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Quantitative analysis of fentanyl in rat plasma by gas chromatography with nitrogen–phosphorus detection

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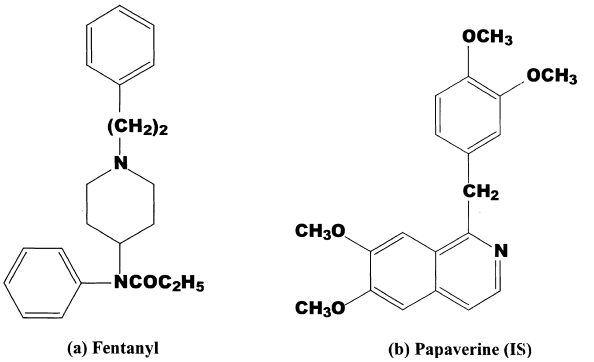
Abstract

A sensitive assay method was developed to determine fentanyl, an opiate agonist, in rat plasma by gas chromatography with nitrogen–phosphorus detection. For the pretreatment of plasma samples, sodium hydroxide was added to denature protein and *n*-butyl chloride was used to extract fentanyl. The calibration curve was linear within the concentration range 0.5 to 50 ng/ml (r=0.9997). The limit of detection was 0.1 ng/ml, and 0.5 ng/ml could be quantified with acceptable precision. Furthermore, fentanyl could be determined in only 200 μl of rat plasma. The method has been successfully applied to an intramuscular pharmacokinetic study at a dose of 10 μg/kg. Therefore, the current method is a valuable analytical tool for investigating the pharmacokinetics of fentanyl at low clinical doses.

1. Introduction

Fentanyl, *N* - (1 - phenethyl - 4 - piperidyl)propio - nanilide (Fig. 1), is a potent synthetic opiate commonly used for surgical analgesia and sedation [1,2]. It is approximately 200 times more potent than morphine and has a rapid onset (1–2 min), but short duration of action (30–60 min) [1–4]. Fentanyl has minor cardiovascular effects but can induce respiratory depression, hypotension, and coma [4,5]. Because of its potency and quick onset, even a very small dose of fentanyl can lead to sudden death



(a) Fentanyl (b) Papaverine (IS)

Fig. 1. Chemical structures of (a) fentanyl and (b) papaverine (I.S.).

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[6–9]; the minimal lethal dose for fentanyl is estimated to be 2 mg [10,11].

Comprehensive pharmacokinetic studies of fentanyl have proven difficult as the blood concentration of fentanyl from single or infrequent doses falls rapidly below the limit of detection (LOD) of most assay procedures. To understand the pharmacokinetics of fentanyl, detection of lower levels of the compound from analgesic doses is important [12,13]. Following the report of Fryira et al. [14], a number of methods have been developed to measure fentanyl concentrations in biological fluids with different levels of sensitivity and usefulness in describing the pharmacokinetics of fentanyl [15]. A high-performance liquid chromatography (HPLC) method with an LOD of 1 ng/ml was described but this analysis is not sensitive enough for pharmacokinetic studies at analgesic doses [16,17]. Enzyme-linked immunosorbent assay (ELISA) methods have also been utilized for detection of fentanyl with lowest detectable concentrations of 100 pg/ml [18,19], but these methods have low precision and do not appear to have yet been used in pharmacokinetic studies. Radiochemical and radioimmunoassay methods are the most commonly employed for pharmacokinetic studies, but suffer from a lack of selectivity, particularly at clinically realistic levels of fentanyl (<10 ng/ml). This lack of selectivity may be partly responsible for the wide variability in kinetic parameters of fentanyl. A number of gas chromatographic (GC) techniques have been reported where several different detectors have been utilized. A method using thermionic specific detection (TSD) had a limit of 250 pg/ml [20] and alkali flame ionization detection had a limit of 3.3 ng/ml [21]. Kowalski et al. recorded an LOD of 2.5 ng/ml using nitrogen–phosphorus detection (NPD) [22] and Watts and Caplan later increased sensitivity to subnanogram levels of fentanyl using mass spectrometry (MS) (100 pg/ml) [23]. GC methods, particularly when coupled with MS, are sensitive but they are time-consuming due to the number of purification and derivation steps required [24–30]. In addition, these methods required more volume of plasma to achieve satisfactory detection levels. For this reason, there have been no pharmacokinetic studies published in rats due to the small amount of plasma available. Therefore, we developed a simple but precise assay using a small volume of rat plasma. The method has been applied to an intramuscular (IM) pharmacokinetic study and some of the kinetic parameters obtained are discussed.

