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Quantitative analysis of two opioid peptides in plasma by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

Quantitative analysis of two opioid peptides, DSLET [(D-Ser^2)Leu-enkephalin-Thr^6] and Met-enkephalin-Arg-Gly-Leu, was performed using microbore liquid chromatography interfaced to electrospray ionization tandem mass spectrometry. Validation of the methodology was demonstrated for each peptide in plasma. Quantitative analyses were performed through the use of a deuterium labelled peptide analog as an internal standard. Linearity was observed for the analysis of DSLET (5–1000 ng/ml) and Met-enkephalin-Arg-Gly-Leu (1–1000 ng/ml) in plasma with a limit of detection of 0.25 ng/ml for Met-enkephalin-Arg-Gly-Leu and 1.0 ng/ml for DSLET. In general, the observed concentrations showed good reproducibility with coefficients of variation of within 15%. In the concentration range studied, only 0.5 ml of plasma was required for optimal detection of Met-enkephalin-Arg-Gly-Leu and 0.25 ml for DSLET. Application of this method was demonstrated by studying the disposition of DSLET in a rat. DSLET administered to a rat exhibited a short half-life and a high clearance value.

Keywords: Opioid peptides; (D-Ser^2)Leu-enkephalin-Thr^6; Met-enkephalin-Arg-Gly-Leu

1. Introduction

Few analytical methods are presently available for pharmacokinetic analysis of peptides in biological fluids at low therapeutic concentrations. Methods currently used to quantitate peptides include immunoassays, bioassays, or derivatization prior to HPLC analysis. These methods are often compromised by significant background interferences or cross-reactivity with related peptides which confounds the quantitation of peptides at pmol levels. As a result, there is considerable controversy in the literature with regard to the results obtained through various analytical methods employed. Therefore, improved analytical methods which can provide high sensitivity and selectivity are needed for quantitative analysis of peptides in plasma at pmol levels.

On-line combination of liquid chromatography (LC) and mass spectrometry (MS) has been demonstrated to be a powerful method for both quantitative and qualitative analysis of peptides. In particular, the use of LC interfaced to tandem mass spectrometry (MS–MS) has shown excellent sensitivity and specificity for rapid analysis of complex mixtures [1]. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are currently the

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most widely used ionization techniques for quantitation of peptides in biological fluids. MS–MS expands the information available through these techniques, in that once charged species are generated, further fragmentation can be induced to yield structurally informative product ions.

The use of micro-LC columns has attracted a great deal of attention in pharmaceutical research [2]. Advantages to using micro-LC columns include enhanced detection limits, higher separation efficiency, improved speed of analysis and less solvent consumption. Straub et al. [2] characterized a mixture of β-lactam antibiotics in bovine milk using a micro-LC column packed with derivatized porous polystyrene coupled to ESI–MS. By virtue of its ability to accommodate flow-rates of 40 μl/min and thus, short retention times, this column was particularly well suited for rapid separation times and ESI–MS. In other studies, Fouda et al. [3] interfaced LC to APCI–MS at flow-rates of 1 ml/min through the use of a heated nebulizer for the quantitative measurements of a renin inhibitor, CP-80794. Detection levels of 50 pg/ml of serum were obtained.

Currently, quantitative analysis of peptides utilizing micro- or standard bore columns in conjunction with LC–MS [2–4] or LC–MS–MS [1] have generated a great deal of interest due to the speed of analysis, selectivity and sensitivity obtained. The work presented here describes the quantitative analysis of two opioid peptides, DSLET [(D-Ser^2)-Leu-enkephalin-Thr^6], a synthetic hexapeptide, and Met-enkephalin-Arg-Gly-Leu, an endogenous octapeptide. DSLET has been shown to enhance feeding in rats, increase dopaminergic activity in the striatum, and decrease dopaminergic activity in the hypothalamus [5] while Met-enkephalin-Arg-Gly-Leu tends to modulate stress, analgesia, eating, reproductive activity, mental disturbances and seizures [6]. As with most studies involving opioid peptides, the published literature on DSLET [5] and Met-enkephalin-Arg-Gly-Leu [6] primarily emphasizes their tissue distribution and various pharmacological effects. Few studies have been done which describe the pharmacokinetic behavior of either DSLET or Met-enkephalin-Arg-Gly-Leu. Therefore in this work, DSLET and Met-enkephalin-Arg-Gly-Leu are evaluated in plasma using micro-LC columns interfaced to ESI–MS–MS. Applications of this method are demonstrated to study the pharmacokinetics of DSLET in rats. This method shows great promise in the detection of peptides in biological fluids at levels in the low pmol/ml range.

2. Experimental

2.1. Materials

The following peptides were synthesized at the University of North Carolina at Chapel Hill using an ABI peptide synthesizer with FMOC technology: Met-enkephalin-Arg-(Leu-d_{10})-Gly [Tyr-Gly-Gly-Phe-Met-Arg-(Leu-d_{10})-Gly] and DSLET-d_3 [Tyr-D-Ser-Gly-Phe-(Leu-d_3)-Thr]. Unlabelled and tritiated DSLET were obtained from NIDA (Baltimore, MD, USA) while unlabelled Met-enkephalin-Arg-Gly-Leu was purchased from Sigma (St. Louis, MO, USA). All solvents employed were HPLC or GC grade purity.

