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Quantitative determination of fentanyl in newborn pig plasma and cerebrospinal fluid samples by HPLC-MS/MS

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In this study, a selective and sensitive high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method requiring low sample volume (≤100 μL) was developed and validated for the quantitative determination of the opioid drug fentanyl in plasma and cerebrospinal fluid (CSF). A protein precipitation extraction with acetonitrile was used for plasma samples whereas CSF samples were injected directly on the HPLC column. Fentanyl and $^{13}C_6$-fentanyl (Internal Standard) were analyzed in an electrospray ionization source in positive mode, with multiple reaction monitoring (MRM) of the transitions *m/z* 337.0/188.0 and *m/z* 337.0/105.0 for quantification and confirmation of fentanyl, and *m/z* 343.0/188.0 for $^{13}C_6$-fentanyl. The respective lowest limits of quantification for plasma and CSF were 0.2 and 0.25 ng/mL. Intra- and inter-assay precision and accuracy did not exceed 15%, in accordance with bioanalytical validation guidelines. The described analytical method was proven to be robust and was successfully applied to the determination of fentanyl in plasma and CSF samples from a pharmacokinetic and pharmacodynamic study in newborn piglets receiving intravenous fentanyl (5 μg/kg bolus immediately followed by a 90-min infusion of 3 μg/kg/h). Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: fentanyl; newborn pig; plasma; cerebrospinal fluid; HPLC-MS/MS

Introduction

Fentanyl (1-N-phenyl-N-(1-(2-phenylethyl)piperidyl)propanamide, FEN) is a synthetic μ-opioid agonist used in neonatal and paediatric critical care units to provide analgesia and/or sedation when administered in continuous intravenous infusion during and after surgery[1] or in mechanically ventilated patients.[2,3]

However, FEN administration is not indicated in infants (i.e., below 2 years of age) according to the manufacturer's product license, and the drug is therefore used off-label in this population. In order to increase the knowledge on the product within this context and to try to reduce the degree of empiricism currently associated with the establishment of dosing regimens in this population, a maturation-physiology-based predictive pharmacokinetic/pharmacodynamic (PK/PD) model for fentanyl in neonatal care was built.[4] The performance of a PK/PD study in a suitable animal species was subsequently deemed convenient, as a complement and preliminary confirmation to the developed theoretical model. Concretely, the newborn piglet was considered a representative model of FEN behaviour in neonates because many of its anatomical and physiological characteristics more closely resemble those of humans than other non-primate species,[5,6] as supported by the frequent use of preterm and term neonate pigs in paediatric research.[7–9] In this respect, cytochrome P450 isoform 3A4 (CYP3A4), the enzyme responsible for hepatic fentanyl biotransformation in humans, is also present in pigs with comparable levels and activity.[5,10,11] Moreover, the differences observed between juvenile and adult pig PK for some drugs were deemed as consistent with ontogenic changes reported for human PK.[12] Additionally, the swine cardiovascular system and its physiological development (related with the PD) are almost identical to those of humans.[6,13]

The general objective of this kind of experimental PK/PD studies is to characterize the systemic exposure of the drug after a given dose (assessed by plasma levels) as well as its relationship with the observed pharmacological effects. Nevertheless, FEN, a centrally acting drug that has to cross the blood-brain barrier to exert the majority of its analgesic and sedative effect, is known to exhibit certain degree of delay between its concentration-time profile in the blood and that observed in the central nervous system (CNS).[14] Under such circumstances, assessment of *in vivo* CNS availability may be of interest, as it is more likely to be directly correlated to the pharmacodynamic effects as compared to blood availability. Cerebrospinal fluid (CSF) is one of the biological matrices that can be sampled to provide an overall index of drug access to the CNS after systemic administration of a compound, thus being considered as a surrogate measure for drug concentrations at the target site within the brain.[15–17] Indeed, CSF penetration studies, often in combination with cerebral microdialysis techniques

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measuring drug concentration in the brain interstitial fluid (ISF), are usually performed in preclinical species to investigate CNS drug distribution, as it is often a good reflection of the situation in humans.[17,18] Consequently, the development of a suitable, selective and sensitive analytical method capable of measuring FEN in both biological fluids is essential for the development of an experimental investigation where CSF and plasma samples are analyzed.