2. Materials and methods

2.1. Materials

Fentanyl base (purity >99%) was purchased from McFarland Smith (Edinburgh, UK). Papaverine hydrochloride (purity >98%), the internal standard (I.S., selected due to structural similarity with fentanyl and the presence of an ionizable nitrogen group [30]), was obtained from Sigma (Steinheim, Germany). *n*-Butyl chloride, toluene, and methanol were obtained from Sigma (St. Louis, MO, USA). Water was obtained by a Milli-Q purification system from Millipore (Molsheim, France). All other chemicals were of analytical grade and used with distilled purification. Blank plasma for the preparation of standard samples was supplied by Korea Research Institute of Chemical Technology (KRICT, Taejon, South Korea) and heparin sodium from Korea Green Cross (Yongin, South Korea). Sample vials (1.5 ml with 30 μl reservoir) and crimp caps (11 mm with PTFE/silicone/PTFE septa) were purchased from Hewlett-Packard (Palo Alto, CA, USA). Microcentrifuge tubes (1.5 ml, siliconized flat-top) and all tips (silanized) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Apparatus and conditions

Chromatography was performed on a Hewlett-Packard 6890 series gas chromatograph, equipped with an autosampler (HP 7683) and an NPD system. High-purity helium was used as the carrier gas at a constant pressure of 25 p.s.i. (1 p.s.i.=6894.76 Pa). A HP-5 5% phenyl–methyl siloxane capillary column (60 m×0.32 mm I.D. and 0.25 μm film thickness) was used. The initial oven temperature was 150°C for 1 min. The oven temperature was programmed to 270°C at 30°C/min, held 2 min, then to 280°C at 5°C/min, and held 9 min (overall run

time 18 min). The temperature of the injector and the detector were maintained at 285°C and 310°C, respectively. Flow rates were 2.0 ml/min for the helium gas, 60 ml/min for air, and 3.0 ml/min for hydrogen.

2.3. Preparation of samples and standards

The fentanyl and I.S. stock solutions were prepared in methanol, at concentrations of 100 μg/ml and 1 μg/ml, respectively. Aqueous samples, quality controls (QCs), and calibration standards were extracted using silanized centrifuge tubes and stored frozen at −20°C. The concentration range of the standard solutions was from 0.5 to 50 ng/ml. Blood samples were obtained from Sprague–Dawley (SD) rats before and after receiving fentanyl, placed in heparinized blood collecting tubes, and centrifuged at 12 879 *g* (Centrifuge 5415C, Eppendorf, Germany). Plasma standards were prepared by adding 0.9 ml drug-free plasma to 10 μl of aqueous working standard to give final concentrations 0.5 to 50 ng/ml. Deactivating all glassware, including disposable culture tubes and the injection port liner, with a 5% solution of dimethyldichlorosilane and vapor of hexamethyldisilazane (HMDS) were necessary to avoid adsorption of the drug onto the glassware in order to achieve optimal recovery.

2.4. Extraction of samples

To extract fentanyl, 10 μl I.S. solution and 50 μl NaOH (10 mol/l) were added to 200 μl of plasma in a centrifuge tube. After being alkalinized, the aqueous phase was extracted with 600 μl of 5% isopropanol in *n*-butyl chloride. The tubes were vortex-mixed (Maxi Mix II, Thermolyne, USA) and centrifuged at 12 879 *g*. The upper organic phase was transferred to a second centrifuge tube. The samples were evaporated in a vacuum concentration system (Spinvac, Hanil, South Korea) at 40°C. Extraction residues were reconstituted in 50 μl toluene, vortex-mixed, sonicated, centrifuged, and transferred to a silanized vial with 30 μl reservoir. These vials were capped, and 2 μl was injected into the GC system via splitless mode.

2.5. Variation

Calibration curves were obtained prior to each batch analysis. QCs were included to validate every calibration curve and to ensure sample stability during analysis. To investigate intra-day and inter-day variation, five calibrations were carried out at five different times (0, 6, 12, 18, and 24 h) and on 5 different days (0, 1, 2, 3, and 5 days) with pooled blank plasma, respectively. The standards were prepared fresh daily.

2.6. Pharmacokinetics

Fentanyl was suspended in 0.2% Tween-80 solution and given to a rat at an IM dose of 10 μg/kg. Blood samples were collected from the tail vein at 10 and 30 min, 1, 2, 4, 8, 12, and 24 h after the injection. Plasma concentration–time data were fitted by one-compartment open model using a pharmacokinetic program ''NONLIN''. The pharmacokinetic parameters including the area under the plasma concentration versus time curve (AUC), the maximum plasma concentration (C_{max}), the time to peak concentration (t_{max}), absorption rate constant (k_{el}), and the elimination half-life ($t_{1/2}$) were calculated from the fitting results.

