Brief/Technical Note

Development of a GC-MS Assay for the Determination of Fentanyl Pharmacokinetics in Rabbit Plasma after Sublingual Spray Delivery

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INTRODUCTION

Fentanyl (*N*-phenyl-*N*-(1-(2-phenylethyl)4-piperidinyl)propanamide) (Fig. 1), is a potent synthetic opioid agent widely used for surgical analgesia and sedation. Fentanyl is approximately 200 times more potent than morphine and has a quick onset (1–2 min) and short duration of action (30–60 min; 1). Sufentanil, a structurally related compound, is about five to ten times more potent than fentanyl, but has an even shorter duration of action (2). Fentanyl has a selective and potent effect on mu opioid receptors, and is used extensively in the treatment of breakthrough pain (3), which is a transitory exacerbation of pain experienced by a patient who has relatively stable and adequately controlled baseline pain (4).

A rapid onset of pharmacological effect is often desired for some drugs, especially those used in the treatment of acute disorders. This can effectively be achieved by parenteral administration. However, this route may not always be the most convenient for the patient. Therefore, there is growing interest in developing new, reliable, and convenient non-parenteral dosage forms utilizing routes of administration where a rapidly dissolved drug is immediately absorbed into the systemic circulation. Although conventional oral tablet formulations are generally the first choice for drug administration, because of the relative ease of production and usage, some drugs undergo significant first pass metabolism in the gastrointestinal tract and the liver. In addition, oral tablet or solution administration of analgesics (i.e. fentanyl) is not considered optimal for management of moderate to severe acute pain or breakthrough pain, principally because of the insufficient intrinsic activity and prolong time-to-peak effect (5). Therefore, there is a growing need for novel dosage forms that can effectively deliver fentanyl in a timely manner, and that have the capability of affording dose titration based on patient need.

There has been much interest expressed in the use of oral cavity membranes as sites of drug administration (6,7). Oral mucosal delivery, especially that utilizing the sublingual mucosa as an absorption site, is a promising drug delivery route which promotes rapid absorption and high bioavailability, with a subsequent, almost immediate onset of pharmacological effect (8). These advantages are the result of the highly vascularized oral mucosa, through which drugs enter the systemic circulation directly, by-passing the gastrointestinal tract and first pass metabolism in the liver (9). Various *in vivo* models for intra-oral drug delivery studies have been evaluated. Rabbit and dog are generally regarded as the most suitable animal models for such studies, since the oral cavities of both animals are histologically similar to that of man (10).

Sublingual drug administration has been applied to a number of drugs, including scopolamine, tetrahydrocannabinol, and oxycodone (11–13). All opioid drugs are absorbed sublingually to some extent; and fentanyl is particularly well absorbed via this route (14). As the citrate salt, fentanyl is sparingly soluble in water and highly lipophilic, and is expected to be rapidly absorbed sublingually, because of its ease of permeation through mucous membranes (15).

Currently, for the treatment of breakthrough pain, fentanyl is marketed in the form of an injectable dosage form, which has always suffered from issues of patient compliance. Fentanyl citrate has also been developed as a solid intra-oral formulation in shape of a lollipop. This formulation was designed for oral transmucosal administration in children, to allow rapid absorption of the drug from the oral cavity. However, it was found that a large proportion of the drug is swallowed using this delivery system (16).

Fentanyl is also administered through the Transdermal Therapeutic Fentanyl-System (TTS-F), which allows for the delivery of a continued and sustained titratable amount of fentanyl without the inconvenience of the typical 24 h administration of other analgesics. Although incidences of respiratory depression have led to TTS-F being contraindicated for postoperative analgesia, this route of delivery is currently in Phase III clinical trials for treatment of nociceptive, neuropathic and chronic moderate to severe pain, in a variety of settings (17).

