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Determination of remifentanil, an ultra-short-acting opioid anesthetic, in rat blood by high performance liquid chromatography with ultraviolet detection[1]

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Abstract

Remifentanil is an ultra-short-acting opioid under evaluation for use during surgical procedures which require opioid analgesia or anesthesia. It has an N-substituted methyl propanoate ester group which is highly susceptible to cleavage by blood and tissue esterases as well as to chemical hydrolysis. A selective and specific high performance liquid chromatography method was developed to quantitate remifentanil in rat blood. A liquid–liquid extraction method using *n*-butyl chloride was used to separate interfering endogenous products from the compound of interest. Reverse phase chromatography with ultraviolet (λ 210 nm) detection was used to quantitate the eluate. The calibration curves were found to be linear in the range 2.5–250 ng ml^{-1}. Intra-day assay variability was 15% or less for all standards evaluated. The method was applied to blood samples collected from a short-term infusion study in rats. Good recovery, linearity, accuracy and precision were achieved with the assay.

Keywords: Esterase metabolism; Rat blood; Remifentanil; UV-HPLC

1. Introduction

Remifentanil is an ultra-short-acting opioid anesthetic agent [1–4]. It is a 4- anilinopiperidine derivative and contains an N-substituted ester group which is the site of metabolism by blood and tissue esterases as well as chemical hydrolysis. Remifentanil is structurally similar to other 4-anilinopiperidine derivatives including fentanyl. A number of analytical methods have been developed to quantitate fentanyl and related agents in biological matrices including HPLC [5,6], radio immunoassays [7], radioreceptor [8] as well as gas chromatography–mass spectrometry methods [9,10].

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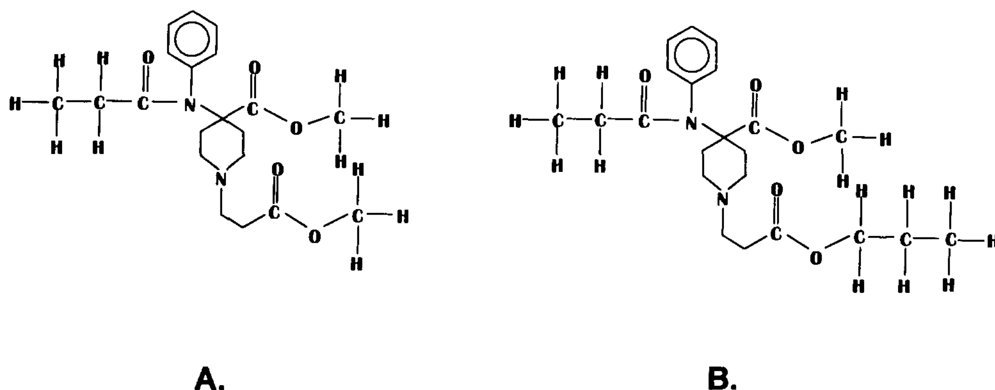

Fig. 1. Chemical structures of (A) remifentanil and (B) the internal standard.

Due to its N-substituted methyl propanoate ester group, remifentanil has been reported to be cleared rapidly in dogs (3.8–8.3 min) [3] and humans (8–20 min) [11]. To evaluate the pharmacokinetics, a GC-HRMS-SIM (high resolution mass spectrometry-selected ion monitoring) [12] and an UV-HPLC method [13] have been previously developed to quantitate remifentanil in human and dog blood. However, there are currently no analytical methods for the quantitation of remifentanil in rat blood. Pharmacokinetic studies in small animals require limited sample volumes. Therefore, to quantitate remifentanil in rat blood it is imperative that a highly sensitive and specific method be developed. The objective of this study was to develop a simple, rugged, sensitive analytical method applicable for the analysis of remifentanil in rat blood.

2. Experimental

2.1. Materials and reagents

Remifentanil hydrochloride and the internal standard (GI97559) were donated by Glaxo-Wellcome Inc. (Research Triangle Park, NC). The structures of remifentanil and the internal standard are shown in Fig. 1. Citric acid was purchased from Sigma Chemical Co. (St Louis, MO), and sodium phosphate monobasic was obtained from Fisher Scientific (Fair Lawn, NJ). Acetonitrile, triethylamine and *n*-butyl chloride were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). All chemicals and solvents were ACS analytical grade or HPLC grade. Deionized water was prepared by an ultrapure water system Pyrosystem Plus® (Hydro, Research Triangle Park, NC).