Methods of high sensitivity and selectivity are especially required in the case of FEN, since due to its higher potency in comparison with morphine,[19–21] effective doses are much lower and, therefore, diminished concentrations (<10 ng/mL) are expected in biological fluids. In addition, the use of high sample volumes is impracticable for PK/PD studies in the newborn, where several samples must be obtained periodically. Consequently, sensitive methods requiring low sample volumes must be used.

Some studies for the analysis of FEN in biological samples,[22–24] using immunoassay methods have been reported, reaching in the best case a limit of detection of 0.0048 ng/mL[25] using 50 μL of plasma sample. However, these methods are prone to suffer from cross-interference of similar molecules such as structurally related compounds or metabolites.[26] Gas chromatography-mass spectrometry (GC-MS) methods for the analysis of FEN in plasma[27–29] have also been reported, obtaining values of lowest limit of quantitation (LLOQ) ranging from 0.05 ng/mL up to 4 ng/mL when using a minimum of 500 μL of plasma. High performance liquid chromatography methods coupled to ultraviolet detection (HPLC-UV) found in literature[30,31] show the same problem, using 1 mL of plasma to reach an LLOQ of 0.2 ng/mL, in the best case. The only method using a suitable volume of plasma (100 μL)[32] is not sensitive enough for this PK/PD analysis (LLOQ equal to 3 ng/mL).

Several high performance liquid chromatography-mass spectrometry (HPLC-MS) methods are available for the determination of FEN and its derivatives in plasma. Methods reported by Koch *et al.*[33] and Huynh *et al.*[34] reached LLOQ values as low as 0.02 ng/mL and 0.025 ng/mL, respectively; however, in order to reach those levels 1 mL of plasma sample and a liquid-liquid extraction (LLE) procedure was required. Lower sample volumes were used by Chang *et al.*[35] and Hisada *et al.*,[36] using an LLE procedure and a simple protein precipitation method respectively.

Studies using HPLC for the quantification of FEN in CSF or brain perfusate samples are scarce,[37,38] and to the best of our knowledge, HPLC-MS/MS has not been yet applied to the analysis of FEN in CSF samples from the newborn. The knowledge of drug concentrations in this biological matrix and their relationship to plasma or urine levels would add relevant information towards the establishment of PK/PD correlation for FEN.

The aim of this work was to develop an HPLC-MS/MS method with electrospray ionization (ESI) in positive mode that would allow rapid, sensitive and reproducible quantification of fentanyl in plasma and CSF, requiring small sample volume and quick sample processing, for its subsequent application on a PK/PD study of FEN in newborn pigs as an animal model of human neonates.

Material and methods

Instrumentation

Chromatographic separation was carried out on an Alliance HPLC 2695 separation module (Waters, Milford, MA, USA). A Luna C18 (150 x 2 mm id, 3 μm) chromatographic column (Phenomenex, Torrance, CA, USA) was used as stationary phase. Mass spectrometric analysis was performed using a tandem mass spectrometer Quattro micro (Waters, Milford, MA, USA) equipped with an electrospray ionization source operating in positive mode. Data acquisition was performed using MassLynx 4.0 software (Waters, Milford, MA, USA). Sample centrifugation was performed using an Eppendorf 5424 centrifuge (Eppendorf, Hamburg, Germany).

Reagents and solutions

FEN and $^{13}C_6$FEN, used as internal standard, were purchased from Alsachim (Illkirch Graffenstaden, France). HPLC quality formic acid and ammonium formate, from Sigma Aldrich (St Louis, MO, USA), were used in the preparation of buffer solutions. LC-MS grade acetonitrile (VWR, Radnor, PA, USA) was used as organic modifier. Purified water from a Milli-Q Element A10 System (Millipore, Billerica, MA, USA) was used in the preparation of buffer and reagent solutions.

