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Journal of Chromatography B, 657 (1994) 243–247

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

High-performance liquid chromatographic determination of intrathecally administered [D-Ala^2-D-Leu^5]-enkephalin concentrations in canine cerebrospinal fluid

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(First received October 22nd, 1993; revised manuscript received March 29th, 1994)

Abstract

A rapid and useful method for high-performance liquid chromatographic analysis of exogenous [D-Ala^2-D-Leu^5]-enkephalin (DADLE) in cerebrospinal fluid (CSF) is described. CSF (0.5 ml) samples were filtered using a 0.22-μm Co-Star filter. Chromatography was performed on a μBondapak C_{18} column using a mobile phase of A, 0.05 *M* sodium phosphate (monobasic, pH 6.0) and B, 60% acetonitrile in 0.05 *M* sodium phosphate (pH 6.0) with a flow-rate of 1 ml/min. Absorbance at 210 *nm* was measured. The procedure produced a linear curve for the concentration range 1–10 μg/ml. The development of the assay produced rapid, repeatable and accurate results for CSF analysis of DADLE at concentrations achieved with therapeutic administration of the peptide. This method could also be used in the future for analysis of compounds like DADLE.

1. Introduction

[D-Ala^2-D-Leu^5]-Enkephalin (DADLE) is a synthetic pentapeptide that has been effective in providing analgesia in cancer patients who have developed a tolerance to systemic opiates such as morphine and whose pain is no longer relieved by high doses of these opiates [1–3]. Little is known about the pharmacokinetic distribution of the peptide when administered therapeutically, particularly the rate of distribution to the brain and the degree of distribution within the brain. Studies are being conducted to determine the distribution and rate of elimination of DADLE from CSF and the appropriate therapeutic dosages necessary to maintain analgesia in experimental animals.

Analyses of endogenous enkephalins, particularly leucine enkephalin and methionine enkephalin, have been performed in samples of human cerebrospinal fluid (CSF) [4,5], brain [6], pituitary [7], jejunal lumen [8] and adrenal effluent [9]. These procedures use C_{18} Sep Pak extractions, and separation of the peptides using high-performance liquid chromatography followed by quantitation of the separated peptides by a radioimmunoassay.

The present article describes a rapid and efficient HPLC procedure for analysis of exogenously added DADLE at concentrations likely to be achieved in CSF following therapeutic administration of the peptide for chronic pain. The

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0378-4347/94/$07.00
SSDI 0378-4347(94)00187-A

assay should facilitate the evaluation of the pharmacokinetic disposition of analgetic compounds such as DADLE administered to humans and animals.

2. Experimental

2.1. Reagents and standards

Methanol and acetonitrile were HPLC grade (Burdick and Jackson Laboratories, Muskegon, MI, USA). Reagent grade sodium phosphate monobasic and [D-Ala^2-D-Leu^5]-enkephalin (98% purity by amino acid analysis) were purchased from Sigma (St. Louis, MO, USA).

A stock standard solution of DADLE (0.1 mg/ml) was prepared by dissolving the peptide in 0.05 *M* sodium phosphate monobasic (pH 6.0). DADLE standards were made by serial dilutions of the stock standard solution with control CSF. Standards were stable at −4°C for one month.

2.2. Apparatus

The analytical system consisted of a 600E solvent delivery system, a Model 700 WISP autosampler, a RCM 10 cm × 8 mm cartridge holder equipped with a 10 cm × 8 mm I.D. μBondapak C_{18} cartridge (10 μm particle size) and a C_{18} Guard-Pak precolumn insert, a Model 990 photodiode array detector (Waters, Milford, MA, USA) and a NEC Powermate computer system (NEC, Foxborough, MA, USA).

2.3. Chromatography

The mobile phase consisted of A, 0.05 *M* sodium phosphate buffer (pH 6.0) and B, 60% acetonitrile in 0.05 *M* sodium phosphate (pH 6.0). The sodium phosphate buffer pH was adjusted with 6.0 *M* sodium hydroxide. The solvent was used in a linear gradient from 100% A to 100% B over 30 min, with an additional 10 min for column re-equilibration after each analysis. Gradients run after sample analysis with no sample injected were found to contain no evidence of retained peptides. The flow-rate was 1 ml/min, column temperature was ambient, and absorbance was measured at 210 nm.

2.4. CSF samples

Cerebrospinal fluid (CSF) for all assays was obtained from healthy beagle-hound or hound-mix dogs housed in AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited facilities. Procedures were performed after obtaining approval from The Institutional Animal Care and Use Committee.