2.2. Instrumentation

The blood samples were analyzed by an HPLC system consisting of a Waters 6000A solvent delivery system (Waters Associates Inc, Milford, MA), a Perkin-Elmer ISS-100 automatic injector (Perkin-Elmer Corp., Analytical Instruments, Norwalk, CT) and a Waters variable wavelength ultraviolet detector ($\lambda = 210$ nm). The detector was coupled with a HP 3394 integrator plotter (Hewlett-Packard Co., Avondale, PA). A high-ligand-density reverse phase C_8 column (15 cm × 0.46 cm, 5 μm; Burdick & Jackson Division, Baxter, Muskegon, MI) and a guard column (Supelco Inc., Bellefonte, PA) containing the same bonded phase were used for the separation of remifentanil and the internal standard.

The buffer component of the mobile phase was prepared with deionized water and the pH was adjusted to 3.5 using concentrated phosphoric acid. Acetonitrile and triethylamine (300 μl l^{-1}) were added to the buffer. The final mobile phase consisted of acetonitrile and 0.05 M NaH_2PO_4 buffer (27:73, v/v). The mobile phase was filtered through a 0.45 μm nylon filter and degassed under ultra-sound and vacuum for 15 min. Sparging with helium gas was used to maintain the

mobile phase in a degassed state. The mobile phase was delivered at a flow rate of 1.5 ml min^{-1}.

2.3. Preparation of the standards

Rat blood containing citric acid as an anti-coagulant was purchased from Hilltop Laboratories (Scottsdale, PA) to be used for preparation of standards. The blood standards (0.4 ml each) contained remifentanil at the following concentrations: 0, 2.5, 5, 25, 125 and 250 ng ml^{-1}. Freshly prepared remifentanil stock solutions in 0.01 M HCl with concentrations of 20, 200 and 1000 ng ml^{-1} were used as spiking solutions. Appropriate volumes (50–100 μl) of the stock solutions were added to each blood sample (0.4 ml) to obtain the desired concentration. Within seconds of the addition of remifentanil, each blood sample was vortexed and then flash frozen with an acetone/dry ice bath. The frozen blood standards were then stored at −85°C until the time of analysis. The internal standard (GI97559) was prepared at a concentration of 1 μg ml^{-1} in 0.01 M HCl.

2.4. Extraction procedure

Blood samples or standards were removed from the freezer (−85°C) and allowed to partially thaw. 50 μl of internal standard solution was added to each sample followed by 1 ml of 1 M sodium phosphate buffer, pH 7.4. The samples were transferred to the cold room (4°C) and vortexed briefly before adding 5 ml of *n*-butyl chloride. A mechanical shaker (Eberbach Corp., Ann Arbor, MI) was used on "high" setting to mix the samples for 10 min. The samples were centrifuged for 2 min (3000 rev min^{-1}) and placed in the freezer (−85°C) for 5–10 min to freeze the aqueous phase. The organic phase was then decanted to 15 ml conical test tubes containing 250 μl of 0.01 M HCl. The samples were vortexed for 3 min, centrifuged for about 2 min (cold room) and the organic phase aspirated. The aqueous phase was transferred to micro-vials and 200 μl was injected onto the HPLC system.

2.5. Assay validation

2.5.1. Linearity and range

The linear detector response for the assay was tested as follows. Three determinations ($n = 3$) from a minimum of five concentration levels (2.5, 5.0, 25, 125 and 250 ng ml^{-1}) of the analyte were made. Detector response was correlated against analyte concentration by least-squares regression. A weight of $1/y$ was used to detemine slopes, intercepts and correlation coefficients. The minimum acceptable coefficient to establish linearity was set at 0.95 a priori.

2.5.2. Method precision and percent recovery

Precision of the assay was established by analysis of three replicates ($n = 3$) of a standard solution of the analyte at the following concentrations: 2.5, 5, 25, 125 and 250 ng ml^{-1}. To determine intra-day precision of the assay, replicate ($n = 3$) blood samples of five different concentrations were analyzed. To determine inter-day precision, replicate blood samples ($n = 3$) were analyzed on three different days. The precent relative standard deviaitons (RSDs) of the assay results were determined.

Extraction efficiency was determined by comparing replicate ($n = 9$) peak height ratios (PHRs) of extracted blood samples vs. unextracted water standards for the 2.5, 25 and 250 ng ml^{-1} concentrations. The percent recovery was determined by the following equation:

$$\%\ \text{recovery} = \frac{\text{PHR of blood standard}}{\text{PHR of water standard}} \times 100$$

Stability of remifentanil in blood at −85°C was evaluated by comparing the mean peak height of three 5 ng ml^{-1} standards which had been in storage for 4 months with the peak height of a freshly prepared standard run under identical assay conditions. The mean peak height of the stored standards was within 5% of the peak height of the freshly prepared standard. The 5 ng ml^{-1} standards were selected because stability problems would have been most apparent with the lower concentrations.