2.2. Instrumentation

2.2.1. Mass spectrometry

Electrospray ionization mass spectra were acquired on a Perkin Elmer Sciex API III mass spectrometer (Ontario, Canada) fitted with an ionspray interface. The MS–MS conditions were: ionspray voltage, 5000 V; orifice voltage, 60 V; collision energy, −20 V; collision gas thickness, 15 Pa. A poly(propylene glycol) mixture was utilized for instrument calibration.

2.2.2. HPLC

Chromatographic separations were carried out on an Applied Biosystems Model 140B solvent delivery system (Foster City, CA, USA) in conjunction with a DBSC C_{18} column (50×1 mm I.D., 5 μm particle size) obtained from Keystone Scientific (Bellefonte, PA) and an Applied Biosystems C_{18} guard column (20×1 mm I.D.). A flow-rate of 50 μl/min was employed. The mobile phase utilized for the separation of both Met-enkephalin-Arg-Gly-Leu and DSLET was A: 2 m*M* ammonium acetate, pH 4.4 and B: 60% acetonitrile. The gradient was from 20 to 80% B in 10 min and then remained at 80% B for 2 min. The analytical column was interfaced to the

Sciex API III mass spectrometer via a Valco switching valve and a fused-silica capillary tubing. A continuous flow of acetonitrile–2 m*M* ammonium acetate, pH 4.4 (1:1) was maintained through the capillary tubing at 10 μl/min by means of a low pressure pump. This replacement flow of solvent was important to maintain the stability of the electrospray ion current. For the first 5 min of the analysis, the LC effluent was directed to waste and thereafter sent to the mass spectrometer.

2.3. Stability in plasma

Stability studies were performed in vitro by incubating either Met-enkephalin-Arg-Gly-Leu, Leu-enkephalin, or DSLET (100 μg/ml) in 25% plasma at 37°C with the remaining 75% composed of RPMI Medium 1640. Details of the experimental design are presented in previous studies by Márquez et al. [7].

2.4. Sample preparation

The C_{18} solid-phase extraction procedure used in this investigation is described in Márquez et al. [7]. Briefly, standard samples of Met-enkephalin-Arg-Gly-Leu and DSLET (0.1–1000 ng of peptide/ml of plasma) were separately prepared in 0.5 ml of human plasma for Met-enkephalin-Arg-Gly-Leu and 0.25 ml for DSLET. The deuterated internal standards were separately added at concentrations of 50 ng/ml for Met-enkephalin-Arg-(Leu-d_{10})-Gly and 10 ng/ml for DSLET-d_3. After application to a C_{18} solid-phase extraction column, the peptide fraction was eluted with 80% acetonitrile–0.1% trifluoroacetic acid. The solvent was evaporated using a stream of nitrogen at 25°C and the residue reconstituted in 25 μl of 2 m*M* ammonium acetate, pH 4.4.

2.5. Analysis by LC–ESI–MS–MS

Aliquots of 5–10 μl were employed for the analysis by LC–ESI–MS–MS. Peak-area measurements were obtained for selected reaction monitoring of the following transitions: m/z 450.5→136 for Met-enkephalin-Arg-Gly-Leu-d_0; m/z 455.6→136 for Met-enkephalin-Arg-(Leu-d_{10})-Gly; m/z 687.0→540 for DSLET-d_0; m/z 690.0→543 for DSLET-d_3. Linearity was assessed by means of either a weighted fit of $1/x$ or $1/x^2$ as well as a non-weighted fit. More detailed information about the fragmentation of the peptides will be discussed below.

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

The disposition of DSLET was examined in rats treated intravenously with 10 mg/kg. Arterial blood samples (0.5 ml) were collected at 0, 1, 2, 3, 4, 5, 10, 15 and 30 min after injection. Peptides were isolated from plasma by C_{18} solid-phase extraction in a similar manner to the standard samples as described by Márquez et al. [7], and analyzed by LC–ESI–MS–MS.

3. Results and discussion

The potent pharmacological activity of Met-enkephalin-Arg-Gly-Leu or DSLET necessitates that methods be available for quantitative determination at low pmol per ml levels. Low concentrations of Met-enkephalin-Arg-Gly-Leu and DSLET in plasma are a result of their short in vitro plasma stability half-lives of 10 and 120 min, respectively [7]. Since these peptides are relatively short lived in biological fluids, it is essential that sample processing be optimized and that methods used to characterize them be highly selective and provide low detection levels. The extraction efficiency from plasma was 65% for Met-enkephalin-Arg-Gly-Leu-d_0, 90% for Met-enkephalin-Arg-(Leu-d_{10})-Gly and 85% for [^{3}H]DSLET [7]. The internal standard (Met-enkephalin-Arg-(Leu-d_{10})-Gly) differs from the parent peptide by reversing the terminal amino acid residues Leu and Gly. However, there were no marked differences observed in the HPLC retention times.

