Isotope Dilution Analysis for Urinary Fentanyl and Its Main Metabolite, Norfentanyl, in Patients by Isotopic Fractionation Using Capillary Gas Chromatography

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Isotope dilution analysis was applied to determine urinary excretion of fentanyl (FT) and its main metabolite, norfentanyl (Nor-FT), by isotopic fractionation using a capillary gas chromatograph equipped with a surface ionization detector (SID). Urinary FT was determined quantitatively in the range of 0.4 - 40 ng/ml using deuterium labeled FT (FT- $^2\text{H}_{19}$), as an internal standard.

We also performed isotope dilution analysis of Nor-FT in urine. N-Alkylation was necessary to sensitively detect Nor-FT with SID. Methyl derivative was selected from 3 kinds of N-alkyl derivatives to increase sensitivity and peak resolution, and to prevent interference with urinary compound. Nor-FT concentration was quantitatively determined in the range of 10 - 400 ng/ml using deuterium labeled Nor-FT (Nor-FT- $^2\text{H}_{10}$). No endogenous compounds or concomitant drugs interfered with the detection of FT and Nor-FT in the urine of patients. The present method will be useful for pharmacokinetic studies and the evaluation of drug interactions in FT metabolism.

Key Words: capillary gas chromatography, isotope dilution analysis, fentanyl, norfentanyl, urinary concentration, deuterium label, isotopic fractionation

1. Introduction

Fentanyl[1-(2-phenethyl)-4-N-(N-propionylanilino)piperidine, FT] is a synthetic narcotic analgesic more potent than morphine, and it has been widely used during surgery. By gas chromatography-mass spectrometry (GC-MS) combine with the stable isotope tracer technique, we have identified five urinary metabolites of FT in rats¹⁾, three of them were also detected in the urine of surgical patients who had received FT²⁾. Furthermore, we have detected some FT metabolites with phenethyl and propionyl group oxidation in isolated hepatocytes from rats and guinea pigs³⁾. Urinary excretion of unchanged FT, N-propionyl-

anilinopiperidine (Nor-FT) and 1-(p-hydroxyphenethyl)-4-(N-propionylanilino)piperidine were excreted, as expressed as dosage percentage, at 1 - 4 %, 8 - 25% and 3 - 6 %, respectively, in surgical patients under high dose FT anesthesia⁴⁾.

GC-MS in conjunction with stable isotope techniques enables structural information to be obtained. Concentration can also be accurately measured using selected ion monitoring. However, the major problems of GC-MS equipment are the limited availability and expensiveness. Recently, we have reported that isotopic fractionation techniques using capillary GC to measure deuterium labeled compounds is an alternative to GC-MS, and we have applied this technique to isotope

dilution analysis of drugs and metabolites⁵⁾⁻⁸⁾. We previously showed the isotopic fractionation of FT and deuterium labeled FT⁹⁾. Furthermore, we observed that the quantitative determination of serum FT in patients using this technique is accurate and reproducible¹⁰⁾. In the present study, this technique was applied to determine urinary FT and its main metabolite, Nor-FT.

2. Experimental

2.1 Materials

FT and 1-nonadeuterophenethyl-4-*N*-pentadeuteropropionyl-pentadeuteroanilino-piperidine (FT-²H₁₉) were prepared as described previously⁹⁾. *N*-Propionyl-anilinopiperidine (Nor-FT) and *N*-pentadeuteropropionyl-pentadeuteroanilino-piperidine (Nor-FT-²H₁₀) were synthesized as described previously⁹⁾ and purified as propionate. Nor-FT propionate and Nor-FT-²H₁₀ propionate have melting points of 116–120°C and 113–120°C, respectively. The molecular ions of the free forms were detected at *m/z* 232 in Nor-FT and *m/z* 242 in Nor-FT-²H₁₀ by their EI-mass spectra. Found values (calculated values in parenthesis) from elemental analysis were H: 8.49 (8.55)%, C: 66.32 (66.64)% and N: 9.05 (9.14)% in Nor-FT propionate, and H: 5.10 (5.10)%, C: 64.30 (64.52)%, N: 8.97 (8.85)% and D: 6.20 (6.36)% in Nor-FT-²H₁₀ propionate. The deuterium labeled position was identified from the disappearance of signal when comparing ¹H-NMR spectrum with Nor-FT. The chemical structures of Nor-FT and its analogues are shown in Fig. 1.

