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Simultaneous solid-phase extraction combined with liquid chromatography with ultraviolet absorbance detection for the determination of remifentanil and its metabolite in dog plasma

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Abstract

To establish pharmacokinetic/pharmacodynamic relationships, a selective and specific high-performance liquid chromatographic method was developed for the quantitation of remifentanil and its metabolite in dog plasma. The assay involves a solid-phase extraction and a reversed-phase chromatographic separation with ultraviolet detection (λ=210 nm). The calibration curves are linear in the range of 7.89–1500 ng ml^{-1}. Intra-day assay variability is less than 7% for all standards evaluated. Good recovery, linearity, accuracy, and precision were achieved with the assay that proved readily applicable to pharmacokinetic studies in dogs.

Keywords: Remifentanil

1. Introduction

Remifentanil, methyl-3-{4-methoxycarbonyl-4-[(1-oxopropyl)-phenylamino]-1-piperidine}-propanoate (GI87084B), is an ultra-short acting μ-opioid anesthetic agent of the fentanyl class with potent analgesic activity (Fig. 1). It has an in vivo elimination half-life of 9 min in patients [1] which is much shorter than that of fentanyl, sufentanil and alfentanil (219, 164, and 94 min, respectively) [2]. Remifentanil is used extensively for short-term anesthesia, often in combination with sedatives [3]. Its labile methyl propanoate ester linkage is sensitive to enzymatic and chemical hydrolysis and rapidly forms the corresponding carboxylic acid (GI90291). This acidic metabolite has only 1/4500 the potency of the parent compound in animal models [4] and will be referred to as demethoxy-remifentanil. *N*-Dealkylation of remifentanil (GI94219) is a minor metabolic pathway in humans (Fig. 1).

Several analytical methods have been developed to quantitate remifentanil either alone or simultaneously with its major metabolite in whole blood. Among the methods are gas chromatography–mass spectrometry (GC–MS) [5,6], high-performance liquid chromatography (HPLC) [7,8], as well as tandem mass spectrometry [9]. In these methods, extraction procedures are mostly based on liquid extractions and are therefore time consuming. In addition, the only solid-phase extraction method reported does not allow the

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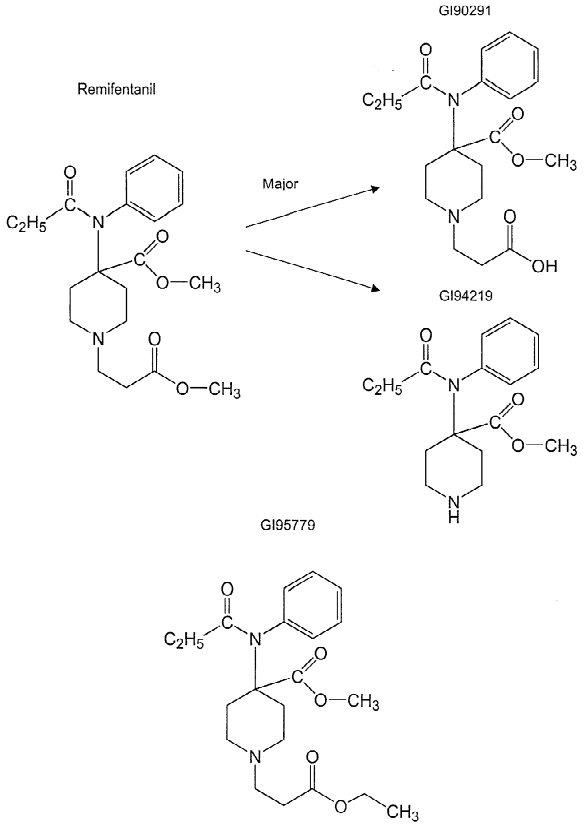

Fig. 1. The metabolic pathways of remifentanil are illustrated. Remifentanil is metabolized by ester hydrolysis, primarily to GI90291. Structure of GI95779, internal standard in HPLC assay. Reproduced with permission [3].

simultaneous extraction of both remifentanil and its major metabolite [4]. Considering the low potency of demethoxy-remifentanil, its contribution to the analgesic effect is not of pharmacological interest. However, in view of the importance of this metabolic pathway its determination will help understanding remifentanil body disposition.