Drug-free pig plasma samples were purchased from Seralab (West Sussex, United Kingdom) and collected in polypropylene tubes to be frozen at -20 °C. Due to the lack of drug-free pig CSF samples, artificial CSF was prepared as an aqueous solution of NaCl (147 mmol/L), KCl (2.7 mmol/L), $CaCl_2$ (1.2 mmol/L) and $MgCl_2$ (0.85 mmol/L).

Preparation of standard solutions and quality control (QC) samples

FEN and $^{13}C_6$FEN were dissolved in dimethyl sulfoxide to give 1 mg/mL primary stock solutions. A 1000-fold dilution of the FEN primary stock solution was made in water to achieve a working solution with a concentration of 1 μg/mL. Aliquots of this working solution were added to drug-free plasma and artificial CSF to obtain quality control samples (QCs) at three concentration levels: low, mid and high QCs; being the low QC three times the concentration at the LLOQ, the mid QC the geometrical mean of the calibration range points, and the high QC the 85% of the upper limit of quantitation (ULOQ). Calibration standards at seven levels ranging from 0.2 to 15 ng/mL for plasma and from 0.25 to 5 ng/mL for CSF were prepared also by dilution of the working solution with drug-free plasma or CSF. A dilution of the internal standard solution with acetonitrile was made to give a 15 ng/mL solution. Primary stock solutions were stored at -20 °C and working solutions were stored at 4 °C until analysis. Calibration standards and QCs were freshly prepared immediately prior to analysis.

Experimental study design

The analytical method developed was used for the quantification of FEN in pig plasma and CSF samples obtained in a prospective study that aimed to investigate the drug PK/PD behaviour when intravenously (i.v.) administered alone (in monotherapy) to mechanically ventilated newborn piglets (2–4 days, 1.7 ± 0.2 kg, n = 6) of each gender. The experimental protocol, which is explained in detail somewhere else,[39] met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and was approved by the Ethical Committee for Animal Welfare of the Cruces University Hospital.

FEN dosage regimen (5 μg/kg bolus immediately followed by a 90-min infusion of 3 μg/kg/h) was estimated as suitable for providing an adequate degree of sedation, measured by amplitude-integrated electroencephalography (aEEG), based on the results

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of a pilot study previously performed in two additional animals (data not shown).

Blood samples (n = 13–15 per animal) for the quantification of FEN were withdrawn at baseline, immediately after bolus administration, at t = 1, 10, 30, 90, 95, 120, 150, and 180 min after the start of the infusion and then every 30 min until experiment was stopped, which occurred at initial signs of awakening shown by each animal (i.e., t = 225–300 min). As restricted by the low volume of CSF in the study population as well as by the short evaluation period (maximum of 5 h), the extraction of a single CSF sample in each animal was considered acceptable from an ethical perspective. CSF sample was drawn either at t = 10, 90, or 150 min (2 animals per time point), in order to allow comparison with the simultaneously extracted blood sample.

Sample collection

Samples were collected by the Research Unit for Experimental Neonatal Respiratory Physiology at Cruces University Hospital (Barakaldo, Biscay, Basque Country, Spain).

Whole arterial blood samples were collected in EDTA tubes, and kept on ice until their immediate centrifugation at 3000 rpm at 4 °C in order to obtain the plasma. The supernatant was transferred to cryovials and stored at -80 °C until analyzed. CSF samples were collected by lumbar puncture and stored in cryovials at -80 °C.