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Fig. 1. Chemical structures of fentanyl and sufentanil

Several analytical methods have been employed to measure fentanyl in biological samples, including HPLC/UV (18,19), radioimmunoassay (20–22), and GC-MS (23–28) methodologies. However, many HPLC methods lack the sensitivity required for application in pharmacokinetic studies, which often involve long blood sampling times and/or small doses of fentanyl. Also, immunoassays tend to suffer from cross-reactivity, and radioanalytical techniques are expensive and not always amenable to human studies. Mass spectrometric methods LC-MS have been also reported (29, 30). GC-MS in the electron impact ionization (EI) mode is one of the most commonly used techniques in drug analysis, and although some GC-MS methods for analyzing fentanyl have been reported, they are time-consuming, due to the number of purification and derivatization steps employed. Nevertheless, GC-MS offers the best sensitivity of the currently available methods for determining fentanyl in biological fluids when utilizing the Single Ion Monitoring (SIM) mode of operation, which affords unparalleled specificity.

The main objective of this current study is two-fold; to develop a simple and sensitive GC-MS-EI method for the quantitation of nanogram levels of fentanyl in support of preclinical pharmacokinetic studies and then to determine the extent of fentanyl absorption from sublingual tissue and distribution to the general circulation compared to intravenous administration in the rabbit.

MATERIALS AND METHODS

Materials

Fentanyl free base was kindly supplied by Insys Therapeutics, Inc (Mundelein, IL), fentanyl citrate and sufentanil free base were obtained from Mallinckrodt Chemicals (St. Louis, MO). 1-Chlorobutane was purchased from Acros Organics (New Jersey), methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). SurfaSil™ siliconizing fluid was obtained from Pierce (Rockford, IL),

hydrogen peroxide 3% USP solution from Hydrox Labs (Elgin, IL), bacteriostatic sodium chloride injection USP 0.9% from APP (Schaumburg, IL), and AErrane™ isoflurane gas was provided by VMC Anesthesia Ohmeda (Waukesha, WI). Heparinized caraway capillary tubes were purchased from Baxter Healthcare Corporation (McGraw Park, IL). Tuberculin slip tip sterile catheters were purchased from J&J Medical (New Brunswick, NJ). All vials, crimp caps with PTFE/silicone septa, and siliconized micro centrifuge tubes, were purchased from Fisher Scientific (Fair Lawn, NJ). Water for HPLC use was passed through a reverse osmosis system (Milli-Q® Reagent Water System) before use. New Zealand rabbits were purchased from Myrtle's Rabbitry, Inc. (Thompson Station, TN).

Analytical Methods

An Agilent 6890 GC, Agilent 7683 autosampler and an Agilent 5973 MSD (Agilent Technologies, Wilmington, DE) was interfaced to a Hewlett-Packard KAYAK XM600 computer (Hewlett-Packard Co., Palo Alto, CA), which utilized MSD Productivity ChemStation Software (Rev. C.00.01; Agilent Technologies, Palo Alto, CA) for analysis and data processing. Separation was achieved using an HP-5MS column (30 m×0.25 mm I.D.×0.25 µm film thickness; Agilent Co., Wilmington, DE) with ultra-pure helium as a carrier gas at a flow rate of 1.8 ml/min and an average velocity of 45 cm/s in the constant flow mode. Temperature at the injection port, MS interface, electron impact source and Mass filter was set constant at 320°C, 280°C, 230°C and 150°C, respectively. Samples (3 µl) were injected in the splitless mode, with the valve on for 6 s. Oven temperature was set at 50°C for 2 min and increased to 240°C at the rate of 30°C/min, and then the temperature was increased to 280°C at a rate of 15°C/min to elute any possible high boiling point interfering impurities. Total run time was estimated at 15 min and post-run time at 9 min at 275°C. The MS analyzer was set in the selected ions monitoring (SIM) mode. The area ratio of selected ions at m/z 245 for fentanyl and m/z 289 for sufentanil was used for

Fig. 2. Selected ion profile of fentanyl and sufentanil

quantitation. Selected ion profiles of fentanyl and sufentanil are shown in Fig. 2.

Fentanyl Adsorption to Glass Surfaces

It has been reported previously that fentanyl detection levels under the same experimental conditions are not always reproducible (29). A possible explanation for this is the potential for fentanyl to bind to active sites on glass surfaces resulting in a considerable loss of fentanyl, and consequently a decrease in the sensitivity and reproducibility of the GC-MS method. To solve this problem, silicone deactivation of all glass surfaces (including sample vials and the GC glass injection port liner) was carried out utilizing SurfaSil™ siliconizing fluid. SurfaSil™ reacts directly with the polar groups on the glass surfaces, and results in the formation of a hydrophobic coating that precludes nonspecific binding. It also makes glass surfaces slippery by covalently attaching a silane chain to render the glass surface inert.

