Sensitive analysis of [D-Pen^{2,5}]enkephalin in rat serum by capillary electrophoresis and laser-induced fluorescence detection

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Abstract

A highly sensitive analytical method based on capillary zone electrophoresis (CZE) coupled with a laser-induced fluorescence (LIF) detector was explored for the analysis of [D-Pen^{2,5}]enkephalin (DPDPE) in rat serum. DPDPE and the internal standard Phe-Leu-Glu-Glu-Ile (P9396) were extracted from serum samples with C_{18} solid-phase extraction disk cartridges, followed by derivatization with tetramethylrhodamine-5-isothiocyanate (TRITC) isomer G before introduction onto the capillary column. Complete resolution of DPDPE and the internal standard from other serum components was achieved within 20 min on a 140 cm×50 μm I.D. capillary column with borate buffer (25 mM, pH 8.3). With the current method, it is possible to detect 1.3E-18 mol of DPDPE on column. The results suggest that CZE–LIF is a promising method for the sensitive and specific quantitation of therapeutic peptides in biological matrices. © 1997 Elsevier Science B.V.

Keywords: Enkephalins; Peptides

1. Introduction

[D-Pen^{2,5}]enkephalin (DPDPE; H-Tyr-D-Pen-Gly-Phe-D-Pen-OH, Pen=Penicillamine), a synthetic opioid peptide, has been used widely as a highly selective delta-receptor agonist in studies of receptor–ligand binding [1,2]. However, the pharmacokinetics and pharmacodynamics of this metabolically stable peptide [3] have been neglected to a large extent, mainly due to lack of a sensitive and selective analytical method for the determination

of DPDPE in biological samples. Several methods have been used for the analysis of DPDPE [3–5]. A radiochemical method was employed for whole-body distribution studies with ^{3}H-DPDPE in mice [3]. One disadvantage of using radiolabeled substrates is the potential for non-specificity [3]. HPLC with UV absorbance detection at 210 nm was developed for in vitro stability studies of DPDPE in mouse serum and applied to analysis of DPDPE at a concentration range of 50–100 μM [4]. Recently, a novel capillary zone electrophoresis (CZE) method with a UV absorbance detector was developed in this laboratory to examine the disposition of DPDPE in rats after an intravenous bolus dose [5]. With the CZE–UV method, DPDPE could be detected in serum for 30–40 min after administration of a 10 mg/kg dose; the detection limit of this method was 0.4 μM of

DPDPE in serum [5]. Since the half-life of this compound is approximately 20 min, an assay with a lower detection limit is desired in order to conduct comprehensive studies of the pharmacokinetics and pharmacodynamics of DPDPE.

CZE with laser-induced fluorescence (LIF) detection may be a highly sensitive analytical tool for compounds that can be derivatized with tetramethylrhodamine-5-isothiocyanate (TRITC). TRITC has been used as a fluorescent tag for DNA analysis with gel electrophoresis [6], for the separation of amino acids and peptides from digests of horse heart cytochrome c with LC–CZE–LIF [7], and in the analysis of tryptic digests of porcine thyroglobulin with LC–LC–LIF [8]. DPDPE contains a single primary amine group, which is associated with tyrosine; this primary amine should react with the thiourea in TRITC to form a single derivative. TRITC–DPDPE has an absorbance wavelength maximum (543 nm) that is well-suited for the 543.5 nm line emitted from helium–neon (He–Ne) lasers.

The present study was conducted to assess the feasibility of applying CZE–LIF technology to the quantitative analysis of DPDPE in biological samples. Successful development of this technique would facilitate determination of the pharmacokinetics of this compound, as well as other peptides with similar chemical characteristics, in experimental animals.

2. Experimental

2.1. Chemicals

DPDPE was a gift from National Institute on Drug Abuse (Baltimore, MD, USA). Based on analysis by both CZE–UV and HPLC–UV, substrate purity was >98%. Therefore, it was used without further purification. P9396 was purchased from Sigma (St. Louis, MO, USA). Tetramethylrhodamine-5-isothiocyanate isomer G (TRITC) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents used in this study were of the highest grade commercially available in the United Sates and were used without further purification.

2.2. Sample pretreatment and derivatization

2.2.1. Solid-phase extraction

The internal standard P9396 (10–20 μl; 1 mg/ml) was added to serum samples (100–200 μl) to achieve an initial concentration of 70 μM in serum. Next, proteins in serum were precipitated with acetonitrile (400 μl). After mixing by vortex and centrifugation (15 000 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 7-mm, 3-ml C_{18} high-performance extraction disk cartridge (referred to as cartridges; 3M Empore, St. Paul, MN, USA) which had been preconditioned with methanol (1 ml) and water (1 ml). The cartridge then was washed with water (0.5 ml). Analytes were eluted from the stationary phase bed with 0.1% TFA–methanol (20:80, v/v, 1 ml). This 1-ml fraction was evaporated to dryness under nitrogen, the residue was reconstituted with sodium borate buffer (150 mM, pH 9.0, 100 μl) and a 75-μl aliquot derivatized as described in Section 2.2.3. Alternatively, the residue was reconstituted with borate buffer (25 mM, pH 8.3, 100 μl) and analyzed by CZE–UV for determination of extraction efficiency of DPDPE from serum as described below.

C_{18} solid-phase extraction columns (referred to as columns; Varian Sample Preparation, Sunnyvale, CA, USA) were compared with C_{18} cartridges for serum sample preparation after precipitation of proteins with acetonitrile. The procedures used were identical to those outlined above except that solvent volumes consumed in preconditioning and washing, as well as elution of components from the column, were doubled compared with those used for cartridges.

In an attempt to improve sensitivity, the extraction efficiency of DPDPE from serum with cartridges and columns was investigated at three concentrations (3.1, 15.5 and 77.4 μM) of DPDPE. Briefly, DPDPE was added to serum (200 μl) to achieve the above three concentrations (n=3 for each concentration). After solid-phase extraction with cartridges or columns as described previously in this section, the residue was reconstituted with borate buffer (25 mM, pH 8.3, 100 μl) and analyzed by CZE–UV, as

described in Section 2.3.1. The extraction efficiency was calculated based on the ratio of peak areas of DPDPE from serum samples vs. those of standards in buffer (25 mM, pH 8.3 borate buffer without any pretreatment and at the corresponding concentrations).

