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Development of a capillary zone electrophoresis assay to examine the disposition of [D-pen^{2,5}]enkephalin in rats

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Abstract

A novel capillary zone electrophoresis (CZE) assay method was developed to evaluate the systemic disposition of [D-pen^{2,5}]enkephalin (DPDPE) in rats. DPDPE was recovered from serum samples (200 μl) by solid-phase extraction. Complete resolution of DPDPE and the internal standard ([D-ser^{2}]leucine-enkephalin; DSLET) from other serum components was achieved within 15 min on a 50-μm I.D. capillary column with borate buffer (25 m*M*, pH 8.3). The peak-height ratio (DPDPE to DSLET) was linear through 100 μg/ml, with a detection limit of 250 ng/ml in serum, when absorbance of the column eluent was monitored at 210 nm. Serum samples obtained from rats after a 10 mg/kg intravenous bolus dose of DPDPE were analyzed with the present CZE method. The results suggest that CZE is a useful technique for quantitating therapeutic peptides in biological matrices.

Keywords: Enkephalin; Pharmacokinetics

1. Introduction

[D-pen^{2,5}]Enkephalin (DPDPE; H-Tyr-D-Pen-Gly-Phe-D-Pen-OH, Pen=penicillamine) is a [Met^{5}]-enkephalin opioid pentapeptide that was first synthesized in 1983 [1]. DPDPE was developed as an antinociceptive drug devoid of the multiple side-effects commonly associated with opioids [2,3]. Extensive pharmacological studies have indicated that DPDPE is highly selective for opioid δ-receptors [4,5] and is capable of penetrating the blood–brain barrier [6]. In addition to producing antinociception, DPDPE also decreases gastrointestinal transit after intrathecal administration [7,8] and inhibits diarrhea through an antisecretory mechanism, when given intracerebroventricularly or subcutaneously [9]. Few studies [2,6] have been conducted to characterize the pharmacokinetic properties of DPDPE, primarily due to the lack of selective and sensitive analytical methods for this class of compounds. One technique utilized [^{3}H]DPDPE with radiochemical detection to examine the stability and distribution of the peptide in mice [2,6]. DPDPE was relatively stable compared to endogenous enkephalin peptides, with an in vitro half-life of up to 60 min when incubated with purified enkephalinase (NEP EC 3.4.24.11), an important enzyme in the in vivo metabolism of enkephalins [10]. The relatively long half-life, and the absence of detectable biotransformation products, have been attributed to the conformational rigidity and the disulfide bond between the two modified amino acids (i.e., D-pen^{2} and D-pen^{5}). However,

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these data should be interpreted cautiously due to the use of a relatively non-specific assay technique and the possibility of ^{3}H exchange during the experiment. Recently, a MS–MS method was reported for the analysis of DPDPE and three other opioid peptides in ovine plasma [11]. This technique provided acceptable sensitivity and reproducibility, but is complicated and expensive to conduct on a routine basis.

Capillary electrophoresis (CE) is a relatively simple and rapid method that offers high separation efficiency for the analysis of complex mixtures [12,13]. Although several applications of CE to the analysis of peptides in biological fluids have been reported [14–18], most have focused on the mechanism of separation, electrophoretic behavior, or component identification; limited effort has been expended on the quantitative analysis of peptides and on the application of capillary zone electrophoresis (CZE) to pharmacokinetic studies of peptides [14]. Thus, the objective of the present study was to establish a quantitative CZE method for the determination of DPDPE in serum, and to test the method under relevant experimental conditions, by examining the disposition of DPDPE in rats after intravenous administration of the peptide.

2. Experimental

2.1. Equipment

Solid-phase extraction of serum samples was performed on 3 ml C_{18} bonded-phase columns (Varian, Sunnyvale, CA, USA) under vacuum (MFG, Benton Harbor, MI, USA), and the eluents were concentrated in an analytical evaporator (Meyer N-EVAP, Oganomation Associates, South Berlin, MA, USA). Electrophoretic separation was achieved with a Dionex capillary electrophoresis system equipped with an unmodified fused-silica capillary column (100 cm × 50 μm I.D.; Dionex, Sunnyvale, CA, USA).

2.2. Chemicals

DPDPE and DSLET (H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH) were provided by the National Institute of Drug Abuse (Baltimore, MD, USA) and were used without further purification. All reagents used in this study were of the highest grade available from commercial sources in the USA.

