



Short communication

HPLC-UV method development for fentanyl determination in rat plasma and its application to elucidate pharmacokinetic behavior after *i.p.* administration to ratsAhmed A. Almousa^a, Rie Ikeda^a, Mitsuhiro Wada^a, Naotoka Kuroda^a, Ruri-Kikura Hanajiri^b, Kenichiro Nakashima^{a,*}^a Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan^b National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

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ABSTRACT

A simple, rapid and validated high performance liquid chromatography method with UV detection for the quantification of an opioid agonist, fentanyl (FEN), in rat plasma was developed. The assay procedure involved chromatographic separation using a ZIC-HILIC SeQUANT column (250 mm × 4.6 mm, i.d., 5 μm) and a mobile phase of acetonitrile and acetate buffer (pH 3.4, 20 mM) of ratio (=65:35, v/v) at a flow rate of 1.2 mL/min and detection wavelength of 201 nm. Plasma sample (100 μL) pretreatment was based on simple deproteinization by acetonitrile spiked with clonidine as an internal standard (I.S.) of 20 ng/mL followed by extraction with *tert*-butyl methyl ether and centrifugation. The organic layer was evaporated under N₂ gas and reconstituted with 100 μL of acetate buffer (pH 3.4, 20 mM), and 50-μL portions of reconstituted sample were injected onto the column. Sample analysis including sample pretreatment was achieved within 35 min. Calibration curve was linear ($r \geq 0.998$) from 5 to 100 ng/mL. Both intra- and inter-day assay precisions that are presented through RSD were lower than 12.6% for intra-day and lower than 12.0% for inter-day assessment. Limit of detection was 0.8 ng/mL at S/N of 3. This method was omitting the use of expensive solid phase extraction and time consuming liquid extraction procedures. Moreover, the present method was successfully applied to study pharmacokinetic parameters of FEN after intraperitoneal administration to male Wistar rat. Pharmacokinetic parameters estimated by using moment analysis were $T_{1/2}$ 198.3 ± 44.7 min, T_{max} 28.3 ± 2.9 min and AUC_{0-180} 15.6 ± 2.9 (× 10²) ng min/mL.

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1. Introduction

Fentanyl [*N*-phenyl-*N*-(1-(2-phenylethyl)piperidyl)propanamide, FEN] is a potent synthetic μ-opioid agonist that is commonly used for anesthesia, analgesia for post-operative surgical procedure and chronic pain management mainly for cancer patients. FEN in its various dosage forms has been illicitly used either alone [1–3] or in combination with other drugs including antidepressants, opioids, benzodiazepines, and amphetamines [3,4].

Due to variation of FEN use, misuse and its high potency; several studies have been reported for its detection in biological samples and different body organs using various separation and detection techniques including high-performance liquid chromatography (HPLC) coupled to tandem mass spectroscopy [5], and some other HPLC-UV methods [6–8]. These methods included different types of extraction procedures such as liquid–liquid extraction

(LLE) utilizing hexane to extract target analytes [6,7], solid phase extraction (SPE) [9] and single-drop liquid–liquid–liquid micro-extraction (LLME) [10]. All these reported methods were developed with attention to sensitivity, method applicability and reproducibility.

This work was conducted to develop and validate a method that is convenient, simple and rapid for FEN determination in rat plasma, coupled to pharmacokinetic study after single intraperitoneal (*i.p.*) administration of FEN 0.1 mg/kg to male Wistar rat.

This method is characterized by the usage of 100 μL of plasma, and the simplicity of not using any derivatization reaction or pre-extraction procedure. Moreover, it is uniquely coupled to pharmacokinetic analysis of FEN in male Wistar rats.

2. Experimental

2.1. Reagents and standards

FEN citrate stock (from Daiichi Sankyo Co. Ltd., Tokyo) was kindly gifted by the National Institute of Health Sciences, Ministry

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of Health and Welfare (Japan). Clonidine-HCl was used as the internal standard (I.S.) of 20 ng/mL. Sodium carbonate, sodium hydrogen carbonate, sodium acetate, acetonitrile were obtained from Wako Pure Chemicals (Osaka, Japan). Acetic acid was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). *tert*-Butyl methyl ether (*t*-BME) was obtained from Sigma–Aldrich, Inc. (MO, USA). Water for buffers was passed through a pure line WL21P (Yamato Science, Tokyo). Nitrogen gas of 99.999% purity was provided by Daiichisanjo (Nagasaki, Japan).

