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Rapid quantification of the δ-opioid receptor selective enkephalin DPDPE in canine cerebrospinal fluid by liquid chromatography–mass spectrometry

Steven Rossi*, Tony Yaksh

Department of Anesthesiology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA, 92093-0818, USA

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Abstract

An atmospheric pressure ionization liquid chromatographic–mass spectrometric assay was developed and validated for the determination of D-penicillamine^{2,5} enkephalin (DPDPE) in cerebrospinal fluid (CSF) from dog. DPDPE and internal standard (D-Ala^2,D-Leu^5 enkephalin=DADLE) were isolated from CSF by reversed-phase C_{18} solid-phase extraction with ZipTip micro-cartridges. Aliquots of extracted eluate were injected onto an Agilent Zorbax SB C_{18} column (30×2.2 mm; 3.5 μm) at a flow-rate of 0.4 ml/min. The isocratic mobile phase of methanol–10 m*M* ammonium formate (pH 3) (75:25, v/v) was then diverted to waste for 45 s after injection, after which time flow was directed to the single quadrupole mass spectrometer. DPDPE was detected by positive mode selected ion monitoring. Standard curves were linear ($r^2 \geq 0.991$) over the concentration range 1–1000 ng/ml. The efficiency of extraction recovery was greater than 97%, and the intra-assay and inter-assay precisions were within 9% relative standard deviation. DPDPE and the internal standard were stable in the injection solvent at 4 °C for at least 48 h. The assay was applied to the pharmacokinetic study of intrathecal DPDPE administration in the dog animal model.

Keywords: D-Penicillamine-enkephalin (DPDPE); D-Ala; D-Leu-enkephalin (DADLE)

1. Introduction

The pentapeptide D-penicillamine^{2,5} enkephalin (Tyr–D-Pen–Gly–Phe–D-Pen–OH, DPDPE, M_r 647.80) was developed in 1983 as a synthetic alternative to endogenous enkephalins for delta opioid receptor specific control of pain [1]. Its penicillamine disulfide bridge confers resistance to enzymatic degradation, resulting in an enhanced pharmacological half-life relative to other enkephalins [2]. DPDPEs disulfide bridge also confers poor immunogenicity; immunoassays for DPDPE quantification are difficult to implement, unlike other enkephalins which exhibit predictable immunogenerative response. Consequently, quantification of DPDPE in most of the in vitro and small animal model studies to date has employed measurement of radiolabeled [^{3}H]DPDPE, instead of direct assay of unlabeled enkephalin [3,4]. Since DPDPE is most effective when administered intrathecally [4–6], continued pharmacokinetic and toxicity studies with large animal models (and eventually human subjects) will require a facile, non-radiochemical assay for

*Corresponding author. Fax: +1-619-5436-070.
E-mail address: ssrossi@ucsd.edu (S. Rossi).

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DPDPE in cerebrospinal fluid (CSF), in the concentration range 0.01 to 100 μg/ml [7]. Quantification of this synthetic opiate in CSF has not been reported. An assay for DPDPE in other biological matrices, utilizing capillary zone electrophoresis, was deemed too laborious and of insufficient sensitivity [8]. A liquid chromatography–tandem mass spectrometry (LC–MS–MS) technique utilizing triple quadrupole methodology for measurement in plasma required large (5 ml) sample volumes and an overnight sample preparation step [9]. We have therefore developed a rapid and economical quantitative assay for DPDPE in CSF, using bench top single quadrupole LC–MS.

2. Methods

DPDPE (peptide free base, E-119) and the internal standard (I.S.) Tyr–D-Ala–Gly–Phe–D-Leu–OH (DADLE—peptide free base, E-116) were obtained from Research Biochemicals International (Natick, MA, USA). Both peptides were >99% pure. MS-grade methanol was obtained from Burdick and Jackson (Muskeson, MI, USA). Water was purified by a Barnsted Nanopure System (Barnsted Thermolyne, Dubuque, IA, USA). HPLC-grade ammonium formate and trifluoracetic acid were procured from Sigma (St. Louis, MO, USA). ZipTip C_{18} pipette tips for sample preparation were from Millipore (Bedford, MA, USA). Male dogs (beagle) were purchased from Marshall Farms (North Rose, NY, USA).