The most effective mobile phase for interfacing LC to ESI–MS–MS is one that has a low ionic strength and can maintain a pH range of 4 to 5. Several mobile phases were examined in this investigation, including acetonitrile–trifluoroacetic acid (1, 0.1, or 0.05%, pH range of 2–4), as well as varied concentrations of acetonitrile–2 m*M* ammonium acetate, pH 4.4. Of these, 60% acetonitrile–2 m*M* ammonium acetate, pH 4.4 was selected since it yielded the best LC chromatographic peak shape for

both DSLET and Met-enkephalin-Arg-Gly-Leu in plasma and generated intense protonated species. In preliminary studies, DSLET was analyzed using LC–ESI–MS by selectively monitoring $[M+H]^{1+}$. However, high background interferences from the plasma matrix hampered quantification. Therefore, LC–ESI–MS–MS was utilized for analysis of both DSLET and Met-enkephalin-Arg-Gly-Leu in order to minimize these interferences.

3.1. DSLET analysis

Electrospray ionization of DSLET was found to produce singly charged species of $[M+H]^{1+}$ (m/z 687 for the unlabelled peptide and m/z 690 for DSLET-d_3). In Fig. 1 and Fig. 2, product ion scans are shown for both DSLET-d_0 and DSLET-d_3 dissolved in acetonitrile–2 m*M* ammonium acetate, pH 4.4 (1:1). The product ion chosen for selected reaction monitoring (SRM) was m/z 540 for DSLET-d_0 and m/z 543 for DSLET-d_3 because it was sufficiently abundant and would contain the deuterium labelled isotope for the internal standard. The MS–MS conditions were therefore optimized for detection of these product ions. It was necessary to decrease the mass window in the first quadrupole in order to minimize mass interferences for DSLET-d_0 and DSLET-d_3. It is important to note that when a stable isotope is 3 mass units higher than the analyte a narrower mass window can be utilized to minimize mass interferences. This is particularly important for compounds that co-elute with the internal standard.

Quantitation of DSLET in mobile phase and in human plasma was accomplished by SRM of the

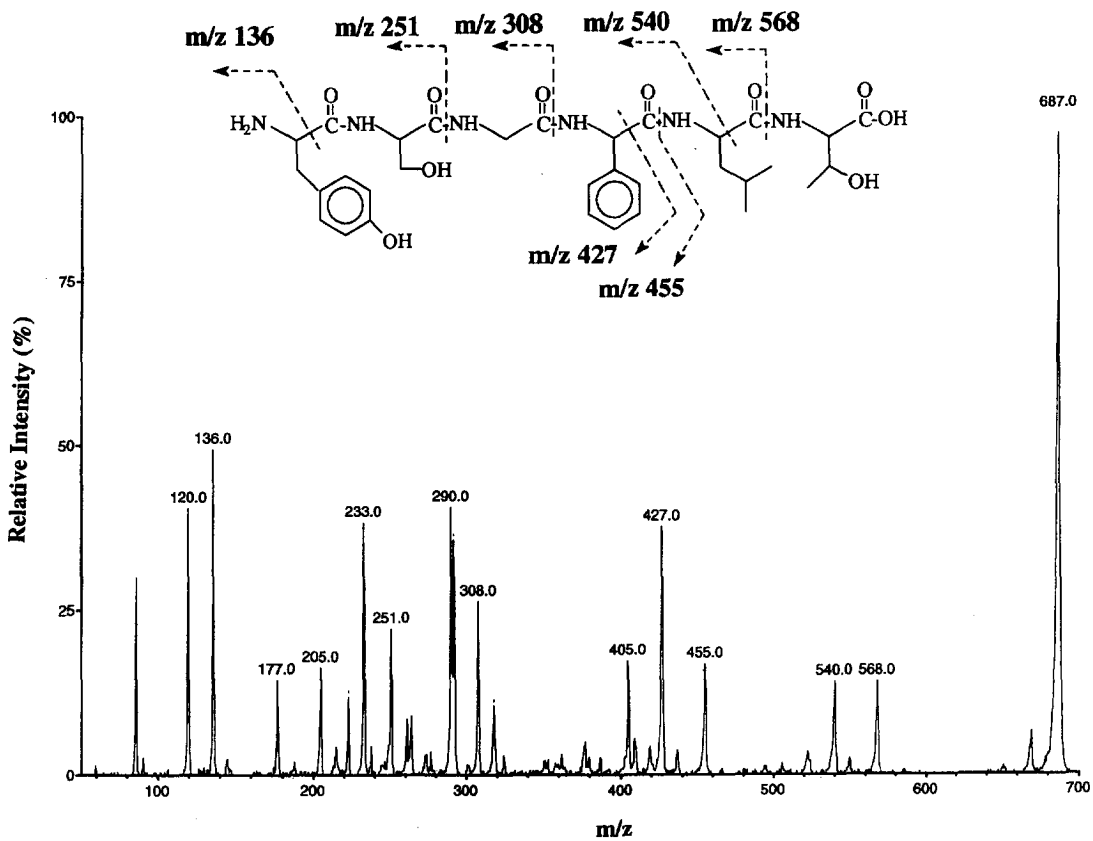

Fig. 1. Product ion spectrum of m/z 687.0 ($[M+H]^{1+}$ for DSLET-d_0) generated by electrospray ionization. The sample was dissolved at a concentration of 10 ng/μl in acetonitrile–2 m*M* ammonium acetate (1:1), pH 4.4 and infused into the mass spectrometer at a flow-rate of 10 μl/min. The spectrum represents the average of 25 scans.