2.3. Sample pretreatment

The internal standard DSLET (2 μl; 1 mg/ml) was added to serum samples (100–200 μl) to achieve a final concentration of 10 μg/ml before sample pretreatment. Proteins were precipitated with acetonitrile (400 μl). After vortex-mixing and centrifugation (1500 *g* for 10 min), the supernatant was transferred to a clean 1.5-ml microcentrifuge tube and evaporated to dryness at 40°C under a stream of dry nitrogen. The residue was reconstituted with water (1 ml) and applied to a solid-phase extraction column that had been preconditioned with methanol (2 ml) and water (2 ml). The column then was washed sequentially with water (1 ml) and 0.1% TFA in methanol (80:20, v/v; 1 ml). Analytes were eluted with 0.1% TFA in methanol (20:80, v/v; 2 ml). The 2 ml eluent was evaporated to dryness under nitrogen, the residue was reconstituted with sodium borate buffer (10 m*M*, pH 8.3, 100 μl) and filtered with a microcentrifuge filter (0.45 μm, Alltech Associates, Deerfield, IL, USA) before analysis by CZE.

2.4. Electrophoretic separation

Separation by CE was achieved on an unmodified fused-silica capillary column (100 cm × 50 μm I.D., 93 cm effective length) with a sodium borate buffer (25 m*M*, pH 8.3). Samples were introduced into the capillary via electrokinetic injection (10 kV, 6 s). Separation was conducted at an applied voltage of 25 kV, and absorbance was monitored continuously at 210 nm. All CZE analyses were conducted at room temperature. Data (peak height of the analyte and internal standard) were acquired with Dionex CE software and recorded on an IBM-compatible personal computer.

2.5. Pharmacokinetic studies

Male Sprague–Dawley rats (250–350 g, Hilltop Laboratory Animals, Scottdale, PA, USA) were used

in this experiment. Silicone rubber cannulae were implanted in the right jugular veins of ether-anesthetized rats 24 h prior to experimentation. The cannulae were filled with saline containing heparin (20 U/ml) to maintain patency. Blood samples (300 μl) were collected through the jugular vein cannula before and at 2, 4, 6, 8, 10, 15, 20, 30 and 40 min after administration of DPDPE in saline (10 mg/ml; 10 mg/kg total dose). Serum was harvested from blood and stored frozen (−20°C) until analysis (within 24 h). Serum samples were prepared for analysis as described in Section 2.3 and Section 2.4. The serum concentration–time data for individual rats were analyzed by fitting a biexponential equation to the data with non-linear least-squares regression to obtain estimates of intrinsic pharmacokinetic parameters.

3. Results and discussion

3.1. Optimization of sample pretreatment conditions

Three methods, i.e., microcentrifugation, acetonitrile precipitation and ultrafiltration, were assessed for precipitation of serum proteins before analysis by CZE. Results (Fig. 1) demonstrated that the former two methods did not provide sufficient sample clean-up prior to CZE analysis, as evidenced by the large amount of impurities eluting after 5 min. Although ultrafiltration did remove significant impurities from serum, the recovery of DPDPE after ultrafiltration was only approximately 30% (data not shown), which would adversely affect both the sensitivity and the reproducibility of the assay. The limited recovery of DPDPE after ultrafiltration may be due to binding of the peptide to serum proteins, which requires further examination.

Solid-phase extraction also was assessed as a sample pretreatment method. An initial experiment was conducted to examine the necessity for protein precipitation with acetonitrile before solid-phase extraction. As shown in Fig. 2, precipitation of proteins with acetonitrile prior to extraction eliminated the potential interference that occurred in samples pretreated only by solid-phase extraction.

The recovery of DPDPE from serum was evaluated at three different concentrations (2, 10 and 50 μg/ml) and under two different pretreatment conditions, namely deproteination with acetonitrile plus solid-phase extraction (method 1) or solid-phase extraction only (method 2). Recovery of the analyte (evaluated relative to DPDPE in buffer without any pretreatment) expressed as mean ± S.D. was approximately 87% for both methods (Table 1). These results, combined with those in Fig. 2, indicated that protein precipitation with acetonitrile removed more impurities from serum, but did not impact significantly on the recovery of DPDPE from serum. Thus, deproteination with acetonitrile before solid-phase extraction was an important and necessary step in the optimized sample pretreatment process.

3.2. Optimization of CZE conditions

3.2.1. Buffer composition and pH

In CZE, selectivity (i.e., the relative order of solute migration) can be altered most readily through changes in buffer composition and pH [19]. Consequently, buffer selection is extremely important to the success of any CZE separation. Most peptide separations by CZE have been conducted under extremely acidic conditions (e.g. phosphate buffers at pH 2) in order to suppress the ionization of silanol groups on the silica surface and thus reduce the electroosmotic flow and the interaction between analytes and the capillary wall [20]. Due to the high chemical stability of the fused-silica capillary, the accessible pH range can vary from below 2 to more than 12, and usually is limited by analyte stability. Two systems, phosphate (6.25, 25, 50 m*M*, pH 2.4) and borate (6.25, 25, 50 m*M*, pH 8.3) buffers were compared as the running buffer in this study. Both buffers provided good resolution for DPDPE and DSLET in aqueous solution (Fig. 3). However, analysis of more complicated matrices (serum) was not possible with phosphate buffer, due to interference from endogenous components (Fig. 4). In addition, phosphate buffer generated a higher current than borate buffer at the same ionic strength. The high current prevented the use of higher voltages or larger diameter capillaries, either of which could increase separation efficiency on the CE system [19]. A buffer with 50 m*M* boric acid, 10 m*M* sodium

