ELSEVIER

Journal of Chromatography B, 780 (2002) 269–281

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantitative analysis of a model opioid peptide and its cyclic prodrugs in rat plasma using high-performance liquid chromatography with fluorescence and tandem mass spectrometric detection

Jerry Z. Yang, K. Chad Bastian[1], Randy D. Moore[2], John F. Stobaugh, Ronald T. Borchardt*

Department of Pharmaceutical Chemistry, The University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047, USA

Received 25 February 2002; received in revised form 31 July 2002; accepted 31 July 2002

Abstract

Two analytical methods were developed for quantitative determination of DADLE (H_2N–Tyr–D-Ala–Gly–Phe–D-Leu–COOH) and its two cyclic prodrugs in rat plasma. For high-performance liquid chromatography with fluorescence detection (LC–FLU), precolumn derivatization of DADLE was accomplished by labeling the N-terminal amino group with the reagent naphthalene-2,3-dicarboxaldehyde in the presence of cyanide (NDA/CN) to form a highly fluorescent 1-cyanobenz[*f*]isoindole (CBI) derivative. A multi-dimensional LC system was employed to improve selectivity, and solid-phase extraction (SPE) was used for plasma sample preparation. The cyclic prodrugs were converted to DADLE prior to their derivatization. With fluorescence detection after derivatization, the limit of quantitation (LOQ) was 6 ng ml^{-1} for the analysis of DADLE, and good linearity was observed up to 6000 ng ml^{-1} in rat plasma. Quantitative analysis of DADLE and its cyclic prodrugs was also performed using liquid chromatography interfaced to electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS). Chromatographic separation was achieved on a C_{18} column using gradient elution in a water–acetonitrile system containing 0.1% (v/v) formic acid. The tandem mass spectrometric analysis was performed in the multiple reaction monitoring mode using internal standardization to improve assay precision and accuracy. For plasma sample pretreatment, acetonitrile was added first to precipitate proteins and SPE was used to minimize matrix effects. Using LC–ESI-MS–MS, the LOQ was 0.5 ng ml^{-1} for DADLE and 2 to 5 ng ml^{-1} for its prodrugs. Good linearity was observed from the LOQ up to 1000 ng ml^{-1} for all compounds. For the analysis of DADLE, both analytical methods showed good precision, accuracy and stability. However, for prodrug analysis, LC–FLU showed some sensitivity and accuracy problems, while the LC–ESI-MS–MS method provided consistent and satisfactory results. In conclusion, LC–ESI-MS–MS is the method of choice for the analysis of DADLE and its cyclic prodrugs in rat plasma samples due to its good selectivity, high sensitivity, and fast analysis. Its application was demonstrated through biodisposition and bioconversion studies of the coumarinic acid-based prodrug after intravenous administration in rats.

Keywords: Derivatization, LC; Opioid peptides; DADLE; Cyclic prodrugs

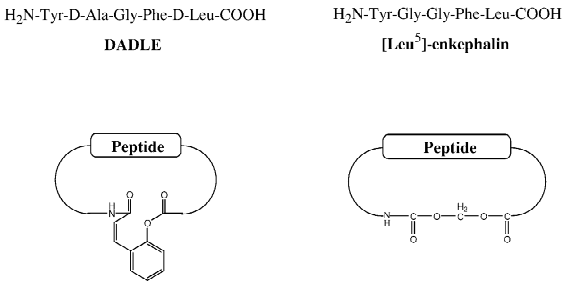
*Corresponding author. Tel.: +1-785-864-3527; fax: +1-785-864-5736.
E-mail address: rborchardt@ku.edu (R.T. Borchardt).
[1]Present address: AAI International, 2320 Scientific Park Drive, Wilmington, NC 28405, USA.
[2]Present address: 7237 Marty Drive, Overland Park, KS 66204, USA.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00536-6

1. Introduction

Recently, the concept of making cyclic prodrugs of opioid peptides to modify their physicochemical properties has been introduced by this laboratory [1–5]. The evaluation of cell membrane permeation characteristics of these cyclic prodrugs was conducted using a cell culture model of the intestinal mucosa [6–8]. While the data from these in vitro experiments allowed the evaluation and correlation of a particular chemical modification on the cell membrane permeation characteristics of these prodrugs, in vivo pharmacokinetic studies were needed to determine the effects of these chemical modifications on other pharmacokinetic properties (e.g., liver clearance, bioconversion). To support these studies, the development of analytical methods with high sensitivity and selectivity for determination of DADLE (H_2N–Tyr–D-Ala–Gly–Phe–D-Leu–COOH) and the cyclic prodrugs (Fig. 1) in biological fluids and tissues was required.

Several high-performance liquid chromatography (LC) methods using different on-line detectors have been reported for the analysis of opioid peptides. In general, these approaches have been based on ultraviolet [9–11], electrochemical [11–13], fluorescence [14–19], or mass spectrometric detection [20,21]. Ultraviolet and electrochemical detection offer the least sensitivity and are not widely used for low concentration determination. Fluorescence is the most commonly used detection method for opioid peptide analysis due to its high sensitivity and simplicity. Different derivatization methods have been employed in attempts to further improve the sensitivity [14–19]. While mass spectrometry (MS) was widely used for structural identification and peptide sequencing in the past, its use as an on-line detector coupled to LC has been relatively limited until recently [22–27]. The advances in interfacing technology between LC and MS have led to the success of MS detection for both qualitative and quantitative analysis of peptides. In particular, LC interfaced to tandem mass spectrometry (MS–MS) has shown excellent specificity and sensitivity for rapid analysis of peptides in biological samples [20,21].



prodrug	Peptide
Ia	DADLE
Ib	[Leu^5]-enkephalin
IIa	DADLE
IIb	[Leu^5]-enkephalin

Fig. 1. Chemical structures of DADLE and its internal standard [Leu^5]enkephalin, the two prodrugs of DADLE (**Ia** and **IIa**) and their internal standards (**Ib** and **IIb**).

In earlier studies, LC methods with ultraviolet- and fluorescence-based detection were developed in support of in vitro studies of DADLE and its prodrugs [6–8]. However, these analytical methods could only provide a limit of quantitation (LOQ) around 50 ng ml^{-1}, which was not sensitive enough for in vivo pharmacokinetic studies where an LOQ of less than 5 ng ml^{-1} was needed. In addition, the in vivo pharmacokinetic studies presented new challenges, including sample cleanup and monitoring conversion from the prodrugs to DADLE in biological media. Therefore, to develop an analytical method for in vivo pharmacokinetic studies, the following issues needed to be addressed: (i) a separation system that could resolve compounds having quite different physiochemical properties (e.g., DADLE that is hydrophilic and charged vs. the cyclic prodrugs that are hydrophobic and uncharged); (ii) a detection system that had both high sensitivity and selectivity; and (iii) a sample handling procedure that could address the enzymatic instability of the prodrugs in biological media.