Rapid hydrolysis of remifentanil in blood has always been a key argument for choosing this matrix for remifentanil analysis, avoiding the time needed to harvest plasma [7]. As the plasma-free fraction is responsible for the pharmacological effect, quantitation of remifentanil in plasma instead of whole blood would appear preferable when establishing concentration–effect relationships. In addition, remifentanil hydrolysis in whole blood is attributed to non-specific red blood cell esterases [10] whereas plasma pseudo-cholinesterases are not involved [11]. For both these reasons, it seemed more appropriate to develop an analytical method to quantitate remifentanil in plasma.

The primary objective of this study was to develop a single procedure based on solid-phase extraction for the rapid determination of both remifentanil and its major metabolite in plasma. For that purpose, stability studies were conducted to document the ex vivo rate of degradation of remifentanil in whole blood. Finally, the applicability of the method to pharmacokinetic studies was verified, using the dog as an animal model.

2. Experimental

2.1. Materials

Remifentanil hydrochloride, demethoxy-remifentanil, and the internal standard (I.S.) (GI97559) were kindly provided by Glaxo-Wellcome (Stevenage, UK). Citric acid was purchased from ACP Chemicals. Potassium phosphate monobasic was obtained from American Chemicals (Montreal, Canada). Concentrated phosphoric acid and potassium phosphate dibasic were purchased from Fisher Scientific (Montreal, Canada). Acetonitrile and methanol were purchased from Anachemia Canada (Montreal, Canada). All solvents were of HPLC grade, and all other chemicals were of analytical grade.

2.2. Instrumentation

The HPLC system consisted of a Constametric 4100 pump, a Spectrasystem autosampler (Thermo Separation Products, Riviera Beach, FL, USA) and a variable-wavelength SM4000 UV detector set at 210 nm (Milton Roy LDC Division, Riviera Beach, FL, USA). Peak integration was performed using the Star Chromatography Varian Software version 4.51 (Walnut Creek, CA, USA). Separation of remifentanil and its metabolite was performed on a 5 μm reversed-phase Spherisorb C_1 column (150×4.6 mm I.D.), equipped with a C_1 guard column (4×3 mm I.D.), both purchased from Phenomenex (Torrance, CA, USA) and preceded by a 0.45-μm in-line filter (State College, PA, USA). The solvent flow-rate was set at

1.5 ml min^{-1} with a resulting backpressure of 1700 p.s.i. (1 p.s.i.=6894.76 Pa). The chromatographic system was operated at room temperature (22 °C).

2.3. Mobile phase

The potassium phosphate buffer (50 m*M*) component of the mobile phase was prepared with deionized water and the pH was adjusted to 3 using concentrated phosphoric acid. The mobile phase was prepared daily by adding 180 ml of acetonitrile, 120 ml of methanol and 48 ml of phosphate buffer into a 1 l volumetric flask. The flask was then completed to volume with water. The solution was stirred and filtered through a 0.2-μm nylon filter (Millipore, Waters, Milford, MA, USA).

2.4. Standard solutions

Pure standard stock solutions of remifentanil and demethoxy-remifentanil (1 mg ml^{-1}) were prepared in 50 m*M* phosphate buffer (pH 3) and stored at 4 °C. Working solutions (100 000 and 10 000 ng ml^{-1}) were made by further dilution in the same buffer.

A standard stock solution of the internal standard (1 mg ml^{-1}) was prepared in phosphate buffer (pH 3) and further diluted in the same medium to yield the working solutions (100 000 and 800 ng ml^{-1}). Working solutions of the I.S. were stored at 4 °C and were stable for at least 6 months (data not presented).

2.5. Remifentanil ex vivo stability in blood

Fresh dog blood aliquots (20 ml) were spiked with remifentanil at a concentration of 1000 ng ml^{-1} and were kept on ice for 15 min. Each aliquot was previously neutralized with 5 mg ml^{-1} of citric acid and/or 40 μg ml^{-1} of echothiophate, and was kept on ice for 4 h. A control aliquot containing neither citric acid nor echothiophate was prepared. Samples were collected at 0, 30, 120 and 240 min, centrifuged (2000 *g* for 15 min) and stored at −70 °C until analysis. Control samples and samples containing echothiophate were acidified with citric acid (5 mg ml^{-1}) prior to storage.

To test the ex vivo stability, a fresh dog blood aliquot (20 ml) previously maintained at 37 °C was spiked with remifentanil at a concentration of 1000 ng ml^{-1}. The aliquot was immediately placed on ice and the temperature was measured regularly. Blood samples were collected and treated as described above.