Sample preparation

Frozen samples from the studied animals were thawed until reaching room temperature. A volume of 150 μL of acetonitrile with a concentration of $^{13}C_6$FEN of 15 ng/mL was added to 100 μL of plasma (final $^{13}C_6$FEN concentration 9 ng/mL) to promote protein precipitation and was vortex mixed for 5 min. Samples were then centrifuged at 10 000 rpm during 5 min. The clean upper layer was transferred to a chromatographic vial to be injected in the HPLC-MS/MS system. CSF samples were injected without any sample preparation except the addition of 5 μL of the $^{13}C_6$FEN solution in acetonitrile at a concentration of 15 ng/mL to 50 μL of sample (final $^{13}C_6$FEN concentration 1.36 ng/mL).

Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using an isocratic method, operating at a flow rate of 0.25 mL/min over a total run time of 3.5 min. The mobile phase was a mixture of acetonitrile and water (40:60 v:v) containing 10 mM of formic acid/ammonium formate buffer, pH 3.5. A sample aliquot of 10 μL was injected into the column. The autosampler temperature was set at 10 °C and the column was kept at 30 °C.

Mass spectrometer source temperature was set at 120 °C. Nitrogen was used as desolvation gas at a temperature of 300 °C and at a flow of 450 L/h. Capillary voltage was set at 0.8 kV. FEN and $^{13}C_6$FEN were detected by multiple reaction monitoring (MRM) mode with a dwell time of 0.20 s. The following transitions were monitored in ESI +: m/z 337.0 → m/z 188.0 – in accordance with the values reported in the literature[34–36] – using a cone voltage (CV) of 35 V and a collision energy (CE) of 25 eV for FEN quantification, m/z 337.0 → m/z 105.0 using a CV of 25 V and a CE of 45 eV for FEN confirmation and m/z 343.0 → m/z 188.0 using a CV of 45 V and a CE of 25 eV for $^{13}C_6$FEN.

Validation of HPLC-MS/MS method

The developed method was validated in terms of selectivity, linearity, sensitivity, accuracy, precision, carryover and matrix effect, following the FDA criteria established in the Bioanalytical Method Validation Guide.[40]

The selectivity of the method for plasma was evaluated by comparing the response of six individual drug-free plasma samples against a sample at the LLOQ, with reference to potential endogenous and environmental interferences. Due to the absence of blank real samples of CSF, the selectivity of the method in this matrix was evaluated analyzing aliquots of artificial CSF. The signal obtained in the blank matrices must be lower than 20% the response of FEN at the LLOQ and 5% the response of $^{13}C_6$FEN.

Calibration curves – consisting of a blank sample (blank matrix), a zero sample (blank matrix spiked with $^{13}C_6$FEN), and six non-zero calibration standards – were built, plotting the corrected peak area of fentanyl (FEN/$^{13}C_6$FEN) against its nominal concentration. The acceptance criterion for the calibration curve was that at least four out of the six non-zero calibration standards had less than 15% deviation from the nominal concentration (20% for LLOQ standard). Sample concentration was calculated by interpolating the resulting corrected area in the regression equation of the calibration curve.

Sensitivity was examined by comparing blank samples with the response of calibration standards at the LLOQ, calculated using Eqn (1)

$$LLOQ = \frac{y_{blank} + 10.s}{b} \quad (1)$$

where y_{blank} is the average signal obtained from six different plasmas or six replicates of artificial CSF, s is its standard deviation and b is the slope of the calibration curve. The analyte response should be at least five times the response obtained from a blank sample.

In order to evaluate the intra-day accuracy, five replicate spiked samples were prepared in plasma and CSF at three concentration levels: low, mid and high QC; they were analysed the same day and their concentration value was obtained from interpolation of the resulting corrected area in the regression equation of the calibration curve. Accuracy was expressed as relative error (%RE). The acceptance criterion for accuracy was %RE $<$15%. Inter-day accuracy was determined by calculating the %RE obtained when repeating intra-day accuracy experiments in three different days.

Intra- and inter-day precision were evaluated as relative standard deviation (%RSD) of five replicates of the low, mid, and high QCs in three different days, following the same procedure as for accuracy assay. The acceptance criterion for precision was %RSD $<$15%.