The subject of this paper is the development, validation, and comparison of two analytical methods, i.e., liquid chromatography with fluorescence detection (LC–FLU) and liquid chromatography with electrospray ionization tandem mass spec-

trometric detection (LC–ESI-MS–MS), which could be used for in vivo pharmacokinetic studies of DADLE and its prodrugs.

2. Experimental

2.1. Materials

The model opioid peptide DADLE and its internal standard [Leu^5]enkephalin were purchased from Sigma (St. Louis, MO, USA). Prodrugs of DADLE (**Ia**, **IIa**) and [Leu^5]enkephalin (**Ib**, **IIb**) were synthesized following procedures described elsewhere [28,29]. All solvents used were HPLC grade, including deionized ultra filtered (DIUF) water (Fisher, Fair Lawn, NJ, USA). All other chemicals were of the highest purity available and used as received. Rat whole blood and plasma were obtained from male Sprague–Dawley rats (Animal Care Unit, The University of Kansas, Lawrence, KS, USA).

2.2. Instrumentation

2.2.1. LC–FLU method

LC with fluorescence detection was performed using a Shimadzu LC-10A gradient system (Shimadzu, Tokyo, Japan) consisting of two LC-10AS pumps, an SCL-10A system controller, an SIL-10A auto injector with a sample cooler, an RF-535 fluorescence detector (xenon lamp: λ_{ex}=420 nm; λ_{em}=490 nm), and two FCV-2AH high-pressure switching valves controlled by an SCL-6A controller. The chromatographic data were acquired and analyzed using the CLASS-VP (version: 4.2) Chromatography Data System (Shimadzu).

A multi-dimensional chromatographic system [15] was implemented for selectivity enhancement. Two columns with different stationary phases were used; both were purchased from Vydac (Hesperia, CA, USA). Column 1 was a diphenyl column (Vydac 219TP54, 250×4.6 mm I.D., 5 μm) with a Vydac diphenyl guard column. Column 2 was a C_{18} reversed-phase column (Vydac 218TP54, 250×4.6 mm I.D., 5 μm). The mobile phase for pump 1 was a mixture of acetonitrile–water (39:61, v/v) with 0.1% (v/v) trifluoroacetic acid. The mobile phase for pump 2 was a mixture of acetonitrile–water–tetrahydrofuran (42:54:4, v/v) with 0.1% (v/v) trifluoroacetic acid. The mobile phases were degassed under vacuum, and each was delivered at a flow-rate of 1 ml min^{-1}. The separation studies were conducted at ambient temperature (approx. 22 °C). For centrifugation, a Micro-Centrifuge Model 59A from Fisher Scientific (Pittsburgh, PA, USA) was used.

2.2.2. LC–ESI-MS–MS method

Liquid chromatography with tandem mass spectrometric detection was performed using a Micromass VG Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA, USA). Liquid chromatography was conducted using a HP 1100 series HPLC System (Agilent Technologies, Palo Alto, CA, USA). A Zorbax SB-C_{18} column (50×2.1 mm I.D., 5 μm) from Agilent Technologies was used as the analytical column; it was protected with a 0.5 μm precolumn filter (MAC-MOD Analytical, Chadds Ford, PA, USA). The column temperature was kept at 25 °C to ensure reproducible separation. Two mobile phases were used to generate a linear gradient with a 0.2 ml min^{-1} flow-rate, which allowed simultaneous analysis of DADLE and its prodrugs in a single run. Mobile phase A was water with 0.1% (v/v) formic acid, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. The linear gradient was from 10 to 100% mobile phase B in the first 8 min; mobile phase B was kept at 100% for 3 min and was then reduced to 10% in 1 min; finally the column was equilibrated in 10% mobile phase B for 8 min. The total run time was 20 min with eluent from the first 5 min being directed to waste to avoid the contamination of the mass spectrometer with salts. For direct infusion of sample solutions, a Harvard ''22'' syringe pump (Harvard Apparatus, Holliston, MA, USA) was used to deliver a continuous and pulse-free flow. A micro-centrifuge Model 59A from Fisher Scientific was used for centrifugation.

The LC system was interfaced to the mass spectrometer via an electrospray interface. The capillary voltage and cone voltage were 3.5 kV and 40 V, respectively. The source temperature was set at 120 °C and the nebulizer gas flow was controlled around 700 l h^{-1} for effective evaporation of the solvent. Both MS1 and MS2 resolutions were set at 15.0 and the multiplier voltage was set at 650 V. For

ion recording, multiple reaction monitoring (MRM) of several channels was used with 0.1-s dwell time and inter-channel delay set at 0.03 s. Data acquisition and analysis were performed using MassLynx v3.4 software (Micromass).

2.3. Standard preparation

2.3.1. LC–FLU method

Separate standard stock solutions (1 m*M*) were prepared in dimethyl sulfoxide (DMSO) for DADLE and its prodrugs. For solution standards, subsequent dilutions were made in 20% (v/v) acetonitrile to give the following concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 μ*M*. For working standards, dilutions were made using DMSO to give a series of solutions with concentrations covering the calibration range. Rat whole blood standards were prepared by adding 4 μl of the working standards containing DADLE or the prodrug to 396 μl blank rat blood. The resulting concentrations were 0.01, 0.1, 1.0, and 10.0 μ*M*. The blood samples were then centrifuged immediately at 4700 rpm (2100 *g*) and 4 °C for 10 min to obtain rat plasma standards.

2.3.2. LC–ESI-MS–MS method

Standard stock solutions (100 μg ml^{-1}) were prepared separately in different solvents. For DADLE and its internal standard, deionized water was used; for the prodrugs and their internal standards, 10% (v/v) DMSO in water was used to improve the solubility of the compounds. A stock solution mixture made up of DADLE and its two prodrugs at 10 μg ml^{-1} each was prepared from individual stock solutions. Subsequent dilutions were made with deionized water to prepare a series of working standards at different concentrations for calibration. An internal standard solution mixture was also prepared at 100 ng ml^{-1} for [Leu^5]enkephalin and 500 ng ml^{-1} for each of the two internal standards **Ib** and **IIb** in deionized water. Plasma standards were prepared by adding 10 μl of the working standard solutions containing DADLE and its two prodrugs to 100 μl blank rat plasma. The resulting concentrations were 0.2, 0.5, 2.0, 5.0, 10.0, 40.0, 200, and 1000 ng ml^{-1} for all three compounds.