2.1. High-performance liquid chromatography (HPLC) and atmospheric pressure ionization mass spectrometry (API-MS) conditions

HPLC was performed on an Agilent (Wilmington, DE, USA) Zorbax SB C_{18} column (30×2.2 mm, 3.5 μm) fitted with a MetaChem (Lake Forest, CA, USA) 3 μm Intertsil guard cartridge (5×2 mm), coupled to an Agilent Model 1100 LC–mass-selective detection (MSD) system. The isocratic mobile phase consisted of methanol–10 m*M* ammonium formate (pH 3) (75:25, v/v), at a flow-rate of 0.4 ml/min. The guard column was replaced after 100 injections, and the HPLC system was rinsed daily with 10 ml 100% methanol.

Mass spectrometric detection was performed with an Agilent G2708AA (MSD 1100) single stage quadrupole instrument operating in the positive electrospray (API) mode. The LC–MS system was programmed to divert column flow to waste for 45 s after injection, after which time flow was directed into the mass spectrometer. The mass spectrometer was programmed to admit protonated molecules at the mass-to-charge ratios (m/z) of 646.2 (DPDPE) and 570.7 (I.S.), beginning 50 s after injection. Ionspray voltage was set to 6.0 kV, and fragmentor voltage was 80 V. Dwell time was 333 ms. Drying gas (nitrogen) flow-rate was 4 l/min, electrospray temperature was 350°C. Nebulizer gas (nitrogen) pressure was 138 kPa. Overall run time was 6 min. Data acquisition and peak area measurement were performed by the Agilent ChemStation software (ver. 6.02).

2.2. Standard solutions

Stock solutions of DPDPE and I.S. were prepared in HPLC mobile phase (1000 μg/ml) and stored at −70 °C in high-density polyethylene cryovials. Working solutions were prepared daily at concentrations of 1 μg/ml with sterile saline and were used to spike samples prior to extraction.

2.3. Standard curve and quality control samples

Serial dilutions were employed to obtain final concentrations of 1–1000 ng/ml of DPDPE in naive CSF. These spiked samples, containing six different concentrations (1, 5, 10, 100, 500, 1000 ng/ml), were used to construct standard curves. Quality control (QC) samples of pooled naive CSF (n=10 animals) were prepared from independent stock solutions to contain concentrations of DPDPE representative of the standard curve range, and were stored at −70 °C. A QC sample pool above the upper limit of the standard curve was additionally prepared to serve as a dilution QC sample.

2.4. Sample preparation

To a 5 μl aliquot of CSF standard, blank, QC, or study sample in a clean 0.5-ml high-density polyethylene micro centrifuge tube, 5 μl of I.S. solution was added and the resultant solution was vortexed

for 5 s. The above solution was loaded onto a sample preparation pipette tip (ZipTip), previously conditioned with 20 μl methanol and then 20 μl aqueous 0.1% trifluoroacetic acid, by 10 cycles of sequential aspiration and expiration through the ZipTip.

The ZipTip was then rinsed 5× with 20 μl water, after which the sample was eluted into an autosampler vial with 5 μl methanol. Duplicate injections of each 1 μl sample were made onto the LC–MS system. Autosampler temperature was maintained at 4±0.5 °C.

2.5. Assay calibration

Calibration curves were produced by plotting peak area ratio of the analyte to the internal standard against the analyte's concentration ratio. The linear regression was fitted to the concentration range 1–1000 ng/ml.

2.6. Assay accuracy and precision

Accuracy and precision were determined by assaying QC samples (5, 100, and 1000 ng/ml) in 10 replicates on 6 different days. In addition, a fourth QC sample (dilution QC) was spiked (5000 ng/ml) above the highest standard and diluted with naive CSF prior to sample preparation. Dilution QC samples were analyzed at the same time as QC samples. The inter-assay precision was evaluated by one-way analysis of variance (ANOVA). Inter-assay precision, expressed as relative standard deviation (RSD), was defined for each of the concentrations as:

$$\mathrm{RSD}\,(\%) = 100 \cdot [(\mathrm{TMS} - \mathrm{EMS})/N]^{0.5}/\mathrm{GM}$$

where TMS = treatment mean square, EMS = error mean square, and GM = grand mean are taken from ANOVA.