Carryover was tested by injection of a blank plasma sample directly after injection of the ULOQ standard. The response in the blank sample following the high concentration standard was then compared with the response at the LLOQ, and was considered acceptable if the signal obtained at the FEN and $^{13}C_6$FEN retention time was under 20% of the signal at the LLOQ and under 5% of the $^{13}C_6$FEN signal.

For the evaluation of matrix effect, five samples of each low QC, mid QC and high QC were prepared spiking five different blank plasmas with FEN and $^{13}C_6$FEN after protein precipitation. Normalized matrix factor (NMF) was determined as follows: NMF = (analyte peak area/IS area) in matrix/(analyte peak area/IS area) in pure solution. %RSD of the results in different plasma samples was calculated in order to demonstrate the absence of "relative" matrix effect, referring to the variability of matrix effect among different sources

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of the same matrix. If %RSD was lower than 15%, the method was considered to be free of relative matrix effect.

Moreover, matrix effect was also qualitatively studied performing the post-column infusion experiments reported by Bonfiglio *et al.*[41] For this purpose, a solution of FEN (10 ng/mL) was infused post-column at a flow rate of 10 μL/min while the analysis of a blank plasma sample was carried out simultaneously. The eluent from the column and the flow from the infusion were combined using a zero-dead-volume Tee union and introduced into the source of the mass spectrometer.

Results and discussion

Chromatographic behaviour of fentanyl

In the optimum chromatographic conditions the mean retention time of FEN was 2.05 min. As expected, the internal standard $^{13}C_6$FEN eluted at the same time as FEN, as can be seen in Figure 1.

Method validation

Selectivity

No interfering peaks were observed at FEN retention time in any of the six individual pig plasma and CSF samples evaluated. For FEN and $^{13}C_6$FEN the response in blank plasma and CSF samples was lower than 20% and 5%, respectively, of the response at the LLOQ values (0.2 ng/mL for plasma and 0.25 ng/mL for CSF).

Calibration curves and sensitivity

Calibration curves met the criteria established for linearity in the range of 0.2 ng/mL to 15 ng/mL for FEN in plasma and 0.25 ng/mL to 5 ng/mL in CSF with values for $R^2 > 0.999$ in all cases. Moreover, the % RE value of all the non-zero standards was lower than 15%.

Accuracy and precision

The results for accuracy and precision are presented in Table 1. Both, in plasma and CSF, the calculated %RE was lower than 15% at the low, mid, and high QC for both the intra and inter-day assays, evidencing an adequate accuracy along the calibration range. Moreover, the %RSD was below 15% in all QC samples for both plasma and CSF samples, indicating that the precision of both methods was also suitable.

Carryover

No quantifiable carryover effect was observed when injecting blank pig plasma or CSF solution immediately after the ULOQ.

Matrix effect

Postcolumn infusion experiments showed a substantial suppression of the ionization of FEN due to matrix interferences as shown in Figure 2. Notwithstanding, this effect was compensated by the isotopically labelled internal standard, with the average NMF among the different plasma sources being 93% and presenting a variability, in terms of %RSD, of 9% (n = 5).

Notably, all parameters (i.e., selectivity, sensitivity, accuracy and precision, carryover and matrix effect) complied with the established acceptance criterion; therefore, the method was successfully validated.

Analysis of samples from PK/PD experimental study

The optimized HPLC-MS/MS method was implemented for the measurement of FEN concentration in pig plasma and CSF samples obtained from a PK/PD experimental study performed in newborn piglets.[39]

The developed method enabled the quantification of FEN concentrations from as low as 0.2 ng/mL, thus allowing the characterization of the plasma profiles in piglets (Figure 3). The majority of the plasma concentrations calculated were above the LLOQ, except for the latest sampling points of two of the pigs in the study (no.3 and no.6), in which FEN has apparently already been eliminated by that time.