2.2.2. HPLC

In addition to the solid-phase extraction method, HPLC was assessed as a clean-up approach for serum samples prior to derivatization. Serum (100–200 µl) containing DPDPE (1.5E-8 to 1.5E-6 M) was treated with acetonitrile (400 µl) to precipitate proteins as described in Section 2.2.1. After evaporation to dryness, the residue was reconstituted with water (20 µl) and centrifuged (15 000 g for 2 min). The clear supernatant (10 µl) was injected onto the HPLC column. The DPDPE fraction (17.5–20 min) was collected in a 7-ml glass tube and evaporated to dryness. This residue was either reconstituted with sodium borate buffer (150 mM, pH 9.0, 20 µl) and an aliquot (18 µl) of the reconstituted sample was used for derivatization as described in Section 2.2.3, or reconstituted with HPLC mobile phase (20 µl) and re-injected onto the HPLC system (10 µl) to investigate the recovery efficiency for DPDPE during fraction collection.

2.2.3. Derivatization

The fluorescent derivatizing reagent TRITC was dissolved in DMSO (1 mg/ml) as a stock solution. The TRITC solution was diluted to the desired concentration, depending on concentrations of DPDPE in serum or in the buffer medium. In all cases, one volume of TRITC solution (e.g., 25 or 6 µl) was combined with three volumes (e.g., 75 or 18 µl) buffer containing DPDPE (either a serum extract reconstituted in buffer, or DPDPE in buffer only). The reaction was allowed to proceed at room temperature for 12 h in the dark. Unless indicated otherwise, all derivatizations were conducted with TRITC in 3-fold molar excess of DPDPE. Following derivatization, the mixture was diluted to the desired concentration of DPDPE and analyzed by CZE–UV, CZE–visible (Vis, at 546 nm), or CZE–LIF.

The conditions used for derivatization of DPDPE with TRITC were optimized with regard to the reaction time, the molar ratio between TRITC and DPDPE and the pH of the borate buffer used as the derivatizing reaction medium. Briefly, to examine the influence of reaction time, 300 µl of DPDPE (15 µM) in borate buffer (150 mM, pH 9.0) was combined with 100 µl of TRITC in DMSO. The reaction proceeded at room temperature in a glass vial which was wrapped with aluminum foil to avoid exposure to light. Aliquots (20 µl) were taken at timed intervals from initiation of the reaction (time 0) until 24 h. The reaction mixture was analyzed by CZE–UV as described in Section 2.3.1. The yield was calculated as follows: (DPDPE peak area before derivatization − DPDPE peak area after derivatization) × 100 / DPDPE peak area before derivatization. The percent yield vs. reaction time data were fit with a first-order or zero-order kinetic equation with the nonlinear least-squares regression program Scientist (MicroMath, Salt Lake City, UT, USA) to recover the corresponding rate constant for the reaction. The molar ratio between TRITC and DPDPE was optimized by comparison of the peak areas of DPDPE–TRITC at different combinations of molar ratio between TRITC and DPDPE, from 1:1, 2:1, 3:1, 4:1 and 10:1. In each case, the reaction was allowed to proceed for 12 h before analysis of the derivative by CZE–UV. To optimize the pH of the borate buffer (150 mM), buffers with pH of 8.0, 8.5, 9.0, 9.5 and 10 were prepared; 75 µl of DPDPE (15 µM) dissolved in these buffers were combined with 25 µl TRITC. After reaction for 12 h in the dark, these mixtures were analyzed by CZE–UV. Peak area ratios between DPDPE–TRITC and DPDPE (unreacted in the reaction medium) were plotted as a function of the pH of the buffers. All optimization experiments were conducted for DPDPE in both buffer and serum samples.

2.3. Instrumentation and separation

2.3.1. CZE–UV and CZE–LIF

The CZE–UV system (Dionex Corp, Sunnyvale, CA, USA) was used primarily to analyze samples during optimization of serum sample pretreatment and derivatization conditions. Samples were injected hydrodynamically (50 mm height, 30 s) onto a fused-silica capillary column (90 cm×50 µm I.D., with an

effective length of 83 cm, Dionex Corp). The samples were separated with a borate buffer (25 mM, pH 8.3) under a voltage of 30 kV. The analytes were monitored with an on-column UV absorbance detector at 210 nm.

The CZE–LIF system was a combination of the above Dionex capillary electrophoresis system and a LIF detector constructed in this laboratory (Fig. 1). Hydrodynamic injections also were used in the CZE–LIF system. Separation was achieved on a 50-μm I.D. capillary column with a total length of 140 cm and an effective length of 72 cm (to the point of LIF). One end of the capillary column was inserted into the autosampler head, and the other end was directed though the capillary holder for LIF to the UV detector cell and the destination vial of the Dionex system. This installment of the capillary utilized the automated rinse system and the high voltage power supply available with the Dionex system to regenerate the capillary between runs and to drive the electrophoresis, respectively.

The LIF detector was a modification of one previously described [8], which utilized a 1.5-mW green He–Ne laser (Uniphase, Menteca, CA, USA).

The TRITC derivative of DPDPE was excited with the green line from the He–Ne laser (543.5 nm) and emitted fluorescent light with a band centered at 570 nm. Incident light from the green He–Ne laser passed though a 546 nm bandpass filter of 10 nm bandwidth (546DF10, Omega Optical, Brattleboro, VT, USA) and was reflected from a dichroic filter (560DRLPO2, Omega Optical) to a 60× microscope objective lens (Edmund Scientific, Barrington, NJ, USA) to the capillary window. Emitted fluorescent light was collected with the same 60× microscope objective lens and was transmitted though the dichroic filter, passed though a 590 nm bandpass filter of 35 nm bandwidth (590DF35, Omega Optical), and directed to the detector. The collected fluorescent light was converted to an electrical signal by a photomultiplier tube (R1477, Hamamatsu, Bridgewater, NJ, USA), and the current was converted to a voltage by an amplifier (570, Stanford Research System, Sunnyvale, CA, USA). The entire optical system was mounted on an optical breadboard (Technical Manufacturing, Peabody, MA, USA) and was covered with a custom-built aluminum housing. The breadboard provided stability via vibrational isolation, while the flat-black painted aluminum housing prevented interference from stray room light.

For the CZE–UV system, data were acquired with Dionex CE software (Dionex Corp) and recorded on an IBM-compatiable personal computer. For the CZE–LIF system, data collection was achieved with a data acquisition board (National Instruments, Austin, TX, USA) and a Power Macintosh 7100/80 (Apple Computer, Cupertino, CA, USA). Labview (National Instruments) was used to execute commands to the data acquisition board.

