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Quantitative analysis of exogenous peptides in plasma using immobilized enzyme cleavage and gas chromatography–mass spectrometry with negative ion chemical ionization

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Abstract

A method is presented for the analysis of peptides in plasma at picomole to femtomole levels. Peptides are isolated from plasma by solid-phase extraction, the peptide of interest is purified by reversed-phase high-performance liquid chromatography (HPLC) and selectively digested using immobilized trypsin or chymotrypsin to yield specific di- or tripeptides. These di- and tripeptides are esterified using heptafluorobutyric anhydride, alkylated with pentafluorobenzyl bromide, then quantified by gas chromatography–mass spectrometry with negative ion chemical ionization. This method has been evaluated for a model synthetic heptapeptide, using a deuterium labeled analog as an internal standard. The half-life of the heptapeptide in human plasma was found to be 2 min. Extraction efficiencies of a tritiated peptide of similar size to the heptapeptide, [^{3}H]DSLET, from plasma using either C_{18} or strong cation-exchange columns were 85±3 and 70±2%, respectively. Quantitation of fragments from the heptapeptide indicated that the analysis was linear from 1–50 ng of the heptapeptide per ml of plasma. This method was subsequently employed for pharmacokinetic studies of the biologically active peptide Met–enkephalin–Arg–Gly–Leu, where linearity was obtained from 50 to 1000 ng/ml in rat plasma. This method demonstrated negligible side reaction by-products due to autolysis, and has potential for extensive use given the wide availability of gas chromatography–mass spectrometry.

Keywords: Peptides; Enzymes

1. Introduction

Many peptides such as the opioid peptides are extremely potent and are present in biological fluids at low concentrations. Therefore, highly sensitive and specific analytical methods are essential for pharmacokinetic and disposition studies of exogenously administered peptides. A variety of mass spectrometric techniques have shown great promise for analysis of peptides in a biological matrix; they include gas chromatography–mass spectrometry (GC–MS) with negative ion chemical ionization (NICI) [1,2] or electron ionization [3] as well as liquid chromatography–mass spectrometry (LC–MS) in conjunction with either electrospray ionization [4], thermospray (TS) [5,6] or atmospheric pressure chemical ionization (APCI) [7].

Peptides analyzed by GC–MS must be cleaved

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into smaller fragments of no more than two or three amino acid residues. In the early 1970s, Caprioli et al. [3] designed a method for peptide mapping in which polypeptides were enzymatically hydrolyzed to dipeptides by dipeptidylaminopeptidase (DAP) and then derivatized with pentafluoropropionic acid anhydride (PFPA) for GC–MS analysis. The amino acid sequence was deduced by overlapping data sets from the DAP-digested peptides and from Edman degradation techniques. However, a limitation of this technique was that 50–100 nmol of sample was required. Liberato et al. [1] employed a derivatization method obtained by Hayashi et al. [2] to quantify the peptide GHR factor in plasma by GC–MS with NICI following total hydrolysis and derivatization with pentafluorobenzylbromide (PFBBr) and heptafluorobutyric anhydride (HFBA). Liberato et al. [1] examined the liberation of Phe from GHR factor; however, they were unable to obtain adequate detection of Phe, presumably due to high background interferences generated from the total hydrolysis of contaminating peptides. Cobb and Novotny [8] evaluated the use of immobilized enzymes to reduce interference peaks caused by autolysis of peptides such as trypsin. They demonstrated that immobilized enzymes like trypsin could be employed where only picomole levels of peptides available to obtain peptide-digested fragments.

In previous studies, on-line procedures for peptide analysis have been performed using immobilized enzyme columns coupled to liquid chromatography (LC) followed by thermospray-mass spectrometry (TS-MS) [5,6]. Kim et al. [5] demonstrated this technique, using on-line immobilized carboxypeptidase Y and trypsin to obtain useful peptide sequencing information at the low picomole range. In 1990, Voyksner et al. [6] demonstrated this technique using on-line immobilized chymotrypsin, thermolysin and V_8 protease in conjunction with LC–TS-MS and demonstrated applications for quantitation of neuropeptides in mobile phase down to 800 femptomole. The work presented here describes a method for quantitative analysis of peptides involving on-line LC fractionation, on-line digestion using an immobilized enzyme column (either trypsin or chymotrypsin), on-line collection of the peptide fragments, and off-line derivatization of di- and tripeptide fragments with PFBBr followed by GC–MS with NICI.