Stock solutions (50 µg/mL) of FEN and I.S. were subsequently diluted to prepare working standards with acetonitrile. Standards of calibration curves were prepared by pipetting suitable amounts of FEN and I.S. (20 ng/mL) and spiking them into rat plasma, and all stock solutions were kept at 4 °C.

2.2. Chromatographic apparatus and conditions

The HPLC system consisted of a Shimadzu LC-10ATvp liquid chromatographic pump (Shimadzu, Kyoto, Japan), a ZIC-HILIC SeQUANT column (250 mm × 4.6 mm i.d.; 5 µm, Merck, Darmstadt, Germany), a Shimadzu SPD-10A utilized as a detector at a wavelength of 201 nm and a 7125 injector with 50-µL sample loop (Rheodyne, CA, USA). The mobile phase was a mixture of acetonitrile and sodium acetate buffer (pH 3.4, 20 mM) with a ratio of (=65:35, v/v). The mobile phase was degassed and pumped into the column at a flow rate of 1.2 mL/min.

2.3. Plasma collection

Male Wistar rat's plasma was used in the experiments (Kyudo Experimental Animal, Saga, Japan). The rats were housed in the conditions of constant temperature (24 ± 1 °C), provided with standard laboratory food (Oriental Yeast, Tokyo) and water ad libitum. All animal procedures and care in this experiment were permitted by Nagasaki University Animal Care Use Committee. The rats were anesthetized with ethyl carbamate (1.5 g/kg, *i.p.*). After operating on rats and inserting the cannula into the femoral artery, rats were stabilized for 1 h and then blood samples were collected into EDTA containing eppendorf tubes. Samples were centrifuged at 2000 g for 10 min at 20 °C, the obtained plasma samples were kept at –30 °C prior to use.

2.4. Plasma pretreatment

To 100 µL of rat plasma 10 µL of I.S. (final concentration is 20 ng/mL in plasma) was spiked and vortex-mixed for 20 s. To this solution, 40 µL of sodium carbonate buffer (pH 11.2, 100 mM) were added and vortex-mixed for 1 min. Followed by the addition of 500 µL *t*-BME, which was vortex-mixed for 1 min and then centrifuged at 2000 g for 20 min at 10 °C, 400 µL organic layer was transferred into a test tube. Collected organic layer extract was evaporated to dryness under N₂ gas, the residue was reconstituted with 100 µL sodium acetate buffer (pH 3.4, 20 mM), and 50 µL of reconstituted volume were injected onto the column.

2.5. Validation of the method

Calibration curves were prepared using plasma samples spiked with known concentrations of FEN (final concentrations in plasma were 10, 20, 50, 100 ng/mL); triplicate for each concentration was used. The limits of detection (LOD) and quantitation (LOQ) were calculated as the peak height at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Intra- and inter-day assay precision, accuracy and recovery were assessed using rat plasma spiked with 10, 50 and 100 ng/mL of FEN and 20 ng/mL of I.S. were used, five replicate measurements for each standard concentration were performed.

The accuracy was calculated as found concentration/nominal concentration, while the recovery was calculated as peak height ratio of FEN to I.S. in plasma/peak height ratio of standard FEN to standard I.S.

2.6. Application to pharmacokinetic study on rat

An *in vivo* pharmacokinetic study was performed in male Wistar rat ($n = 3$, weight 295–320 and 7–8 weeks old). Blood sampling after administration of FEN (0.1 mg/kg, *i.p.*) was held at 0, 10, 15, 20, 25, 30, 40, 90, 120 and 180 min, the used dose of FEN is consistent with previously reported method for rat treatment [11,12]. Blood was collected as previously explained, followed by spiking of 10 µL I.S. solution to each plasma sample, and pretreated as mentioned earlier. The concentrations of FEN in rat plasma were calculated from the corresponding calibration curve. The peak concentration (C_{max}) and concentration peak time (T_{max}) were obtained directly from the original data. The elimination half-life ($T_{1/2}$) was calculated using the equation $0.693/k$ (k = rate constant), also clearance (CL) was calculated as the dose/area under the curve for concentration versus (vs.) time (AUC_{0-180}). Other parameters such as AUC_{0-180} , and the mean residence time (MRT_{0-180}) were calculated by implicating moment analysis model.