2.4. Sample preparation

2.4.1. LC–FLU method

Solid-phase extraction (SPE) was used for plasma sample preparation. Aqueous guanidine·HCl (6 *M*), which was acidified with H_3PO_4 (0.01%, v/v), was initially added (160 μl) to the plasma sample (80 μl) to denature plasma proteins and release bound DADLE and its prodrugs. Subsequently, SPE was performed using Sep-Pak Vac C_{18} cartridges (1 cc/100 mg, Waters, Milford, MA, USA) according to the following protocol. An SPE cartridge was conditioned with 1 ml acetonitrile followed by a 1 ml water wash. The plasma sample (240 μl) was loaded onto the cartridge. After washing with 1.5 ml deionized water, 1 ml of acetonitrile–water (70:30, v/v) was used to elute DADLE and its prodrugs from the cartridge, and borosilicate glass tubes were used for eluent collection. The final solution was evaporated to dryness with nitrogen gas and the residue reconstituted in 150 μl of 20% (v/v) acetonitrile. A 5000 nominal molecular mass limit (NMWL) membrane filter unit (Millipore, Bedford, MA, USA) was used to filter the reconstituted solution to remove any remaining proteins.

Precolumn derivatization was accomplished using a 50 μl sample aliquot, to which was added 30 μl of sodium phosphate buffer (200 m*M*, pH 6.8). The reaction was initiated by adding 10 μl of freshly made potassium cyanide (CN) solution (50 m*M*) and 10 μl of freshly made naphthalene-2,3-dicarboxaldehyde (NDA) solution in acetonitrile (50 m*M*). After mixing twice and waiting 30 s, the reaction was complete and 50 μl of the final product solution was injected for analysis. The whole precolumn derivatization procedure was automated and performed by the autosampler at 4 °C. Before the cyclic prodrugs could be analyzed, they first had to be converted to DADLE since the prodrugs do not react with NDA/CN. The conversion process was conducted at 37 °C with 0.1 *M* NaOH and HCl aqueous solutions. Briefly, a small aliquot of NaOH solution was added to a 50 μl sample solution to hydrolyze the cyclic prodrugs to DADLE. After 10 to 30 min, an equal amount of HCl solution was added to quench the reaction and to neutralize the solution before the derivatization of DADLE was conducted. The amounts of NaOH and HCl solutions used and

the reaction time were adjusted accordingly for the two prodrugs due to their different conversion kinetics.

2.4.2. LC–ESI-MS–MS method

For the preparation of plasma samples for LC–ESI-MS–MS, an SPE method similar to that used for LC–FLU with some modifications was employed. To a 110 μl plasma sample, 200 μl of acetonitrile was added to precipitate the plasma proteins along with 10 μl of the internal standard solution mixture. After vortex mixing, the precipitated proteins were removed by centrifugation at 6000 rpm (3300 *g*) and 4 °C for 10 min. Since the supernatant had high organic content, it had to be evaporated and reconstituted with water for compatibility with SPE. A Centrivap Concentrator (Labconco, Kansas City, MO, USA) was used, and the organic solvent was evaporated under vacuum. After 1 h, the concentrated supernatant (approx. 30 μl) was diluted with 1 ml of deionized water, and the solution was loaded onto the cartridges for extraction. The SPE procedure was similar to the one used in the LC–FLU method with four steps: conditioning; loading; washing; and eluting. It was performed using Waters Oasis HLB extraction cartridges (1 cc/30 mg, Waters) according to the following protocol. An SPE cartridge was conditioned with 1 ml methanol followed by a 1 ml water wash. The plasma sample (approx. 1 ml) was loaded onto the cartridge. After washing with 2 ml deionized water and 1 ml 5% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid, 1 ml of acetonitrile with 0.1% (v/v) trifluoroacetic acid was used to elute DADLE and its prodrugs from the cartridge. The final solution was evaporated to dryness and the residue was reconstituted in 100 μl of 10% (v/v) acetonitrile. Then it was centrifuged again at 10 000 rpm (9300 *g*) and 4 °C for 1 min, and a 50 μl supernatant was injected for LC–ESI-MS–MS analysis.

2.5. Method validation

2.5.1. LC–FLU method

Limited method validation was done for the LC–FLU method due to the success of the LC–ESI-MS–MS method. The LC–FLU method validation was focused on the analysis of DADLE and prodrug **Ia**. The standard curves were constructed by plotting the peak areas of the analytes versus the theoretical concentrations, and a weighted $(1/c^2)$ linear least-square regression was used. The precision and accuracy of the method were assessed within the entire concentration range. Other validation parameters including recovery, specificity, and stability were also studied.

2.5.2. LC–ESI-MS–MS method

The LC–ESI-MS–MS method validation was more extensive, with important validation parameters including precision, accuracy, recovery, specificity and stability being established. The calibration curves were constructed from the peak area ratios of the analytes to the respective internal standards versus the theoretical concentrations with a weighted $(1/c)$ linear least-square regression. Unknown sample concentrations were calculated using linear equations $(y = ac + b)$ fitted for different calibration curves. The precision of the method was determined by replicate analyses $(n = 5)$ of rat plasma samples containing DADLE and its prodrugs at different concentrations within the calibration range and presented as the relative standard deviation (RSD). The accuracy of the method was expressed as [(mean calculated concentration)/(spiked concentration)]× 100. The limit of detection (LOD) was defined as the lowest sample concentration that could be detected $(S/N > 3)$. The limit of quantitation (LOQ) was defined as the lowest sample concentration that could be quantified with good precision (RSD < 20%) and accuracy (75–125%). The recoveries of DADLE and its prodrugs along with their internal standards were determined by comparing the peak areas of the analytes extracted from plasma to those of standards made with blank plasma extract injected directly. The results were obtained from different concentrations and the averages were taken as mean recoveries. The specificity of the assay was established by analyzing blank rat plasma samples from six different sources. Quality control samples at low and high concentrations were prepared and analyzed on three separate days. Finally, the sample stability before and during the analysis was also assessed at different concentrations. This included freeze–thaw stability after three cycles and long-term storage stability at −60 °C for up to 1 month.

2.6. Application of the LC–ESI-MS–MS method

Disposition and conversion of prodrug **Ia** to DADLE was studied in rats after a 1 mg kg^{-1} intravenous injection ($n=3$). Blood samples (0.2 ml) were taken from the jugular vein at 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, and 120 min after injection. The SPE method described above was used for plasma sample preparation, and final solutions were analyzed by the LC–ESI-MS–MS method.