2.3.2. HPLC system

Reconstituted serum samples were injected into a 7125 Rheodyne injection valve (Cotati, CA, USA) with a 20-μl injection loop, after precipitation of proteins with acetonitrile. The sample was eluted with acetonitrile–water (28:72, v/v, with 0.05% TFA) from a 5-μm C_6 HPLC column (25 cm×4.6 mm I.D., Spherisorb, Phase Separation, Norwalk, CT, USA) with an LKB Model 2150 HPLC pump (Bromma, Sweden) at a flow-rate of 1.0 ml/min. Absorbance of column eluent was monitored with an
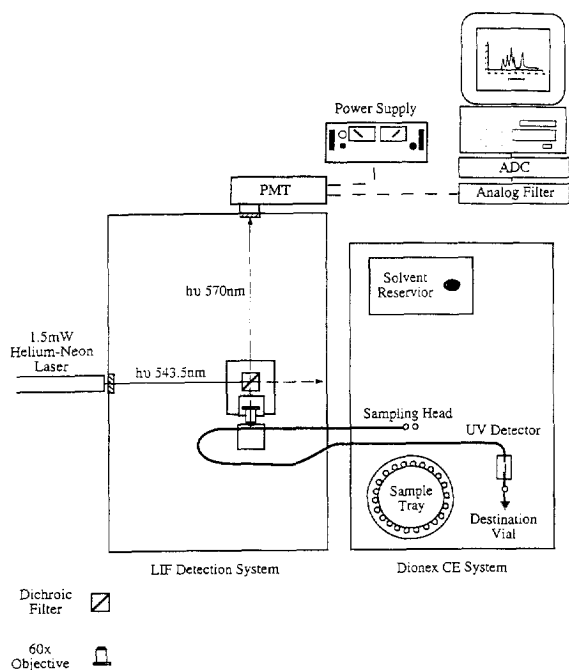

Fig. 1. Schematic of the CZE–LIF system.

LKB model 2151 variable wavelength detector at 210 nm. Detector response was recorded and quantitated with a CR601 integrator (Shimadzu, Columbia, MD, USA).

2.3.3. Mass spectrometry systems

Mass spectrometry (MS) was used to determine the stoichiometry of the reaction between DPDPE and TRITC, as well as to study the completeness of the reaction. A 75-µl aliquot of DPDPE standard $(1.6 \cdot 10^{-3}\ M)$ dissolved in borate buffer (150 mM, pH 9.0) was combined with a 25-µl aliquot of TRITC $(2.3 \cdot 10^{-2}\ M$ in DMSO), giving a molar ratio of TRITC–DPDPE of approximately 5:1. After a 12-h reaction, the reaction mixture was desalted with a C_{18} trapping cartridge (Michom Bioresources, Auburn, CA, USA). Briefly, 50 µl of the reaction mixture was loaded onto the cartridge, the cartridge was rinsed with 200 µl of 0.05% TFA, and the derivative was eluted from the cartridge with 100 µl of 66% acetonitrile in 0.05% TFA. The eluent was diluted 50:1 with 66% acetonitrile in 0.05% TFA prior to MS analysis. Mass spectra were acquired on a Sciex API-1 single quadrupole spectrometer (Thornhill, Ontario, Canada) equipped with an ion spray interface (pneumatically assisted electrospray). The instrument was calibrated with a standard mixture of polypropylene glycols. The tuning of the instrument was optimized on the m/z 1089 ion of the DPDPE–TRITC complex. Full scan mass spectra were acquired from m/z 200 to 2000 at a scan rate of 4.7 s/scan (1.00 ms dwell, 0.40 m/z step), with an orifice potential of 60 V. Tandem mass spectra were acquired on a Sciex API-3 triple-quadrupole spectrometer (Thornhill) from the m/z 1189 precursor ion over the m/z range of 50 to 1100 at a scan rate of 6.1 s/scan (1.1 ms dwell, 0.2 m/z step) with an orifice potential of 60 V, and collision gas thickness of $245 \cdot 10^{12}$ molecules/cm^2 of argon–nitrogen (90:10).

3. Results and discussion

3.1. Optimization of serum sample pretreatment

Solid-phase extraction columns and cartridges were compared for the pretreatment of serum sam-
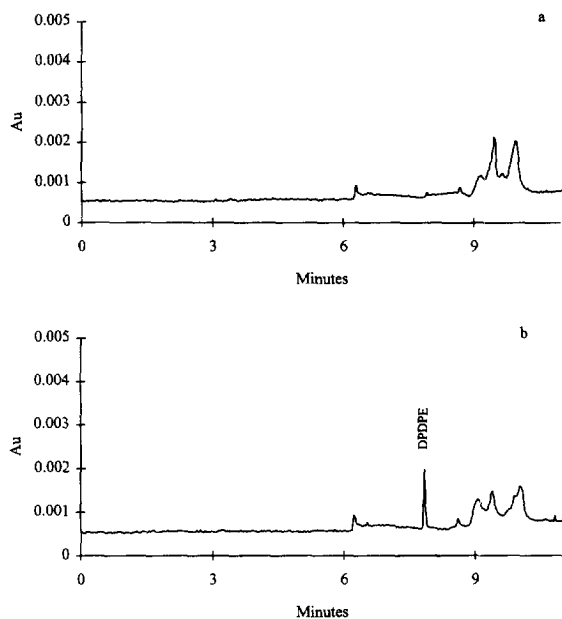

Fig. 2. Electropherogram of (a) blank serum and (b) serum containing 7.74 µM DPDPE pretreated with a solid-phase disk cartridge. Eluent was monitored by UV absorbance. Other conditions were as described in Section 2.2.1.
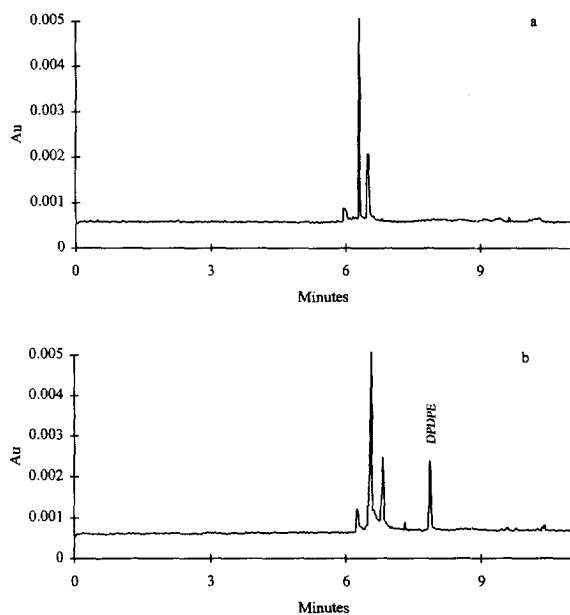

Fig. 3. Electropherogram of (a) blank serum and (b) serum containing 7.74 µM DPDPE pretreated with a solid-phase extraction column. Eluent was monitored by UV absorbance. Other conditions were as described in Section 2.2.1.