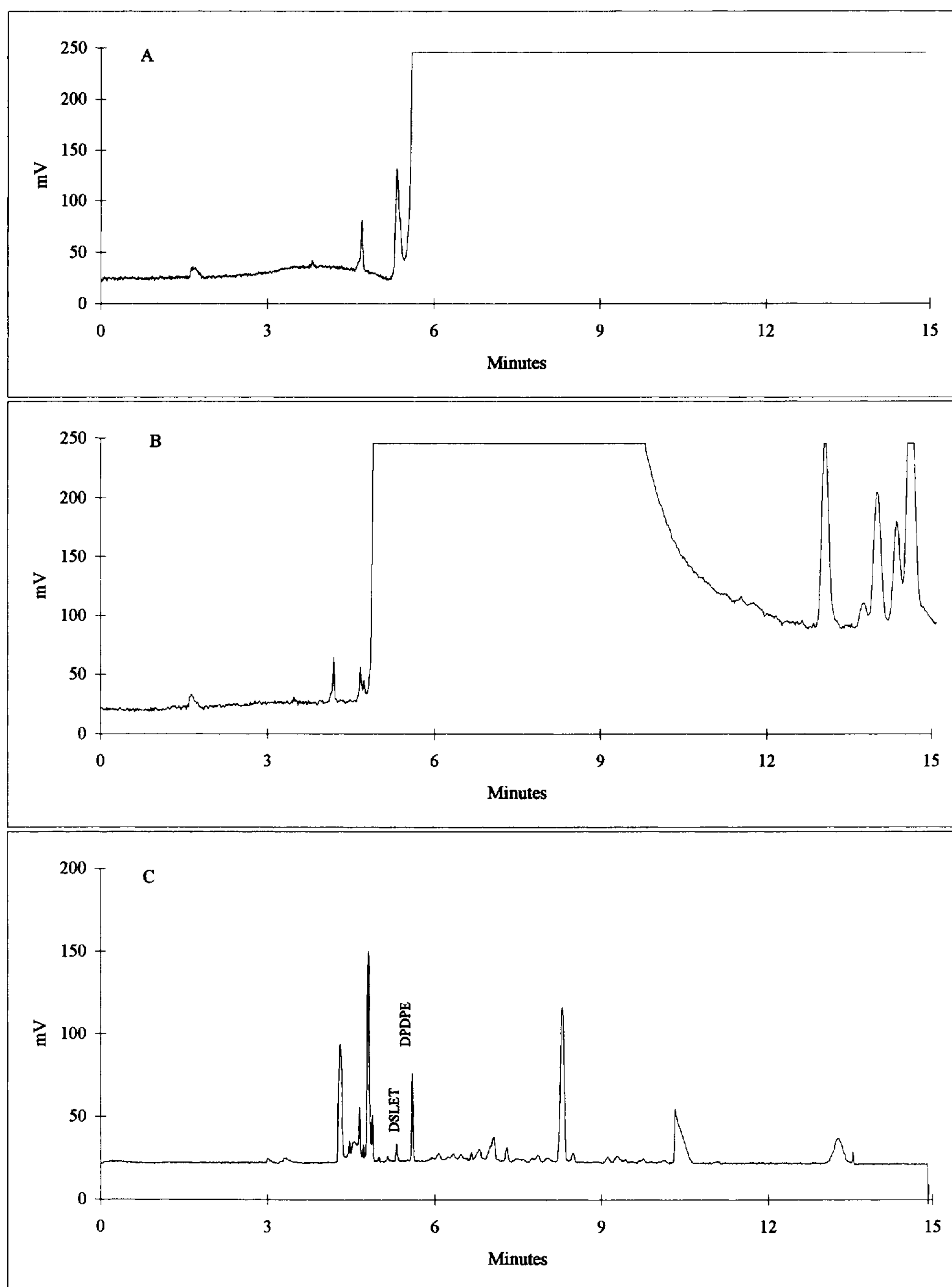

Fig. 1. Electropherogam of blank rat serum pretreated with (A) microcentrifugation, (B) protein precipitation with acetonitrile or (C) ultrafiltration (770 *g* for 10 min). Column, 67 cm × 50 μm I.D.; buffer, 50 m*M* boric acid, 10 m*M* sodium borate and 10 m*M* sodium dodecyl benzene sulfonate, pH 7. Other conditions were as described in Section 2.