2.5.3. Accuracy

Method standards in the concentration range 2.5–250 ng ml^{-1} from three different runs performed over several days were used to check for accuracy. The means of the three runs were calculated and compared to the spiked value to determine the percentage difference between the mean and the spiked value (amount added). The percentage relative error was determined as

$$\% \text{ relative error (RE)} = \frac{[\text{mean} - \text{spiked}]}{[\text{spiked}]} \times 100$$

Between- and within-run accuracy were also determined.

2.6. Pharmacokinetic study

A study was designed to investigate the pharmacokinetics of remifentanil in Sprague Dawley rats. The protocol was approved by the Animal Research Committee at the University of Maryland at Baltimore, School of Pharmacy. A male rat (wt. = 325 g) was administered a 50 μg kg^{-1} min^{-1} intravenous infusion for 21 min via a jugular vein cannula. Serial blood samples (0.4 ml) were collected via a femoral artery cannula over a 25 min period. Blood samples were collected into test tubes containing 20 μl citric acid (50%). Samples were flash frozen and stored at − 85°C until assayed.

3. Results and discussion

3.1. Resolution

Figs. 2a and 2b represent chromatograms of extracted blank rat blood and a calibration standard containing remifentanil at 25 ng ml^{-1} respectively. Representative chromatograms of an extracted rat blood sample from the pharmacokinetic study are presented in Figs. 3a and 3b respectively. Fig. 3a illustrates a chromatogram of an extracted pre-dose rat blood sample and Fig. 3b displays a chromatogram from the 1.5 min sample (26 ng ml^{-1}) during the infusion. The assay was found to be specific for remifentanil and no interfering peaks from degradation products, citric acid, internal standard or blood were detected. Moreover, the peaks of remifentanil and the internal standard were sufficiently separated with typical retention times of 6 min for remifentanil and 10 min for the internal standard.

Retention times of 8.5 min for remifentanil and 13 min for the internal standard were reported in the remifentanil assay method by Selinger et al. [13]. The differences in retention times may be explained by column and mobile phase differences. For example, in the study reported by Selinger et al. [13] a Zorbax SB-CN (25 cm × 0.46 cm) column was used and the mobile phase components included methanol. Additionally, the column was kept at 30°C in a column heater. In this study, a high ligand-density C_8 column was used and the mobile phase only contained acetonitrile, buffer and triethylamine and the column was maintained at room temperature.

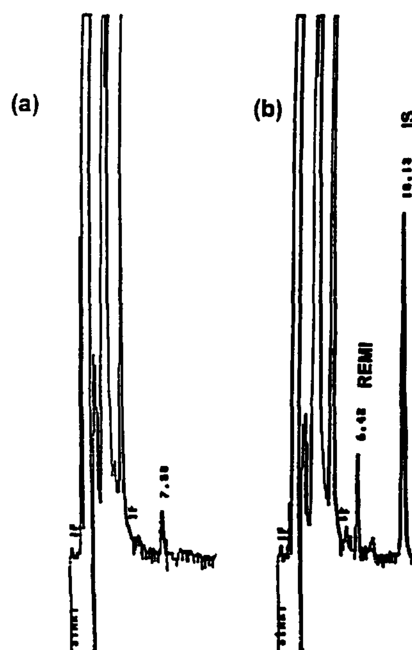

Fig. 2. Extracted rat blood chromatograms: (a) extracted blank rat blood; (b) extracted rat blood standard (25 ng ml^{-1}).

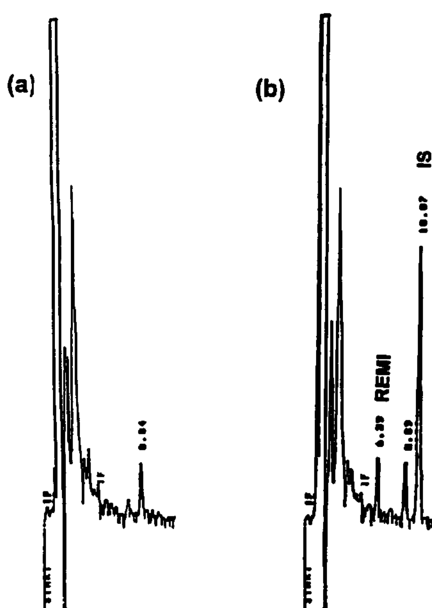

Fig. 3. Extracted rat blood chromatograms from pharmacokinetic study: (a) extracted rat blood pre-dose sample; (b) extracted rat blood 1.5 min sample (26 ng ml^{-1}).

3.2. Extraction recovery

After the comparison of the PHRs of extracted blood standards and water standards, the extraction recovery was found to be 88.2, 102 and 99.2% for the 2.5, 25 and 250 ng ml^{-1} standards respectively.