ples prior to derivatization. The electropherograms obtained from these pretreated serum samples are shown in Figs. 2 and 3, respectively. The results indicate that the solid-phase extraction columns and cartridges used have different desalting capabilities: the cartridges remove components from the serum that migrate before DPDPE (retention time of 7.8 min; Fig. 2), while the columns eliminate those components migrating after DPDPE (Fig. 3). The sera pretreated with these two methods were used further for derivatization [Figs. 4–6, (I) and (II) corresponding to cartridge and column extracts, respectively] and the resulting derivatives were analyzed by CZE–UV (Fig. 4), CZE–VIS (Fig. 5) and CZE–LIF (Fig. 6). Pretreatment of serum samples with cartridges resulted in an acceptable level of
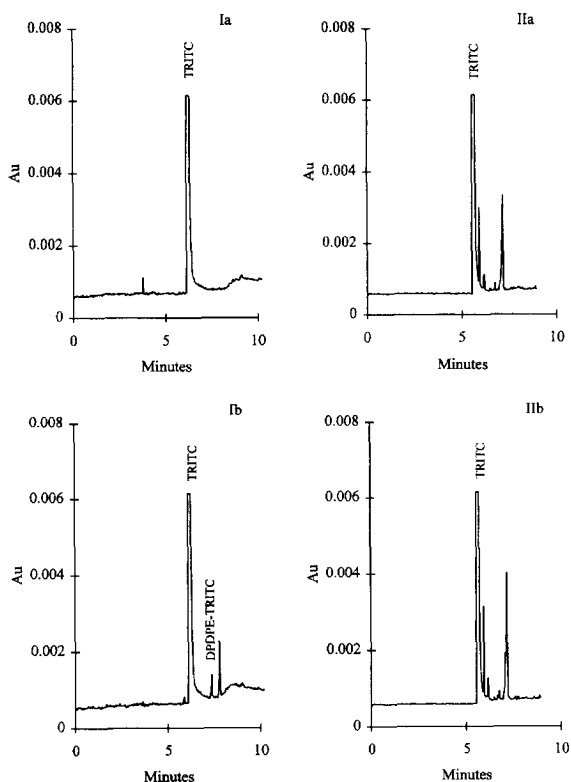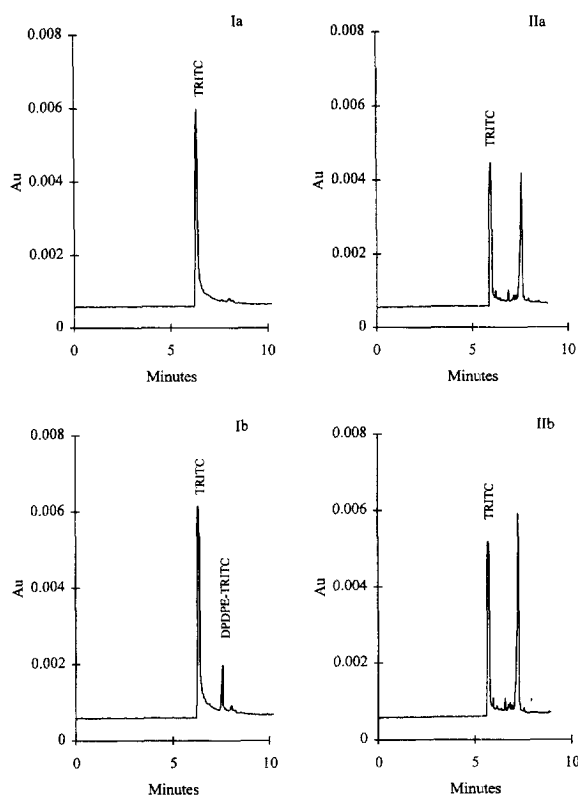

Fig. 5. Electropherogram of derivatives from serum samples pretreated with (I) a solid-phase disk cartridge or (II) a solid-phase extraction column and analyzed by CZE with visible absorbance at 546 nm [(a) blank, (b) serum containing DPDPE, 15 μM]. The reaction time was 4 h. Other conditions were as described in Section 2.2.3 Section 2.3.1.

Fig. 4. Electropherogram of derivatives from serum samples pretreated with (I) a solid-phase disk cartridge or (II) a solid-phase extraction column and analyzed by CZE with UV absorbance at 210 nm [(a) blank, (b) serum containing DPDPE, 15 μM]. The reaction time was 4 h. Other conditions were as described in Section 2.2.3 Section 2.3.1.

clean-up; good resolution was achieved between DPDPE–TRITC (retention time 7.3 min in CZE–UV or Vis and 15 min in LIF) and other components in the derivatizing media. In contrast, the columns did not remove contaminants from serum sufficiently; there was no difference between the electropherograms from derivative reaction mixtures with blank serum and serum containing DPDPE. A slight difference in the retention time of TRITC also was observed between derivatives generated from serum extracts pretreated with the column versus the cartridge (Fig. 6, part I and II); the retention time of TRITC from a derivative mixture originating from serum pretreated with the column generally was smaller than that pretreated with the cartridge (13 vs.
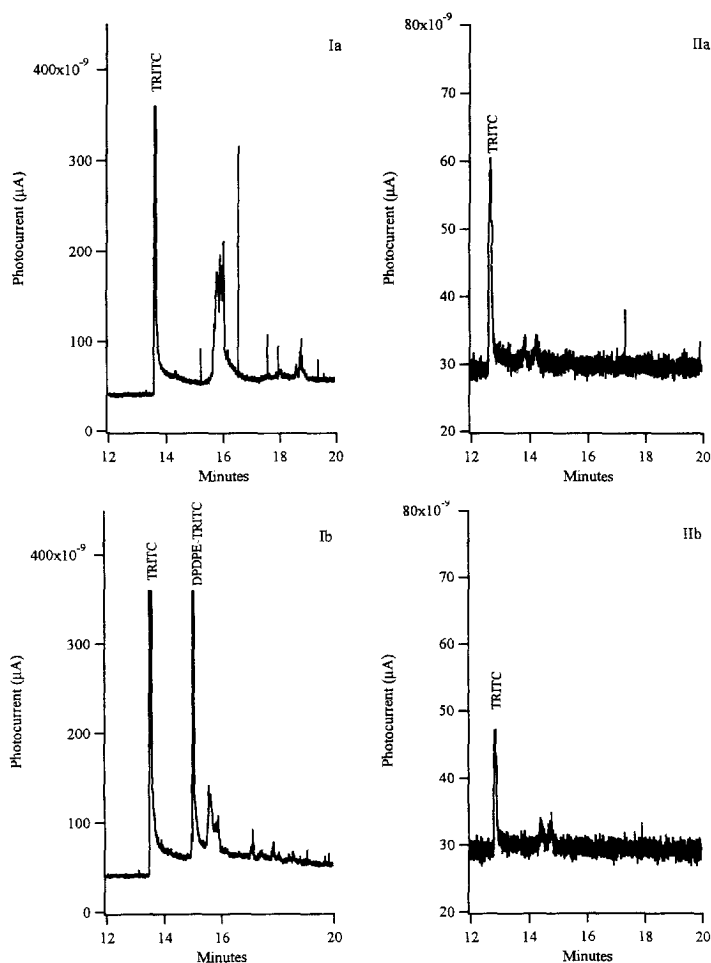
Fig. 6. Electropherogram of derivatives from serum samples pretreated with (I) a solid-phase disk cartridge or (II) a solid-phase extraction column and analyzed by CZE with LIF detection [(a) blank, (b) serum containing DPDPE, 10^{-8} M]. Other conditions were as described in Section 2.2.3 Section 2.3.1.