Prior to use, glassware was prepared by the following procedure: (1) The SurfaSil™ fluid was diluted in methylene chloride to 5%. (2) The glassware was then completely immersed in the SurfaSil™ solution for 5–10 min allowing a thin film to immediately coat the glass surface. (3) The glass apparatus was then removed and rinsed with methylene chloride. (4) The glass apparatus was rinsed with methanol to prevent interaction of the SurfaSil™ coating with water. (5) The glass apparatus was finally oven-dried at 100°C for 45 min.

Preparation of Standards

A stock solution of 10 µg/ml was prepared by dissolving 5 mg of fentanyl in 500 ml of GC-grade methanol. Working range concentrations of 1 to 1500 ng/ml were made by serial dilution of the stock solution in methanol. Aliquots of these standard solutions were added to blank plasma to study the extraction recovery of fentanyl. Methanol was chosen as the extraction solvent because of the high solubility of fentanyl and sufentanil in this solvent. Solutions were kept protected from light and stored refrigerated until analyzed. Sufentanil,

the internal standard, was chosen due to its structural similarity to fentanyl, and because it was readily available as a pure crystalline solid. A 10 µg/ml stock solution of sufentanil was prepared in methanol and a 100 µl aliquot part was added to plasma samples.

Standard curves were prepared with plank plasma samples spiked with fentanyl to cover the desired concentration range. Calibration curves were obtained by plotting fentanyl concentration against the peak-area ratio of fentanyl/sufentanil. The concentration of unknown fentanyl samples was determined by utilizing the linear regression line of the standard curve.

Preparation of Samples

Blood was collected from rabbits in 3 ml tubes containing 7.5% EDTA and centrifuged at 600×g-force for 15 min to remove blood cells and other insoluble material. Samples were immediately placed on ice, and plasma was separated by centrifugation within 1–2 h of blood collection. The plasma was decanted into silanized tubes, which were kept frozen at –20°C until assay.

Individual plasma samples (0.5 ml) were each transferred to a 2 ml silanized Eppendorf tube, and 100 µl of sufentanil (IS) was added to the sample. Fentanyl was extracted with 1 ml of acetonitrile: 1-chlorobutane (1:1 v/v) by vortex-mixing for 5 min. After centrifuging at 600×g-force for 5 min, the upper organic layer was decanted and evaporated to dryness using a gentle stream of nitrogen. The extraction residue was reconstituted in 100 µl methanol followed by vortex-mixing for 5 min and sonication for 1 min; a 3 µl volume was then injected into the GC-MS system.

In vivo Sublingual Study

Male New Zealand white rabbits (3.5±0.5 kg) were used to study the bioavailability of sublingually administered fentanyl by comparison with i.v. administered fentanyl (*n*=3 rabbits/formulation per route). Rabbits were reused again after 2 weeks waiting period. All surgical procedures were performed under isoflourane general anesthetic gas. Follow-

ing induction of anesthesia, a catheter was placed in the marginal ear vein for blood sample collection. After the collection of each blood sample, the cannula was flushed with 0.1 ml of 10% (v/v) heparin/normal saline solution to keep the cannula patent.

Two formulation solutions were tested *in vivo*, which contained either fentanyl free base or fentanyl citrate salt (0.5 mg/ml fentanyl free base equivalent) together with propylene glycol (6%), absolute alcohol (40%), and deionized water.

Upon induction of anesthesia, the fentanyl dose (50 µg) was applied to the sublingual mucosa of the rabbit through a spray bottle (0.1 ml). The same protocol was followed for the intravenous delivery of fentanyl, where the dose was delivered as a bolus through the cannulated ear vein of the rabbit. Aliquots of 1 ml blood samples collected in tubes containing 7.5% EDTA were obtained at 5 min before drug delivery, and then at 5, 10, 20, 45, 60, 90, and 120 min following fentanyl administration. Blood samples were immediately placed on ice. Plasma was separated by centrifugation at 3,000 rpm for 10 min, placed in polypropylene tubes, and frozen at -20°C until the time of analysis.