2.1.1 Synthesis of Nor-FT and Nor-FT-²H₁₀ derivatives

Methyl derivatives of Nor-FT (Nor-FT-Me) and Nor-FT-²H₁₀ (Nor-FT-²H₁₀-Me) were synthesized as follows. Nor-FT or Nor-FT-²H₁₀ (0.3 g) was dissolved in 9 ml of methanol. Dimethyl sulfate (140 μl) and 60 mg of calcium oxide was

Fig. 1 Chemical structures of Nor-FT and its analogues.

added and the solution was refluxed for 1 h. The reaction mixture was evaporated off, dissolved in 3 ml of ethyl acetate, and washed with 3 ml of H₂O. This solution was evaporated and the residue was sublimated at 150–200°C under reduced pressure to produce 0.07 g of Nor-FT or Nor-FT-²H₁₀. Ethyl derivatives of Nor-FT (Nor-FT-Et) and Nor-FT-²H₁₀ (Nor-FT-²H₁₀-Et) were synthesized using the above procedure using diethyl sulfate instead of dimethyl sulfate.

Benzyl derivatives of Nor-FT (Nor-FT-Ben) and Nor-FT-²H₁₀ (Nor-FT-²H₁₀-Ben) were synthesized using a similar method for 1-benzyl-4-*N*-(*N*-propionylanilino)piperidine as previously reported⁹⁾.

Chemical structures were identified by EI-mass spectra and the molecular ions were detected at *m/z* 246 in Nor-FT-Me, *m/z* 256 in Nor-FT-²H₁₀-Me, *m/z* 260 in Nor-FT-Et, *m/z* 270 in Nor-FT-²H₁₀-Et, *m/z* 322 in Nor-FT-Ben, and

m/z 332 in Nor-FT- $^2\text{H}_{10}$ -Ben. The purities of these compounds were confirmed by gas chromatography. The chemical structures of these derivatives are shown in Fig. 1.

2.2 Gas chromatographic conditions

Gas chromatographic analysis was performed with a fused-silica capillary column (25 m, 0.22 mm i.d.) with chemically bounded methylpolysiloxane and a film thickness of 0.25 mm (Hicap-CBP1-M25-025, Shimadzu). Theoretical plates for the CBP1 columns were 100 000 (25 m, pentadecane). A Shimadzu model GC-14A gas chromatography equipped with a surface ionization detector (SID) was used. The inlet system used was solvent less of moving-needle type. The retention time was measured by a data processor, Shimadzu model C-R5A Chromatopac. The injection port was kept at 290°C, the column was operated at 180°C for Nor-FT or 260°C for FT and the detector temperature was 320°C. The carrier gas was helium at 1.4 ml/min.

2.3 Deactivation of glassware surface

Glassware surface was deactivated to prevent FT losses by adsorption. The glassware was silanized. The glass vials and tubes for sample preparation were coated with 1 or 2 drops of dichlorodimethylsilane (Tokyo Kasei). After being in contact with the vapor of the reagent for overnight, the glass vials were rinsed with dry toluene and absolute methanol. Subsequently, they were rinsed with ethyl acetate and air-dried.

2.4 Extraction of FT from urine

The determination of FT concentration in urine was performed by a similar method in previous report¹⁰⁾. Urine (1 ml) was added to 0.5 ml of 1 N NaOH and was extracted with 1 ml of ethyl acetate, which contained 20 ng of FT- $^2\text{H}_{19}$, by

shaking for 10 min. After centrifugation (4 000 rpm/5 min), the supernatant put in a glass vial and was back-extracted with 0.5 ml of 1 N HCl using a vortex mixer. After centrifugation (3 000 rpm/5 min), the ethyl acetate layer was removed. The residual aqueous layer was added to 0.5 ml of 2 N NaOH and was extracted by 200 μl of hexane using a vortex mixer. After centrifugation (3 000 rpm/5 min), the hexane layer was transferred to another glass vial and was evaporated until dry by a N_2 stream. The residue was dissolved in 10 μl of methanol and the methanol solution (1–3 μl) was injected in to a gas chromatograph.