3. Results and discussion

This HPLC-UV method for FEN determination in rat plasma represents considerably simple and rapid bioanalytical technique. Moreover, among many of analytical techniques, HPLC-UV is the most abundant technique and the least costly, especially when combined to simple liquid extraction unlike SPE [9]. Portier et al. [7] reported a method utilizing simple LLE, using 1 mL of human plasma and an injection volume of 100 µL, while this method is using a small plasma volume that equals to 100 µL and injection volume of 50 µL. LLME that was reported previously is requiring pre-extraction and back-extraction method combined to the tedious micro-droplet acceptor phase, and takes 50 min for extraction procedure only [10], whereas this method using simple liquid extraction makes it the handiest method to perform, that takes 35 min to obtain the final peak on the chromatogram. With these qualities, this method can be used for FEN pharmacokinetic assessment in rats, FEN drug interactions studies and will give possible use for forensic purposes after slight modifications.

3.1. Chromatography

During the development of the method, various mobile phases were assessed. Eventually, the ratio of acetonitrile and sodium acetate buffer (pH 3.4, 20 mM) (=35:65, v/v) provided the optimal separation of FEN and I.S. with retention times of 6.6 and 8.0 min, respectively. Fig. 1 represents typical chromatograms obtained from (A) plasma free drug and (B) that is spiked with FEN 50 ng/mL and I.S. 20 ng/mL. There were no interferences from the blank plasma. Also for FEN treated rats, no interfering peaks were detected. The implementation of 201 nm as detection wavelength was considered after practically achieving better sensitivity. Additionally, the UV spectrum of FEN in mobile phase showed two maximum absorption peaks at 210 nm and 258 nm, these results are consistent with the previously reported methods that are using detection wavelengths ranging from 200 to 210 nm [6–8].

Upon the usage of a ZIC-HILIC column with its unique zwitterionic functional group, a clear relationship between the ionization capacities of FEN and examined internal standards to their corresponding retention times was established. Additionally, the column ability to retain FEN and I.S. for short time made it favourable to utilize for this method. The usage of *t*-BME overcame the presence of