2.6. Calibration curves

Blank dog plasma from dogs used for the preparation of standards and quality control (QC) samples contained citric acid (5 mg ml^{-1}, pH 5) to prevent the degradation of remifentanil. Remifentanil and demethoxy-remifentanil solutions at concentrations of 10 000 and 100 000 ng ml^{-1} were used as working solutions. Appropriate volumes of the working solutions were added to stabilized plasma to yield both standards of 1500 and 1000 ng ml^{-1}. The pool of plasma containing 1000 ng ml^{-1} of each analyte was then serially diluted (1:1) with drug-free plasma to yield plasma standards containing remifentanil and demethoxy-remifentanil at the following final concentrations: 500, 250, 125, 62.5, 31.25, 15.62 and 7.89 ng ml^{-1}. Very low, low, medium, and high QC samples containing remifentanil and its metabolite at concentrations of 20, 40, 200 and 1250 ng ml^{-1}, respectively, were prepared. Following a bolus dose of 5 mg kg^{-1} of remifentanil in vivo, plasma concentrations are expected to rise well above 1500 ng ml^{-1} after. Therefore, the technique used to dilute the samples was validated. QC samples at concentrations of 10 000 ng ml^{-1} were diluted 1:10 and were analyzed for precision and accuracy. After mixing, 0.7 ml aliquots of standards and QC samples were dispensed into polypropylene tubes and stored at −70 °C until analysis.

2.7. Extraction procedure

Plasma samples, standards and QC samples were allowed to thaw at room temperature. All samples were centrifuged at 4 °C (2000 *g* for 15 min) prior to manipulation. Bond Elut phenyl solid-phase extraction cartridges (Varian, Harbor City, CA, USA) were used for the extraction of remifentanil and its metabolite. GI97559 (120 ng ml^{-1} plasma) was used as internal standard. The cartridges were conditioned with a sequence of methanol (1 ml) and phosphate

buffer, pH 3 (1 ml). Volumes of 0.5 ml of plasma and 0.75 ml of the I.S. (800 ng ml^{-1}) were sequentially added to the cartridges and allowed to be adsorbed on the column packing before applying the vacuum. For the highest plasma concentration, samples (50 μl) were diluted 1:10 with blank plasma (450 μl) directly onto the solid-phase extraction cartridges. The cartridges were then washed successively with 1 ml of phosphate buffer, pH 3 and 1 ml of acetonitrile under a weak vacuum (≤50 kPa). Finally, the analytes were eluted with 1 ml of methanol into glass tubes. The eluent was evaporated to dryness using a Speed-Vac concentrator (Model SC210A, Savant Instruments, Farmingdale, NY, USA). The dry residues were reconstituted in 0.2 ml of mobile phase. Volumes of 50–100 μl were injected into the chromatographic system.

2.8. Assay validation

2.8.1. Recovery

The recovery of remifentanil and demethoxy-remifentanil from plasma was evaluated in quadruplicate at two different drug concentrations (40 and 1250 ng ml^{-1}). Blank plasma spiked with known amounts of analytes and 0.75 ml of I.S. (800 ng ml^{-1}) was extracted and compared with blank plasma and I.S. extracts subsequently spiked with the same amounts of analytes. The two sets of extracts were then evaporated to dryness using the Speed-Vac system and reconstituted in mobile phase. The recovery was assessed by comparing the analyte/I.S. peak-height ratios for both sets of extracts.

2.8.2. Linearity and limit of quantitation

Calibration curves were generated from the nine standards using remifentanil or GR90291/I.S. peak-height ratio. Linearity was assessed by weighted least square regression ($1/x^2_{observed}$) of the analyte/internal standard peak-height ratio against standard concentration.

The limit of quantitation was defined as the lowest plasma concentration of the standard curve. In order to estimate the lower limit of detection at a signal-to-noise ratio of 5, a 1000 ng ml^{-1} solution of remifentanil and its metabolite was serially diluted in the mobile phase.

2.8.3. Reproducibility

Inter-assay precision and accuracy were determined using QC samples spiked with four different concentrations of remifentanil. The concentrations used were, 20, 40, 200 and 1250 ng ml^{-1} for the very low, low, medium, and high levels of the calibration range, respectively. Two replicates of each QC concentration were assayed over 6 consecutive days. The QC concentrations were determined from six different calibration curves which were run along with the QC samples. Precision was expressed as the relative standard deviation (RSD) and accuracy was measured as the bias (%).