2.5 Extraction and derivation procedure of Nor-FT

Urine (1 ml) was added to 0.5 ml of 1 N NaOH and was extracted with 1 ml of ethyl acetate, which contained 200 ng of Nor-FT- $^2\text{H}_{10}$, by shaking for 10 min. After centrifugation (4 000 rpm/5 min), the supernatant was put in a glass tube and evaporated until dry by a N_2 stream. The residue was dissolved in 2 ml of methanol. To the solution, 50 μl of methanol containing 4 % of dimethyl sulfate was added. After heating at 35°C for 60 min, the reaction mixture was evaporated until dry. The residue was dissolved in 1 ml of 5 N NaOH and the solution was extracted with 1 ml of ethyl acetate using a vortex mixer. After centrifugation (4 000 rpm/5 min), the supernatant was put in a glass vial and was back-extracted with 0.5 ml of 1 N HCl using a vortex mixer. After centrifugation (3 000 rpm/5 min), the ethyl acetate layer was removed. The residual aqueous layer was added to 0.5 ml of 2 N NaOH and was extracted with 200 μl of ethyl acetate using a vortex mixer. After centrifugation (3 000 rpm/5 min), the ethyl acetate layer was transferred to another glass vial and the solution (1–3 μl) was injected in to a gas chromatograph.

2.6 Collection of urine samples

All urine samples were obtained from surgical patients that received FT, who had normal hepatic and renal functions. Anesthesia was carried out as previously described¹⁰⁾. FT was i.v. administered ($5\mu\text{g/kg}$) by a bolus injection as fentanyl citrate (Fentanest, Sankyo). Urine samples were collected 7 times across 3 h after FT administration by urine bag exchange. Collected urine samples were transferred to silanized tubes, capped, sealed with Parafilm, and was stored for 9–15 days at 4°C until analysis.

3. Results and Discussion

In our previous report¹⁰⁾, the advantage of the isotope dilution analysis for serum FT determination was demonstrated by comparison with other FT analogues which substituted a propionyl group with an acetyl group or a phenethyl group with a benzyl group as the internal standard. Furthermore, we indicated that the isotopic fractionation method using capillary GC with SID is accurate for the isotope dilution analysis of FT.

In this study, we applied isotope dilution analysis to determine urinary excretion of FT from patients. Six standard urine samples, which contained 0.4 to 40 ng/ml of FT with 20 ng/ml of $\text{FT-}^2\text{H}_{19}$, were prepared. The extraction procedure used was similar to that used for serum FT analysis. The calibration curve was estimated for the amount ratio of FT to $\text{FT-}^2\text{H}_{19}$, x and the peak area ratio of FT to $\text{FT-}^2\text{H}_{19}$, y was obtained. The

calibration curve was expressed as $y=1.022x+0.027$ with a correlation coefficient of $r=0.999$. The linear relationship was determined and the slope was almost equal to the case of serum. The reproducibility of the FT concentration in urine is shown in Table 1. The relative standard deviation (RSD) was 16.7% at the minimum quantifiable concentration (0.4 ng/ml), 3.7% at 4 ng/ml, and 3.0% at 40 ng/ml.

The main metabolite of FT, Nor-FT has previously been measured by GC-MS^{11)–13)}, GC using an ECD¹⁴⁾, and enzyme linked immunosorbent assay (ELISA)¹⁵⁾. However, these methods have mostly been used for detection of FT abuse, and are not accurate enough for pharmacokinetic studies.