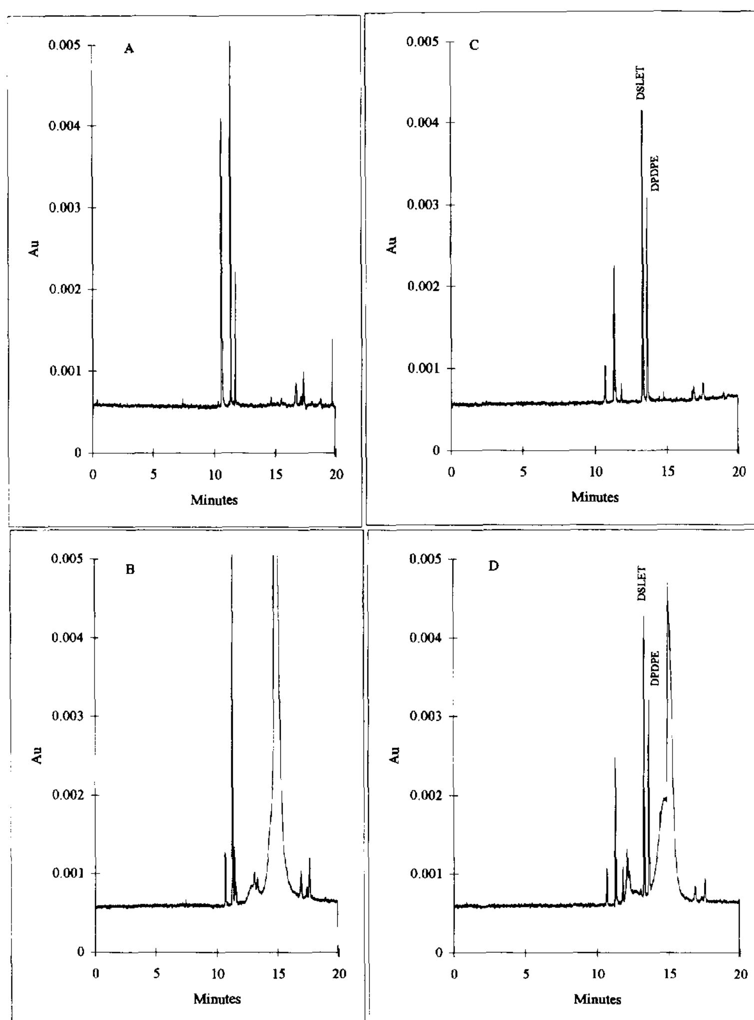

Fig. 2. Electropherogram of drug-free rat serum (A, B) or serum spiked with 10 μg/ml DPDPE and 20 μg/ml DSLET (C, D), with (A, C) or without (B, D) protein precipitation prior to extraction. Conditions were as described in Section 2.

Table 1
Extraction efficiency of DPDPE from rat serum[a]

Amount added (μg)	Method 1		Method 2	
	Amount recovered (μg)	Recovery (%)	Amount recovered (μg)	Recovery (%)
0.4	0.34 ± 0.02	86 ± 3.6	0.35 ± 0.02	88 ± 5.0
2.0	1.70 ± 0.01	86 ± 3.9	1.80 ± 0.10	90 ± 5.0
10.0	8.70 ± 0.02	87 ± 2.0	8.70 ± 0.20	87 ± 1.5
Mean ± S.D.	87 ± 3.4		88 ± 3.8	

[a]Data represented as mean ± S.D. for $n=3$ per experimental condition.

