 summarizes the summary of the individual standard data obtained in the five replicates. These calibration

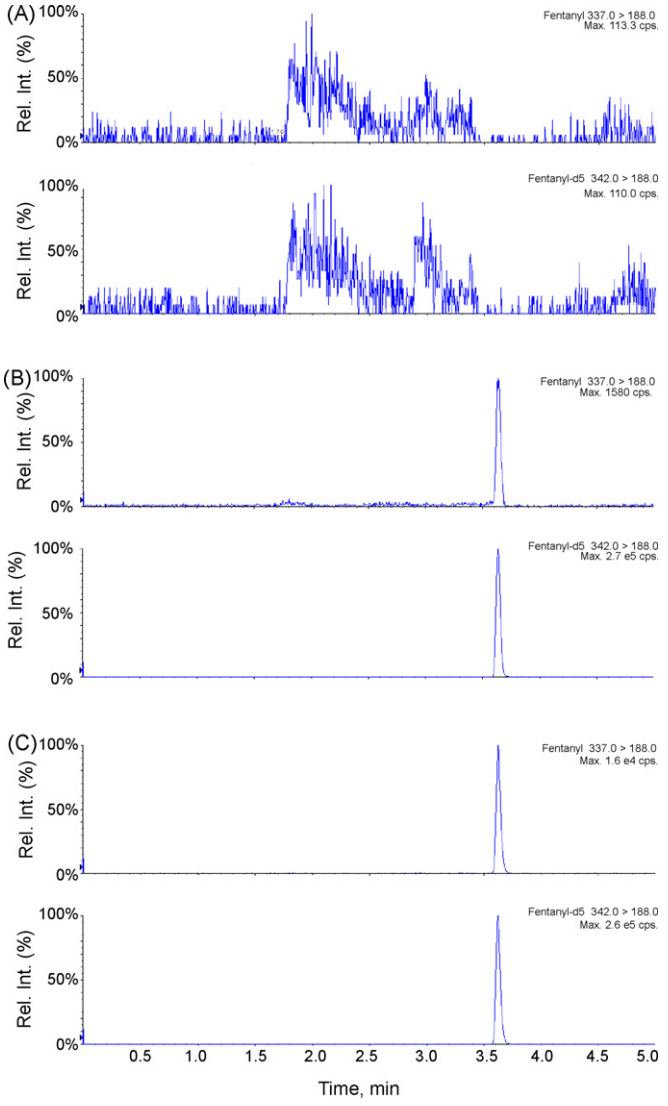

Fig. 2. Representative MRM chromatograms of fentanyl. (A) Blank dog plasma. (B) Blank dog plasma spiked with fentanyl (0.01 ng/mL) and internal standard (15 ng/mL). (C) Dog plasma sample 4 h after removing the transdermal fentanyl patch.

curves were found to be linear within the concentration range of 0.01–50 ng/mL in dog plasma with correlation coefficient ($r > 0.99$) when evaluated by weighted ($1/x$) linear regression. The mean slope of the calibration curves used in the method validation is 0.558, with %CV of 3. The typical calibration

Table 1
Summary of calibration curves ($n = 5$)

Curve no.	Slope	Intercept	*r*
1	0.547	0.00159	0.9966
2	0.548	0.00160	0.9967
3	0.576	0.00149	0.9990
4	0.572	0.00138	0.9992
5	0.547	0.00165	0.9968
Mean	0.558	0.00154	0.9977
S.D.	0.015	0.00011	0.0013
%CV	3	7	0

curve is $Y = 0.558X + 0.00154$. The result indicated good linear relationships between the peak areas and concentrations.

3.4.2. Limit of quantification (LOQ) and detection (LOD)

The limit of quantitation (LOQ) and the limit of detection (LOD) were 0.010 and 0.003 ng/mL, respectively. At the LOQ, the R.S.D. ($n = 5$) of the measured concentration was 0% and the accuracy was 109%. Representative chromatograms from a LOQ calibration sample (0.01 ng/mL) are shown in Fig. 2. Previous reports by Shou et al. [22], Naidong et al. [17], and Day et al. [14] demonstrated that their analytical methods has a LOQ of 0.05 ng/mL. In addition, Lennernas et al. [15] improved the LOQ to 0.02 ng/mL. Our analytical method with regard to the limit of quantification was 0.01 ng/mL. Compared to these previously published methods, our analytical method is highly sensitive. Although an assay method based on automated SPE LC/MS/MS has been reported by Marier et al., its linear range of the standard curve is narrow (0.01–4 ng/mL) [18]. On the other hand, our calibration curves were linear over three orders of magnitude (0.01–50 ng/mL) indicating the high reliability of the whole analytical process for various conditions, such as variety of different doses of fentanyl delivered among individuals.

3.4.3. Accuracy and precision

The accuracy and precision of intra- and inter-day assay data for QC samples are summarized in Table 2. Accuracy was the percentage of the concentration found compared with the theoretical target concentration. Precision was based on calculation of the R.S.D. The intra-day accuracy ranged from 102 to 112% throughout the four concentrations. The precision of fentanyl for the four concentrations examined was 1, 3, 3 and 1%, respectively. The inter-day accuracy and precision was studied over 5 days. The inter-day accuracy ranged from 102 to 110% and the inter-day precision ranged form 1 to 3% across the four concentrations examined.

3.4.4. Recovery and matrix effect

In on-line SPE HILIC LC/MS/MS assays, it is challenging to directly determine the matrix effect. We monitored the concentration-dependent matrix effect for this assay. The recovery of fentanyl was calculated by comparing the peak area ratios of fentanyl and internal standard in the extracted plasma samples with that of plasma-free standards prepared in pure solvent. The recovery of fentanyl from dog plasma was 90, 93, 95 and 105% at concentrations of 0.04, 0.04, 4 and 40 ng/mL, respectively. These observations indicate that no endogenous substances significantly affect the ionization of fentanyl suggesting through the rigorous washing procedure no matrix effect was observed.