We examined isotope dilution analysis of Nor-FT in urine using isotopic fractionation by capillary GC. Formerly, sensitive detection of Nor-FT was attempted by using an ECD after conversion to halogenated derivatives. However, the sensitivity and stability of this method were insufficient to determine the urine level of Nor-FT. The pentafluoropropionic derivative of Nor-FT was reported to have the highest sensitivity of the halogenic derivatives used for ECD¹⁴⁾, but the sensitivity was less than by use of SID, and reproducibility was insufficient due to interference with urinary compounds.

In our previous report¹⁰⁾, we demonstrated highly sensitive detection of FT by SID that was specific detector for the tertiary amino compound. However, Nor-FT is a secondary amino

Table 1 Reproducibility of FT concentration in urine

Amount added (ng/ml)	Amount found (ng/ml)					Mean	RSD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
0.4	0.481	0.372	0.592	0.512	0.454	0.482	16.7%
4	3.89	3.50	3.68	3.67	3.68	3.68	3.7%
42	42.4	40.2	43.4	43.1	41.9	42.2	3.0%

compound, and *N*-alkylation is necessary for detection by SID. The sensitivity of 3 kinds of *N*-alkyl derivatives, Nor-FT-Me, Nor-FT-Et, and Nor-FT-Ben was compared. The relative sensitivity of Nor-FT-Me, Nor-FT-Et, and Nor-FT-Ben were 2.12, 1.68, and 0.59, respectively, by comparison with FT after injection of 2 ng of each into GC. These results demonstrate that the introduction of methyl or ethyl group to Nor-FT increase the sensitivity on SID analysis. Suzuki et al. reported this is due to an increase in surface ionization by the electron donating effect of the added groups¹⁶⁾.

The isotopic fractionation of Nor-FT derivatives from their deuterium labeled analogues at column temperature 160–260°C or 220–270°C are shown in Fig. 2. The retention times of deuterium analogues were shorter than non-labeled analogues at all temperatures. We found linear relationships between resolution (R_s) and the reciprocal of the absolute temperature ($1/T$), as shown in Fig. 3. The resolution of peaks ($R_s > 1$) within 30 min was achieved at 170–180°C for Nor-FT-Me, 170–210°C for Nor-FT-Et, and 230–250°C for Nor-FT-Ben.

The determination of Nor-FT in spiked urine using ethyl derivative was not accurate, therefore, the methyl derivative was adopted in the determination of Nor-FT by the reason for its sensitivity, peak resolution and accuracy. The calibration curve was estimated from 1 ml of the urine spiked with 10–400 ng of Nor-FT and 200 ng of Nor-FT-²H₁₀ on 180°C, and was expressed as $y = 1.372x - 0.044$ with a correlation coefficient of $r = 0.999$. The slope of this line was differ from that of FT for overlapping the peaks ($R_s = 1.1$) in this column temperature. The methyl derivative yield was approximately 60% under these conditions.

The reproducibility of the Nor-FT concentra-

Fig. 2 Chromatograms of Nor-FT derivatives and their deuterated analogues Nor-FT-Me(A), Nor-FT-Et(B) and Nor-FT-Ben(C).

tion in urine is shown in Table 2. Intra-day precision was evaluated by analyzing the standard urine 5 times. The relative standard deviation (RSD) was 4.0% at the minimum quantifiable concentration (10 ng/ml).

To assess the possibility of formation of Nor-FT from FT during the derivation procedure, we examined the effect of FT concentration on the determination of Nor-FT. Nor-FT was measured in urine, which contained 200 ng/ml of Nor-FT and Nor-FT-²H₁₀ and FT at a 0.1–2 molar ratio to Nor-FT. The effect of FT on the determination of Nor-FT is shown in Fig. 4. Nor-FT concentration increased in relation to FT

Fig. 3 Effect of column temperature (T in Kelvin) on the separation of Nor-FT derivatives and their deuterated analogues.

Nor-FT-Me(□), Nor-FT-Et(△) and Nor-FT-Ben(○)

amount. Nor-FT was confirmed after treatment of FT (50 μ g) with dimethyl sulfate by GC-MS at same conditions. FT might have been degraded to Nor-FT by dimethyl sulfate treatment. Regardless of FT concentration, $25.4 \pm 7.8\%$ ($n=6$) of FT was converted to Nor-FT. Since our previous report⁴⁾ showed the urinary concentration of FT was lower than Nor-FT, the methyl derivation procedure might cause a slight change on Nor-FT concentration, especially at earlier time points after FT administration. Therefore, we corrected Nor-FT concentrations by subtracting the contribution of FT.

