Mechanism-based pharmacokinetic-pharmocodynamic modelling of opioids : role of biophase distribution and target interaction kinetics

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Section 2

CHARACTERISATION OF THE ROLE OF BIOPHASE DISTRIBUTION

Chapter 3

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NALBUPHINE, BUTORPHANOL AND MORPHINE IN BLOOD AND BRAIN MICRODIALYSATE SAMPLES: APPLICATION TO PHARMACOKINETIC-PHARMACODYNAMIC STUDIES IN RATS

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ABSTRACT

A rapid and sensitive assay for quantification of nalbuphine, butorphanol and morphine in blood (50µl) and brain microdialysate (~40µl) samples was developed. Blood samples were extracted with ethyl acetate. Analysis was performed with high performance liquid chromatography coupled to an electrochemical detector. The mobile phase was a mixture of 0.1M sodium phosphate buffer, methanol and octane-sulfonic acid with ratio and pH depending on compound and matrix. The limits of quantification in blood samples were 25, 50 and 25 ng/ml for nalbuphine, butorphanol and morphine, respectively and 0.5 ng/ml for morphine in microdialysate samples. Based on sample volume, sensitivity and reproducibility, these assays are particularly suitable for pharmacokinetic-pharmacodynamic studies in rodents.

INTRODUCTION

Opioids are widely used in clinical anaesthesia, analgesia and treatment of drug abuse. For example, the natural opioid morphine, the semi-synthetic nalbuphine and the synthetic butorphanol are used in analgesia, whereas the synthetic opioids alfentanil, fentanyl, sufentanil and remifentanil have been developed for use in anaesthesia. However, optimal dosing for these drugs is difficult, due to the development of tolerance, risk of addiction and side effects like respiratory depression.

At present there is a considerable interest in the development of μ-opioid receptor partial agonists, since these compounds in theory have a much-improved selectivity of action. A mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) approach can provide insight into factors that determine pharmacodynamic behaviour of μ-opioid receptor agonists *in vivo* by distinction between drug and biological system characteristics (van der Graaf & Danhof 1997). Recently, the effects of the opioids alfentanil, fentanyl and sufentanil have been studied *in vivo* in a chronically instrumented rat model, using the amplitude in the 0.5-4.5 Hz frequency band of the electroencephalogram (EEG) as a pharmacodynamic endpoint (Cox *et al.* 1998). On the basis of mechanism-based PK-PD analysis, it was shown that these opioids all behave as full agonists *in vivo.* Subsequently, the model has been successfully applied to characterise the *in vivo* pharmacodynamic properties of the novel synthetic opioid remifentanil and its active metabolite GR90291 (Cox *et al.* 1999), showing that they also behave as full agonists at the μ-opioid receptor. Current research on the PK-PD correlations of opioids focuses on nalbuphine, butorphanol and morphine (figure 1).

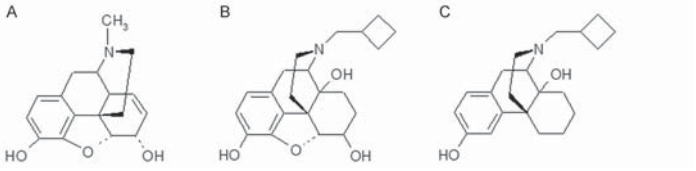

Figure 1: Chemical structures of the opioids morphine (A), nalbuphine (B) and butorphanol (C)

Nalbuphine and butorphanol were selected because they behave as partial agonists at the μ-opioid receptor (Cherny 1996; Emmerson *et al.* 1996; Garner *et al.* 1997; Pallasch & Gill 1985). An important feature of morphine is that blood-brain barrier (BBB) transport is a major determinant of its *in vivo* effect (Bouw *et al.* 2000).

To be able to study the PK-PD correlations of nalbuphine, butorphanol and morphine in the rat EEG model a convenient, rapid and sensitive analytical assay should be developed for the analysis of concentrations in small blood samples. In addition, for morphine the free concentrations in brain microdialysate should be obtained to get insight into the BBB transport, but because of the small sample volume and the low concentrations a highly sensitive HPLC method is required (Benveniste & Huttemeier 1990; Bouw *et al.* 2000; de Lange *et al.* 1999).