3.4.5. Stability

The stability of fentanyl in dog plasma under different storage conditions (at room temperature and −70 °C) is summarized in Table 3. There was no significant degradation occurred at ambient temperature for 6 h and during three freeze/thaw cycles for fentanyl dog plasma samples because of the concentrations deviated by no more than 3% relative to the reference nominal

Table 2
Accuracy and precision of fentanyl in dog plasma

Nominal concentration (ng/mL)	Intra-day precision ($n = 5$)				Inter-day precision ($n = 5$)			
	0.04	0.4	4	40	0.04	0.4	4	40
Measured concentration (ng/mL)	0.041 ± 0.000	0.427 ± 0.011	4.483 ± 0.133	41.867 ± 0.208	0.041 ± 0.000	0.421 ± 0.010	4.420 ± 0.123	41.267 ± 1.115
Accuracy (%)	102 ± 1	107 ± 3	112 ± 4	104 ± 1	102 ± 1	105 ± 3	110 ± 3	103 ± 3
R.S.D.	1	3	3	1	1	2	3	3

Table 3
Stability of fentanyl in dog plasma ($n = 5$)

Measured concentration (ng/mL)	Spiked concentration (ng/mL)			
	0.04	0.4	4	40
Three freeze/thaw cycle stability (−70 °C)				
Mean	0.041 ± 0.000	0.426 ± 0.010	4.476 ± 0.116	41.540 ± 0.879
R.S.D. (%)	1	2	3	2
Post-preparative stability (6 h at room temperature)				
Mean	0.041 ± 0.000	0.424 ± 0.011	4.455 ± 0.122	41.400 ± 0.949
R.S.D. (%)	1	3	3	2

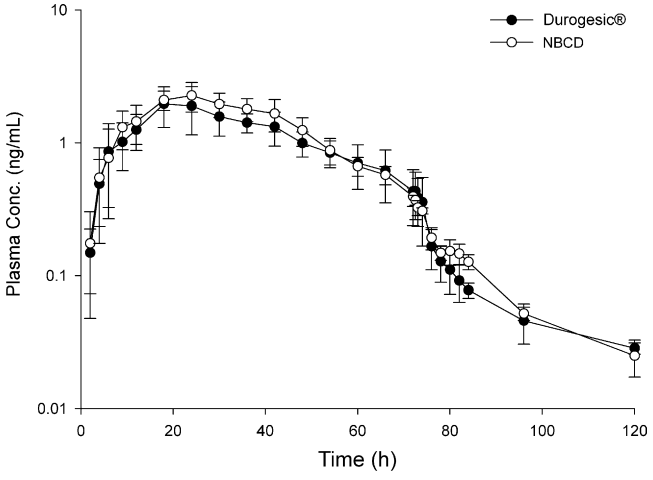

Fig. 3. Mean plasma concentration–time profiles of fentanyl after applying with Durogesic® and National Bureau of Controlled Drugs (NBCD) fentanyl transdermal patches to beagle dogs.

concentrations. These results indicated that fentanyl was stable under routine laboratory conditions.

3.5. Pharmacokinetic study

The developed and validated method was applied to a pharmacokinetic study of two different fentanyl transdermal patches in dogs. The plasma concentration versus time curves are shown in Fig. 3 and the mean values of pharmacokinetic parameters are depicted in Table 4. Both fentanyl transdermal patches showed similar PK profiles in dogs. After 2.5 mg fentanyl per patch (25 μg/h) was dermal applied to each dog on the clipped site for 72 h, the concentration of the parent drug in plasma could be measured up to 120 h after applying both patch. At that time it averaged 0.028 ± 0.003 ng/mL for Durogesic® fentanyl transdermal patch and 0.025 ± 0.008 ng/mL for NBCD fentanyl transdermal patch. The mean T_{max} of both fentanyl transdermal patches were about 20.0 h. The overall systemic drug exposure (AUC) and the maximal plasma concentrations (C_{max}) appear to be similar. The Kel was 0.042 ± 0.021 h^{-1} for Durogesic® fentanyl transdermal patch and 0.047 ± 0.012 h^{-1} for NBCD fentanyl transdermal patch. There were no significant differences between these two formulations.

Table 4
Pharmacokinetic parameters of fentanyl after applying with Durogesic® and National Bureau of Controlled Drugs (NBCD) transdermal patches to dogs

Parameter	Unit	Durogesic®	NBCD
Dose	mg/patch	2.5	2.5
$AUC_{(0-t)}$	ng/mL h	84.99 ± 26.06	107.16 ± 33.60
$AUC_{(0.inf)}$	ng/mL h	85.78 ± 26.28	107.76 ± 33.59
$t_{1/2}$	h	19.1 ± 7.5	15.6 ± 4.7
C_{max}	ng/mL	2.06 ± 0.66	4.00 ± 3.44
T_{max}	h	20.0 ± 3.5	20.0 ± 9.2
Kel	1/h	0.042 ± 0.021	0.047 ± 0.012

4. Conclusion

This on-line SPE HILIC LC/MS/MS method is rapid and sensitive for quantification of fentanyl in dog plasma. The method fulfilled all bioanalysis validation criteria. Compared with the previous reports, our method features are more sensitive and have a wider linear range (0.01–50 ng/mL). It is expected that the method would be efficient in analyzing biological samples obtained for pharmacokinetics and bioequivalence studies.

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