3. Results and discussion

3.1. LC–FLU method

For the LC–FLU method, DADLE was derivatized with NDA/CN to produce the fluorescent N-substituted CBI derivative (Fig. 2) [16]. The derivatized DADLE gave at least a 10-fold increase in fluorescence sensitivity over DADLE itself. The LOD of the LC–FLU assay was approx. 1 ng ml^{-1}, which made the fluorescence method sensitive enough for detection of this peptide in biological samples (e.g., plasma) generated during in vivo pharmacokinetic studies. The whole precolumn derivatization procedure was performed by the autosampler, which gave consistent results with minimal human manipulation. However, with the increase in sensitivity, a significant drop in selectivity was observed with the LC–FLU method. Unfortunately, during derivatization, the NDA/CN reagent reacts with other compounds containing primary amine groups, which leads to increased background interferences, especially for rat plasma samples. This problem was solved by using a two-dimensional (2D) LC system following the guidelines of previous workers [16]. Two switching valves were used to control the flow between two different analytical columns; thus, chromatographic separation occurred on both columns, resulting in improved selectivity. With this two-column system, an improved separation of the CBI-DADLE derivative peak from the background interferences was achieved (Fig. 3). However, even with this multi-dimensional LC system, interfering peaks from rat plasma still existed and compromised the selectivity of the assay at low concentrations (<5 ng ml^{-1}).

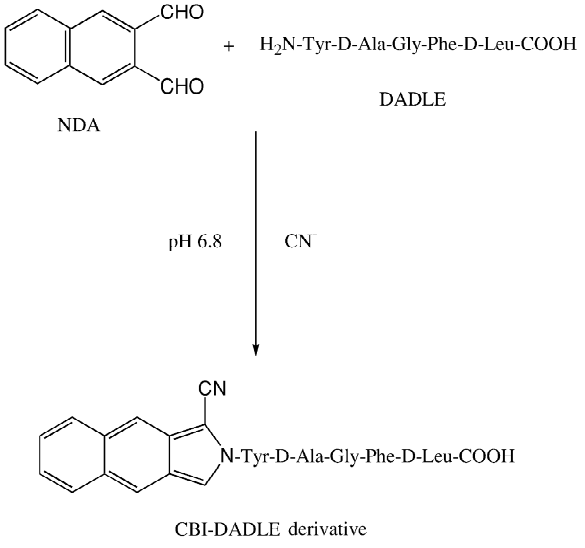


Fig. 2. Derivatization reaction of DADLE with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion (CN^-) to produce an N-substituted 1-cyanobenz[*f*]isoindole (CBI-DADLE derivative).

Using the 2D-LC–FLU method, the LOQ for DADLE was 6 ng ml^{-1} in rat plasma, which despite its limitations was a significant improvement compared to methods that use UV or native fluorescence to detect DADLE. Good linearity (correlation coefficient $r>0.99$) was observed over the 6 to 6000 ng ml^{-1} concentration range. The LC–FLU method also provided good precision and acceptable accuracy (87–114% for DADLE analysis; Table 1). High and reproducible DADLE recovery (79±11%) was achieved by the SPE method for rat plasma samples. The extraction method also reduced plasma background interferences significantly and ultimately led to improved sensitivity and selectivity for the LC–FLU method. Since the derivatization procedure was performed by the autosampler and the CBI-DADLE product was injected immediately onto the LC column, no degradation of the derivatization product was observed. All these results indicated that the LC–FLU method was reliable and applicable for DADLE analysis in rat plasma samples.

While the NDA/CN derivatization reaction improved the sensitivity of the analysis for DADLE, it could not be used with the prodrugs since they do not have free N-terminal amino groups for reaction

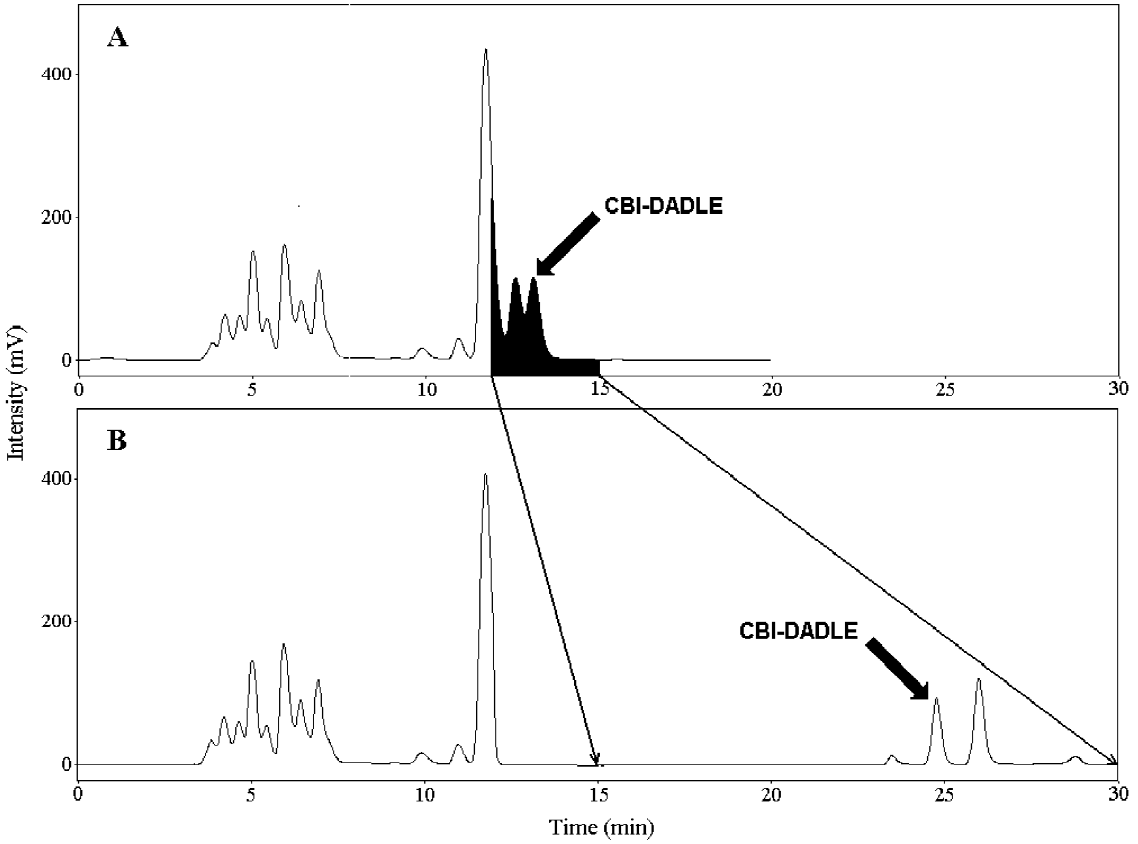


Fig. 3. Chromatograms showing optimized separation of CBI-DADLE derivative using the column switching system. The DADLE concentration in plasma was 6 μg ml^{-1}. (A) Separation with column 1 only; (B) separation with columns 1 and 2 after column switching.