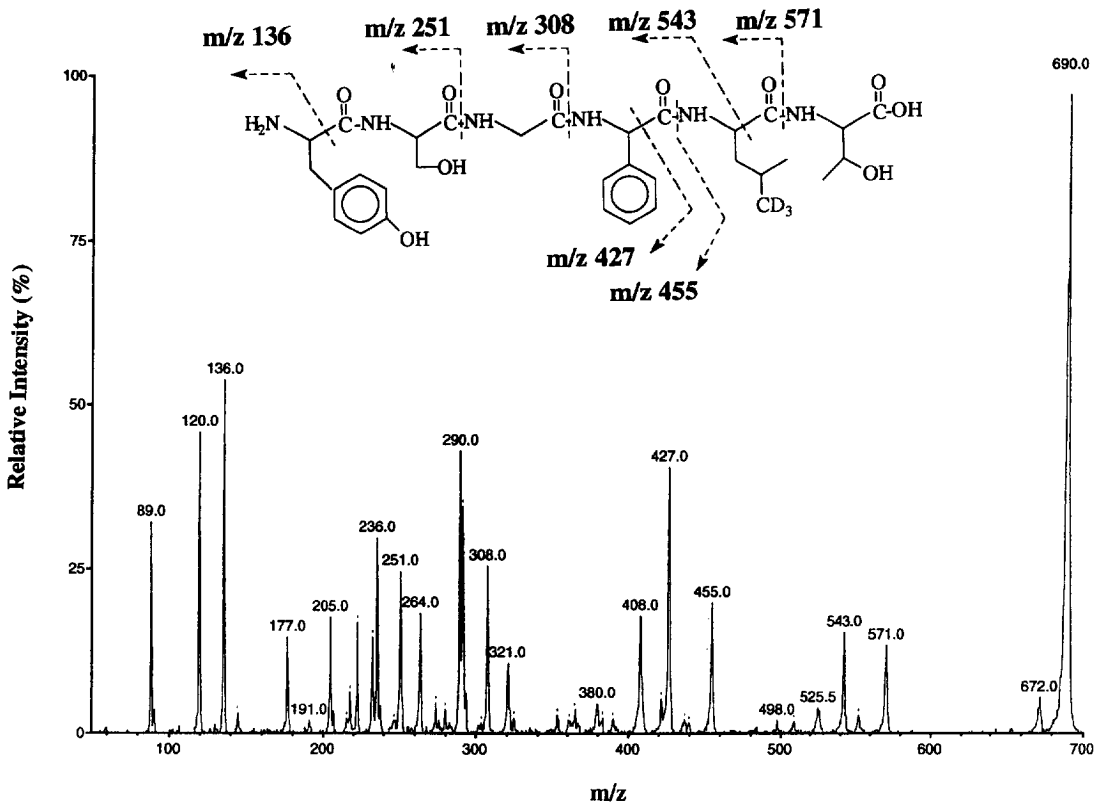

Fig. 2. Product ion spectrum of m/z 690.0 ($[M+H]^{1+}$ for DSLET-d_3) generated by electrospray ionization. The sample was dissolved at a concentration of 10 ng/μl in acetonitrile–2 m*M* ammonium acetate (1:1), pH 4.4 and infused into the mass spectrometer at a flow-rate of 10 μl/min. The spectrum represents the average of 25 scans.

following transitions, m/z 687.0→540 for DSLET-d_0 and m/z 690.0→543 for DSLET-d_3. Linearity was observed for DSLET in mobile phase using two standard curves from 0.5–100 ng/ml and from 1–1000 ng/ml with correlation coefficients greater than 0.99. In some instances in quantitative analysis by ESI–MS, it is necessary to use multiple standard curves to cover the concentration range of interest [3]. In Fig. 3, SRM profiles are shown for DSLET-d_0 (10 ng/ml) and DSLET-d_3 (10 ng/ml) in plasma. Standard curves for DSLET in plasma showed linearity from 5–1000 ng/ml. The data were fitted using $1/x$ weighting and resulted in a correlation coefficient of 0.998. In Table 1 are the results of three separate analyses. The results showed that acceptable coefficients of variation and percentage nominal difference (% ND) were obtained for most of the concentrations measured. The accuracy of the concentrations measured was deemed acceptable at 20% of their nominal concentration. The limit of quantitation (LOQ) and limit of detection (LOD) for DSLET were determined at 5 and 1 ng/ml of plasma.