The plasma concentration time curves obtained in all animals revealed multi-exponential disposition kinetics as expected, displaying a rapid initial distribution phase (compatible with high lipophilicity of FEN) followed by a slower decline. Although FEN plasma levels showed quite a large inter-individual variability, the drug had, overall, been cleared up by the end of the experiments (225–300 min), which is consistent with animals showing initial signs of awakening at this point.

Fentanyl is primarily eliminated from the body by hepatic N-dealkylation via CYP3A4 to the inactive metabolite norfentanyl,[42–44] which is subsequently excreted in urine accounting for roughly 94% of the dose. The remaining percentage of the dose is excreted unchanged in urine and stool,[45,46] so that quantification of the metabolites in study samples was deemed purposeless.

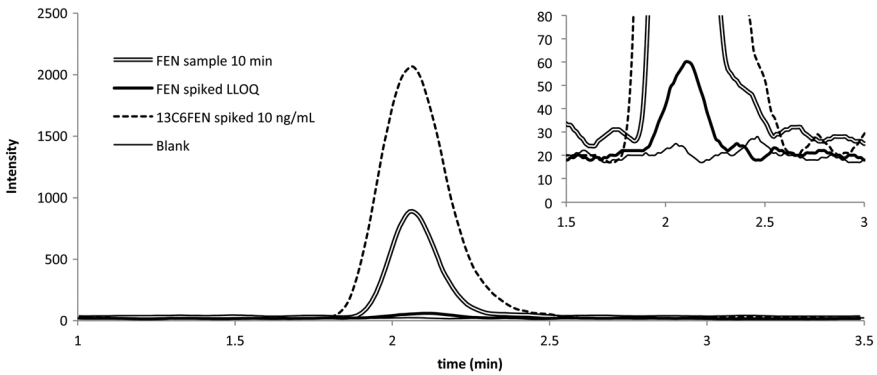

Figure 1. Chromatograms of a blank pig plasma sample, the same sample spiked with 10 ng/mL of $^{13}C_6$FEN and with 0.2 ng/mL of FEN at the LLOQ, and a pig plasma sample from the pharmacokinetic study taken 10 min after the fentanyl bolus dose.

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Table 1. Intra- and inter-day accuracy and precision in terms of %RE and %RSD, respectively, for plasma and CSF samples at low, mid and high QC concentration values

Plasma					**CSF**				
	Intra-day			Inter-day		Intra-day			Inter-day
	Day 1	Day 2	Day 3	Days 1-3		Day 1	Day 2	Day 3	Days 1-3
Low QC					**Low QC**				
(0.5 ng/mL)					(0.6 ng/mL)				
Mean	0.44	0.54	0.43	0.47	Mean	0.53	0.62	0.62	0.59
%RE	**8.17**	**10.61**	**2.40**	**5.67**	%RE	**12.04**	**2.58**	**3.47**	**2.00**
%RSD	**9.23**	**7.45**	**8.72**	**12.32**	%RSD	**14.23**	**12.69**	**9.06**	**8.89**
Mid QC					**Mid QC**				
(3 ng/mL)					(1.2 ng/mL)				
Mean	2.80	2.96	3.33	3.03	Mean	1.12	1.29	1.18	1.20
%RE	**6.60**	**1.25**	**10.90**	**0.96**	%RE	**6.56**	**7.33**	**1.86**	**0.37**
%RSD	**12.53**	**3.75**	**9.48**	**8.88**	%RSD	**7.62**	**9.80**	**11.66**	**7.09**
High QC					**High QC**				
(12 ng/mL)					(4.25 ng/mL)				
Mean	12.32	10.73	12.29	11.78	Mean	4.26	4.14	4.41	4.27
%RE	**2.67**	**7.71**	**13.35**	**1.85**	%RE	**0.25**	**2.53**	**3.68**	**3.09**
%RSD	**12.05**	**6.55**	**10.87**	**7.73**	%RSD	**4.26**	**6.43**	**7.85**	**0.47**