2. Experimental

2.1. Materials

Peptides (Met–enkephalin–Arg–Gly–Leu and Leu–enkephalin) and enzymes in solution or immobilized (trypsin, chymotrypsin, Leu–aminopeptidase, or dipeptidylaminopeptidase) were obtained from Sigma (St. Louis, MO, USA). Reagents obtained from Pierce (Rockford, IL, USA) included pentafluorobenzyl bromide (PFBBr), heptafluorobutyric acid anhydride (HFBA), diisopropylethylamine, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and triethylamine (TEA). HPLC-grade solvents (acetonitrile, 2-propanol, ethyl acetate) employed were stored over Na_2SO_4 to maintain dryness. The following peptides were synthesized using an ABI peptide synthesizer with FMOC technology: d_0- and d_3-heptapeptide [(d_3-Ala)–Tyr–(d_3-Ala)–Phe–Lys–(d_3-Ala)–Thr] as well as Met–enkephalin–Arg–(d_{10}-Leu)–Gly and d_3-DSLET [Tyr–D– Ser–Gly–Phe–(d_3-Leu)–Thr], an enkephalin analog. Deuterium labeled isotopes were on the amino acid side chain for all stable isotopes employed. Unlabeled and tritiated DSLET [^{3}H-ring Tyr] were obtained from NIDA.

2.2. Instrumentation

2.2.1. GC-FID detection

Gas chromatography (GC) with flame ionization detection (FID) was performed with a Hewlett-Packard Model 5880 gas chromatograph equipped with a DB-1 (15 m×0.32 mm I.D.), fused-silica capillary column (J&W Scientific, Folsom, CA, USA). The GC conditions were: injector temperature, 300°C; detector temperature, 300°C; helium flow-rate, 1 ml/min; splitless injection. The initial oven temperature was 75°C; 1 min after injection, the temperature was increased at 15°C/min to 275°C.

2.2.2. GC–NICI-MS

Analyses were performed on a Fisons Trio 1000 quadrupole mass spectrometer, using negative ion detection with selected ion monitoring and methane as a chemical reagent gas. GC conditions were as described in Section 2.2.1. MS conditions were: electron energy, 900 eV; ion source temperature, 200°C; methane pressure, 65 Pa.

2.2.3. HPLC system

A Hewlett-Packard 1040 Model HPLC system was equipped with a Waters Model 441 UV detector at 214 nm, a Bio-Rad AS-100 autosampler and a Gilson FC 204 fraction collector. Chromatographic separations were carried out on a Spherisorb C_{18} column (15 cm×2.0 mm I.D.) at a flow-rate of 0.3 ml/min. Also, a Shimadzu low-pressure pump was incorporated to supply a continuous flow of buffer to the immobilized enzyme column.

2.3. Selection of a model peptide

The proposed analytical method was established and characterized in human plasma using a peptide that possessed low-molecular mass, lipophilic properties, as well as trypsin and chymotrypsin cleavage sites. A heptapeptide (Ala–Tyr–Ala–Phe–Lys–Ala–Thr) was selected such that cleavage by trypsin or chymotrypsin would produce small peptide fragments as shown in Table 1. The pentapeptide has a high molecular mass and as a result is not detected by GC–MS. A deuterium-labeled heptapeptide was synthesized using deuterated alanine (d_3-Ala); the peptide fragments containing d_3-Ala served as the deuterium labeled internal standard. Application of the analytical method was demonstrated in a rat plasma matrix using a synthetic exogenous peptide, Met–enkephalin–Arg–Gly–Leu (Fig. 1). A deuterium-labeled internal standard was synthesized using d_{10}-Leu such that when cleaved by trypsin, a dipeptide containing d_{10}-Leu would be formed to serve as the internal standard (Table 1). Unfortunately, it was not possible to prepare a deuterated peptide that was directly analogous to Met–enkephalin–Arg–Gly–Leu. Currently available procedures for peptide synthesis require that the C-terminal amino acid be bound to a resin at the carboxyl group. However, d_{10}-Leu is not commercially available in this form and in order to alleviate this problem, Leu and Gly residues were reversed in the peptide sequence, thereby forming Met–enkephalin–Arg–(d_{10}-Leu)–Gly.

2.4. Stability in plasma

Stability studies were performed in vitro by incubating the heptapeptide, Met–enkephalin–Arg–Gly–Leu, Leu–enkephalin, or DSLET (100 μg/ml) in 1.0 ml of 25% human plasma at 37°C [9]. Aliquots of each sample were removed at 0, 5, 10, 15, 30 min and combined with 200 μl of 6% TFA; the quantity of the unhydrolyzed peptide was determined by HPLC with UV absorbance detection at 214 nm. Chromatographic separations are as in Section 2.7. The proteolysis in plasma is assumed to be at a first order process; preliminary assessment of the plasma half-life was obtained from a log-linear concentration–time profile.