Dogs were anesthetized using halothane or isoflurane, hair was clipped and a surgical scrub was performed of the skin over the base of the skull and proximal cervical spine for immediate collection of control CSF and over the lumbar spine for pharmacokinetic studies. Percutaneous puncture of the cervical dural membrane was performed with the dogs in lateral recumbency and the neck ventro-flexed at a 90 degree angle using a 20 gauge two and one-half inch spinal needle (Monoject). Using fluoroscopic guidance, Arrow-Racz spring wire reinforced catheters (Theracath, Arrow International, Reading, PA, USA) were placed between lumbar vertebrae 5 and 6 (L_{5-6}) and advanced to L_{1-2} in the intrathecal (subarachnoid or spinal) space. The catheters were used for CSF collection immediately prior to and following the subsequent administration of DADLE.

DADLE (1 mg in 1 ml sterile H_2O) was administered intrathecally to dogs via spinal needle between the third and fourth lumbar vertebrae (L_{3-4}) for pharmacokinetic studies. CSF was collected between L_{1-2} at 5, 10, 15, 30, 45, 60, 120, 180 and 240 min after intrathecal injection for determination of DADLE concentration. CSF samples (1 ml) were placed in tubes containing 20 μl concentrated formic acid (final sample pH 4.0) and immediately frozen at −20°C.

Previously frozen CSF samples were thawed and vortex-mixed before use. CSF (0.5 ml) was filtered using a 0.22-μm CoStar filter (CoStar Corporation, Cambridge, MA, USA). A 175-μl

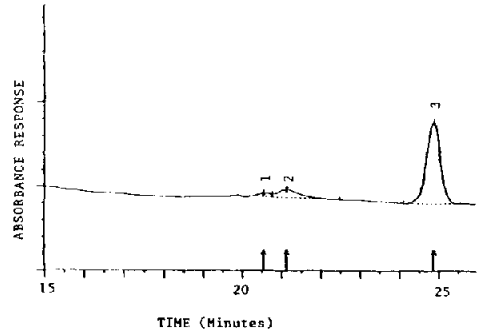

Fig. 1. Chromatogram of DADLE added to control CSF (7.5 μg/ml). Peaks: 1 and 2 = endogenous components of CSF, and 3 = DADLE.

aliquot of this solution was injected into the liquid chromatograph.

Variability in measured DADLE concentrations was determined by calculating the concentration means ± standard deviations and coefficients of variation using Statgraphics version 2.1 (Statistical Graphics Corporation, Rockville, NY, USA).

3. Results

A representative chromatogram of DADLE added to control CSF (7.5 μg/ml) is shown in Fig. 1. The retention time for DADLE was 24.8 min. Fig. 2 is a chromatogram of canine CSF collected 60 min after intrathecal administration

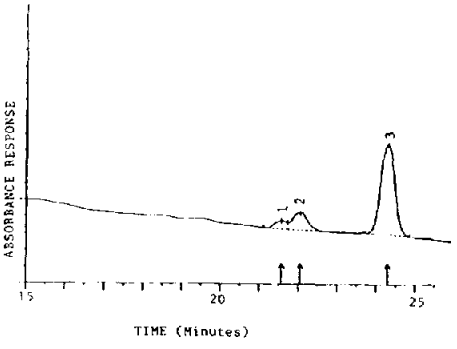

Fig. 2. Chromatogram of CSF sample collected from a canine 60 min after intrathecal administration of DADLE (1.0 mg in lumbar subarachnoid space). Peaks: 1 and 2 = endogenous components of CSF, and 3 = DADLE.

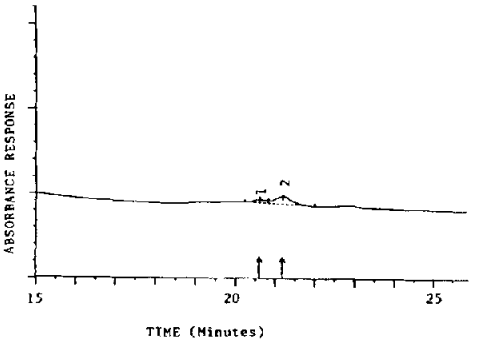

Fig. 3. Chromatogram of control canine CSF. Peaks: 1 and 2 = endogenous components of CSF.

of 1 mg of DADLE. The retention time for DADLE in the sample was 24.5 min. Endogenous CSF components did not interfere with the elution of DADLE in normal blank CSF (Fig. 3) at the level of sensitivity of the assay.

The method produced a linear curve for the concentration range used in this study, with the correlation coefficients ranging from 0.998 to 0.999. Replicate analyses performed on the same day for CSF samples spiked with specific concentrations showed intra-assay coefficients of variations to be 10.1% for 2 μg/ml, 3.0% for 4 μg/ml and 1.9% for 8 μg/ml (Table 1). Inter-assay variability for DADLE quantification in CSF on four different days is shown in Table 2. The mean percent recoveries of DADLE added to CSF on the same day as analysis were 88%, 95%, 96%, 96% and 97% for 1, 2.5, 5, 7.5 and 10 μg/ml (μg of DADLE added per ml of CSF), respectively (Table 3). These values were

Table 1
Intra-assay precision for DADLE standards in CSF

Concentration added[a] (μg/ml)	n^b	Measured concentration (μg/ml)[a,c]	C.V. (%)
2.0	4	1.9 ± 0.19	10.1
4.0	4	3.7 ± 0.12	3.0
8.0	4	8.3 ± 0.16	1.9

[a] μg of DADLE added to 1 ml CSF.
[b] n = number of samples.
[c] Mean ± standard deviation of 4 samples.
C.V. = coefficient of variation.