3.2. Met-enkephalin-Arg-Gly-Leu analysis

Electrospray ionization of Met-enkephalin-Arg-Gly-Leu was found to produce singly and doubly charged species of $[M+H]^{1+}$ and $[M+2H]^{2+}$ [m/z 900.5 and m/z 450.5 for Met-enkephalin-Arg-Gly-Leu-d_0 as well as m/z 910.6 and m/z 455.6 for Met-enkephalin-Arg-(Leu-d_{10})-Gly]. The MS conditions were optimized to obtain maximum abundance of the $[M+2H]^{2+}$ ions in the first quadrupole. In Fig. 4 and Fig. 5, product ion scans for Met-enkephalin-Arg-Gly-Leu-d_0 and Met-enkephalin-

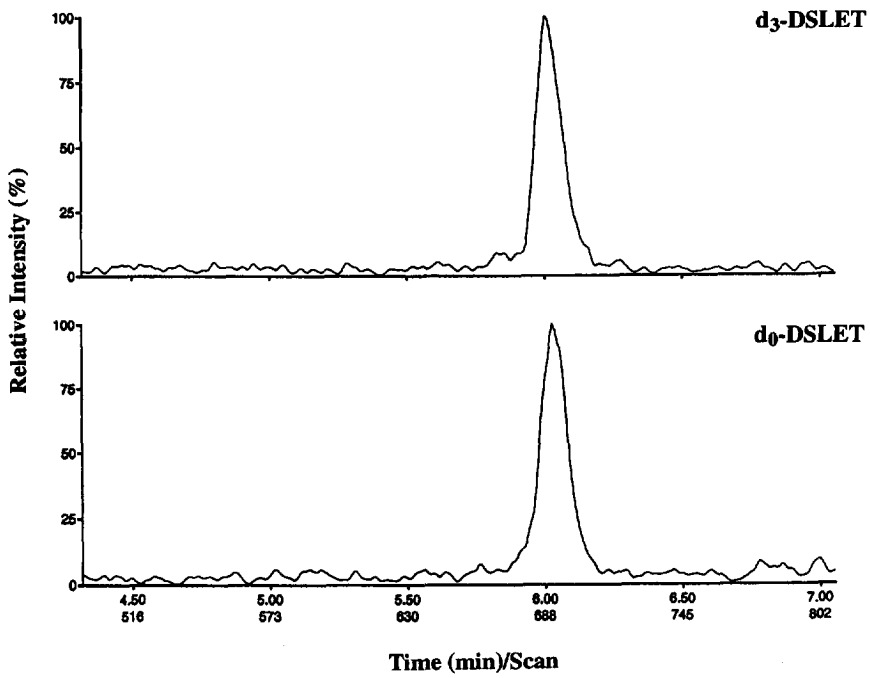

Fig. 3. Reconstructed ion chromatogram for selected reaction monitoring of DSLET-d_0 (10 ng/ml) and DSLET-d_3 (10 ng/ml) extracted from plasma and measured by LC–ESI–MS–MS. HPLC conditions are described above.

Arg-(Leu-d_{10})-Gly are shown. The product ion selected for further optimization was m/z 136 for both Met-enkephalin-Arg-Gly-Leu-d_0 and Met-enkephalin-Arg-(Leu-d_{10})-Gly. Unlike the product ions generated from DSLET-d_3, Met-enkephalin-Arg-(Leu-d_{10})-Gly did not yield fragments that included the deuterium labelled isotope. However, in MS–MS analyses it is not essential that the product fragments contain the stable isotope because initial mass separation in the first quadrupole is able to resolve the precursor ions. Also, since there is a 5 mass unit difference between the doubly charged precursor ions of Met-enkephalin-Arg-Gly-Leu-d_0 and Met-enkephalin-Arg-(Leu-d_{10})-Gly, SRM studies were done using a wider mass window with no cross contamination from one mass channel to the other.

Table 1
Regression analysis of LC–ESI–MS–MS quantification of DSLET

Concentration added (ng/ml)	Concentration observed[a] (ng/ml)		
	Day 1	Day 2	Day 3
5.0	5.2±0.7 (13.4/104)	4.7±0.1 (1.2/94)	5.0±2.1 (41.4/101)
10.0	8.7±1.0 (11.5/87.3)	8.8±3.0 (33.5/88)	9.8±2.7 (27.1/98)
50.0	51.4±1.8 (3.5/103)	50.5±1.4 (2.7/100)	51.9±6.2 (12.0/104)
100.0	102.9±4.9 (4.8/103)	95.8±2.2 (2.3/96)	93.5±11.8 (12.7/94)
500.0	529.0±40.6 (7.7/106)	525.4±38.9 (7.4/105)	526.2±21.2 (4.0/105)
1000.0	967.6±49.9 (5.2/97)	977.2±39.5 (4.0/98)	946.8±12.4 (1.3/98)

[a] The values shown represent the mean±S.D. for 2 determinations with the percent coefficient of variation/accuracy given in parenthesis. Analyses were performed using $1/x$ weighted fit. Selected reaction monitoring of the following transitions: m/z 687→540, DSLET-d_0; m/z 690→543, DSLET-d_3.