with NDA/CN. A two-step analysis was developed to permit detection of both DADLE and its prodrugs in rat plasma. After SPE, the reconstituted solution, which contained DADLE and the prodrug, was split into two equal aliquots. The first aliquot was analyzed by the LC–FLU method after derivatization with NDA/CN in order to determine DADLE concentrations. The second aliquot was treated with NaOH and HCl solutions to convert the prodrug to DADLE and was then analyzed by the NDA/CN derivatization method to determine ''total'' DADLE concentration. Because DADLE is stable during

Table 1
Precision and accuracy data for the determination of DADLE and prodrug **Ia** in rat plasma using the LC–FLU method

Concentration (μM)	DADLE		Prodrug **Ia**	
	Precision[a]	Accuracy[b]	Precision[a]	Accuracy[b]
0.01	96–106	101	N.D.	N.D.
0.1	83–91	87	95–98	96
1.0	96–99	98	135–145	140
10.0	111–116	114	58–69	64

N.D., Not determined because it was below the limit of quantitation.

[a] Precision expressed as the range of [(calculated concentration)/(spiked concentration)]×100, $n=2$.

[b] Accuracy expressed as [(mean calculated concentration)/(spiked concentration)]×100.

treatment with NaOH and HCl solutions, the ''total'' DADLE concentration included DADLE present in plasma and DADLE formed by chemical conversion from the prodrug. Taking into consideration the dilution factors and subtracting the original DADLE concentration, the concentration of DADLE chemically converted from the prodrug could be calculated. Assuming that the chemical conversion from the prodrug to DADLE was constant throughout the concentration range of the prodrug, the concentration of DADLE detected by this method is reflective of the prodrug concentration. Even though it was not necessary to know the exact percentages of conversion of the prodrugs to DADLE in NaOH and HCl solutions, the numbers were needed for validation purposes. These values could be determined through repeated conversion experiments at various concentrations. The percentages of conversion of prodrugs **Ia** and **IIa** to DADLE were approx. 60 and 90%, respectively. Although percentages of conversion close to 100% were desirable, values as low as 50 to 60% were acceptable as long as the process was precise, accurate, and reproducible.

Limited method validation was done for prodrug **Ia**; the results are presented in Table 1. The LOQ for prodrug **Ia** (70 ng ml^{-1}) was much higher than that of DADLE (6 ng ml^{-1}). The two-step assay for prodrug **Ia** showed some accuracy problems (64–140%), although reasonable precision was achieved. There were two problems associated with this prodrug analytical method. First, strong binding between prodrug **Ia** and the C_{18} stationary phase of SPE cartridges led to low and inconsistent prodrug recovery (around 50%) from rat plasma. The second and the most significant problem was the error-prone conversion process due to the formation of a stable intermediate. Inconsistent conversions from prodrug **Ia** to DADLE and multi-step sample dilutions resulted in reduced sensitivity, precision, and accuracy for prodrug analysis. Overall, the LC–FLU method was suitable for DADLE analysis in biological media, but significant improvements in sensitivity and accuracy were needed in order to accurately measure prodrug levels.

3.2. LC–ESI-MS–MS method

The LC–ESI-MS–MS method employed ESI for DADLE and its prodrugs. Formic acid (0.1%, v/v) was used for pH control in the ESI positive mode. The protonated molecules of DADLE and prodrugs **Ia** and **IIa** were observed at m/z 570.3, 698.3, and 626.3, respectively. The representative parent ion spectra of DADLE and prodrug **Ia** are shown in Fig. 4. Interestingly, the sodium and potassium ion adducts for the prodrugs gave responses much higher than those of DADLE. Metal ion adduct formation suppresses the response of the molecular ions and leads to reduced sensitivity and reproducibility [30–32]. This problem is very difficult to eliminate due to the abundance of metal ions. However, the formation of metal ion adducts could be reduced through sufficient chromatographic separation of the analytes from the solvent front by coupling the mass spectrometry to LC. The molecular ions were chosen for product ion formation and the product ion selection was accomplished by adjusting collision energy and

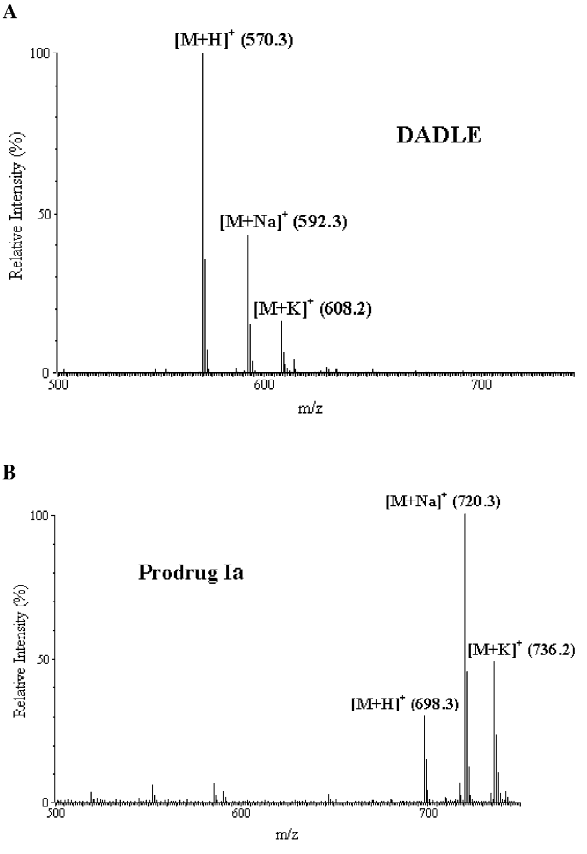


Fig. 4. Parent ion spectra of the protonated molecules in ESI positive mode: (A) DADLE (m/z 570.3); (B) prodrug **I** (m/z 698.3).

collision cell gas pressure. A typical fragmentation pattern for DADLE is shown in Fig. 5. It was not surprising to see that the most stable fragments after high-energy collision were the ones containing aromatic groups since they have the most rigid structures. The product ions chosen for MRM function were 120.1 for DADLE, 136.1 for prodrug **Ia**, and 120.1 for prodrug **IIa** with a 60 V collision energy providing the best response. For LC, a linear gradient program was developed to effectively separate DADLE and its prodrugs in a single run.