Several methods of analysis have been reported for nalbuphine, butorphanol and morphine. These methods include radio-immunoassay and HPLC combined with electrochemical, ultraviolet or fluorescence detection (Pittman *et al.* 1980; Willey *et al.* 1994). More recently, analysis methods with gas chromatography and liquid chromatography coupled to mass-spectrometric detection have been developed (Grinstead 1991; Kanazawa *et al.* 1998; Volk *et al.* 1996). These methods are exceptionally robust and sensitive, but the access to the instrumentation is often limited.
For the analysis of nalbuphine, the published reports focus on HPLC with electrochemical detection (Aitkenhead *et al.* 1988; Nicolle *et al.* 1995; 1997), but these methods require relatively large sample volumes (500 µl). In addition, for analysis of morphine and its metabolites often HPLC analysis with electrochemical and fluorescence detection is described for detection of morphine, the metabolite morphine-6-glucoride (M6G) and morphine-3-glucoronide (M3G), respectively (Drost *et al.* 1984; Joel *et al.* 2002; Liaw *et al.* 1998; Svensson 1986). However, for most assays relatively large plasma volumes are required (1 ml) which precludes application in pre-clinical animal investigations. Therefore, a rapid and highly sensitive HPLC assay was developed which requires only small blood samples (50 – 200 µl) to quantify nalbuphine, butorphanol, morphine. This assay was also able to quantify morphine concentrations in microdialysate samples (20 – 60 µl).

EXPERIMENTAL

Materials

Morphine hydrochloride was purchased from Pharmachemie (Haarlem, The Netherlands), nalbuphine hydrochloride and nalorphine hydrochloride were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) and butorphanol tartrate was purchased from Sigma Aldrich (St. Louis, MI, USA). Millipore water (resistivity 18.2 MΩ.cm) was obtained from a Milli-Q® PF Plus system (Millipore B.V., Amsterdam, The Netherlands). Methanol (HPLC grade) was obtained from Biosolve BV (Valkenswaard, The Netherlands). Ethyl acetate was purchased from Fischer Scientific ('s Hertogenbosch, The Netherlands) and distilled prior to use. All other chemicals were of analytical grade (Baker, Deventer, The Netherlands).

General instrumentation

The HPLC system consisted of an LC-10AD HPLC pump (Shimadzu, 's Hertogenbosch, The Netherlands), a Waters 717 plus autosampler (Waters, Etten-Leur, The Netherlands), a pulse damper (Antec Leyden, Zoeterwoude, The Netherlands) and a digital electrochemical amperometric detector (DECADE, software version 3.02, Antec Leyden, The Netherlands). The electrochemical detector consisted of a VT-03 electrochemical flow cell combined with a 25 µm spacer and an *in situ* Ag/AgCl (ISAAC) reference

electrode operating in the DC mode. For morphine analysis, a standard Ag/AgCl reference electrode, filled with a saturated KCl solution was used. Data acquisition and processing was performed using the Empower® data-acquisition software (Waters, Etten-Leur, The Netherlands).

Extraction procedure for blood samples

For determination of nalbuphine and butorphanol blood concentrations, 50 µl of internal standard solution (butorphanol for nalbuphine analysis and vice versa) was added to hemolysed blood samples (50 – 200 µl blood + 400 µl Millipore water) in glass centrifuge tubes. Next, 500 µl of 1.7 mM phosphoric acid (pH 2.3) and 3 ml of ethyl acetate were added and the mixture was vortexed for 5 min. After centrifugation for 10 min at 4000 rpm, the organic layer was discarded and 500 µl of a 0.15 M carbonate buffer (pH 11) supplemented with EDTA (2.7 mM) was added. Next, 5 ml of ethyl acetate was added and the mixture was vortexed for 5 min. After centrifugation (10 min at 4000 rpm), the organic layer was transferred into a clean glass tube and evaporated to dryness under reduced pressure on a vacuum vortex evaporator (Buchler Instruments, Fort Lee, NJ, USA) at 37°C. The residue was dissolved in 100 µl mobile phase of which 10 – 75 µl was injected into the HPLC system.