New Zealand rabbits were used as the animal model due to the similarity of the sublingual mucosa composition to that of humans. After recovering from anesthesia, animals were returned to their cages. All the animal work was conducted at the University of Kentucky Chandler Medical Center, Division of Laboratory Animal Resources (DLAR), and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Pharmacokinetic Analysis

Concentration-time profiles of fentanyl after i.v. and sublingual administration of the two spray formulation solutions were evaluated by a non-compartmental model (WinNonlin Professional, version 4.1, Pharsight Corporation, Mountain view, CA). Pharmacokinetic parameters, such as terminal elimination half life ($t_{1/2}$), and area-under-the-curve from 0 to infinity ($AUC_{0-\infty}$) were estimated using this software.

After a single dose, maximum plasma concentration (C_{max}), and time to reach maximum concentration (T_{max})

were also determined. The absolute bioavailability of the fentanyl spray formulation was calculated from Eq. 1:

$$F = ((AUC_{SL}/AUC_{IV}) \times (DOSE_{IV}/DOSE_{SL})) \times 100 \quad (1)$$

where F is the percent absolute bioavailability, and AUC_{SL} , AUC_{IV} , $DOSE_{IV}$, $DOSE_{SL}$ are the areas-under-the-curve for the sublingual and i.v. administrations and the corresponding doses for these routes of administration, respectively. Data were evaluated utilizing a two-tailed t test statistical analysis.

RESULTS

Method Validation

To establish the validity of the method, the following analytical parameters were investigated: selectivity, linearity, limit of quantitation, accuracy, precision, recovery and stability of the analytes.

Blank plasma samples were tested for endogenous interferences, and the anticipated fentanyl and sufentanil retention time regions were shown to be free from interference from endogenous components in the plasma sample. The chromatographic conditions for the GC-MS method afforded a well-defined separation of drug, internal standard, and endogenous components. Typical chromatograms are shown in Fig. 3; retention times for fentanyl and sufentanil were 13.2 and 14.0 min, respectively.

Linearity was defined from seven calibration standard solutions ranging from 1 to 1,500 ng/ml. The resulting calibration curve for fentanyl in rabbit plasma was linear from least square linear regression analysis with a correlation coefficient (r^2) of greater than 0.99.

A limit of quantitation (LOQ) of 4 ng/ml for fentanyl in rabbit plasma was obtained. At the LOQ, the RSD ($n=3$) of the measured concentration was 9.4% and the accuracy of measurement was 7.2%. Based on a signal-to-noise ratio of 3, a limit of detection (LOD) of 1 ng/ml was achieved.

The accuracy of the method was determined through the back-calculations of three plasma samples spiked with fentanyl to afford concentration of 1, 100 and 500 ng/ml; the experimentally determined values were within 10.5% of the theoretical values. Precision was determined from repetitive

Fig. 3. Representative chromatogram of extracted fentanyl and sufentanil in rabbit plasma

Fig. 4. Fentanyl plasma concentration *versus* time after i.v. injection and sublingual spray administration of the two spray formulations containing a 50 µg dose in the rabbit

analysis of plasma samples spiked with 1, 100, and 500 ng/ml fentanyl, and was below an RSD of 7%.

Compared to other solvents, 50:50 *v/v* 1-chlorobutane: acetonitrile provided the maximal extraction efficiency. The absolute extraction efficiency of fentanyl was determined by comparing the final extracted fentanyl concentration to that of the initially spiked plasma. An average extraction recovery of at least 70% was achieved for all three fentanyl QC concentrations (i.e. 1, 100, and 500 ng/ml). The overall mean recovery of sufentanil was 77%. Stability tests carried out on fentanyl stock solutions in plasma and on the extracted samples were determined to be stable at 4°C for at least 3 days.

Sublingual Delivery of Fentanyl

Figure 4 illustrates the mean plasma concentration *versus* time curves for fentanyl obtained after i.v. injection and sublingual spray administration of the two spray formulations containing a 50 µg dose in the rabbit. Following i.v. injection, the disposition of fentanyl was best described by a semi-log curve, with a terminal elimination half-life of 20 ± 1.9 min.

The concentration-time profiles were analyzed by a non-compartmental method, the pharmacokinetic parameters of the two sublingual formulations; i.e. formulation no. 1 (fentanyl SL base) and formulation no. 2 (fentanyl SL salt), together with the i.v. fentanyl pharmacokinetics are presented in Table I.