To achieve good precision and accuracy for the quantitative analysis of DADLE and its prodrugs, internal standards were used. As shown in Fig. 1, the internal standards chosen were [Leu^5]enkephalin (an analog of DADLE), and prodrugs of [Leu^5]enkephalin (**Ib**, **IIb**). Internal standards **Ib** and **IIb** have the same linkers as the prodrugs of DADLE **Ia** and **IIa**. For plasma sample cleanup, SPE was used to eliminate background interferences before the samples were injected onto the LC–ESI-MS–MS system. Contrary to a commonly held belief, the reliability of quantitative analysis in biological fluids using LC–ESI-MS–MS is not always absolute. One of the main reasons for this is the lack of specificity and selectivity due to the ion suppression caused by matrix effects, especially under ESI conditions [33]. Coeluting, undetected matrix components from biological samples may interfere with the ionization of the analytes and reduce their ion intensity, therefore affecting the reproducibility and accuracy of the

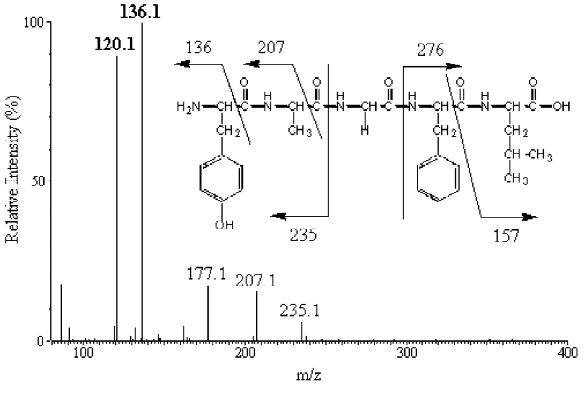


Fig. 5. Product ion spectrum of the protonated molecule of DADLE (m/z 570.3) in ESI positive mode. The collision energy was 60 V.

assay. Matrix effects are especially dependent on the extent of sample cleanup and the degree of analyte retention during LC analysis. Therefore, SPE and gradient separation were improved for the LC–ESI-MS–MS method to eliminate the matrix effect. First, Waters Oasis HLB extraction cartridges were used instead of C_{18} cartridges to eliminate irreversible binding of the prodrugs to the sorbent. Protein precipitation with acetonitrile was employed for more effective plasma protein removal. Last, a gradient program for LC was developed for effective separation and retention of these compounds.

Using the LC–ESI-MS–MS method, the LOQs from rat plasma were 0.5 ng ml^{-1} for DADLE, 5 ng ml^{-1} for prodrug **Ia**, and 2 ng ml^{-1} for prodrug **IIa**. Representative chromatograms obtained with the LC–ESI-MS–MS method are presented in Fig. 6. Overall, DADLE had better sensitivity than its prodrugs; this could be due to the different physiochemical properties of the compounds (e.g., DADLE, hydrophilic, charged; cyclic prodrugs, hydrophobic, uncharged). The LC–ESI-MS–MS method showed less than 20% intra-day assay precision and 83–122% assay accuracy at all concentrations within the calibration range studied (Table 2). Similar inter-day precision (RSD$<$15%) and accuracy (80–125%) results were also obtained. With a $1/c$ weighting function, a linear calibration curve was generated for each of the three compounds from their respective LOQs up to 1000 ng ml^{-1} with correlation coefficients $r>0.98$. In terms of specificity, no background interferences were observed from six different plasma blanks and excellent selectivity was achieved through tandem mass spectrometric detection. SPE provided high and reproducible recoveries for all three compounds from rat plasma samples. The extraction recoveries were 76±4% for DADLE, 88±7% for prodrug **Ia**, and 100±6% for prodrug **IIa** ($n=6$). The internal standards also had consistent recoveries comparable to their respective compounds, 63±9% for [Leu^5]enkephalin, 82±6% for internal standard **Ib**, and 84±2% for internal standard **IIb** ($n=6$). Finally, the stability of DADLE and its prodrugs during sample storage and preparation was established. After over 1 month period stored at −60 °C and three freeze–thaw cycles, the compounds remaining were 98±8% for DADLE, 97±7% for prodrug **Ia**, and 97±8% for prodrug **IIa**