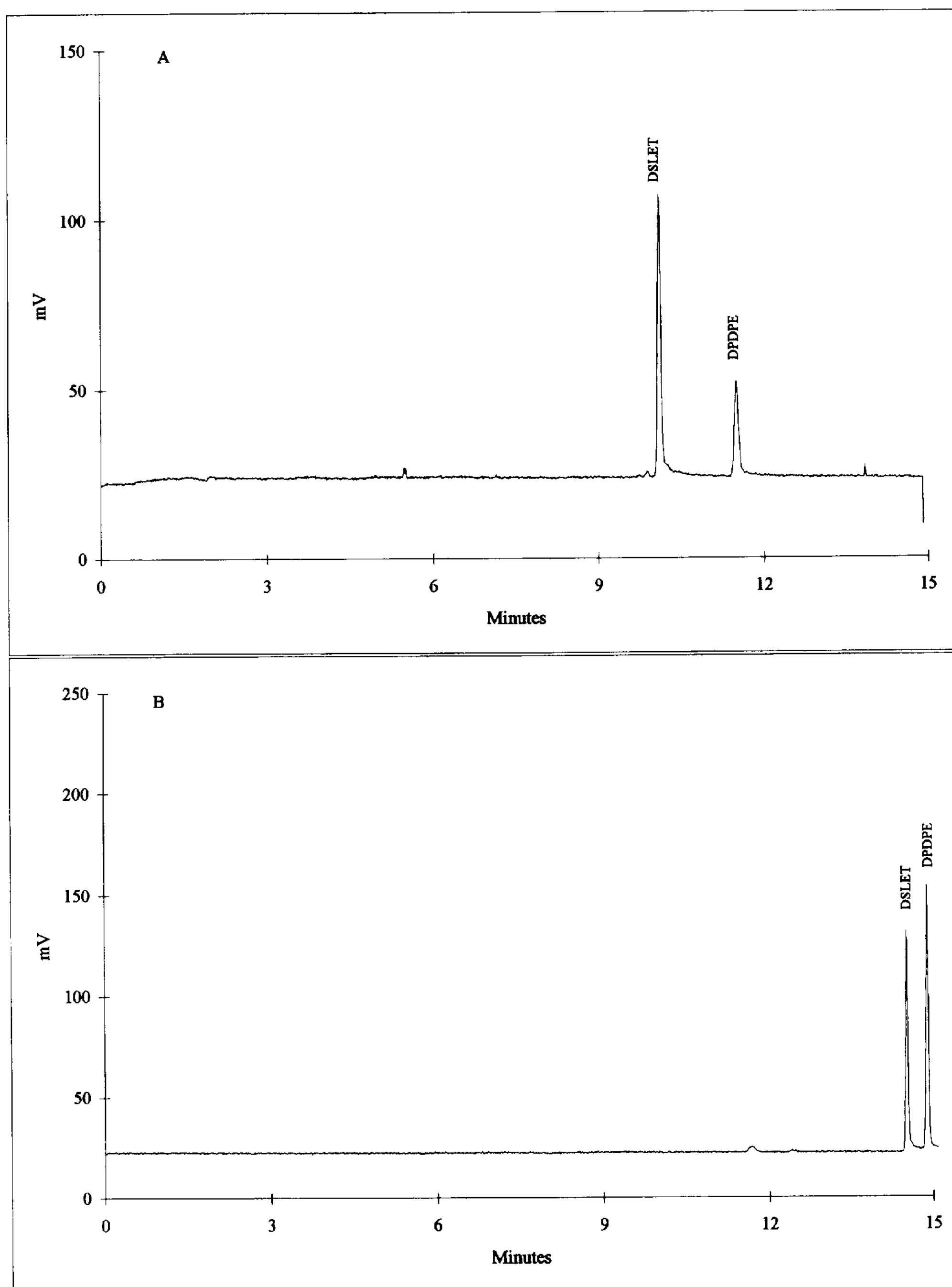

Fig. 3. Representative electropherogram of DPDPE and DSLET eluted with (A) phosphate buffer (6.25 m*M*, pH 2.4) or (B) borate buffer (25 m*M*, pH, 8.3). Conditions were as described in Section 2.