For determination of morphine blood concentrations, 50 µl of internal standard solution (nalorphine) was added to hemolysed blood samples (50 – 200 µl blood + 400 µl Millipore water) in glass centrifuge tubes. Next 500 µl 0.15 M carbonate buffer (pH 11) supplemented with EDTA (2.7 mM) and 5 ml of ethyl acetate was added and the mixture was vortexed for 5 min. After centrifugation (10 min at 4000 rpm), the organic layer was transferred into a clean tube and evaporated to dryness under reduced pressure on a vacuum vortex evaporator at 37°C. The residue was dissolved in 100 µl mobile phase of which 10 – 75 µl was injected into the HPLC system.

Analysis of nalbuphine, butorphanol and morphine concentrations in blood samples

Chromatography of blood samples was performed on an Ultrasphere® C18 5 µm column (4.6 mm I.D. x 150 mm) (Alltech, Breda, The Netherlands) equipped with a refill guard column (2 mm I.D. x 20 mm) (Upchurch Scientific, Oak Harbor, WA, USA), packed with C18 (particle size 20-40 µm) (Alltech, Breda, The Netherlands), at a constant temperature of 30 °C.

The mobile phase was a mixture of 0.1 M sodium phosphate buffer (pH 5.5) and methanol (65:35, v/v) for nalbuphine and butorphanol, whereas for morphine a mixture of 0.1 M sodium phosphate buffer (pH 4) and methanol (75:25, v/v). All mobile phases were supplemented with a total concentration 20 mg/l EDTA (sodium salt). The mobile phase for nalbuphine and butorphanol also contained 5 mM KCl whereas for morphine analysis 2 mM octane-sulfonic acid was added. Mobile phase solvents were filtered through a 0.2 µm nylon filter (Alltech, Breda, The Netherlands), mixed and degassed

continuously with helium. The flow rate was set at 1 ml/min. The optimal working potential for nalbuphine, butorphanol and morphine were +0.85 V, +0.85 V and +0.75 V, respectively, as determined by a voltammogram and sensitivity plot.

Analysis of morphine concentrations in brain microdialysate samples

For analysis of morphine brain microdialysate concentrations, 2 µl of internal standard (nalorphine) solution was added per 5 µl of sample. The samples were injected into the HPLC system without further sample pre-treatment. Chromatography of brain microdialysate samples was performed on a Ultrasphere® C18 column (2 mm I.D. x 150 mm) (Alltech, Breda, The Netherlands) at a constant temperature of 35 °C. The mobile phase was a mixture of 0.1 M sodium phosphate buffer (pH 2.5) and methanol (75:25, v/v), supplemented with 20 mg/L EDTA (sodium salt) and 10 mM octane-sulfonicacid. Mobile phase solvents were filtered through a 0.2 µm nylon filter, mixed and degassed continuously with helium. The flow rate was set at 0.2 ml/min. The optimal working potential for morphine was +0.80 V, as determined by a voltammogram and sensitivity plot.

Reagents and standard solutions

For analysis of blood samples, the stock solutions of nalbuphine, butorphanol, morphine and nalorphine were prepared at a concentration of 1 mg/ml (free base) in Millipore water. The stock solutions were diluted with Millipore water to obtain calibration solutions (range 25 – 10000 ng/ml). Internal standard solutions were prepared by dilution of the stock solutions to a concentration of 250, 2500 and 500 ng/ml for nalbuphine, butorphanol and nalorphine, respectively.

For analysis of brain microdialysate samples, a stock solution of morphine was prepared at a concentration of 1 mg/ml (free base) in microdialysis perfusion fluid. Internal standard solution was prepared by dilution of the stock solution to 500 ng/ml nalorphine in perfusion fluid. Microdialysis perfusion fluid comprised of phosphate buffer (2 mM, pH 7.4) containing 145 mM sodium, 2.7 mM potassium, 1.2 mM calcium, 1.0 mM magnesium, 150 mM chloride and 0.2 mM ascorbate (Moghaddam & Bunney 1989). The stock solutions were stored at –20 °C up to three months. The assay solutions were stored at 4 °C up to four weeks.