3.3. Validation assay precision

The standard curves for remifentanil showed linearity over the selected concentration range (2.5–250 ng ml^{-1}), with consistent slopes and excellent correlation coefficients ($r \geqslant 0.99$) throughout the validation runs.

The intra-day and inter-day precision data for remifentanil in rat blood are listed in Table 1. The intra-day %RSD was 15% or less for all standards whereas the inter-day precision was 21% or less. Table 2 summarizes the intra-day and inter-day accuracy data for remifentanil. The intra-day and inter-day accuracy, as indicated by RE, ranged from −8.2 to 18.0% for remifentanil. The precision of the assay, expressed as the %RSD, was

Table 1
Intra-day and inter-day assay precision for remifentanil in rat blood

Concentration (ng ml^{-1})	Precision (%RSD)	
	Intra-day	Inter-day
250.0	6.6	11
125.0	7.1	13
25.0	3.4	12
5.0	5.6	5.9
2.5	15	21

< 15% at all concentrations except at the lower limit of quantitation (2.5 ng ml^{-1}) where it went up to 21%.

A major challenge faced during the development of this assay was the inhibition of esterases in rat blood. The precise method of completely inhibiting the hydrolysis of remifentanil by esterases is unknown for rat blood. Selinger et al. [13] have developed a method whereby the metabolism of remifentanil by esterases is inhibited in human and dog blood. This is performed by lowering the pH of the blood through the addition of 20 μl of 50% citric acid to 1 ml of human or dog blood. This method was not adequate to stop the metabolism of remifentanil in rat blood. The addition of larger amounts of citric acid resulted in the precipitation of blood proteins. The highly rapid clearance of remifentanil from rat blood may be related to the presence of additional esterases which are not found in human or dog blood [14]. However, limiting processing time and adhering to the following

Table 2
Intra-day and inter-day assay accuracy for remifentanil in rat blood

Concentration (ng ml^{-1})	Accuracy (%RE)	
	Intra-day	Inter-day
250.0	2.9	2.5
125.0	−8.2	−7.3
25.0	−0.7	−2.8
5.0	11	−6.7
2.5	−18	3.6

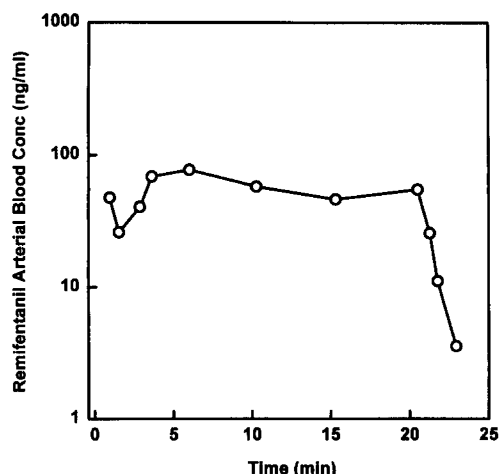

Fig. 4. Remifentanil blood concentration vs. time profile during and after an iv infusion of 50 μg kg^{-1} min^{-1} to a male rat.

steps should adequately control esterase and chemical hydrolyses: (1) limit the number of samples processed at a time (e.g. 6–8 samples); and (2) perform all steps of the extraction at 4°C.

3.4. Pharmacokinetic study

A blood concentration vs. time profile for remifentanil during and after a 50 μg kg^{-1} min^{-1} infusion to a male Sprague Dawley rat is illustrated in Fig. 4. This profile highlights the sensitivity of the assay as well as its utility since it is possible to follow the time course of remifentanil during and after the infusion. This is important in evaluating the pharmacokinetics of this agent since its elimination is extremely fast ($t_{1/2} < 1$ min) [15]. A sensitive and specific method has been developed for measuring remifentanil in rat blood. Sensitivity in critical in small rodent studies due to the limited blood volume of each sample. The limit of quantitation for this method was 2.5 ng ml^{-1} with recovery greater than 88% across all concentrations.

In summary, one of the major concerns with pharmacokinetic studies involving remifentanil is its highly rapid metabolism. Previous reports [1–3] have indicated that this agent is metabolized by esterases in the blood and other tissues. After blood sample collection it is imperative that enzymatic degradation of remifentanil is halted immediately. In this study, this was accomplished by flash freezing each sample followed by storage at −85°C. As a part of methods development as well as sample analysis it was imperative that all extraction steps be performed at 4°C. The accuracy and precision data show that the method reported herein is consistent and reliable with low values of relative error and RSD for standards over the entire concentration range (2.5–250 ng ml^{-1}) examined. This assay can be utilized for animal pharmacology studies aimed at defining the pharmacokinetic and pharmacodynamic (hemodynamic and EED) characteristics of remifentanil. Studies are in progress in this laboratory to evaluate the concentration-effect relationship of remifentanil and the possible interaction with other agents metabolized by esterases.

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