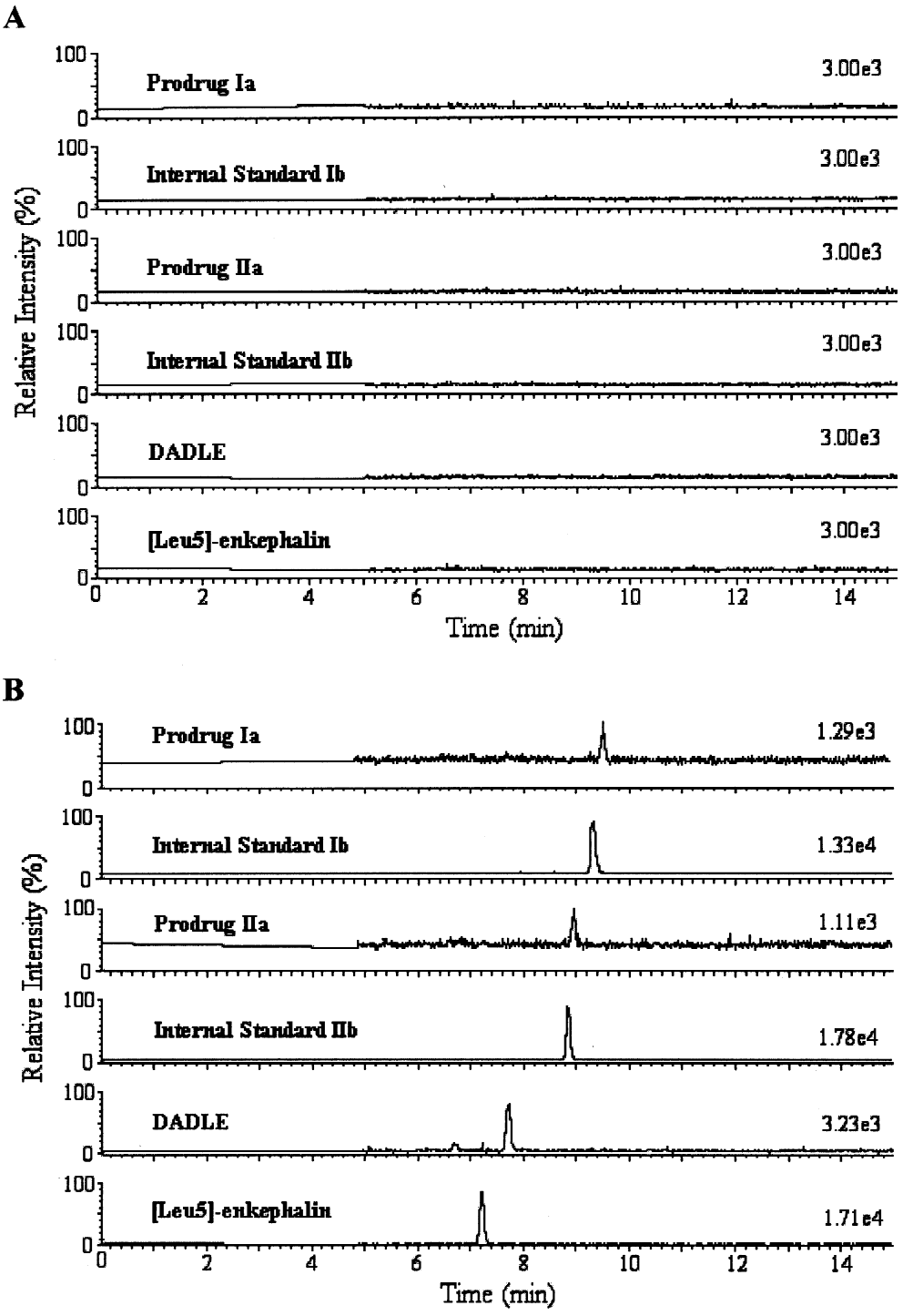


Fig. 6. Representative LC–ESI-MS–MS chromatograms of plasma samples obtained by MRM of six channels. From bottom to top, the six channels are [Leu^5]enkephalin (m/z 556.3→120.1), DADLE (m/z 570.3→120.1), internal standard **IIb** (m/z 612.3→120.1), prodrug **IIa** (m/z 626.3→120.1), internal standard **Ib** (m/z 684.3→136.1), and prodrug **Ia** (m/z 698.3→136.1). (A) Chromatograms of blank plasma sample; (B) LOQ chromatograms of plasma sample spiked with 0.5 ng ml^{-1} DADLE, 5 ng ml^{-1} prodrug **Ia**, and 2 ng ml^{-1} prodrug **IIa** along with their internal standards (10 ng ml^{-1} [Leu^5]enkephalin, 50 ng ml^{-1} internal standard **Ib**, and 50 ng ml^{-1} internal standard **IIb**). The numbers in the upper right-hand corners of the chromatograms are the peak heights expressed in arbitrary units.

Table 2
Precision and accuracy data for the determination of DADLE and its prodrugs in rat plasma using the LC–ESI-MS–MS method

Concentration (ng ml^{-1})	DADLE		Prodrug **Ia**		Prodrug **IIa**	
	Precision[a]	Accuracy[b]	Precision[a]	Accuracy[b]	Precision[a]	Accuracy[b]
0.5	14	119	N.D.	N.D.	N.D.	N.D.
2	12	94	N.D.	N.D.	18	102
5	6	97	5	122	10	100
10	15	88	5	104	6	97
40	7	98	7	83	7	103
200	19	104	8	88	7	105
1000	14	99	10	103	7	99

N.D., Not determined because it was below the limit of quantitation.
[a] Precision expressed as the RSD (%), $n=5$.
[b] Accuracy expressed as [(mean calculated concentration)/(spiked concentration)]×100.

($n=6$). Overall, the LC–ESI-MS–MS method proved to be sensitive and reliable enough for quantitative analysis of DADLE and its prodrugs in rat plasma.

3.3. Comparison of the LC–FLU method with the LC–ESI-MS–MS method

A side-by-side comparison of the two analytical methods is presented in Table 3. The LC–ESI-MS–MS method offered better sensitivity, excellent selectivity and, very importantly, simultaneous determination of DADLE and its prodrug. It did not require derivatization or prodrug conversion, and a less complicated LC system was needed. For the LC–FLU method, direct analysis of the prodrugs was not feasible due to the substrate restriction of the NDA/CN derivatization reaction; the prodrugs must be converted to DADLE for analysis. The conversion process and two-step analysis were time-consuming and led to reproducibility problems for the LC–FLU method; thus, its utilization for routine analysis of the prodrugs was limited. Overall, the LC–ESI-MS–MS method was a better choice for analysis of DADLE and its prodrugs in rat plasma than the LC–FLU method because of its sensitivity, specificity, and efficiency. However, LC–ESI-MS–MS also had its limitations. The instrument was expensive, complex, and required high technical expertise for maintenance and operation. Sample preparation was still the rate-limiting step for fast analysis due to the labor-intensive SPE procedure. The matrix effect encountered in biological samples when using LC–ESI-MS–MS could lead to precision and accuracy problems, and good internal standard selection, careful sample preparation, and efficient chromatographic separation were required. Therefore, a through validation had to be conducted to ensure the reliability and reproducibility of the LC–ESI-MS–MS method.

Table 3
Comparison between the LC–FLU and LC–ESI-MS–MS methods for the determination of DADLE in rat plasma

Parameter	LC–FLU method	LC–ESI-MS–MS method
Sensitivity (LOQ, ng ml^{-1})	6.0	0.5
Specificity	Some background interferences	No background interferences
Precision and accuracy	Within 15%	Within 20%
Plasma preparation	SPE	SPE
Derivatization	Yes	No
Chromatography (No. columns)	2	1
Run time (min)	30	20
Prodrug analysis	Indirect through conversion	Direct

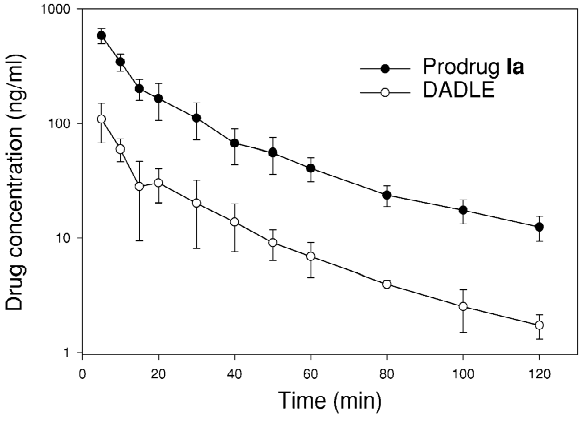


Fig. 7. Concentration–time profile of prodrug **Ia** and DADLE after intravenous administration of prodrug **Ia** (1 mg kg^{-1}) to rats ($n=3$).