Calibration and validation

On each day of blood sample analysis, a 10-point calibration curve was prepared by spiking 50 µl of blood hemolysed in 400 µl water with 50 µl of calibration solution and 50 µl of the internal standard solution. For analysis of brain microdialysates, a 10-point calibration curve was prepared with 40 µl of calibration solutions in perfusion fluid and 16 µl of internal standard solution in Millipore water.

Samples were processed as described above and peak ratios of nalbuphine-butorphanol, butorphanol-nalbuphine or morphine-nalorphine were calculated. Calibration curves

were constructed by weighted linear regression [weight factor = 1/ (peak height ratio)^2] according to the method implemented in the data-acquisition program Empower®.
Quality control samples of fixed concentrations were prepared to determine intra- and inter-assay variability. Extraction yields were determined by comparing the peak ratios after extraction from blood with the peak ratios of not-extracted standards.

Pharmacokinetic-pharmacodynamic study in rats

Chronically instrumented male Wistar rats, weighing 250 – 300 g were used in the experiments. Nine days before the experiment, seven cortical EEG electrodes were implanted into the skull. A number of rats used for the morphine studies were implanted with four cortical EEG electrodes and a CMA/12 microdialysis guide (Aurora Borealis Control, Schoonebeek, The Netherlands) which was replaced by the microdialysis probe (CMA/12, 4 mm) 24 hours before the experiment. Two days before the experiments three cannulas were implanted for drug administration and serial blood sampling. Two cannulas were implanted in the right jugular vein for opioid and midazolam infusion and one cannula was implanted in the left femoral artery to collect blood samples. The surgical procedures were performed under anaesthesia of 0.1 mg/kg Domitor® (intramuscular injection, 1 mg/ml medetomidine hydrochloride, Pfizer, Capelle aan de IJssel, The Netherlands) and 1 mg/kg Ketanest® (subcutaneous injection, 50 mg/ml ketamine base, Parke Davis, Hoofddorp, The Netherlands). After surgery, rats received a single dose of ampicilline trihydrate (0.6 ml/kg of a 200 mg/ml solution, A.U.V., Cuijk, The Netherlands).
At the day of the experiment, the rats received an intravenous infusion of midazolam (5.5 mg/kg/h) and either nalbuphine (10 mg/kg in 10 min), butorphanol (10 mg/kg in 10 min) or morphine (4 mg/kg in 10 min). Midazolam was administered continuously to prevent opioid induced seizures (Cox *et al.* 1997). To reach steady state rapidly, midazolam was administered with a Wagner infusion (Wagner 1974). The midazolam infusion was started 30 min before opioid infusion. A total number between 15 and 20 arterial blood samples were collected over a period of 4 hours at fixed time intervals and immediately hemolysed in Millipore water. The samples were stored at – 20°C until analysis.
During the experiment, the EEG was recorded continuously. After off-line fast Fourier transformation using the data analysis software Spike2 version 4.60, (Cambridge Electronic Design limited, Cambridge, UK), the absolute amplitude in the delta-frequency range in 5 s epochs were averaged over 1 min intervals.
The pharmacokinetics of nalbuphine, butorphanol and morphine were quantified for each individual rat using the least squares minimisation algorithm (weight = 1/(y predicted)^2) of the WinNonlin Pro package V.1.5 (Pharsight Corporation, Mountain View, CA, USA). For nalbuphine, butorphanol and morphine a standard two-compartment model (Gibaldi & Perrier 1982) best described the concentration-time profile by the Akaike Information Criteria (Akaike 1974).

RESULTS AND DISCUSSION

Chromatography

The sample pre-treatment by liquid-liquid extraction provided a good sample clean up as shown in figure 2. For nalbuphine and butorphanol a two-step extraction procedure was required because of interfering peaks, whereas for morphine a one-step extraction was sufficient (figure 3).