Intra-assay precision and accuracy were determined in the same manner as the inter-assay, except that four replicates of each QC concentration (very low, low, medium, and high) were assayed. Their corresponding back-calculated concentrations were extrapolated from a single calibration curve which was run on the same day.

Inter-assay and intra-assay precision and accuracy were determined similarly for demethoxy-remifentanil at three different concentrations: 40, 200 and 1250 ng ml^{-1} for the low, medium and high QC, respectively.

2.8.4. Statistical analysis

Statistical analysis includes calculation of the mean, standard deviation, RSD (% precision) and bias (% accuracy) for the QC samples. The mean standard deviation and RSD are also calculated for the slope (m), intercept (b) and coefficient of determination (r^2) obtained from each curve.

2.9. Pharmacokinetic study

The plasma concentration–time profile of remifentanil was determined after administration of a 4.4 mg kg^{-1} intravenous dose of the drug in an anesthetized dog. Arterial blood samples (3 ml) were collected prior to remifentanil administration and then at 2, 5, 10, 15, 20, 30, 45, 60, 80, 100 and 120 min post dose in tubes containing 20 units of heparine and 30 μl of citric acid (50%). Samples were kept in an ice-water bath for less than 1 h before centrifugation. Centrifugation was applied at 2000 g for 15 min and the

plasma was immediately stored at −70 °C until analysis.

3. Results and discussion

Remifentanil's poor stability has always been an argument put forth against choosing whole blood as the assay matrix. It was considered that the time required for plasma separation from whole blood prolonged exposure of the ester to conditions favoring hydrolysis. However, in pharmacokinetic/pharmacodynamic relationship studies, we usually consider that only plasma concentrations are accessible to the bio-phase.

In order to develop an analytical method using plasma as the biological matrix, we first tested the ex vivo stability of remifentanil in fresh blood to ensure adequate handling of samples and optimal conditions for the assay. Blood aliquots were kept on ice and contained either citric acid, echothiophate, or both stabilizing agents. The citric acid concentration required for stabilization was found to be twofold lower than previously recommended [7]. At higher concentrations of citric acid (10 mg ml^{-1}), blood hemolysis resulted in plasma contamination by red blood cell constituents or active non-specific esterases. It was previously shown that human blood samples preserved with citric acid (10 mg ml^{-1}) could be safely stored on ice for 20 h, although for some reason this time interval was considered too short for adequate sample handling [7]. Our results show that remifentanil is stable for up to 4 h when kept on ice either in the presence of a stabilizing agent or not (Table 1). Citric acid or an esterase inhibitor like echothiophate are therefore not required to maintain remifentanil integrity when samples are kept on ice for at least 4 h, which, in our opinion, allows enough time to separate plasma from blood. The ex vivo remifentanil stability was also tested by spiking a blood aliquot pre-equilibrated at 37 °C with remifentanil and subsequently placing it on ice. Approximately 20 min were required for the sample to cool down to 5 °C, which did not alter remifentanil integrity (Table 1). In contrast to other methods, I.S. stability was not a concern because plasma samples were only acidified and frozen as such.

Since most of the procedures described for remifentanil and demethoxy-remifentanil extraction were based on liquid–liquid extractions, we first attempted to find an organic solvent that would show a great affinity for both compounds. Several studies described remifentanil extraction from blood using *n*-butyl chloride followed by a back extraction in 0.01 *M* HCl [7]. The recovery of remifentanil by this method was shown over 88%, however the major metabolite could not be extracted under these conditions. We therefore tried methylene chloride, a more polar solvent commonly used for the simultaneous extraction of remifentanil and demethoxy-remifentanil [5,6] but the recovery of the metabolite was also found to be low (50%). We then tried solid-phase extraction of the analytes from plasma. Hoke et al. used C_8 cartridges for the solid-phase extraction of remifentanil from blood and demethoxy-remifentanil from plasma [4]. With this procedure, washing with acetonitrile resulted in a very low recovery of remifentanil and its metabolite. Since a final wash with an organic solvent was deemed

Table 1
Stability of remifentanil (1000 ng ml^{-1}) added to fresh dog blood containing citric acid (5 mg ml^{-1}) and/or echothiophate (40 μg ml^{-1})