14 min). This difference in retention time of TRITC was consistent throughout the experiment (Figs. 4–6,8,13), and could be due to differences in components eluting from the column versus the cartridge. However, the exact reason of this behavior was unknown.

There was no significant difference in extraction efficiency between the cartridges and the columns (Table 1). The differences observed in the electropherograms between blank serum and serum containing DPDPE (Figs. 4–6) were not due to extraction efficiency, but rather to interference from serum components extracted along with DPDPE. As

Table 1
Extraction efficiency of DPDPE from rat serum

Amount added (μg)	Recovery (%)	
	C_{18} disk cartridge	C_{18} column
0.4	84±3.6	88±3.5
2.0	86±3.6	89±3.9
10.0	87±1.9	87±2.0
Mean±S.D.	86±3.4	88±3.8

Data presented as mean±S.D. for $n=3$ per experimental condition.

there was no significant difference in the extraction efficiency between cartridges and columns, and the cartridges provided better sample clean-up, the cartridges were selected for further use in evaluation of the CZE–LIF method for quantitation of DPDPE.

The HPLC method was tested as a potentially more efficient method than the disk cartridge in removing interfering substances from serum. A baseline separation of DPDPE from other components in serum was achieved under the conditions described in Section 2.3.2 (Fig. 7). DPDPE eluted at ~18 min under the conditions used for HPLC separation. The peak at this migration time was present in the chromatogram from serum containing DPDPE (Fig. 7b) but not from blank serum (Fig. 7a). The fraction containing DPDPE, collected between 17.5 and 20 min, was evaporated and reconstituted prior to re-injection onto the HPLC column. Several peaks were observed in this re-injected fraction (Fig. 7c). These peaks originated from components in the

mobile phase that were retained after fraction collection, evaporation, reconstitution and re-injection (data not shown). The sample recovery was estimated to be 50%, based on a comparison of the peak area of DPDPE before and after fraction collection. No difference between the electropherograms of the TRITC-derivatized fractions from blank serum (Fig. 8a) and the serum containing DPDPE (Fig. 8b) was apparent. The HPLC method therefore was associated with insufficient clean-up for analysis of DPDPE in serum.

3.2. Optimization of derivatization conditions

3.2.1. Duration of reaction

The time required for reaction between DPDPE and TRITC in 150 mM borate buffer at pH 9.0 was optimized with regard to the following two aspects: percentage conversion of DPDPE to DPDPE–TRITC and stability of the derivative. As shown in Fig. 9, an
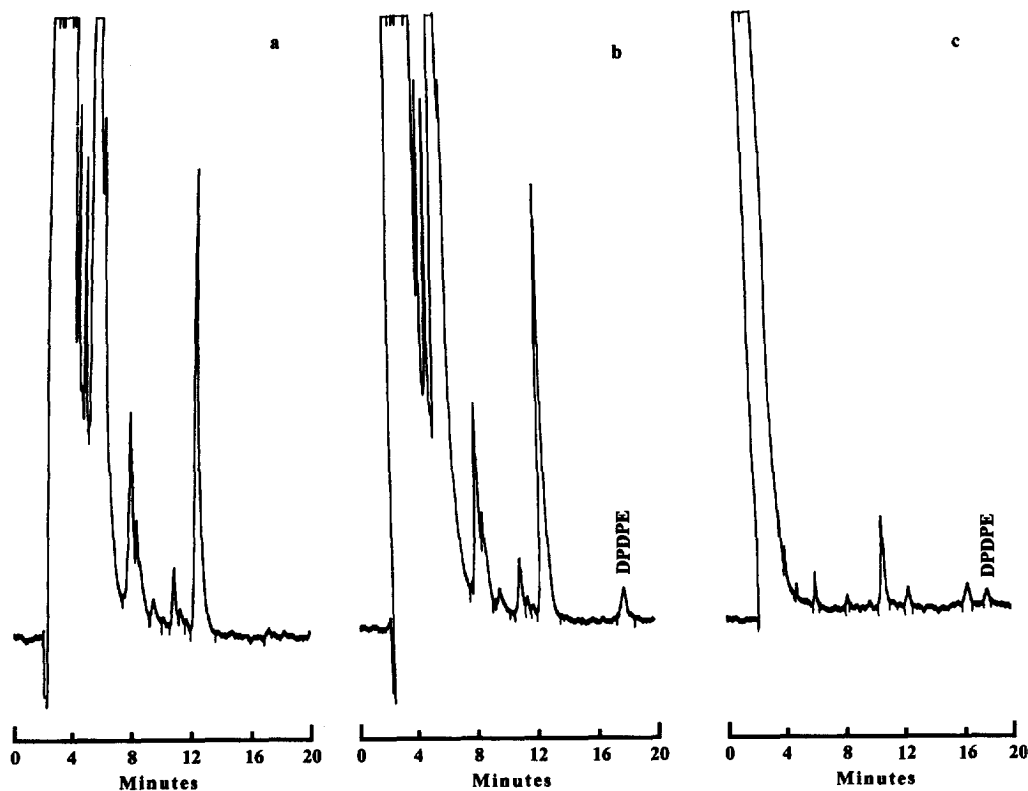