Retention times for nalbuphine and butorphanol were 6 and 11 min, respectively with a mobile phase containing 35 % methanol and 65 % 0.1 M phosphate buffer pH 5.5. Retention times for morphine and internal standard nalorphine were 5 and 11 min,

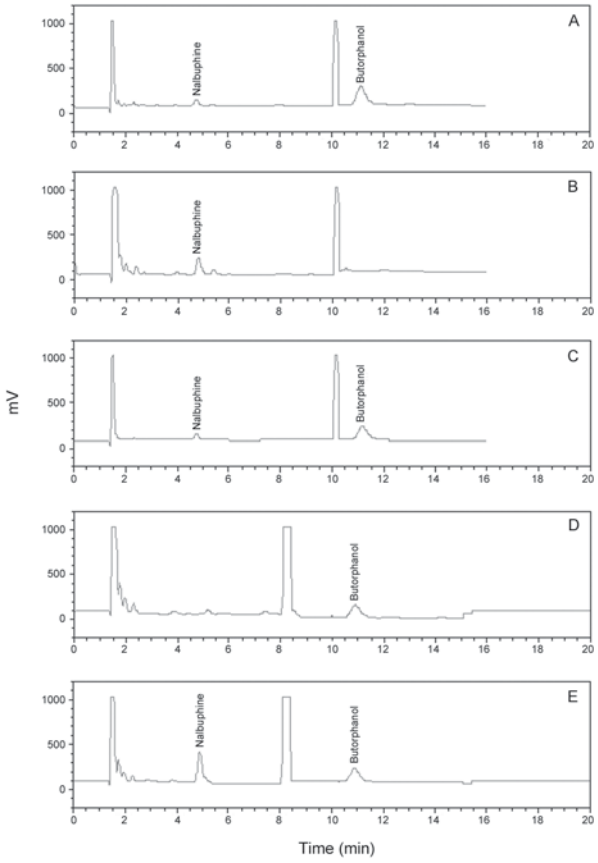

Figure 2: Chromatograms of an extract of blank blood spiked with nalbuphine (250 ng/ml) and butorphanol (2500 ng/ml)(A), blank blood spiked with either internal standard nalbuphine (250 ng/ml) or internal standard butorphanol (2500 ng/ml) (B and D) and blood obtained from a rat at 12 min after start of an infusion of 10 mg/kg butorphanol in 10 min (concentration 1931 ng/ml)(C) or after having received and infusion of 10 mg/kg nalbuphine in 10 min (concentration 1955 ng/ml)(E).

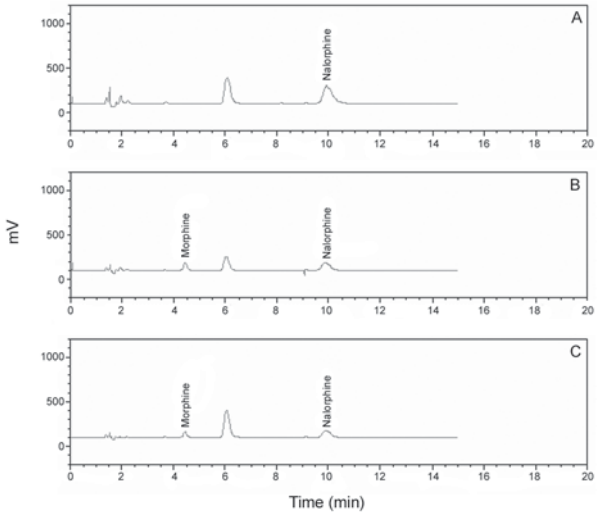

Figure 3: Chromatograms of an extract of blank blood spiked with internal standard nalorphine (500 ng/ml) (A), blank blood spiked with morphine (1000 ng/ml) and internal standard nalorphine (500 ng/ml) (B) and blood obtained from a rat at 12 min after the start of an infusion of 4 mg/kg morphine in 10 min (morphine concentration 767 ng/ml) (C).

respectively with a mobile phase containing 25% methanol, 75 % 0.1 M phosphate buffer pH 4 and 2 mM octane-sulfonicacid. For the analysis of morphine, the mobile phase was adjusted because morphine did not have enough retention on the column with the conditions used for nalbuphine and butorphanol. To improve retention, 2 mM octane-sulfonic acid was added as an ion-pair. The pH was adjusted to improve the peak shape.