3.4. Application of the LC–ESI-MS–MS method

After a 1 mg kg^{-1} intravenous dose in rats, prodrug **Ia** was shown to be eliminated rapidly following a two-phase exponential decay (Fig. 7). The prodrug had a half-life of 25 min with a relatively high clearance of 16 ml min^{-1}. At the same time, the conversion from prodrug **Ia** to DADLE was observed. The disappearance of DADLE followed the decline in prodrug **Ia** concentrations since DADLE was cleared faster than conversion from the prodrug. The LC–ESI-MS–MS method allowed simultaneous determination of DADLE and prodrug **Ia** concentrations in rat plasma, and it was sensitive enough for in vivo pharmacokinetic studies. Therefore, its application for supporting pharmacokinetic studies was demonstrated.

4. Conclusions

Quantitative analysis of DADLE and its prodrugs in rat plasma using LC–FLU and LC–ESI-MS–MS was studied. The excellent sensitivity and selectivity from tandem mass spectrometric detection simplified sample extraction procedures and chromatographic separation and produced significant improvements in comparison with the conventional method based on LC with fluorescence detection. LC–ESI-MS–MS proved to be a reliable technique for the analysis of DADLE and its prodrugs in rat plasma. Applicability to biological samples was demonstrated through disposition and conversion studies of the prodrug **Ia** in rats. In conclusion, as compared to derivatization, 2D-LC, fluorescent detection, LC–ESI-MS–MS is the bioanalytical method of choice for pharmacokinetic studies.

Acknowledgements

The authors would like to thank Oread Laboratories (Lawrence, KS, USA) for the use of the LC–ESI-MS–MS instrument and Drs. Susan Lunte and Annette Bak for their helpful discussions and comments. This research was supported by grants from the United States Public Health Service (DA09315).

References

[1] S. Gangwar, G.M. Pauletti, B. Wang, T.J. Siahaan, V.J. Stella, R.T. Borchardt, Drug Discov. Today 2 (1997) 148.
[2] S. Gangwar, G.M. Pauletti, T.J. Siahaan, V.J. Stella, R.T. Borchardt, J. Org. Chem. 62 (1997) 1356.
[3] G.M. Pauletti, S. Gangwar, F.W. Okumu, T.J. Siahaan, V.J. Stella, R.T. Borchardt, Pharm. Res. 13 (1996) 1615.
[4] B. Wang, S. Gangwar, G.M. Pauletti, T.J. Siahaan, R.T. Borchardt, J. Org. Chem. 62 (1997) 1363.
[5] G.M. Pauletti, S. Gangwar, B. Wang, R.T. Borchardt, Pharm. Res. 14 (1997) 11.
[6] O.S. Gudmundsson, G.M. Pauletti, W. Wang, D. Shan, H. Zhang, B. Wang, R.T. Borchardt, Pharm. Res. 16 (1999) 7.
[7] A. Bak, O.S. Gudmundsson, G.J. Friis, T.J. Siahaan, R.T. Borchardt, Pharm. Res. 16 (1999) 24.
[8] O.S. Gudmundsson, K. Nimkar, S. Gangwar, T. Siahaan, R.T. Borchardt, Pharm. Res. 16 (1999) 16.
[9] F. Nyberg, C. Pernow, U. Moberg, R.B. Eriksson, J. Chromatogr. 359 (1986) 541.
[10] V. Pacakova, J. Suchankova, K. Stulik, J. Chromatogr. B 681 (1996) 69.
[11] V.P. Ranta, K.M. Hamalainen, S. Auriola, A. Urtti, J. Chromatogr. B 709 (1998) 1.
[12] S. Shibanoki, S.B. Weinberger, K. Ishikawa, J.L. Martinez, J. Chromatogr. 532 (1990) 249.
[13] L.S. Monger, C.J. Olliff, J. Chromatogr. 577 (1992) 239.
[14] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[15] M. Mifune, D.K. Krehbiel, J.F. Stobaugh, C.M. Riley, J. Chromatogr. 496 (1989) 55.

[16] P. de Montigny, C.M. Riley, L.A. Sternson, J.F. Stobaugh, J. Pharm. Biomed. Anal. 8 (1990) 419.
[17] M. Kai, A. Nakashima, Y. Ohkura, J. Chromatogr. B 688 (1997) 205.
[18] H. Kanazawa, T. Nagatsuka, M. Miyazaki, Y. Matsushima, J. Chromatogr. A 763 (1997) 23.
[19] D. Shangguan, Y. Zhao, H. Han, R. Zhao, G. Liu, Anal. Chem. 73 (2001) 2054.
[20] J.L. Tseng, L. Yan, G.H. Fridland, D.M. Desiderio, Rapid Commun. Mass Spectrom. 9 (1995) 264.
[21] C.D. Marquez, S.T. Weintraub, P.C. Smith, J. Chromatogr. B 694 (1997) 21.
[22] J. Zagrobelny, C. Chavez, M.L. Constanzer, B.K. Matuszewski, J. Pharm. Biomed. Anal. 15 (1997) 1427.
[23] C.M. Chavez-Eng, M. Schwartz, M.L. Constanzer, B.K. Matuszewski, J. Chromatogr. B 721 (1999) 229.
[24] J. Peng, S.P. Gygi, J. Mass Spectrom. 36 (2001) 1083.
[25] H. Zhang, J. Henion, J. Chromatogr. B 757 (2001) 151.
[26] T.K. Majumdar, S. Wu, F.L. Tse, J. Chromatogr. B 759 (2001) 99.
[27] Y. Hsieh, M.S. Bryant, J.M. Brisson, K. Ng, W.A. Korfmacher, J. Chromatogr. B 767 (2002) 353.
[28] B. Wang, K. Nimkar, W. Wang, H. Zhang, D. Shan, O. Gudmundsson, S. Gangwar, T. Siahaan, R.T. Borchardt, J. Pept. Res. 53 (1999) 370.
[29] A. Bak, T.J. Siahaan, O.S. Gudmundsson, S. Gangwar, G.J. Friis, R.T. Borchardt, J. Pept. Res. 53 (1999) 393.
[30] P. Eichhorn, T.P. Knepper, J. Mass Spectrom. 36 (2001) 677.
[31] J. Yamaguchi, Y. Matsuno, K. Hachiuma, N. Ogawa, S. Higuchi, Rapid Commun. Mass Spectrom. 15 (2001) 629.
[32] D.A. Volmer, C.M. Lock, Rapid Commun. Mass Spectrom. 12 (1998) 157.
[33] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.