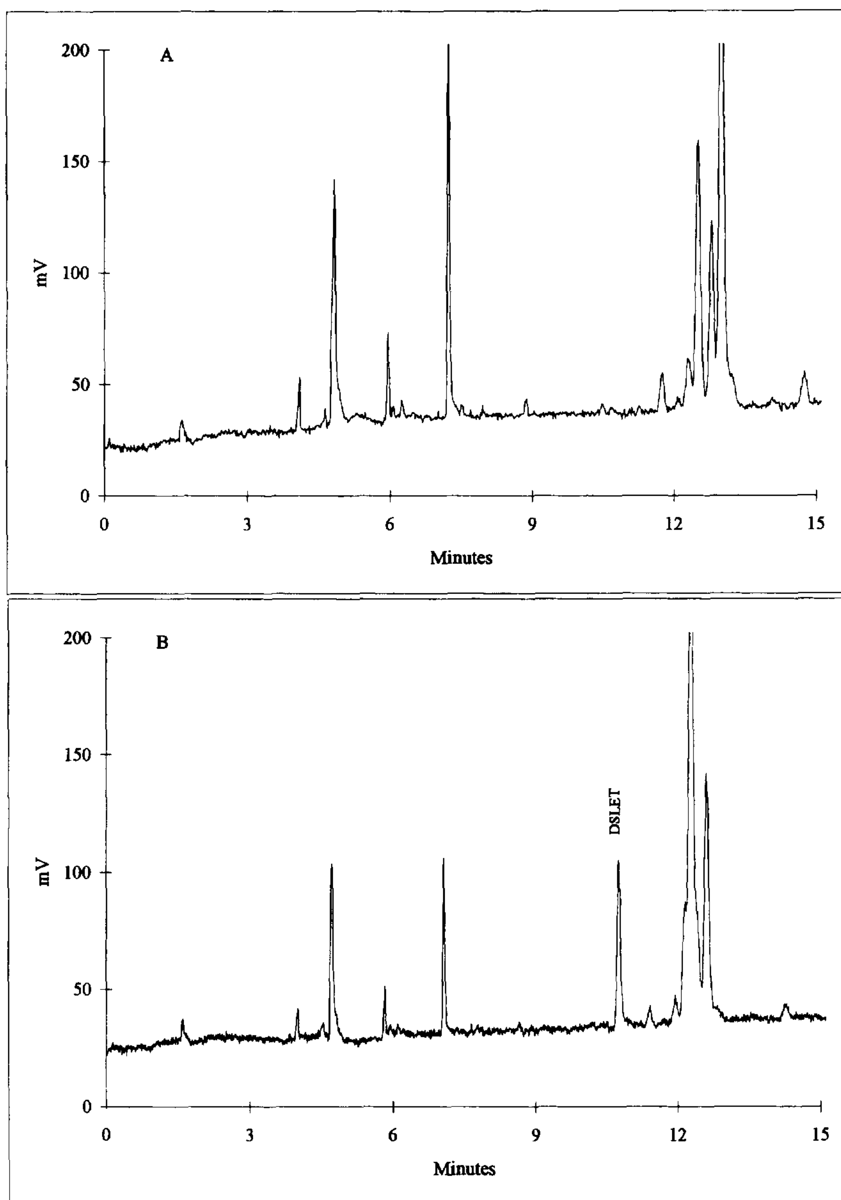

Fig. 4. Electropherogram of (A) drug-free rat serum and (B) serum spiked with DPDPE (10 μg/ml) and DSLET (10 μg/ml), eluted with phosphate buffer (25 m*M*, pH 2.4). Other conditions were as described in Section 2.

borate and 10 m*M* sodium dodecyl benzene sulfonate (pH 7) also was used initially for analysis of serum samples with different pretreatment approaches, as indicated in Fig. 1. However, this buffer did not provide sufficient resolution of DPDPE and DSLET from other serum components (data not shown). Ultimately, borate buffer was selected due to the fact that it can be used in high concentrations without generating significant current or Joule heat, and it provided good resolution of DPDPE and DSLET from serum impurities. The sensitivity of electroosmotic flow to pH requires the use of buffers that maintain a constant pH; since effective buffer systems should have a range of approximately two pH

units centered around the pK_a value, a pH of 8.3 (pK_a 9.24) was selected.

The complete resolution of DPDPE and DSLET from contaminants in rat serum after i.v. administration of DPDPE (10 mg/kg) was achieved under the above optimized conditions, as shown by the representative electropherograms in Fig. 5.

3.2.2. Selection of injection method and capillary diameter

Three injection methods are available on the Dionex CE system (pressure, gravity and electrokinetic injection). The major difference between the former two, referred to as hydrodynamic injection methods, and the electrokinetic method is that the