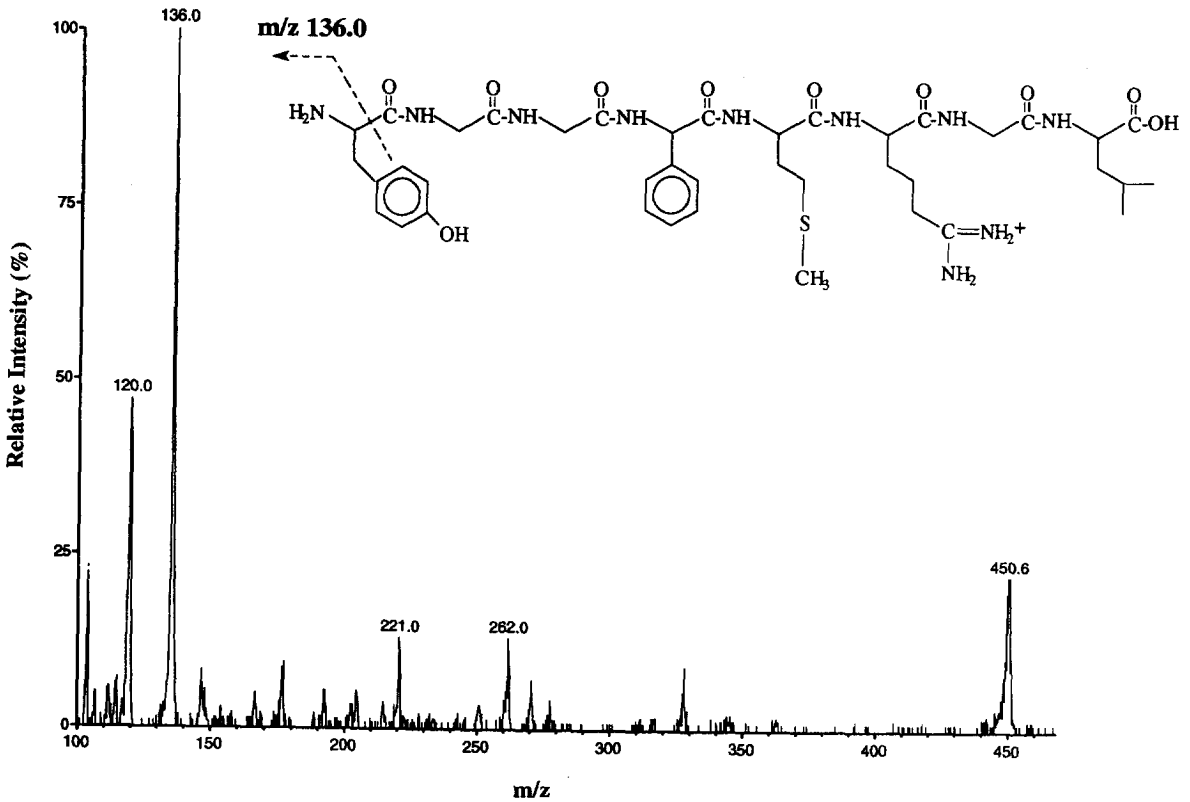

Fig. 4. Product ion spectrum of m/z 450.6 ($[M+2H]^{2+}$ for Met-enkephalin-Arg-Gly-Leu-d_0) generated by electrospray ionization. The sample was dissolved at a concentration of 10 ng/μl in acetonitrile–2 m*M* ammonium acetate (1:1), pH 4.4 and infused into the mass spectrometer at a flow-rate of 10 μl/min. The spectrum represents the average of 25 scans.

Using a wider mass window is important because it contributes to an increased detection of the analyte. No measurable difference in the fragmentation pattern by ESI–MS–MS was observed for Met-enkephalin-Arg(Leu-d_{10})-Gly as compared to the unlabelled peptide, despite the fact that there is a slight difference in sequence. Furthermore, it was possible to utilize a single set of conditions and still obtain optimal detection of product ions for both Met-enkephalin-Arg-Gly-Leu-d_0 and Met-enkephalin-Arg-(Leu-d_{10})-Gly; this is of particular importance for quantitative analysis when using SRM.

Quantification of unlabelled Met-enkephalin-Arg-Gly-Leu in mobile phase and in human plasma was accomplished using SRM of the following transitions, m/z 450.5→136 for Met-enkephalin-Arg-Gly-Leu-d_0 and m/z 455.6→136 for Met-enkephalin-Arg-(Leu-d_{10})-Gly. Linearity was observed for Met-enkephalin-Arg-Gly-Leu in mobile phase from 1–1000 ng/ml with a correlation coefficient greater than 0.99. Unlike the quantitative analysis of DSLET in mobile phase, the standard curves for Met-enkephalin-Arg-Gly-Leu concentrations in mobile phase were linear across the entire range. The data which were fitted using $1/x$ weighting resulted in a correlation coefficient greater than 0.994. In Fig. 6, SRM profiles can be seen for LC–ESI–MS–MS analysis of unlabelled Met-enkephalin-Arg-Gly-Leu (10 ng/ml) and Met-enkephalin-Arg-(Leu-d_{10})-Gly (50 ng/ml) in plasma. Good chromatographic peak shape was observed for both Met-enkephalin-Arg-Gly-Leu-d_0 and its internal standard. Linearity was observed from 1–1000 ng/ml of Met-enkephalin-Arg-Gly-Leu in plasma, with a LOD of 0.25 ng/ml and a LOQ at 1 ng/ml of plasma. The results for any of the three separate analyses can be seen in Table 2.