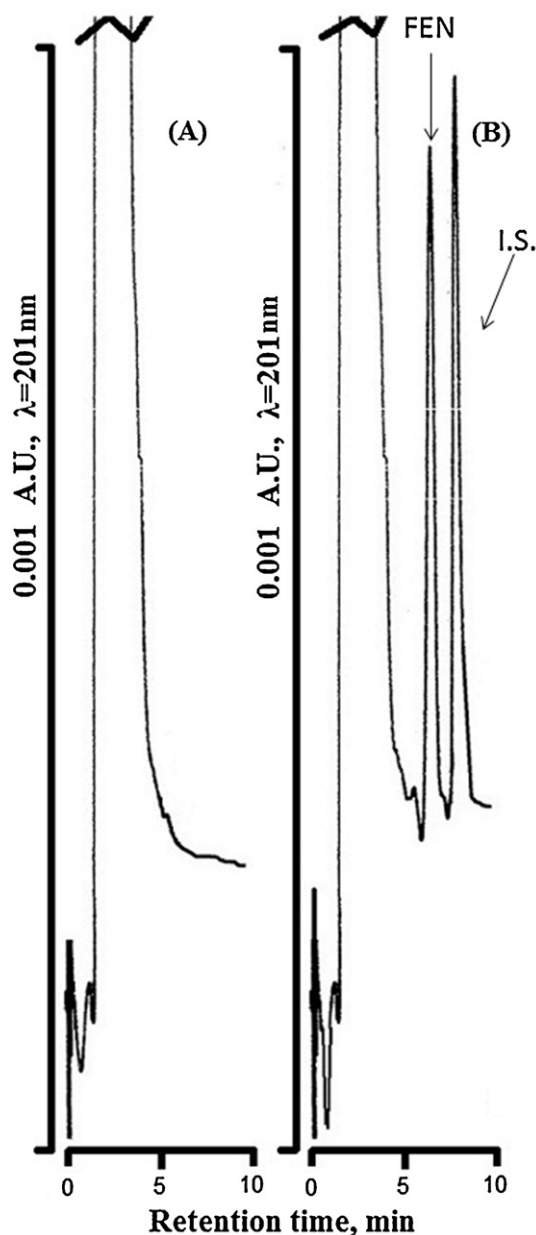


Fig. 1. Chromatograms of (A) plasma and (B) spiked plasma with FEN (50 ng/mL) and I.S. (20 ng/mL).

early peaks that were appearing when hexane or dichloromethane was examined; this helped us to obtain a clear chromatogram as shown in Fig. 1.

3.2. Stability of FEN in blood sample

The stability of FEN at -30°C was examined using rat plasma containing 20 ng/mL of FEN; four cycles of freeze–thaw were

utilized. Furthermore, the stability of FEN for a long period freezing was studied over a period of three months. The results showed FEN concentrations for freeze–thaw and freezing cycles were 18.5 ± 1.6 and 17.8 ± 1.5 ng/mL (mean \pm SD, $n = 3$), respectively.

3.3. Method validation

The calibration curve in rat plasma spiked with FEN and I.S. was obtained by plotting the peak height ratio of FEN to I.S. of 20 ng/mL vs. the concentration in ng/mL, with a replicate of three times. The calibration curve was linear in the range of 5–100 ng/mL with a correlation coefficient of ($r = 0.998$). LOD and LOQ at S/N ratio of 3 and 10 were 0.8 and 3.0 ng/mL, respectively. Parameters of calibration curve for the proposed method are summarized in Table 1.

Intra-day and inter-day precisions of the proposed method were evaluated by analyzing five replicates of plasma samples spiked with known concentrations of FEN (10, 50 and 100 ng/mL) and I.S. (20 ng/mL). The intra-day assay precision (relative standard deviation, RSDs) were 12.0 (10 ng/mL), 6.3 (50 ng/mL) and 3.9% (100 ng/mL). Inter-day assay precision (RSDs) were 12.6% (10 ng/mL), 7.3% (50 ng/mL) and 2.3% (100 ng/mL). The accuracies were ranging from 92 to 101%, and recoveries ranging from 92 to 96% (Table 2). The guidelines for the international conference of harmonization (ICH) were utilized for the validation of this method [13].

3.4. Pharmacokinetics of FEN in rats

The proposed method was applied on male Wistar rats to investigate pharmacokinetic behavior after *i.p.* administration of FEN. Several sampling schedules were assessed till we achieved the most appropriate duration of detection. A dose of 0.1 mg/kg was used for treatment though it represented relatively higher plasma concentration than that of therapeutic in human, but same time it satisfied the proposed goal of pharmacokinetic determination. The obtained pharmacokinetic parameters are summarized in Table 3.

The concentration of FEN in rat plasma was monitored after *i.p.* administration (Fig. 2). Time to achieve maximum concentration in plasma was 28.3 ± 2.9 min (T_{max}), the maximum peak concentration (C_{max}) was 21.3 ± 4.9 ng/mL, the elimination half-life ($T_{1/2}$) was 198.3 ± 44.7 min, area under the curve for concentration vs. time (AUC_{0-180}) was $15.6 \pm 2.9 \times 10^2$ ng min/mL, the mean residence time (MRT_{0-180}) was 75.3 ± 8.7 min and CL was 19.4 ± 2.6 mL/min.