2.7. Limit of quantitation (LOQ)

Pooled naive CSF ($n = 10$ animals) was analyzed simultaneously with aliquots from this same pool that had been spiked with DPDPE to a concentration of 1 ng/ml. Assay performance on accuracy at the LOQ was calculated as the percentage deviation (%DEV) for the mean observed concentration from the nominal concentration for 10 aliquots. Assay precision was expressed as the RSD of the observed concentration in the 10 aliquots.

2.8. Extraction efficiency

Two sets of standards, within the concentration range of 1–1000 ng/ml were prepared in naive CSF and in mobile phase. CSF standards were extracted with ZipTips as previously described and then chromatographed. Standards in mobile phase were injected without extraction. Extraction recovery was calculated by the following equation:

% recovery = peak area slope of CSF standard curve/peak area slope of mobile phase standard curve

2.9. Stability

Stability of DPDPE and I.S. in injection solvent was determined by periodically injecting replicate preparations ($n = 3$) of extracted samples at 0, 12, 24, and 48 h. Peak areas obtained at the 0 h were used as reference in calculating the relative ratios for each analyte at the different time points.

3. Results and discussion

3.1. Specificity

Selected ion monitoring chromatograms of CSF obtained from naive and post-dose animals are presented in Figs. 1 and 2. Throughout this study no significant interfering peaks were detected at the retention times of the analytes (DPDPE and I.S.) in naive samples. Fig. 1 illustrates a baseline signal typical of analysis near the method's LOQ. The nominal retention times for I.S. and DPDPE were 1.67 and 2.75 min, respectively. A single column (with the above noted guard column replacements and daily methanol rinses) was employed for the duration of this study (6 months).

3.2. Linearity

Linear regression of the peak area ratios versus standard concentrations revealed that peak area ratios

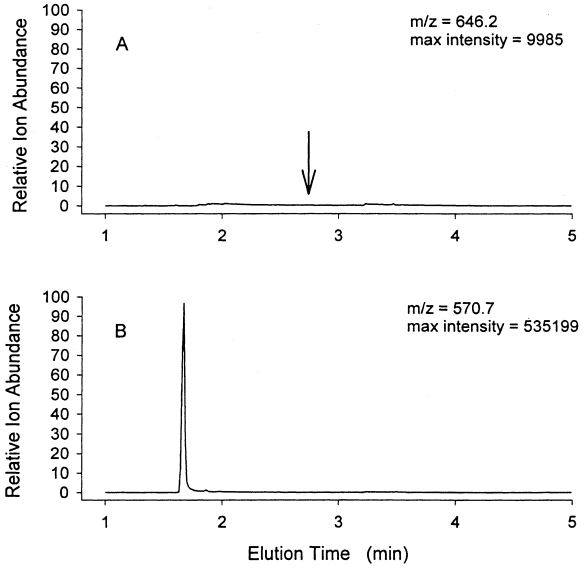

Fig. 1. Typical single ion monitoring chromatograms obtained from dog pre-dose cerebrospinal fluid (A) DPDPE and (B) internal standard (arrow indicates the elution time of DPDPE).

were linear for the concentration range of 1–1000 ng/ml. The correlation coefficient was 0.9909, with an intercept value of 0.0008. Values for r^2 ($\geq$0.991)