Lapsed time (min)	Stability on ice				Ex vivo stability* Control
	Citric acid	Echothiophate	Citric acid+Echothiophate	Control	
0	100	100	100	100	100
30	113 (3)	111 (3)	122 (6)	102 (2)	102 (6)
120	111 (3)	108 (3)	117 (1)	105 (3)	102 (7)
240	112 (4)	102 (2)	118 (2)	96 (4)	94 (5)

The results are presented as mean percent of baseline value with standard deviation in parentheses ($n=3$).
*Remifentanil was spiked in a blood aliquot maintained at 37 °C. The aliquot was subsequently placed on ice.

necessary to maintain a clean chromatographic separation (essentially between the demethoxy-remifentanil and the solvent front), we considered the use of columns having more polar functional groups i.e., C_1 and C-phenyl. Both supports provided an excellent compromise between retention and elution parameters, but the latter yielded cleaner plasma extracts. Under these conditions, the recovery of remifentanil was complete (106–101%) and reproducible (RSD under 8%) for both concentrations (40 and 1250 ng ml^{-1}) (Table 2). Although a lower recovery was obtained for the metabolite (Table 2), it was quite acceptable (76.8–80.0%) and reproducible (RSD under 6%) for both QC samples. The overall recovery of the I.S. was estimated to be 80% (data not shown).

Fig. 2 shows representative chromatograms of blank dog plasma unspiked (A) or spiked (B) with remifentanil and demethoxy-remifentanil. Good resolution of remifentanil from its metabolite and I.S can be seen. The mean retention times of demethoxy-remifentanil, remifentanil and the I.S. ranged from 3.7–4.5, 8.3–9.4 and 9.8–11 min, respectively. The total time for a chromatographic run, including the time required for re-equilibration, was less than 15 min. Peaks of interest were free of any interfering peak with the exception of the metabolite, where a small peak was constantly observed and was attributed to co-extracted endogenous substances. The actual peak height of demethoxy-remifentanil was determined by introducing a valley-baseline event in the automatic integrator. This represents a time window within which all valley points are forced as baseline points for peak integration. This technique yielded excellent precision.

The on-column limits of detection were 0.5 and 0.2 ng ml^{-1} for remifentanil and its metabolite, respectively. The lower limit of quantitation in plasma was 7.89 ng ml^{-1} for remifentanil and 15.62 ng ml^{-1} for demethoxy-remifentanil. The corresponding ratios are more than three times the intercept value, which represents the biological matrix value. The sensitivity of our method is comparable to that obtained by Selinger et al. using an HPLC method with UV detection after liquid–liquid extraction [7]. Under the conditions used in their study, the validated analytical ranges for remifentanil were 10 to 60 135 ng ml^{-1}, 1 to 200 ng ml^{-1} and 2.5 to 250 ng ml^{-1} in dog, human and rat blood, respectively. Obviously, GC coupled with mass spectrometry [5,6] and LC–MS–MS [9] could provide greater sensitivity for both remifentanil [lower limit of quantitation (LLOQ)=0.1 ng ml^{-1}] and demethoxy-remifentanil (LLOQ=1 ng ml^{-1}) determination in human blood. In our opinion, our method could easily be coupled to high-resolution mass spectrometry to meet the sensitivity requirements of clinical studies.

Table 2
Recovery of remifentanil and demethoxy-remifentanil from dog plasma

	Remifentanil		Demethoxy-remifentanil	
Concentration (ng ml^{-1})	40	1250	40	1250
Mean recovery (%)	106	101	77	80
SD	3.7	7.2	3.9	3.6
RSD (%)	3.5	7.1	5.0	4.4
n	4	4	4	4

The intra-assay precision and accuracy were less than 13% for the four remifentanil QC concentrations (Table 3) and less than 8% for the three demethoxy-remifentanil QCs (Table 3).

The mean parameters for the calibration curves are shown in Table 4. Calibration curves for each analyte were linear over a wide concentration range. However, because of the interfering endogenous peaks, at least one concentration of the demethoxy-remifentanil was excluded in all calibration curves. Despite this exclusion, the calibration curves still included at least six standard concentrations. The mean regression equations for remifentanil and demethoxy-remifentanil were $y=0.01081x+0.04400$ ($r^2=0.9957$) and $y=0.02132x+0.15770$ ($r^2=0.9955$), respectively. Good inter-assay reproducibility was obtained for the slopes (m) of the six calibration curves for both remifentanil and its metabolite (RSD less than 8%). However, a higher variability was observed for the intercepts (b).