The amount of data discussing FEN pharmacokinetics after *i.p.* administration is few. An IV administration of 200 μg FEN was conducted on humans, this study reported the pharmacokinetic parameters as follows: (C_{max}) of 1.46 ± 0.66 ng/mL, median (T_{max}) of 0.17 h and ($T_{1/2}$) of 18.03 ± 10.08 h [14]. This data shows a widely different change compared to rat, which can be ascribed to many factors; first: the different rout of administration and small blood volume of rat compared to human, second: amount of fatty tissue that enhance the distribution of FEN as it is considered to be highly lipophilic drug. Other reported methods for FEN determination are using animals that are difficult to handle and operate, these methods are assessing the pharmacokinetic parameters after subcutaneous administration of FEN in Greyhounds and horses [15,16].

Table 1
Calibration range and limits of detection and quantitation of FEN in spiked rat plasma.

Calibration range, ng/mL	r^a	Regression equation ^{b,c}	LOD ^d , ng/mL	LOQ ^d , ng/mL
5–100	0.998	$y = (0.02 \pm 0.001)x + (0.043 \pm 0.007)$	0.8	3.0

^a Correlation coefficient.

^b y = peak height ratio of FEN to I.S.; x = sample concentration, ng/mL.

^c Slope and y -intercept were expressed as mean \pm SD ($n = 3$).

^d Limits of detection and quantitation at S/N = 3 and 10.

Table 2
Method precision, recovery and accuracy of rat plasma spiked with FEN.

Added conc., ng/mL	Precision % (RSD) ^a (n = 5)		Recovery %	Accuracy % ^b
	Intra-day	Inter-day		
10	12.0	12.6	96	93
50	6.3	7.3	92	92
100	3.9	2.3	95	101

^a Relative standard deviation.

^b Accuracy % = (found concentration/nominal concentration) × 100 (n = 5).

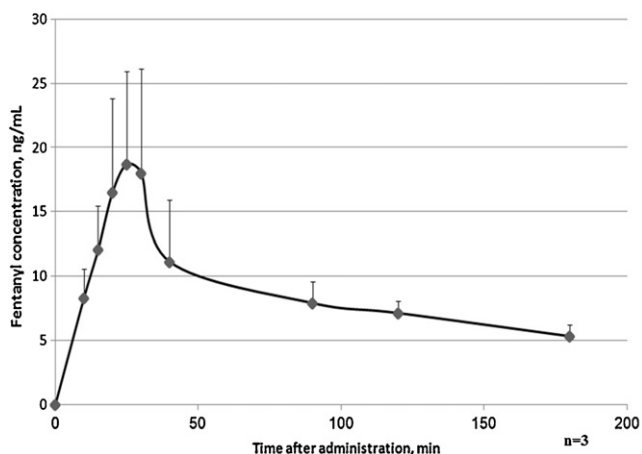


Fig. 2. Concentration–time profile of FEN in rat plasma after single administration of FEN (0.1 mg/kg).

Table 3
Pharmacokinetic parameters of FEN after single administration of 0.1 mg/kg intraperitoneally.^a

Parameter	Mean ± SD (n = 3)
T_{max} , min	28.3 ± 2.9
C_{max} , min	21.3 ± 4.9
$T_{1/2}$, min	198.3 ± 44.7
AUC_{0-180} , ng min/mL	$15.6 \times 10^2 \pm 2.9 \times 10^2$
MRT_{0-180} , min	75.3 ± 8.7
CL, mL/min	19.4 ± 2.6

^a Moment analysis was used to determine pharmacokinetic parameters.

None of the method reported studied FEN determination in male Wistar rats. This would show the suitability of this method for different application.

4. Conclusion

As FEN has many effects over human health, it has been studied with great emphasis in order to maximize its effectiveness and reduce the amount of side effects related to this potent

opioid. Therefore, the availability of a method that can improve the understanding of FEN interaction with other drugs would definitely be of a great advantage. The currently available methods using the simple method of HPLC-UV are either consuming a large volume of plasma sample or has multiple augmentation of concentrations while reconstitution which render them tedious and improbable to handle easily. On the other hand, the highly sensitive methods of LC-MS are of restricted accessibility to research laboratories. Our HPLC-UV detection method is considerably simple, time saving, reproducible and relatively sensitive. This method is suitable for complete pharmacokinetic analysis in small animals with possible utilization in FEN interaction with other drugs.

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