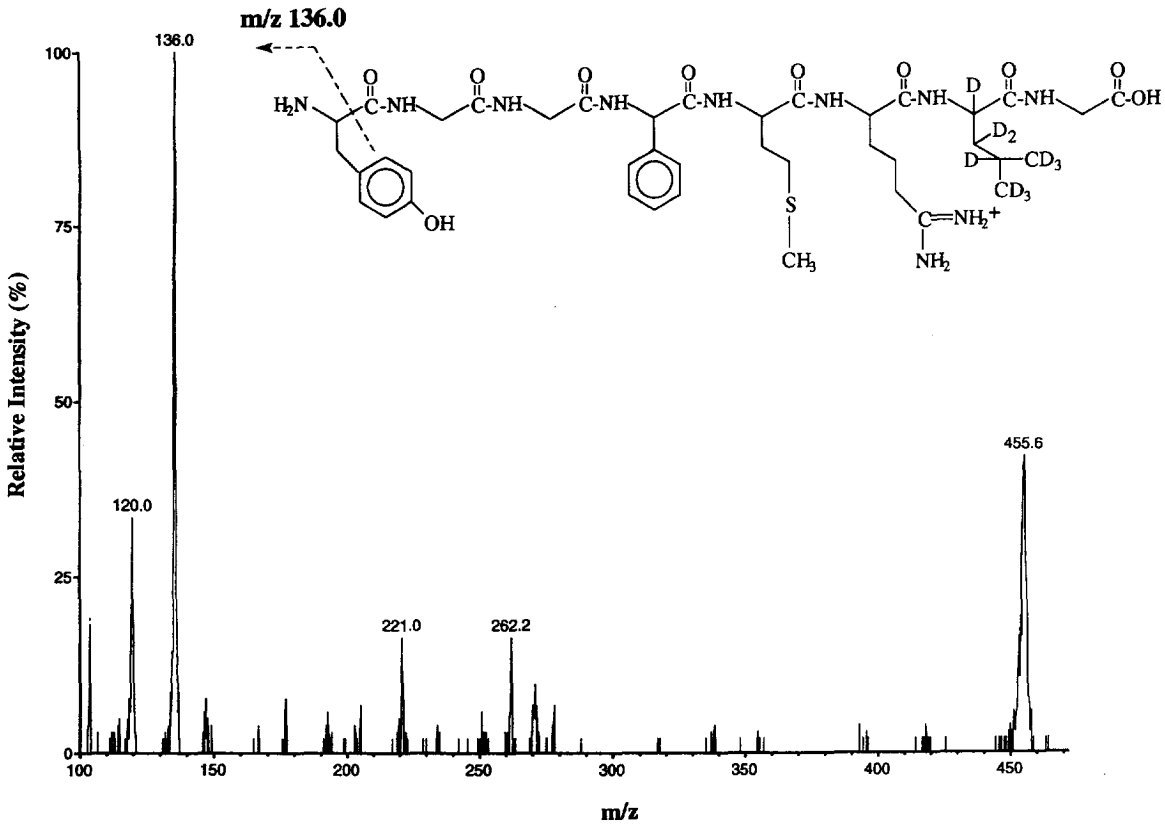

Fig. 5. Product ion spectrum of m/z 455.6 ($[M+2H]^{2+}$ for Met-enkephalin-Arg-(Leu-d_{10})-Gly) generated by electrospray ionization. The sample was dissolved at a concentration of 10 ng/μl in acetonitrile–2 m*M* ammonium acetate (1:1), pH 4.4 and infused into the mass spectrometer at a flow-rate of 10 μl/min. The spectrum represents the average of 25 scans.

Acceptable C.V. values and % ND below 20% were obtained for most samples.

3.3. Application of the LC–ESI–MS–MS method

Following a 10 mg/kg intravenous dose, the peak concentration achieved for DSLET was 793 ng/ml of plasma. DSLET was rapidly eliminated with a half-life of only 4 min and the apparent clearance of 1400 ml/min/kg which exceeded possible organ blood flow for the rat, suggesting extra-organ clearance mechanism. A representative concentration–time profile of DSLET is shown in Fig. 7. In previous published studies on DSLET [5], the pharmacokinetic parameters were not addressed since few methods exist which can detect picomole levels of DSLET in plasma. These studies used radioimmunoassays or bioassays for measurement and did not include validation of the analytical methods employed [5].

4. Conclusions

Methods for the quantitative analysis of DSLET and Met-enkephalin-Arg-Gly-Leu were developed using C_{18} solid-phase extraction followed by micro-LC separation and ESI–MS–MS analysis. Linearity was routinely observed in plasma over the range of 1–1000 ng/ml for Met-enkephalin-Arg-Gly-Leu and 5–1000 ng/ml for DSLET. In most instances, the C.V. for replicate samples was less than 15%, although an occasional lack of reproducibility was observed. This method permitted the detection of