3. Results

3.1. Specificity of chromatographic analysis

High-purity helium as carrier gas and a capillary column of phenyl–methyl siloxane were used in the GC–NPD system. The temperatures of the injector and the detector were maintained at 285 and 310°C, respectively. Under this condition, fentanyl was well separated on the GC chromatogram with a run time of 12.4 min (Fig. 2). In the blank plasma, there were no peaks that interfered at the retention time of the compound.

3.2. Extraction efficiency

Because fentanyl is approximately 80% bound to plasma proteins [31–33], the high concentration of

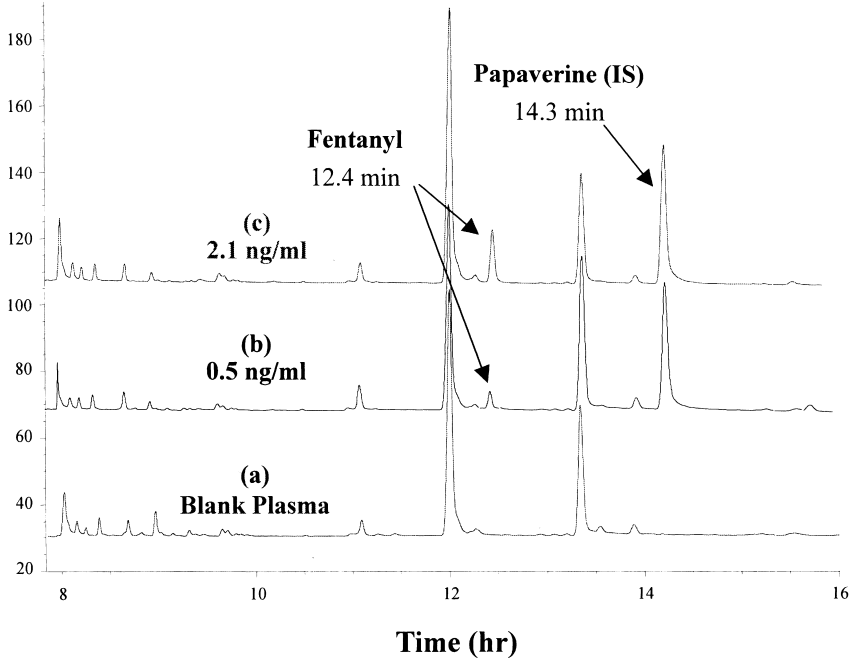

Fig. 2. Chromatograms of (a) blank plasma sample, (b) the LOQ peak (0.5 ng/ml), and (c) the C_{max} peak (2.1 ng/ml).

sodium hydroxide was also added to basify the plasma samples. Of the several extraction solvents such as *n*-hexane, cyclohexane, ethyl acetate, acetonitrile, and *n*-butyl chloride, the most positive results were obtained from 5% isopropanol in *n*-butyl chloride which a solvent to increase the extraction efficiency of fentanyl from the plasma.

3.3. Linearity

The standard curve of fentanyl spiked in the rat plasma was linear over the concentration range from 0.5 to 50 ng/ml. The average slope of standard curves was 1.761 and the average correlation coefficient was 0.9997.

3.4. Accuracy and precision

The accuracy was determined by comparing the means of measured concentrations with the nominal concentration for three levels of QC solutions. The precision was expressed as a mean percentage of the relative standard deviation (RSD). The results of the intra-day and inter-day variation tests are presented in Table 1. The RSD was less than 2%. The LOD and limit of quantitation (LOQ) in rat plasma were 0.1 ng/ml and 0.5 ng/ml, respectively.

3.5. Pharmacokinetics

To confirm the suitability of the assay method, an IM kinetic study was performed at a dose of 10

Table 1
Intra-day and inter-day reproducibility of the GC analysis

Concentration (ng/ml)	Intra-day (n=5)			Inter-day (n=5)		
	Mean±SD	RSD (%)	Accuracy (%)	Mean±SD	RSD (%)	Accuracy (%)
5	5.57±0.11	2.05	111.4	5.59±0.12	2.06	111.8
10	9.53±0.15	1.60	95.3	9.43±0.18	1.95	94.3
50	49.5±0.72	1.46	99.0	49.5±0.91	1.83	99.0