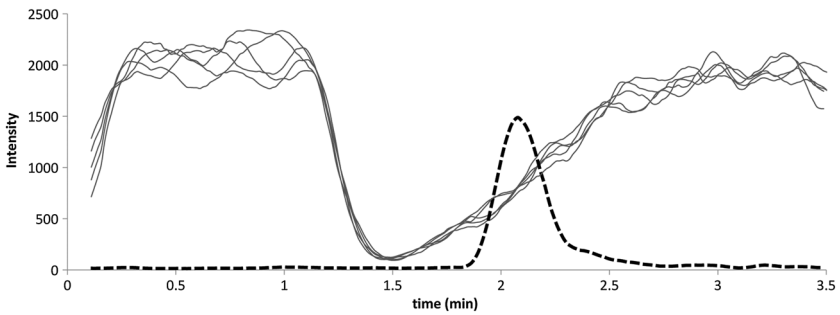

Figure 2. Injection of 5 blank plasmas with postcolumn infusion of FEN (continuous lines) and injection of a blank plasma spiked with FEN at a concentration of 10 ng/mL (dashed line).

FEN was also determined in CSF samples and was detected from all of them, with the exception of the one taken at 150 min from pig no. 6, whose plasma FEN concentration was also below the LLOQ by that time. In the remaining CSF samples, FEN was detected even at the first time point (10 min post-dose) (Figure 3), thus confirming the rapid access of the compound to the CNS, in line with its high lipophilicity.

The CSF/plasma ratio provides insight into the CNS drug exposure or availability of centrally active compounds, thus serving as a reference for assessing the extent of delivery to the pharmacological targets within the CNS (biophase or effect site). This is especially true for those drugs crossing the blood brain barrier (BBB) mainly by diffusion via the transcellular route after systemic administration,[12,14] which seems to be the case for FEN in line with its high lipophilicity and the apparent lack of active transport at the level of BBB. Indeed, FEN has proved not to behave as a substrate of main transporters including efflux P-glycoprotein or influx organic anion-transporting polypeptide (OATP).[47,48]

The comparison of CSF and plasma concentrations is particularly applicable in elucidating the lag in the time course of a central pharmacologic effect relative to that of drug concentration in circulation, under the assumption that CSF is in equilibrium with the biophase.[14] Even if care should be taken when interpreting data with only a single time point CSF and plasma concentration available, this is, to the best of our knowledge, one of the first reports on the temporal inter-relationship of FEN plasma and CSF kinetics after i.v. administration of such low doses in preclinical species. Up to date, two single reports have been found in scientific literature describing this relationship in experimental animal models, but they refer to the administration of doses far higher than the ones concerned herein. The first one was performed in dogs injected tritium-labeled ^{3}H-FEN (10 or 100 μg/kg),[49] and the second one applied HPLC-UV to the quantification of FEN only at steady state conditions in piglets administered 30 μg/kg bolus followed by infusion at 10 μg/kg/h.[38]

In this sense, there also seems to be a paucity of published data on methods of analysis using HPLC-MS/MS for the quantification of FEN in CSF or cerebral microdialysis samples, despite the importance of determining the drug levels in the CNS with a sufficient degree of sensitivity. Even though in the present study only one CSF sample was obtained from each animal, the low volume of CSF needed (50 μL) allows the applicability of the method in future and more specific pharmacokinetic studies aimed to further evaluate the CSF distribution of FEN in larger preclinical populations and/or under different dosing protocols. The low volume of CSF