Following sublingual spray administration, the formulation containing fentanyl free base showed superior bioavailability over the fentanyl citrate salt formulation (i.e. at least 30% greater bioavailability) with a higher $C_{\max}$ and an almost

identical $T_{\max}$ (20 min). The bioavailabilities of the fentanyl free base and fentanyl citrate formulations were summarized in Table I.

Although the fentanyl citrate sublingual spray formulation showed acceptable bioavailability, it was found that the fentanyl free base formulation, with 20% higher alcohol content, had superior physico-chemical properties, making the free base an attractive choice for sublingual dosage form development.

DISCUSSION

The described GC-MS method has the advantage of using one calibration curve for both low and high concentrations of fentanyl. Furthermore, extraction is a one-step procedure that is quick and has high degree of recovery of the analytes. The validation results indicate that the method is sensitive, specific, and reliable for the quantitation of high number of plasma samples.

Considering the shortcomings of the approaches currently available for administration of fentanyl to patients, we have shown in a rabbit model that a sublingual dosage form provides a rapid and reproducible onset of action that may be convenient for use by patients. This dosage form, being a homogeneous solution, allows for rapid absorption of the active ingredient, so as to avoid the potential intra- and inter-individual variability resulting from swallowing an oral dosage form.

The terminal half-life of fentanyl after i.v. administration was found to be close to the terminal half-life after sublingual administration. This result implies that there is a relatively fast absorption phase, since the existence of flip-flop kinetics was not evident. Furthermore, the time to reach the maximum

Table I. Pharmacokinetic Parameters Following i.v. and Sublingual Spray Administration of 50 µg Fentanyl Equivalent Dose of Formulation No. 1 and Formulation No. 2 in Rabbits ($n=3$), Mean $\pm$ SD

Parameter	Formulation No. 1	Formulation No. 2	Intravenous
$C_{\max}$ (ng/ml)	440 ± 103	366 ± 63	730 ± 98^a
$t_{\max}$ (min)	20.0 ± 1.9	20.0 ± 0.9	Not applicable
$t_{1/2}$ (min)	57.0 ± 2.3	33.0 ± 1.9	43 ± 3.9
$AUC_{0-\infty}$ ($\mu\text{g min}^{-1} \text{ml}^{-1}$)	32.7 ± 0.1	23.8 ± 0.5	40.6 ± 0.9
Absolute Bioavailability (%)	82.0 ± 2.1	58.5 ± 3.3	100

^a Extrapolated concentration at time 0

concentration ($T_{\max}$) was 20 min after application of either of the spray formulations to the rabbit sublingual mucosa. This short sublingual $T_{\max}$ value indicates that a sublingual spray formulation of fentanyl will likely afford a rapid onset of action in the treatment of breakthrough pain.

It is also likely that the fraction of fentanyl free base dose that is swallowed during sublingual delivery is smaller when compared to other mucosal delivery systems. This was further supported by calculating the area under the plasma concentration–time curves (AUC) and comparing the data with the pharmacokinetic data from intravenous fentanyl administration. Based on this comparison, at least 70% of the sublingual dose administered reached the systemic circulation.

The stability of the formulated spray solutions was also determined. Both formulations were found to be stable for at least 6 months when stored in a tightly closed, silanized glass container, protected from light, at room temperature.

The sublingual spray formulation described in this study may also be useful for the delivery of drugs other than fentanyl, where a rapid onset of action is desired. However, the spray formulation would probably be less useful for hydrophilic drug molecules, since this class of drug will not undergo sufficiently rapid absorption across the sublingual mucosa.

CONCLUSIONS

In summary, a simple, selective, accurate and precise method for the analysis of fentanyl in rabbit plasma has been developed and partially validated over the concentration range 1–1,500 ng/ml. The method requires only 0.5 ml of plasma with good extraction efficiency. The method was successfully applied to the determination of fentanyl content in more than 400 plasma samples.

Sublingual spray administration of fentanyl afforded a promising pharmacokinetic profile, resulting in rapid absorption with good bioavailability, suggesting the potential for a new approach to the treatment of breakthrough pain. This route may represent a suitable alternative route of delivery for fentanyl in clinical practice. Further studies are needed to fully evaluate the clinical potential of this novel route of delivery for fentanyl.

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