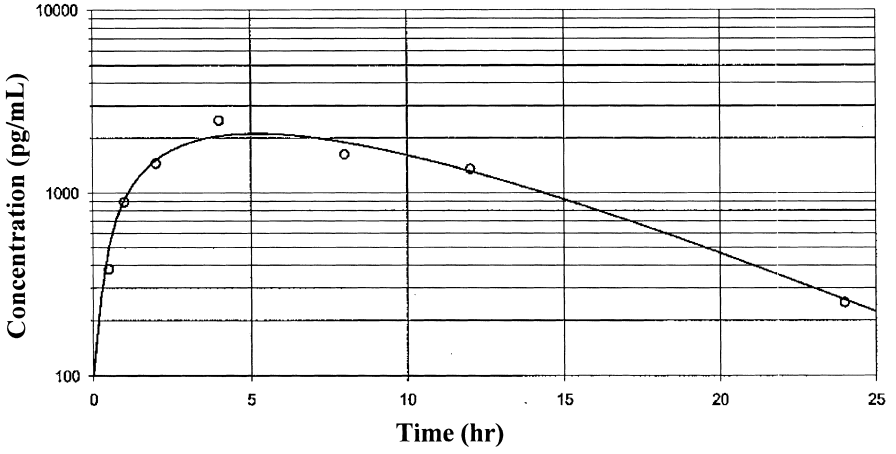

Fig. 3. Plasma concentration–time profiles after a 10 μg/kg intramuscular dose of fentanyl.

μg/kg. The compound was measurable at least at 24 h after injection. The pharmacokinetic parameters were well modeled by a one-compartmental model analysis and the time–concentration profile showed a mono-exponential decline in the rat plasma as shown in Fig. 3. The AUC, C_{max}, t_{max}, k_{el}, and $t_{1/2}$ were 29.73 ng h/ml, 2.11 ng/ml, 5.18 h, 0.1862 h^{-1}, and 3.72 h, respectively. The pharmacokinetic data were similar to those reported for monkeys and humans [37,38].

4. Discussion

The development of a sensitive and selective detection method is required because fentanyl appears extremely small amount in the plasma, less than 1 ng/ml, at the therapeutic doses. We employed GC with NPD which has a good sensitivity for fentanyl, compared with other detection systems. Yuansheng et al. reported that NPD has about 10 to 50 times greater sensitivity for nitrogen containing compounds than standard flame ionization detection (FID) and the selectivity of the detector is about 5000 times greater for nitrogen-containing compounds than for simple hydrocarbons [34].

There have been lots of efforts to minimize the complex and time-consuming steps to extract fentanyl from biomaterials [12–30]. We obtained a simple set of extraction conditions and instrumentation for fentanyl analysis. The extraction solvent of 5% isopropanol in *n*-butyl chloride showed significantly high extraction efficiency for the compound from the rat plasma. In addition, only about 3 h was needed to finish all the extraction steps. Adding a small volume of a high concentration solution of sodium hydroxide resulted in satisfactory protein denaturation. Since the adsorption of fentanyl on the surface of glassware is well known, all glassware was initially silanized with a 5% solution of dimethyldichlorosilane and vapor of HMDS.

For the previous investigations, at least 1 ml of plasma was required to detect fentanyl because of low plasma fentanyl concentration. For instance, Watts and Caplan detected subnanogram concentrations of fentanyl using 2 ml of blood sample with GC–MS [23]. Our method requires only 200 μl plasma. Therefore, it is possible to assess an individual pharmacokinetics by collecting of blood in the same rat repetitively. To the best of our knowledge, there has been no pharmacokinetic report for fentanyl in rats.

Quantitation of fentanyl was achieved by using a weighted linear regression analysis with a weighting factor of $1/x$. The standard curve of fentanyl spiked in rat plasma was linear over the concentration range 0.5 to 50 ng/ml. There was no significant variation in the linear regression parameters between inter-day and intra-day reproducibility studies. The accuracy and precision expressed as a mean percentage of nominal values and RSD were $>90\%$ and $<2\%$,

respectively. Furthermore, the LOQ was 0.5 ng/ml. From the results, the present extraction process is efficient and rapid for the determination of fentanyl in rat plasma.

Recently, Shou et al. reported a high sensitive LC–MS–MS method for the determination of fentanyl in human plasma [24]. This method was validated to measure as low as 0.05 ng/ml of fentanyl by using only 0.25 ml of plasma sample. However, the GC–NPD method might be useful for laboratories where LC–MS–MS is not available. Therefore, this method was carried out to develop a new method for determining fentanyl in rat plasma using GC–NPD. As shown in Fig. 3, a typical fentanyl concentration–time profile was well obtained by one-compartmental model fitting. The AUC was around 30 ng h/ml. The k_{el} of the compound in a single rat was 3.72 h, which is similar to that value with in other animals, 1.20 ± 0.78 h in goats [35], 2.35 ± 0.57 h in cats [36], 3.64 ± 0.59 h in monkeys [37], and 3.7 ± 0.4 h humans [38]. Therefore, it is concluded that the current method is a valuable analytical tool in rats for assessing the pharmacokinetic properties of fentanyl at low dose levels.

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