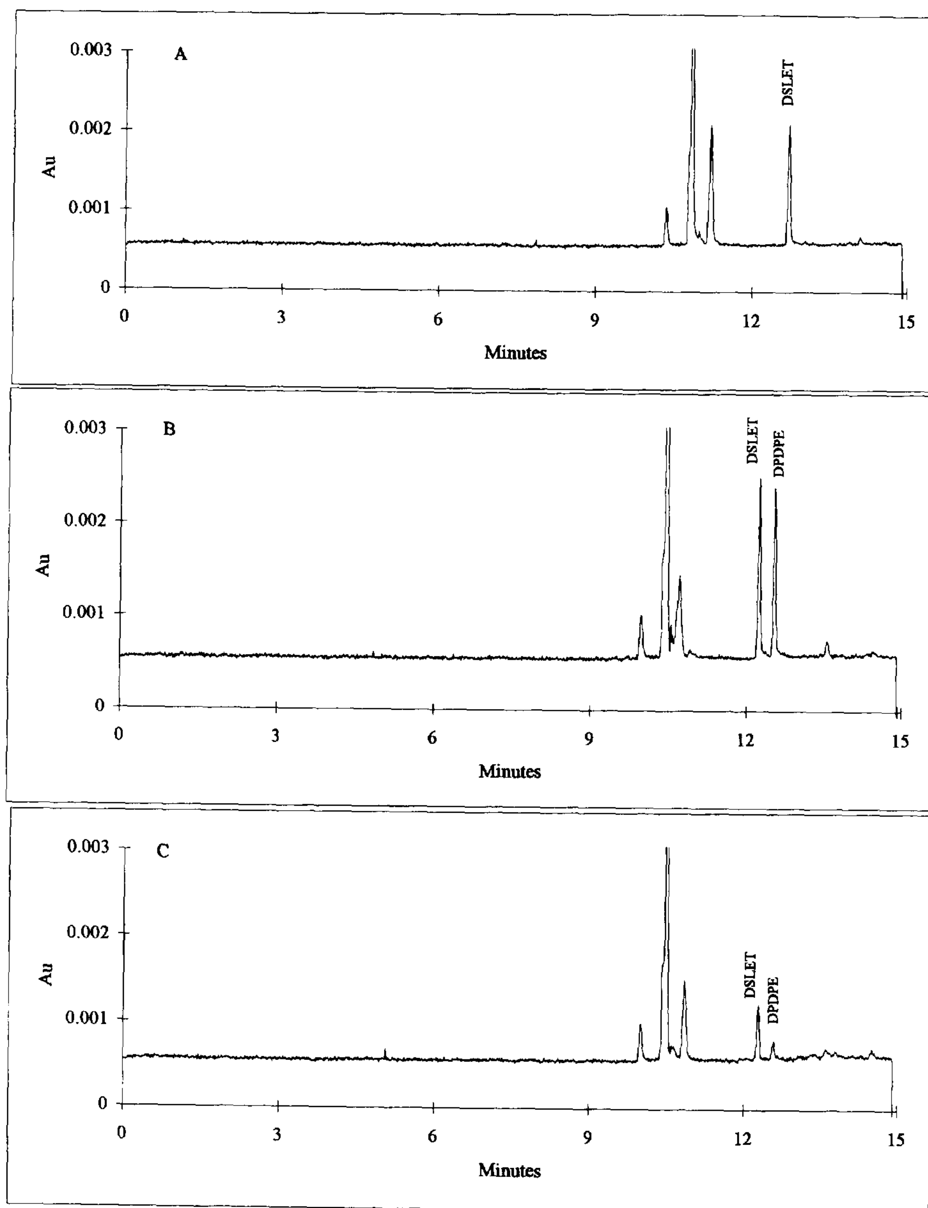

Fig. 5. Electropherogram of DPDPE and DSLET from Rat #1: (A) serum obtained prior to DPDPE administration with DSLET added, (B) serum at 2 min and (C) at 25 min after administration of DPDPE (10 mg/kg). Other conditions were as described in Section 2.

hydrodynamic methods have no "bias" towards specific components (analytes or impurities) in the sample matrix. Thus, these methods are expected to introduce a representative aliquot of the original sample. In contrast, electrokinetic injection is based on the electrophoretic migration and electroosmotic flow of the electrolyte into the capillary. Thus, the amount of each component introduced into the capillary is determined selectively by the electrophoretic mobility of each species in the sample medium [19]. Based on the above rationale, electrokinetic injection should be avoided in cases in which determination of complete sample composition is important. However, in the present study DPDPE and DSLET were the only analytes of interest, and discrimination of these two compounds from impurities was desired. In addition, the use of diluted sample buffer (10 m*M*), as opposed to running buffer (25 m*M*), can improve assay sensitivity due to sample stacking when electrokinetic injection is employed [21]. A comparison between the gravity and electrokinetic injection methods for DPDPE was conducted; results indicated that the latter method yielded a 2- to 4-fold higher sensitivity than the former (data not shown).

In addition to the injection method, sensitivity in CZE analysis can be increased by increasing the diameter of the capillary; in CE with an on-column detection window, the optical path length of the detector equals the I.D. of the capillary. Two different diameter capillaries (75 and 50 μm) were evaluated with respect to assay sensitivity. Results showed that the 75 μm capillary was associated with an approximately 5-fold higher sensitivity than the 50 mm capillary, for DPDPE in a simple matrix (water or buffer) (Table 2). However, use of the larger diameter capillary was associated with a high generated current (>230 μA), which led to significant baseline drift. The 50 μm I.D. capillary was superior to the larger column with respect to separation and heat dissipation.

Table 2
Comparison of sensitivity for DPDPE (1.25 μg/ml) between capillaries with different diameters

Trial	Peak height		Ratio (75:50)	Peak area		Ratio (75:50)
	50 μm	75 μm		50 μm	75 μm	
1	12 665	81 936	6.45	68 481	331 466	4.84
2	12 001	81 736	6.81	68 396	332 402	4.86

3.3. Assay validation

Linearity of the assay procedure was examined for DPDPE in buffer and serum. In both matrices, peak-height ratios (DPDPE to DSLET) varied linearly with DPDPE concentrations through 100 μg/ml. The intercept and slope of the regression equation for DPDPE in rat serum were 0.24 ± 0.12 and 15.2 ± 0.05 (mean ± S.D., $n=5$), respectively. A representative standard curve for DPDPE in serum is presented in Fig. 6. Reproducibility in the retention times for DPDPE (13.8 ± 1.5 min) and DSLET (13.5 ± 1.4 min) was ~10%. However, the ratio of DPDPE to DSLET retention times was extremely reproducible (1.03 ± 0.002). The lower variability in the ratio of retention times, as compared to the DPDPE retention time per se, confirms the requirement to incorporate an internal standard in the assay. In this case, the internal standard serves as a marker for identifying the analyte peak as well as for potential loss of substrate during sample purification. Rinsing the capillary with sodium hydroxide (0.5 *M*), water and eluent buffer sequentially between assays contributed to the good reproducibility in retention time. The intra-day and inter-day coefficients of variation overall were less than 8.5% (Table 3). The minimum detectable concentration under the present experimental conditions was 250 ng/ml

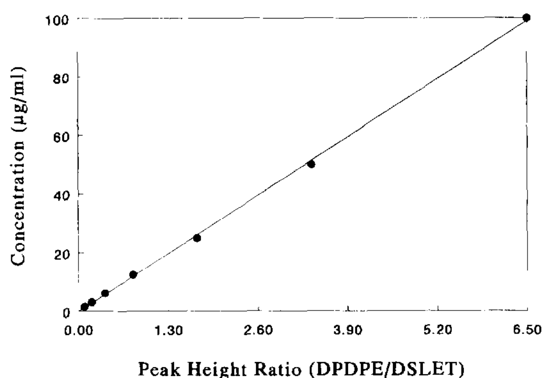

Fig. 6. Representative standard curve for DPDPE in serum. Symbols indicate observed data; line represents the result of linear least-squares regression of the data.