Typical gas chromatograms of the extracts

Fig. 4 Effect of FT on determination of Nor-FT in urine.

from patient urine after i.v. administration of FT are shown in Fig. 5. No endogenous compounds or concomitant drugs interfered with the detection of FT or Nor-FT. The urinary concentration of FT and Nor-FT are shown in Table 3. These results demonstrate the potential utility of this isotope dilution analysis for the clinical studies.

Individual differences in excretion rates of FT and Nor-FT were found, confirming to results from serum FT concentration studies¹⁰⁾. Recently, the *N*-dealkylation enzyme of FT has been identified as CYP3A4, *in vitro*^{17), 18)} and drug interactions *in vivo* with nifedipine¹⁹⁾, midazolam and triazolam²⁰⁾, erythromycin²¹⁾, lovastatin²²⁾ and lidocaine²³⁾, has been predicted. The present method will be useful for evaluation of these interactions in FT metabolism.

Table 2 Reproducibility of Nor-FT concentration in urine

Amount added (ng/ml)	Amount found (ng/ml)					Mean	RSD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5		
10	12.85	12.15	12.72	11.75	11.93	12.28	4.0%
40	37.26	45.40	36.15	42.72	45.23	41.35	10.6%
399	371.3	419.1	429.8	393.5	407.4	404.2	5.6%

Table 3 Urinary concentration of FT and Nor-FT

Time (min)	Subject A			Subject B		
	FT (ng/ml)	Nor-FT (ng/ml)		FT (ng/ml)	Nor-FT (ng/ml)	
		Measured	Corrected		Measured	Corrected
0 - 60	0.37	0.0	0.0	1.54	10.3	10.1
60 - 120	8.03	84.1	82.7	1.81	46.3	45.9
120 - 180	4.97	86.6	85.7	1.75	50.5	50.2
180 - 240	2.49	63.7	63.2	0.36	39.5	39.4
240 - 360	0.59	52.8	52.7	0.59	20.8	20.7
360 - 480	1.74	71.1	70.8	1.61	51.1	50.8

Fig. 5 Chromatograms of urine extracts from patients using the deuterated analogue FT- $^2\text{H}_{19}$ (A), of Nor-FT- $^2\text{H}_{10}$ (B) as an internal standard. Collection time of urine after the administration of FT ($5\mu\text{g/kg}$ i.v.) : 60 min, FT 7.27 ng/ml(A) and 480 min, Nor-FT 71.14 ng/ml(B).

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要 旨

キャピラリガスクロマトグラフィを用いた同位体希釈分析による患者尿中 フェンタニルおよび主代謝物ノルフェンタニルの定量

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安定同位体希釈分析により, 麻薬性鎮痛薬フェンタニル (FT) とその主代謝物である Nor-FT の尿中濃度を測定した。19 個の重水素を標識した FT- $^3\text{H}_{19}$ と FT は, 表面電離型イオン化検出器 (SID) 装備キャピラリ GC により分離測定可能であり, 尿中 FT を 0.4 - 40 ng/ml の範囲で再現性よく測定できた。Nor-FT は二級アミンであり SID では高感度検出ができないが, *N*-アルキル誘導体化により三級アミンとすることで高感度検出を試みた。3 種類の誘導体を用い分離, 感度, 再現性から検討したところ, *N*-メチル誘導体が最適であった。尿中の Nor-FT を 10 - 400 ng/ml の範囲で Nor-FT- $^3\text{H}_{10}$ を用い誘導体化後測定したところ, 優れた直線性と良好な再現性が得られた。FT 投与患者尿中の FT および Nor-FT の測定を行ったところ, 生体内物質や併用薬物の影響を受けず正確な測定が可能であり, FT の体内動態解析に有用であると考えられる。