The suitability of the method for studying the pharmacokinetics of remifentanil was verified in one anesthetized dog. A plasma sample collected before the injection of an intravenous bolus dose of 4.4 mg kg^{-1} of remifentanil was found to be free of any interference peaks, except for the usual endogenous

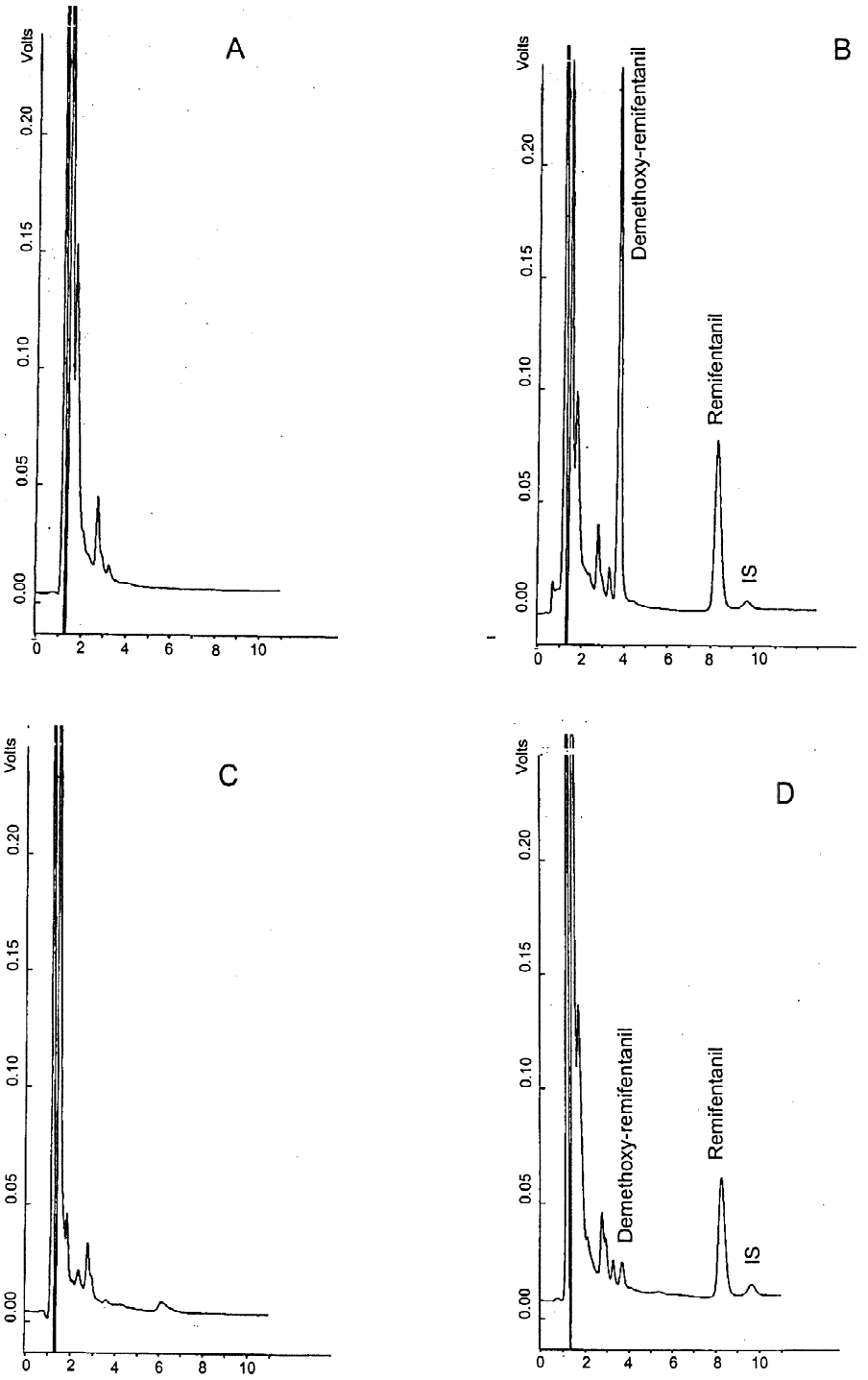

Fig. 2. Representative chromatograms of dog plasma extracts. (A) Drug-free dog plasma sample, (B) calibration standard 1000 ng ml^{-1}, (C) and (D) dog plasma collected from pentobarbital anesthetized dog before bolus administration (C), and 5 min after the injection of an intravenous bolus of 4.4 mg kg^{-1} of remifentanil chloride (D).