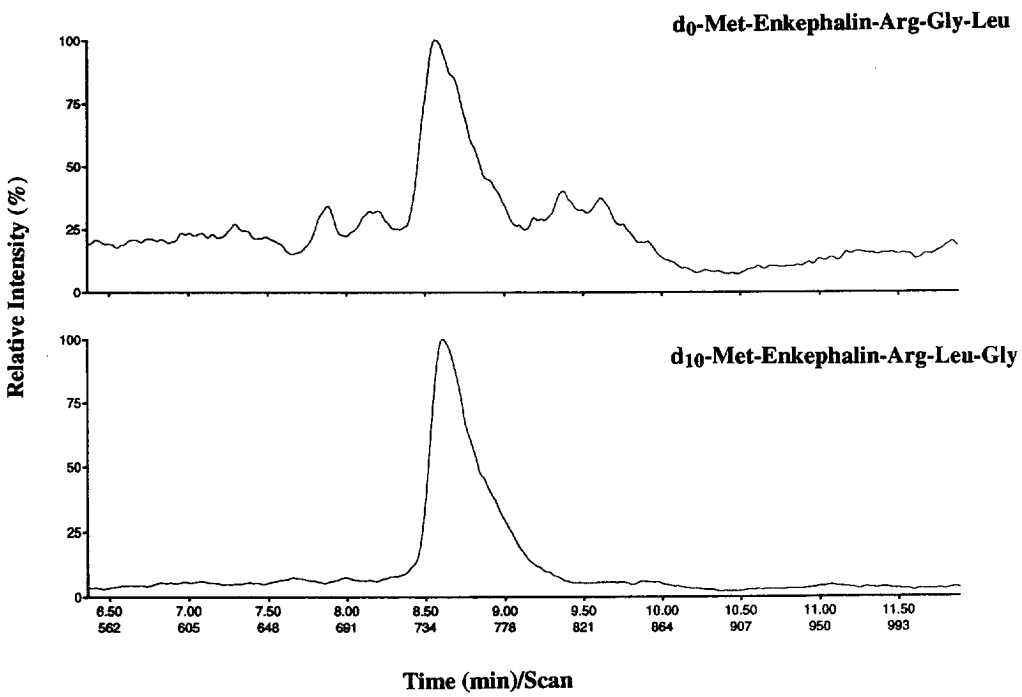

Fig. 6. Reconstructed ion chromatogram for selected reaction monitoring of Met-enkephalin-Arg-Gly-Leu-d_0 (10 ng/ml) and Met-enkephalin-Arg-(Leu-d_{10})-Gly (50 ng/ml) extracted from plasma and measured by LC–ESI–MS–MS. HPLC conditions are described above.

DSLET and Met-enkephalin-Arg-Gly-Leu using only 0.25–0.5 ml of plasma. A 5-fold lower LOQ was observed for Met-enkephalin-Arg-Gly-Leu as compared to DSLET. Although in some cases quantitative analysis by LC–ESI–MS–MS exhibits problems associated with a limited linear dynamic range [3] such that more than one standard curve is needed to describe the data, the results shown here document

Table 2
Regression analysis of LC–ESI–MS–MS quantification of Met-enkephalin-Arg-Gly-Leu

Concentration added (ng/ml)	Concentration observed[a] (ng/ml)		
	Day 1	Day 2	Day 3
1.0	0.9±0.01 (1.4/93)	n.a.	0.4±0.1 (32.3/40)
5.0	n.a.[b]	5.6±1.2 (21.0/112)	3.5±0.1 (4.0/70)
10.0	11.0±0.3 (2.2/109)	9.5±1.5 (15.8/95)	10.3±0.7 (6.7/65)
50.0	48.4±3.6 (7.4/97)	39.3±3.3 (8.4/78)	53.3±0.8 (1.4/107)
100.0	100.8±1.4 (1.4/101)	116.1±1.6 (1.4/116)	97.9±12.3 (12.5/98)
500.0	n.a.	488.8±42/7 8.7/98)	n.a.
1000.0	n.a.	1005.9±41.5 (4.1/100)	n.a.

[a] The values shown represent the mean±S.D. for 2 determinations with the percent coefficient of variation/accuracy given in parenthesis. Analyses were performed using $1/x$ weighted fit. Selected reaction monitoring of the following transitions: m/z 455.5→136, Met-enkephalin-R(L-d_{10})G; m/z 450.5→136, Met-enkephalin-RGL-d_0.

[b] n.a. – not available; only individual analyses were performed for these samples.

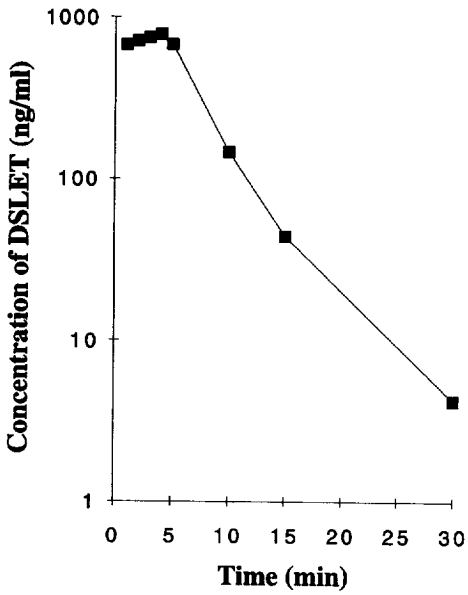

Fig. 7. Pharmacokinetic concentration time profile of DSLET administered intravenously (10 mg/kg) to a rat.

that excellent selectivity and sensitivity can be obtained for detecting peptides in plasma. Application of this method showed that, as expected, DSLET administered to rats exhibited a short half-life and a high clearance value.

Acknowledgments

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