Fig. 7. Analysis of DPDPE in rat serum by HPLC after precipitation of proteins with acetonitrile: (a) blank serum, (b) serum with DPDPE at an initial concentration of 1.5 μM and (c) re-injected fraction from (b). Other conditions were as described in Section 2.2.2 Section 2.3.2.
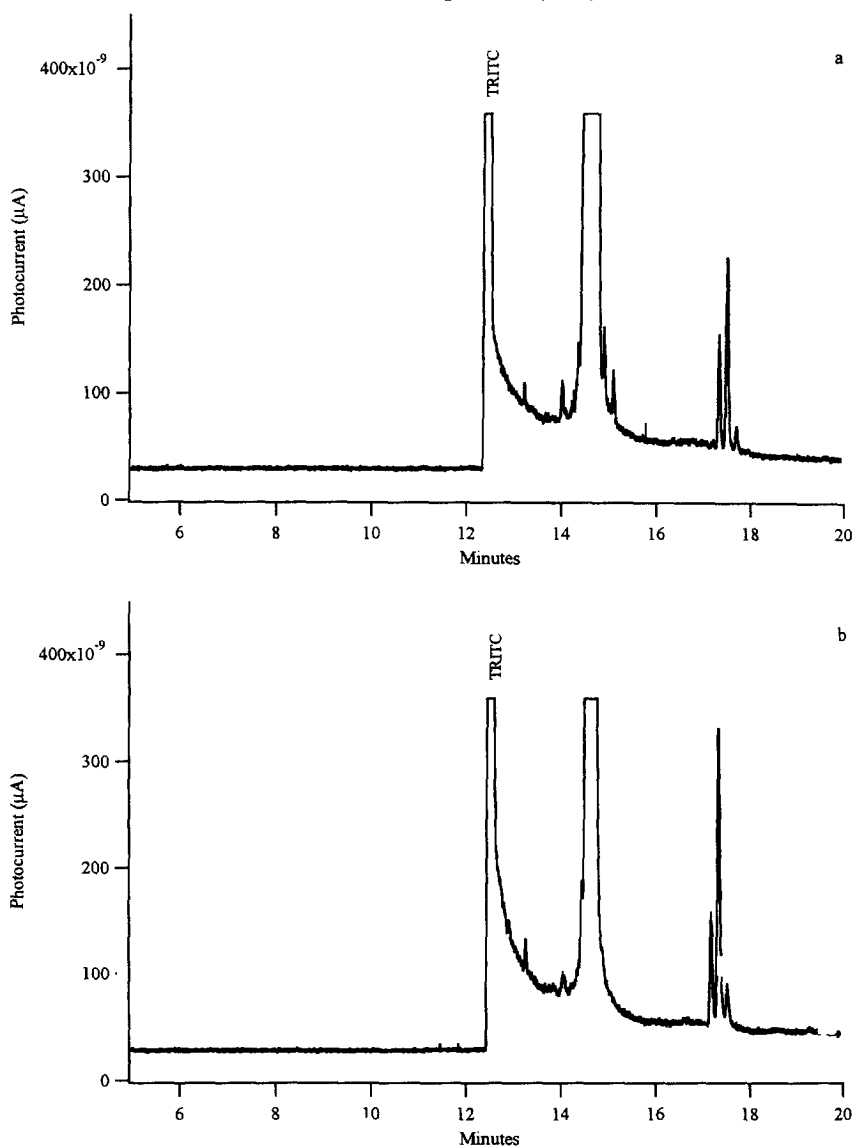
Fig. 8. CZE–LIF analysis of derivatives from (a) blank and (b) spiked serum samples corresponding to Fig. 7a and b, respectively, after collection of the 17.5–20 min HPLC fractions. The derivatizing mixtures were diluted 1000-fold with CZE buffer before injection. Other conditions were as described in Section 2.2.3 Section 2.3.1.

overnight reaction (12 h) at room temperature (21°C) in the dark produced maximal yield (90% conversion of DPDPE to DPDPE–TRITC). The yield of DPDPE–TRITC remained at this level from 12 to beyond 24 h, indicating that the derivative was sufficiently stable under the stated reaction conditions. In addition, the percent yield versus reaction time data were described better by a first-order kinetic equation with a rate constant of 0.31 h^{-1},

indicating that a 12-h reaction duration was sufficient (i.e., >5 half-life).

3.2.2. Molar ratio between DPDPE and TRITC

In order to obtain maximum conversion of DPDPE to the TRITC derivative, an excess derivatizing reagent is required. However, the presence of excess TRITC can interfere with the derivative peak during electrophoretic separation, if no steps are taken to
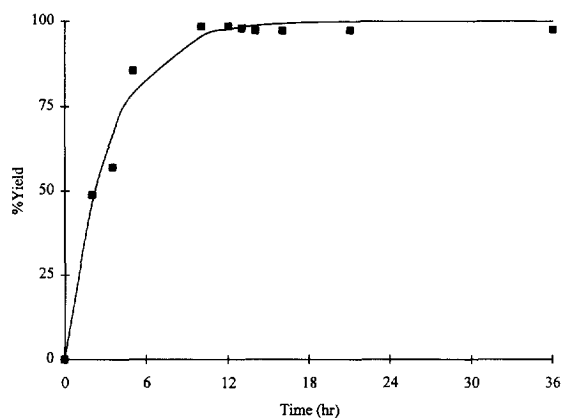
Fig. 9. Reaction of DPDPE with TRITC vs. time. Symbols represent the observed data; the line indicates the fit of the data with a first-order kinetic equation. Other conditions were as described in Section 2.2.3.

Fig. 10. Buffer pH optimization for DPDPE derivatization with TRITC. Conditions were as described in Section 2.2.3.

eliminate excess TRITC after the reaction has come to completion. The molar ratio between TRITC and DPDPE was optimized from this perspective, i.e., to provide high yield and good resolution. For DPDPE in an aqueous medium, a 3-fold molar ratio between TRITC and DPDPE was optimal; a 10-fold molar excess of TRITC was required for DPDPE in serum, since amino acids and endogenous peptides remaining in the serum after pretreatment also consume TRITC (data not shown).

3.2.3. pH of the borate buffer

The pH of the borate buffer used for the derivatization between DPDPE and TRITC is critical. Fig. 10 shows that a pH 9.0 borate buffer was optimal with regard to the reaction yield. The reaction duration experiment (Fig. 9) also indicated that the derivative was stable at this pH. Electropherograms of DPDPE after derivatization under the optimized conditions and monitored with LIF detection are displayed in Fig. 11.

3.3. Mass spectrometry of the DPDPE derivative

In the mass spectrum of the derivative reaction mixture there were two ions related to DPDPE, the m/z 646 ion corresponding to the $(M+H)^+$ of the native (underivatized) peptide, and the ion at m/z 1089, corresponding to the M^+ ion of the TRITC–

DPDPE derivative (Fig. 12A and B). Since TRITC is a quaternary amine, it does not require the addition of a proton to obtain a positive charge for MS analysis. These results indicate a 1:1 stoichiometry between DPDPE and TRITC, as would be expected. A product ion scan of the m/z 1089 precursor ion (Fig. 12C) gives a base peak at m/z 444, corresponding to a neutral loss of m/z 645, that is, the loss of DPDPE as a neutral ion (Fig. 12C).

No evidence for multiple derivatives was observed in the mass spectra. A reaction generating a single derivative has obvious advantages for the quantitative analysis of any substrate requiring derivatization.

The mass spectrum of the reaction mixture using a 2-fold excess of TRITC contained the m/z 646 and 1089 ions at a 1.2:1 ratio, indicating a significant amount of the DPDPE was underivatized (Fig. 12A), whereas the use of a 5-fold molar excess has these ions at a ratio of 0.079:1, indicating essentially all of the DPDPE was present as the TRITC derivative (Fig. 12B). This finding is consistent with results obtained in Section 3.2.2 from CZE–UV and CZE–LIF.