Table 3
Intra-assay and inter-assay precision and accuracy of remifentanil and demethoxy-remifentanil

	Remifentanil								Demethoxy-remifentanil					
	Intra-assay				Inter-assay				Intra-assay			Inter-assay		
Concentration (ng ml^{-1})	20	40	200	1250	20	40	200	1250	40	200	1250	40	200	1250
Mean	21	44	202	1268	21	43	204	1292	37	203	1315	42	195	1296
SD	1	1.5	3	59	1	2	10	65	2	3	34	3	15	55
RSD (%)	2	3	2	5	5	5	5	5	5	1	3	8	8	4
Bias (%)	3	11	1	1	5	9	2	3	−7	1	5	4	−3	4
n	4	4	4	4	11	12	11	11	4	4	4	10	12	11

Table 4
Remifentanil and demethoxy-remifentanil curve parameters summary

Curve	Remifentanil				Demethoxy-remifentanil			
	r^2	*m*	*b*	*n*	r^2	*m*	*b*	*n*
1	0.9978	0.00978	0.05477	9	0.9987	0.02008	0.31209	7
2	0.9927	0.01155	0.05201	9	0.9955	0.02314	−0.01988	8
3	0.9975	0.01173	0.03500	9	0.9929	0.02264	−0.25480	7
4	0.9920	0.01085	0.03012	9	0.9989	0.01929	0.17668	6
5	0.9964	0.01062	0.01308	9	0.9933	0.02078	0.04975	8
6	0.9978	0.01030	0.07930	9	0.9939	0.02196	0.68255	8
Mean	0.9957	0.01081	0.04405		0.9955	0.02132	0.15773	
SD	0.00266	0.00074	0.02305		0.00268	0.00151	0.32032	
RSD (%)	0.27	6.87	52.33		0.27	7.09	203.08	

peaks eluting before that of demethoxy-remifentanil (Fig. 2C). A chromatogram obtained from a plasma sample collected 2 min after injection of remifentanil in the same dog is shown in Fig. 2D. The plasma concentration–time profile for this dog reveals that the remifentanil plasma concentration declined from 18 300 ng ml^{-1} at 2 min to 13.4 ng ml^{-1} at 2 h after injection. For the metabolite, a maximum concentration of 6054 ng ml^{-1} was reached at 15 min followed by a slower decline (Fig. 3). Most samples required dilution to fall well within the limit of quantification of either the parent drug or metabolite.

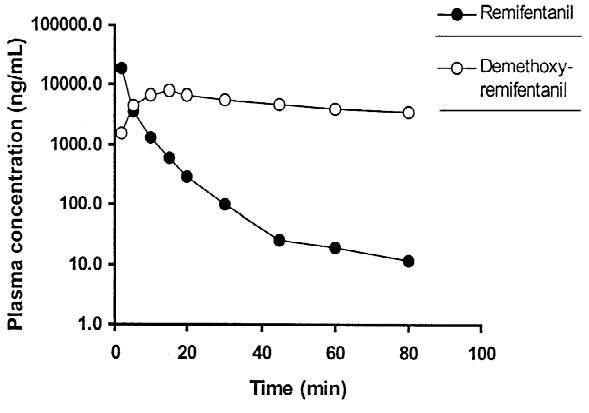

Fig. 3. Plasma concentration–time profiles of remifentanil and demethoxy-remifentanil after a bolus dose of 4.4 mg kg^{-1} of remifentanil chloride in an anesthetized dog.

4. Conclusion

In summary, this is the first solid-phase assay that to allow simultaneous extraction of remifentanil and its major metabolite in plasma. Solid-phase extraction offers significant improvement in the speed of execution without sacrificing of ease, precision, sensitivity or selectivity, therefore offering a good alternative to liquid–liquid extraction. A definite advantage of using plasma as the biological matrix instead of blood is that extraction does not have to follow immediately sample collection. Moreover, pharmacokinetic parameters can be readily compared with those obtained for the other opioids.

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