Table 2
Inter-assay precision for DADLE standard curve in CSF

Concentration Added[a] (μg/ml)	n[b]	Measured concentration (μg/ml)[a]	Mean area[b] ($\cdot 10^{-4}$)	C.V. (%)
1.0	4	0.9	41.1 ± 3.3	8.1
2.5	4	2.3	117.0 ± 10.4	8.9
5.0	4	4.9	256.9 ± 12.2	4.8
7.5	4	7.2	413.8 ± 13.3	3.2
10.0	4	9.7	552.6 ± 15.3	2.8

[a] μg of DADLE added per ml of CSF.
[b] Mean ± standard deviation of chromatogram peak areas for 4 days.
[c] n = number of days.
C.V. = coefficient of variation.

significantly greater than recoveries achieved in our laboratory using C_{18} Sep Pak extraction techniques previously published [5] (data not shown). The detection limit for DADLE was 0.01 μg/ml; this concentration would represent a peak approximately three times greater than baseline noise.

4. Discussion

Pharmacokinetic studies of therapeutically administered substances like DADLE require a quantitative method of analysis. The procedure described here is for HPLC analysis of exogenously administered DADLE concentrations in CSF. The assay is not sensitive enough for quantitation of endogenous concentrations of enkephalins which are in the fmol/ml range [4].

Table 3
Recovery of DADLE from CSF

Concentration added (μg/ml)[a]	n[b]	Recovery[c]	C.V. (%)
1.0	4	88 ± 7.6	8.7
2.5	4	95 ± 8.3	8.8
5.0	4	96 ± 9.0	9.4
7.5	4	96 ± 2.1	2.2
10.0	4	97 ± 3.9	4.0

[a] μg of DADLE added per ml of CSF.
[b] n = number of replicates.
[c] Mean ± standard deviation for 4 replicates.
C.V. = coefficient of variation.

Procedures have been reported [4–9] for CSF and tissue analysis of leucine enkephalin and methionine enkephalin, both of which are structurally similar to DADLE. The procedures rely on radioimmunoassay techniques to achieve the sensitivity required for measurement of naturally occurring enkephalins in CSF. Capper *et al.* [4] and Venn [5] both used a C_{18} Sep Pak extraction with a trifluoroacetic acid–methanol solution, followed by HPLC separation of peptides and quantitation by RIA. We attempted to modify these procedures to our assay without the use of RIA quantitation, since we were not measuring endogenous levels of an enkephalin. However, preliminary studies in our laboratory showed significant loss of DADLE with C_{18} Sep Pak extractions in the concentration range studied. Subsequent studies indicated that sample extraction was not required.

Although specificity of this assay for neuropeptides was not evaluated for all naturally occurring enkephalins, the presence of endogenous concentrations of enkephalins could potentially bias measurements of therapeutically administered peptides to a very limited extent. Endogenous concentrations of enkephalins are reported to be in the fmol/ml range and are not

detectable by absorbance alone following HPLC isolation [4], whereas concentrations of exogenous DADLE measured here were in the nmole/ml range.

DADLE, a delta opioid-receptor agonist, has been shown to be anti-nociceptive when injected intraspinally [1,3]. Total intrathecal doses ranging between 125 μg and 1.6 mg [1,3] have been administered to cancer patients for management of pain. DADLE concentrations in CSF of human patients following intrathecal administration have not been reported; however, the method described here was found to be suitable for measurement of CSF concentrations in canines administered 1 mg DADLE intrathecally in the sublumbar space.

Intrathecal DADLE administration in man has been shown to produce analgesia for 5–12 h [1]. CSF sample collection from dogs was terminated at 4 h after injection of DADLE in this study. Preliminary pharmacokinetic studies suggest that this assay is appropriate for expected concentrations of DADLE in CSF up to 8–12 h following intrathecal administration of DADLE (1 mg) to canines.

In conclusion, a rapid and useful HPLC procedure has been developed to detect CSF levels of intrathecally administered DADLE in the range of reported therapeutic doses. This procedure would be appropriate for large numbers of samples.

Acknowledgements

The authors would like to thank Ms. Pam Carter and Ms. Betsy Cagle for preparing the manuscript. We would also like to thank Anita Genova and Michelle Richardson for their technical assistance in sample analysis.

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