3.4. Selection of an internal standard

The internal standard method minimizes errors introduced during sample pretreatment due to non-specific loss of analyte. In the present case, an
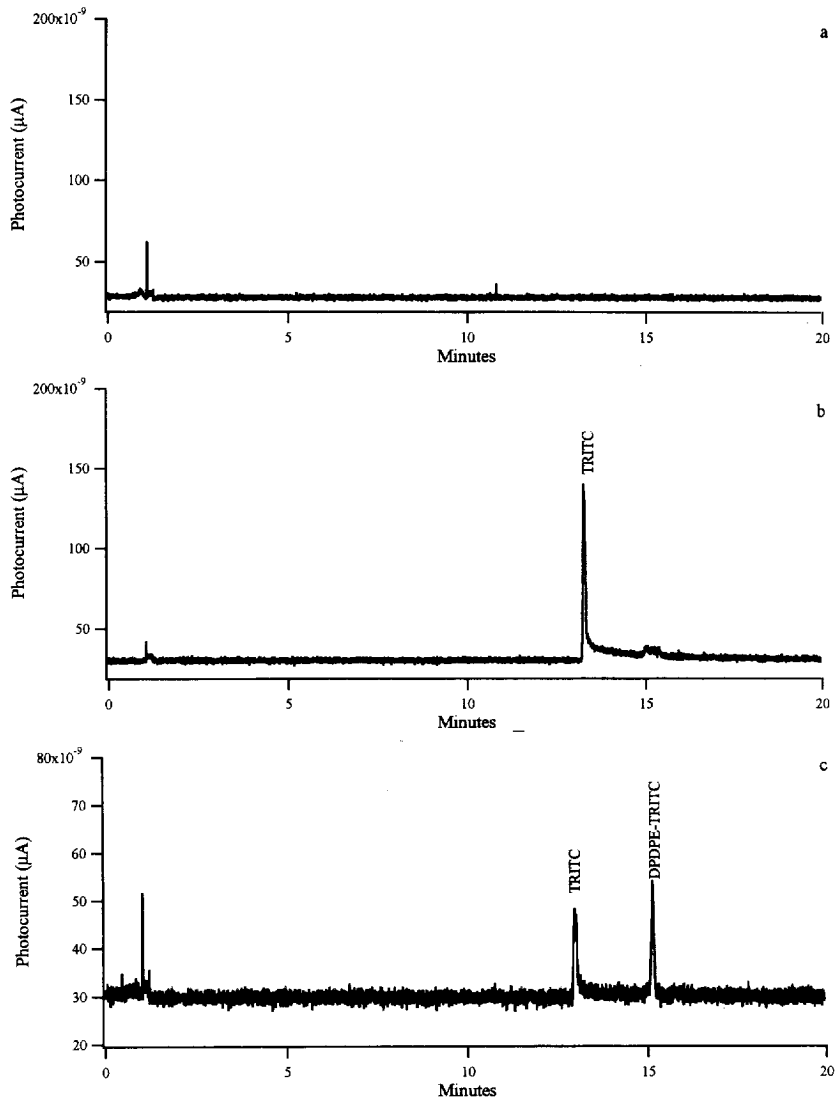
Fig. 11. CZE–LIF analysis of DPDPE–TRITC derivatized under the optimized conditions. (a) DMSO containing no TRITC combined with borate buffer containing no DPDPE (25:75, v/v), (b) TRITC combined with borate buffer containing no DPDPE and (c) DPDPE (10^{-10} M) with TRITC. Other conditions were as described in Section 2.2.3 Section 2.3.1.

internal standard with the following features was required: reproducible extraction efficiency and stability (both native peptide and the TRITC-derivative); a single primary amine group in the structure for derivatization, with no additional functional group(s) that could result in side reactions between the internal standard and DPDPE and/or TRITC; and adequate resolution between the TRITC derivatives

of DPDPE and the internal standard (i.e., differing electrophoretic mobilities of the derivatives).

Several peptides were tested, namely Tyr-D-Ser-Gly-Phe-Leu-Th (DTLET), [D-ser^2]leucine-en-kephalin (DSLET, which was used in a previous assay of DPDPE in rat serum [5]), Tyr-D-Ala-Gly-Me-Phe-Gly (DAGO), P9396, and Val-Gly-Asp-Glu (V6253). P9396 was selected because it conforms
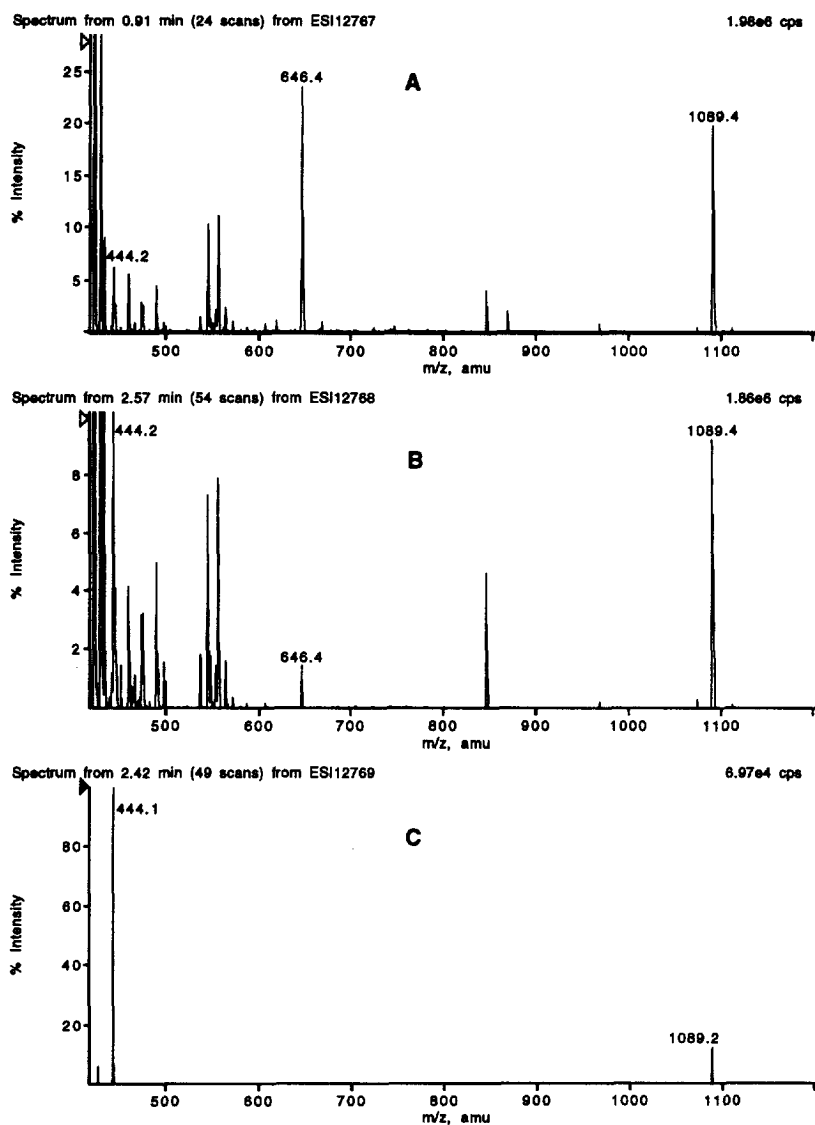
Fig. 12. Mass spectrum of (A) reaction mixture with 2-fold molar excess of TRITC to DPDPE, (B) reaction mixture with 5-fold molar excess of TRITC to DPDPE and (C) a product ion scan of the m/z 1089 precursor ion. Other conditions were as described in Section 2.3.3.