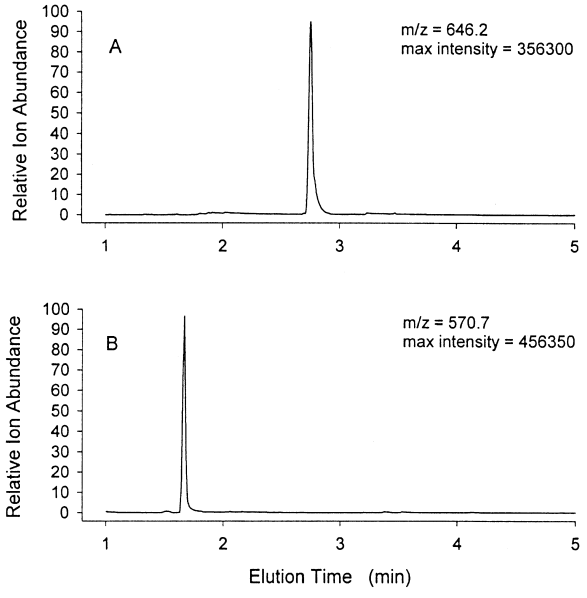

Fig. 2. Typical single ion monitoring chromatograms obtained from dog cerebrospinal fluid after administration of DPDPE: (A) DPDPE, 492 ng/ml and (B) internal standard.

and the ANOVA between slopes from individual curves (n=10, data not shown) demonstrated that standard curves were reliable for the studied concentration range.

3.3. Limit of quantitation

The nominal concentration in LOQ samples was 1.00 ng/ml, which deviated less than ±7.42% from observed values. Precision for the LOQ samples was 8.1% RSD. Since differences between observed and nominal concentrations below 1.00 ng/ml exceeded ±15% (data not shown), the LOQ for DPDPE in canine CSF was therefore established at 1.0 ng/ml.

3.4. Intra- and inter-assay accuracy and precision

Table 1 presents intra- and inter-assay accuracy and precision data for QC samples at three different concentrations of DPDPE. Intra- and inter-day precision values (RSD) for DPDPE were ≤9.3%. The accuracy (%DEV) for all concentrations tested deviated by ≤10.2% from the corresponding nominal concentrations.

3.5. Extraction recovery

Results of the comparison of neat standards versus CSF extracted standards for DPDPE indicated that the extraction of DPDPE from 5 μl of dog CSF samples was greater than 97%, but less than 102% (data not shown).

3.6. Stability

Absolute peak area at three concentration levels of DPDPE and I.S. were found to be within ±7.4% RSD from the corresponding peak areas at time zero during the course of this 48 h stability experiment (Table 2).

3.7. Application

The method was applied to measure levels of DPDPE in dogs (n=3). Following the intrathecal administration of 0.3 mg/kg dose of DPDPE with a single needle lumbar puncture, CSF samples were drawn at timed intervals via an indwelling intrathecal

Table 1
Intra- and inter-assay accuracy and precision results for DPDPE[a]

[Nominal] (ng/ml)	[Observed] (ng/ml)	Accuracy (% DEV)	Precision (RSD, %)	
			Within run	Between run
5	5.10	10.20	7.16	9.33
100	103.7	3.72	4.99	5.39
1000	1016.5	1.65	0.56	1.51
5000[b]	5414.5	8.29	5.93	7.48

[a] $n=10$, for 6 different days, duplicate injections each sample.
[b] Dilution QC samples (analyzed in a single run) with 10-fold dilution.

Table 2
Sample stability for DPDPE and internal standard (DADLE)[a]

[Nominal] (ng/ml)	Time (h)	DPDPE		DADLE	
		Peak area	RSD (%)	Peak area	RSD (%)
5	0	5946	0.00	852 829	0.00
	12	6041	1.60	863 434	4.39
	24	6331	6.48	869 445	0.24
	48	6386	7.40	844 416	4.43
50	0	61 620	0.00	884 177	0.00
	12	62 309	1.12	848 465	1.55
	24	62 269	1.05	882 116	3.47
	48	62 217	0.97	829 354	2.31
500	0	622 058	0.00	855 864	0.00
	12	594 667	−4.40	856 242	3.26
	24	627 550	0.88	874 917	2.94
	48	628 753	1.07	835 944	0.05

[a] $n=3$, duplicate injections each sample.

lumbar catheter, and immediately frozen at −70 °C. Samples were analyzed within 90 days of collection. The mean (±SD) lumbar CSF concentration versus time profile for DPDPE is depicted in Fig. 3. Intrathecally administered DPDPE exhibited a pharmacokinetic profile in dogs which was identical to that observed for similar hydrophilic opioids in other species, consistent with a bi-exponential model of distribution and elimination [10,11]. Fits performed using iteratively re-weighted least-squares analysis produced the following model equation:

$$\text{concentration}_{(t)} = 21.9e^{-0.014t} + 3.25521^{-0.004t}$$

where t is the time in minutes, and concentration units are μg/ml. The elimination half-life for DPDPE from the cerebrospinal space in the dog model from the above equation was 131 min.