Table 1 summarises the recovery after extraction, the accuracy and reproducibility of the analysis. For nalbuphine, butorphanol and morphine intra- and inter-assay were less than 20 % in the concentration range of 25–10000 ng/ml. The weighted linear regression equations (mean ± SEM) for nalbuphine (N=9), butorphanol (N=5) and morphine (N=15) were y = (1.212 ± 0.055)x + (-7.195 ± 2.394), y = (0.0020 ± 0.0003)x + (-0.0629 ± 0.0127) and y = (0.0011 ± 0.0001)x + (-0.0045 ± 0.0015), respectively. Corresponding coefficients of correlation were (0.978 ± 0.003), (0.993 ± 0.001) and (0.996 ± 0.001), indicating the linearity of the methods. Using 50 µl blood, the limit of detection for nalbuphine, butorphanol and morphine was 25, 50 and 25 ng/ml (signal to noise ratio = 3), respectively. The main difference of the methods described here and the methods described in the literature is the sample size. All methods are described for studies in humans or relatively large laboratory animals (dogs, pigs, rabbits), whereas the method described here was especially for application to studies in small laboratory animals (rats). For example, for the analysis of nalbuphine Nicolle and co-workers (1995; 1997) used plasma samples

Table 1: Validation of the determination of nalbuphine (N), butorphanol (B) and morphine (M): recovery, intra-assay and inter-assay variability, coefficients of variation and accuracy. Results are expressed as mean ± SEM.

	Added (ng/ml)	Recovery (N=3)	Intra-assay (N=3)			Inter-assay (N=5)		
			Found (ng/ml)	CV (%)	Accuracy (%)	Found (ng/ml)	CV (%)	Accuracy (%)
N	100	67±2	-	-	-	-	-	-
	250	-	265±17	11	106	263±12	13	105
	1000	71±5	-	-	-	-	-	-
	2500	-	2553±28	1.9	102	2936±80	7	117
	10000	78±4	-	-	-	-	-	-
B	100	64±14	-	-	-	-	-	-
	250	-	248±6	4.7	99	213±10	10	86
	1000	85±8	-	-	-	-	-	-
	2500	-	2530±51	3.5	101	2550±62	5	102
	10000	80±8	-	-	-	-	-	-
M	250	62±4	239±5	4.4	96	257±5	6	103
	3000	58±4	2630±97	8.2	88	3268±82	9	109

of 500 µl whereas in our studies blood samples of 50-200 µl were used. When whole blood samples are used for drug analysis, more samples can be collected from a subject and therefore more information about the individual pharmacokinetic profiles can be obtained. Another advantage of our method is that one general method is applicable for three opioids. Nalbuphine and butorphanol samples can be analysed with the same HPLC-conditions and sample pre-treatment, whereas for morphine only slight modifications are required.

For morphine administration, drug concentrations were also determination in brain microdialysate. No sample pre-treatment was required to clean up the samples as is shown in figure 4. The weighted linear regression equation (mean ± SEM) for morphine (N=9) was $y = (16.644 \pm 0.269)x + (-6.484 \pm 0.565)$ and corresponding coefficient of correlation was (0.994 ± 0.001), indicating the linearity of the method. Using 40 µl microdialysate, the limit of detection for morphine was 0.5 ng/ml (signal to noise ratio = 3).

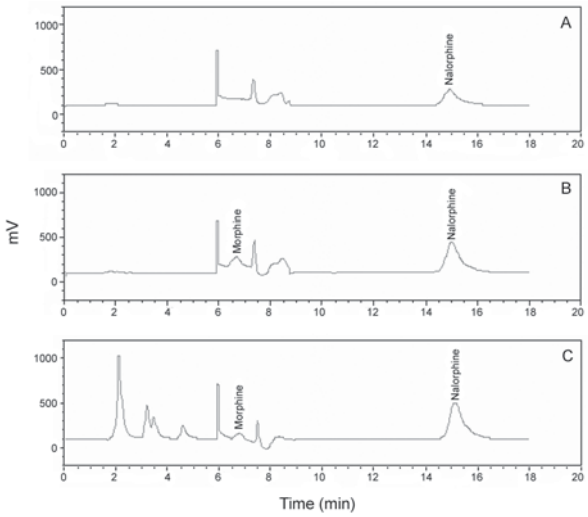

Figure 4: Chromatograms of blank microdialysate spiked with internal standard nalorphine (500 ng/ml) (A), microdialysate spiked with morphine (10 ng/ml) and internal standard nalorphine (500 ng/ml) (B) and a microdialysate fraction obtained from a rat 40-60 min after the start of an infusion of 4 mg/kg morphine in 10 min (morphine concentration 6.1 ng/ml) (C).