most closely to the requirements as discussed above. Although the extraction efficiency of P9396 was low (20%) under the extraction conditions described in Section 2.2.1 as optimized for DPDPE [5], it was reproducible (extraction efficiency: $20\pm3.5\%$, $n=6$) at the concentration appropriate for this assay. In addition, the electropherogram of derivatives of DPDPE (10^{-9} M) and P9396 in serum indicated that the two were well resolved (Fig. 13).

4. Conclusions

In summary, a CZE–LIF detection method was established for the analysis of DPDPE in rat serum. The serum samples were pretreated with acetonitrile to precipitate proteins, followed by further clean-up with solid-phase extraction cartridges. After elution and evaporation, the residue was dissolved with borate buffer (150 mM, pH 9.0) prior to derivatiza-
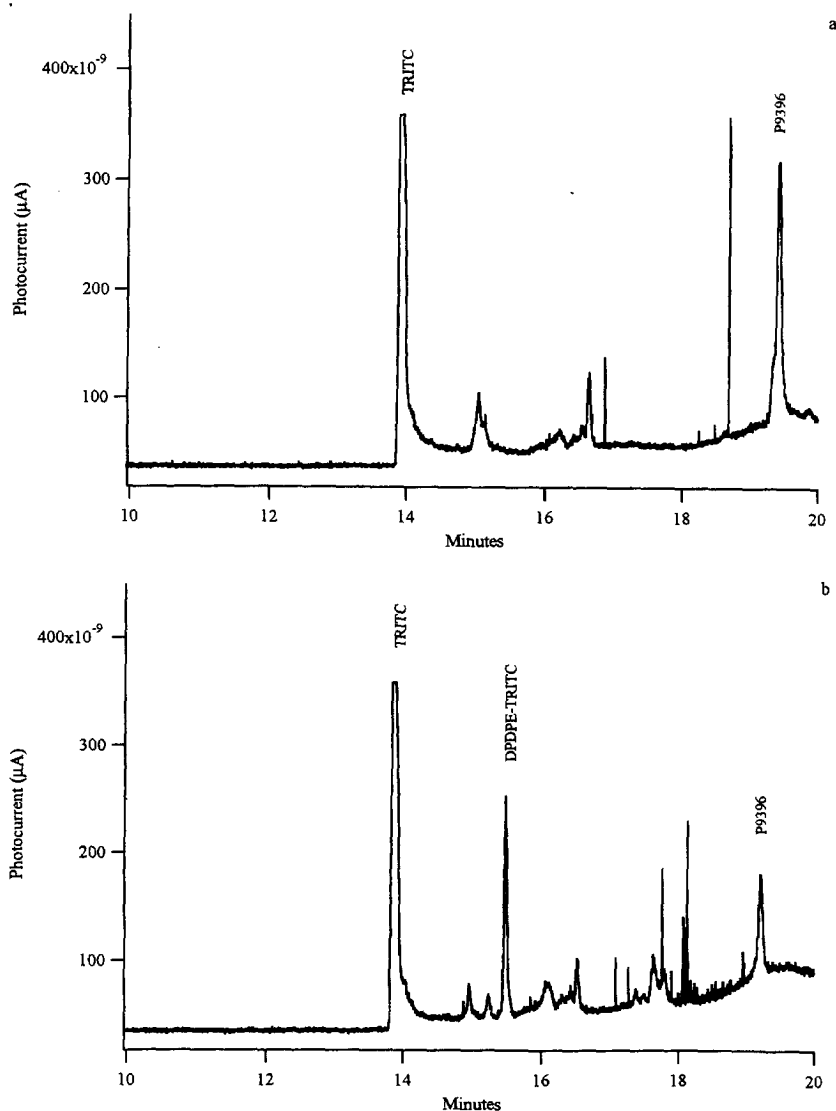
Fig. 13. Electropherogram of derivatives from DPDPE in (a) blank serum with the internal standard P9396 (140 μM) and (b) serum containing both DPDPE and P9396 (70 μM). Other conditions were as described in Section 2.2.1 Section 2.2.3 Section 2.3.1.

tion with TRITC in DMSO. A 3- to 10-fold molar ratio between TRITC and DPDPE was optimal for derivatization in water and serum, respectively, with the reaction proceeding for 12 h at room temperature. The stoichiometry and completeness of the reaction was established with mass spectrometry. P9396 can serve as a useful internal standard. The current CZE–LIF method has a limit of detection of 10^{-18} mol for DPDPE in serum, corresponding to a concentration of DPDPE of 10^{-9} M. This method potentially can be developed further for pharmacokinetic experimentation in laboratory animals.

Acknowledgments

References

[1] H.I. Mosberg, R. Hurst, V.J. Hruby, K. Gee, H.I. Yamamura, J.J. Galligan and T.F. Burks, Proc. Natl. Acad. Sci. USA, 80 (1983) 5871.

[2] G. Toth, T.H. Cramer, R. Knapp, G. Lui, P. Davis, T.F. Burk, H.I. Yamamura and V.J. Hruby, J. Med. Chem., 33 (1990) 249.

[3] S.J. Weber, D.L. Greene, S.D. Sharma, H.I. Yamamura, T.H. Kramer, T.F. Burks, V.J. Hruby, L.B. Hersh and T.P. Davis, J. Pharmacol. Exp. Ther., 259 (1991) 1109.

[4] S.J. Weber, D.L. Green, V.J. Hruby, H.I. Yamamura, F. Porreca and T.P. Davis, J. Pharmacol. Exp. Ther., 263 (1992) 1308.

[5] C. Chen and G.M. Pollack, J. Chomatogr. B, 681 (1996) 363.

[6] D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang, N.J. Dovichi, J. Chomatogr., 559 (1991) 237.

[7] J.P. Larmann Jr., A.V. Lemmo, A.W. Moore Jr. and J.W. Jorgenson, Electrophoresis, 14 (1993) 439.

[8] L.A. Holland and J.W. Jorgenson, Anal. Chem., 67 (1995) 3275.