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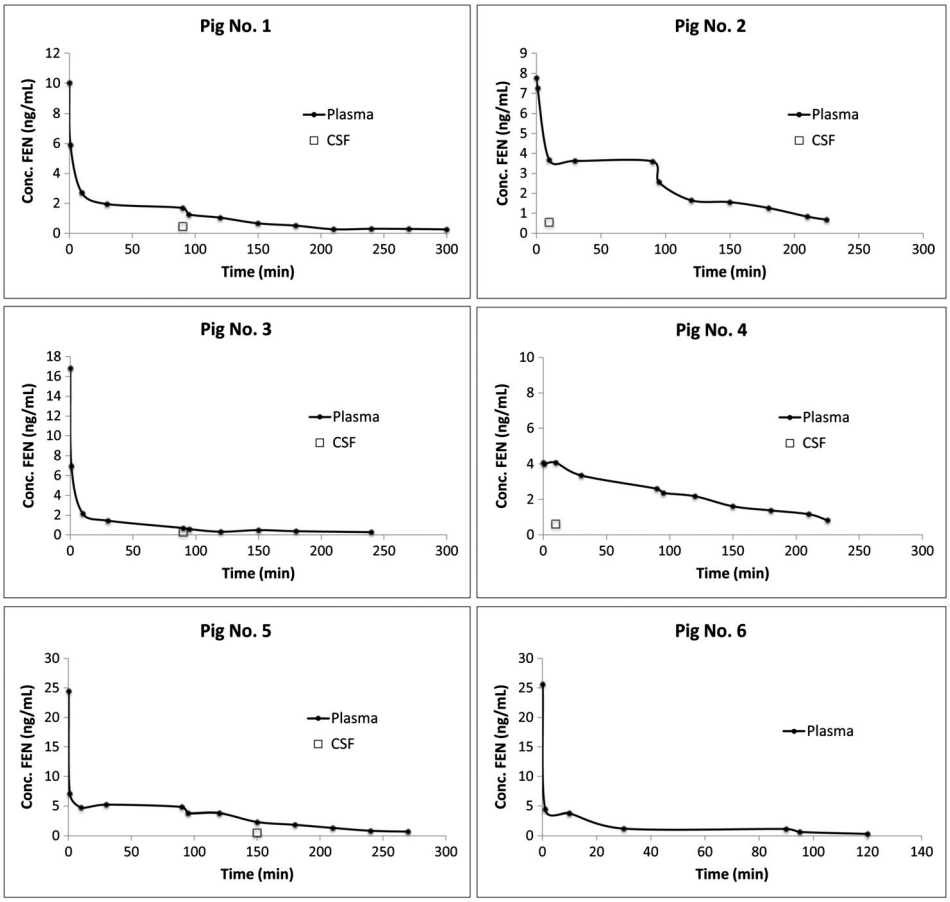

Figure 3. Individual plasma profiles (black dots) and concentration measured in the available single CSF sample (white squares) of FEN in piglets as quantified by the developed HPLC-MS/MS method. FEN level in the CSF sample extracted from pig No. 6 was below the LLOQ and could therefore not be displayed.

needed (50 μL) eases the application of this method to the analysis of samples of the newborn. For instance, the performance of frequent serial CSF sampling over time would allow the calculation of the relative CSF exposure as compared to plasma, which is given by the ratio between the corresponding areas under the curve (AUC_{CSF}/ AUC_{plasma} ratio). Moreover, this HPLC-MS/MS method could also be applied to the quantification of FEN levels in brain ISF samples obtained via microdialysis techniques, thus providing the tool for the joint assessment of PK disposition in both matrices. This could help elucidating the existing PK inter-relationship of FEN concentrations in plasma, CSF and brain ISF, against the observed pharmacodynamic effects in suitable animal models.[17,18,50] This PK/PD correlation may then be extrapolated to humans based on the well-described predictive capacity of some preclinical species,[14,15] which is of great value in view of the extremely restricted access to sampling of these biophase surrogate markers (i.e., CSF and brain ISF as indicative of drug levels at the effect site) in humans.

Conclusion

A simple, selective and sensitive HPLC-MS/MS method was developed and validated for the quantitative determination of FEN in pig plasma and CSF samples, which could be applied in future pharmacokinetic/pharmacodynamic assays.

This assay requires only a small volume of plasma (100 μL) and CSF (50 μL), which is of particular advantage in cases where sample volumes are limited (e.g. paediatric preclinical studies). The suitability of the method was assessed by its successful application to samples of both types of biological fluid from a pharmacokinetic study performed in newborn piglets.

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