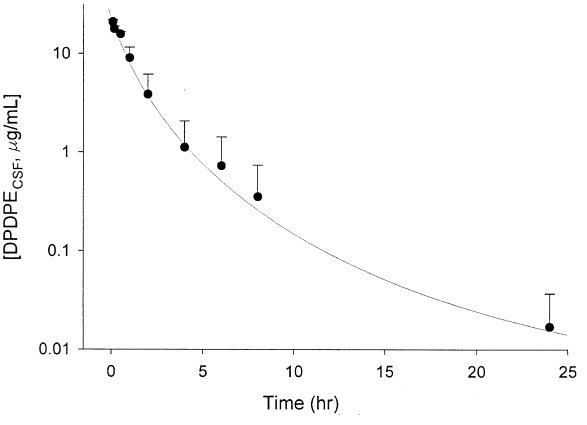

Fig. 3. Mean (±standard deviation) cerebrospinal fluid concentration versus time profile of DPDPE in dogs ($n=3$) following a single 0.3 mg/kg intrathecal dose administration.

Clearance and volume of distribution could be calculated from the above, however, these concepts are of limited utility for intrathecal drug delivery.

4. Discussion

In the course of method development, attempts were made to reduce sample preparation time by injection of unprocessed CSF directly into the HPLC system. This involved utilization of the system's effluent switching capability to divert column flow prior to the analyte elution, under the assumption that constituents which elute before the analytes are those components of CSF most capable of inhibiting ionization within the atmospheric pressure spray chamber. However, whenever CSF was injected directly into the system, including experiments with different buffer systems, ion abundance and overall sensitivity was decreased. Accurate quantification of DPDPE at lower concentrations ($\leq$100 μg/ml) therefore required removal of ion suppressing constituents in CSF by solid-phase extraction prior to sample injection.

Maximum sensitivity for the detection of DPDPE in canine CSF was obtained by use of smaller sample volumes, rather than larger sample volumes with sample enrichment. When larger sample volumes (up to 100 μl CSF) were extracted with larger solid-phase cartridges (up to 500 mg gel) sensitivity was reduced, even after sample enrichment (re-constitution with a volume of methanol less than the original sample volume). Experiments with quality control samples and spiked reagent blanks containing know amounts of DPDPE demonstrated that significant reductions in sensitivity resulted from the use of larger solid-phase cartridges (data not shown). It was not determined whether the reduced sensitivities from larger solid-phase extraction devices were due to ionization suppression, non-specific adsorption (low recovery), or both. Cartridges from several different vendors exhibited this behavior. ZipTip micro extraction cartridges, on the other hand, did not exhibit this behavior and allowed the use of very small sample volumes, down to 5 μl. The ability to accurately measure DPDPE in sample volumes less than 10 μl will be particularly beneficial to the analysis of CSF from small animal models, such as the rat and mouse. Including sample preparation, the method was capable of analyzing 40 samples per day. Automation of the (manual) sample extraction procedure would significantly increase sample throughput.

5. Conclusions

A HPLC procedure with single ion monitoring by single quadrupole mass spectrometry was developed and validated for determination of the synthetic opioid analog, D-penicillamine^{2,5} enkephalin, in dog cerebrospinal fluid. The procedure was shown to be sensitive, selective, accurate and precise. The reported method offers multiple advantages such as a rapid and simple extraction regime, improved sensitivity, and the ability to work with extremely small sample volume, as compared to previous methods. Further, the technique is performed with contemporary supplies and widely available bench top HPLC–MS instrumentation.

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