Study in rats

Figure 5a and 5b show representative blood concentration-time profiles for an intravenous administration of 10 mg/kg nalbuphine in 10 min and 10 mg/kg butorphanol in 10 min. The values for clearance, volume of distribution at steady state and terminal half-life were estimated for each individual rat (table 2).

Table 2: Average pharmacokinetic parameter estimates (Mean ± SEM) obtained with a two-compartment pharmacokinetic model for nalbuphine, butorphanol and morphine after a 10-min intravenous infusion.

Compound	Dose (mg/kg)	N	Cl (ml/min)	Vd_{ss} (ml)	Elimination half life (min)
Nalbuphine	10	8	38.7 ± 3.3	1917 ± 385	56.0 ± 7.0
Butorphanol	10	6	22.8 ± 3.3	1242 ± 193	62.4 ± 14.0
Morphine	4	14	24.1 ± 2.1	881 ± 117	44.1 ± 4.7

Figure 5c shows a representative blood and brain microdialysate concentration-time profile for an intravenous infusion of 4 mg/kg morphine in 10 min. To emphasise the application to PK-PD studies, figure 5 also shows the time-course of the change in amplitude of the delta-frequency band (0.5 - 4.5 Hz) of the EEG during and after administration of nalbuphine, butorphanol or morphine. Combination of both the detailed concentration-time and effect-time relationship revealed a complex concentration-effect relationship, which is currently being investigated by PK-PD modelling.

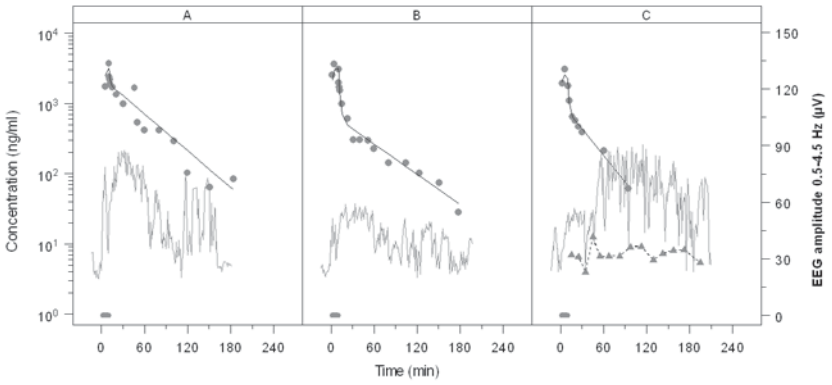

Figure 5: Typical blood concentration-time profiles (filled circles, left ordinate) and EEG amplitudes in delta-frequency range versus time (grey solid line, right ordinate) in rats following intravenous infusion of 10 mg/kg nalbuphine (A), 10 mg/kg butorphanol (B) or 4 mg/kg morphine (C) in 10 min. Panel C also shows the brain microdialysate concentration-time profile of morphine (dotted line). The solid line represents the best description of the plasma concentrations according to a two-compartment pharmacokinetic model.

CONCLUSIONS

A simple and sensitive HPLC method has been developed for the analysis of nalbuphine, butorphanol and morphine in biological samples. The short duration of the analysis, the sample size, the sensitivity, the reproducibility and the simplicity of the methods used make these assays particularly useful for PK-PD studies in small laboratory animals in which large numbers of samples have to be analysed.
In combination with the EEG measurements, concentration-effect profiles can be obtained in individual rats, which can then be used for quantitative analysis of μ-opioid receptor mediated responses *in vivo*. The analysis of the brain microdialysate concentrations of morphine allows the characterisation of the BBB transport of morphine and its influence on the concentration-effect relationships.

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