Table 3
Intra-day and inter-day variability for quantitation of DPDPE in serum

Concentration (μg/ml)	Coefficient of variation (%)	
	Intra-day	Inter-day
3.125	4.7	8.5
6.25	4.2	7.0
50	2.1	4.9

Table 4
Pharmacokinetic parameters of DPDPE after administration of DPDPE (10 mg/kg) to rats (n=4)

Parameter[a]	Mean ± S.D.
α (min^{-1})	0.31 ± 0.15
β (min^{-1})	0.06 ± 0.02
$t_{1/2}$, β (min)	13.1 ± 5.6
Cl (ml/min/kg)	22.5 ± 6.3
V_c (ml/kg)	155 ± 66
V_{ss} (ml/kg)	296 ± 85
MRT (min)	14.3 ± 7.4

[a]The following parameters were recovered by standard techniques [22]: α=distribution rate constant. β=terminal elimination rate constant. $t_{1/2}$, β=terminal elimination half-life. Cl=systemic clearance. V_c=volume of central compartment. V_{ss}=steady-state volume of distribution. MRT=mean residence time.

(signal-to-noise ratio of 5), equivalent to a mass of 80 pg of DPDPE on-column.

3.4. Pharmacokinetic studies

The DPDPE concentration–time profile in rats (n=4) after a 10 mg/kg i.v. bolus dose was biphasic; a two-compartment model was fitted to the data from individual rats by non-linear least-squares regression (Fig. 7). The pharmacokinetic parameters describing DPDPE disposition in the rat were calculated according to standard techniques [22] and are presented in Table 4. The terminal half-life of unchanged DPDPE was approximately 15 min, which differed from a previous report that significant quantities of DPDPE (>45%) remained 120 min after administration of radiolabeled peptide to mice [6]. The discrepancy between the two studies may be due to the different assay approaches taken (CZE for quantitation of authentic DPDPE in the present study versus total radioactivity in the former). However, this difference also may be indicative of species differences in DPDPE disposition. Consistent with the literature, no detectable metabolites (i.e., no unidentified peaks) were observed in the electropherograms in the present study [2]. There was no difference in retention times or number of peaks between the serum samples from naive animals with DPDPE added in vitro and serum samples obtained from rats after DPDPE administration (Fig. 5). However, the fact that no metabolites were observed by CZE does not necessarily indicate that metabolites were not formed. Biotransformation products may have been excluded during sample pretreatment or were not observed due to detection limitations. The rapid disappearance of DPDPE from blood, as indicated by the short half-life, could be due to rapid biliary excretion, as has been shown for somatostatin analogue peptides that have a cyclic structure similar to that of DPDPE [23,24]. Rapid biliary excretion of DPDPE also was suggested by Weber et al. [6], based on the fact that large amounts of ^{3}H were recovered in the intestine after i.v. administration of [^{3}H]DPDPE to mice.

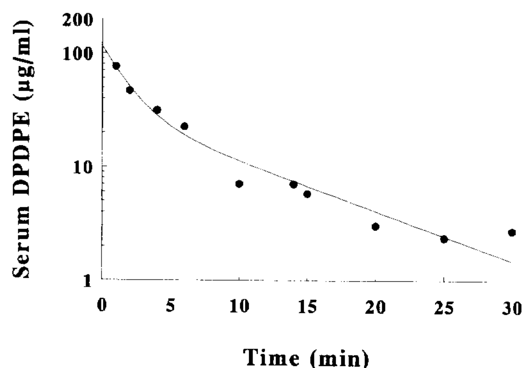

Fig. 7. Disposition of DPDPE after a 10 mg/kg bolus dose in a representative rat. Symbols indicate observed data; line represents fit of a two-compartment model to the data.

The present CZE method also could be applied to the determination of peptides with physical and chemical properties similar to DPDPE, in order to assess the pharmacokinetic characteristics of these agents. This method offers greater selectivity than assessment of total radioactivity, which has been used for pharmacokinetic analysis of most en-

kephalin peptide drugs [25–28]. This approach also provides greater simplicity and economy than multidimensional analysis, which also has been used for the quantitative analysis of peptide drugs in biological matrices [29]. The detection limit achieved in this study was comparable to that reported for other peptides by HPLC [30].

In summary, a CZE method was developed for the quantitation of DPDPE in rat serum, and was applied successfully in the evaluation of DPDPE pharmacokinetics in rats. This study showed that CZE is a reliable alternative approach for peptide drug pharmacokinetic analyses.

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