2.5. Sample preparation

A schematic diagram of the analysis of a synthetic peptide that was added to and extracted from human or rat plasma is shown in Fig. 2. The initial sample preparation involves the addition of the internal standard, either the d_3-heptapeptide (10 ng/ml) in human plasma or Met–enkephalin–Arg–(d_{10}-Leu)–Gly (100 ng/ml) in rat plasma.

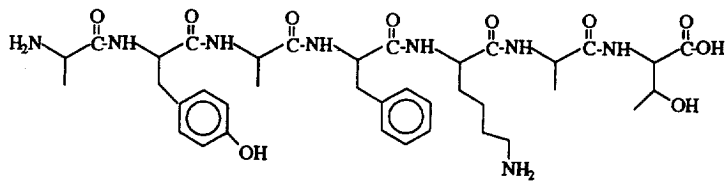
2.6. Extraction

A comparison between C_{18} and strong cation-exchange (SCX) cartridges was evaluated for the

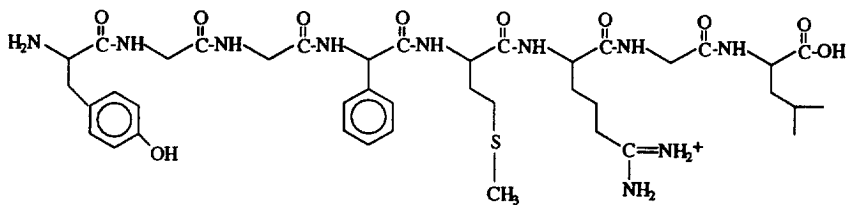
Table 1
Trypsin and chymotrypsin peptide fragments

	Trypsin	Chymotrypsin
Heptapeptide	Ala–Tyr–Ala–Phe–Lys, Ala–Thr	Ala–Tyr, Ala–Phe, Lys–Ala–Thr
Met–Enkephalin–Arg–Gly–Leu	Tyr–Gly–Gly–Phe–Met–Arg, Gly–Leu	Tyr, Gly–Gly–Phe, Met–Arg–Gly–Leu
Met–enkephalin–Arg–(d_{10}-Leu)–Gly	Tyr–Gly–Gly–Phe–Met–Arg, d_{10}-Leu–Gly	Tyr, Gly–Gly–Phe, Met–Arg–(d_{10}-Leu)–Gly

Heptapeptide (ALA-TYR-ALA-PHE-LYS-ALA-THR)



MET-ENKEPHALIN-R-G-L (TYR-GLY-GLY-PHE-MET-ARG-GLY-LEU)



DSLET (TYR-D-SER-GLY-PHE-LEU-THR)

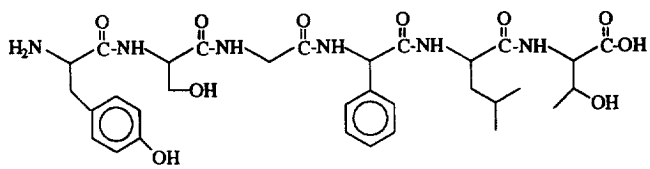


Fig. 1. Structures of the model heptapeptide, Met–enkephalin–Arg–Gly–Leu and DSLET.

extraction recovery of the heptapeptide from plasma. [^{3}H-Tyr]DSLET, an HPLC-purified heptapeptide, was employed for this effort because it possesses lipophilic properties, similar to the model heptapeptide, based on LC retention with C_{18} stationary phase. Also, DSLET was selected because it is available radiolabeled, which enabled the peptide to be measured by scintillation counting. Fig. 3 outlines the procedure used for the extraction of [^{3}H]DSLET from human plasma using C_{18} and SCX columns. Plasma spiked with exogenous peptides were placed on ice, combined with the internal standard, and treated with CH_3CN for protein precipitation. The supernatant was transferred to the solid-phase columns, which were appropriately treated as described in Fig. 3. Extraction recovery was determined for [^{3}H]DSLET (10^5 dpm per sample) by scintillation counting. The method that provided the highest recovery of [^{3}H]DSLET was selected and employed for future studies. Because DSLET did not appear to be a good model for recovery studies of Met–enkephalin–Arg–Gly–Leu, recovery studies for unlabeled Met–enkephalin–Arg–Gly–Leu (10 μg) and Met–enkephalin–Arg–(d_{10}-Leu)–Gly (10 μg) in plasma following C_{18} solid-phase extraction (SPE) were done by measuring the remaining parent peptide using HPLC with UV detection. Following the extraction of peptides from plasma, the sample was evaporated to dryness and reconstituted in 50 μl of mobile phase in preparation of LC analysis.

2.7. Fractionation/enzyme digestion

A continuous flow apparatus was assembled in which the HPLC effluent could be directed to an immobilized enzyme column and then to the fraction collector (Fig. 4). Samples were injected onto the analytical column through the use of an autosampler;

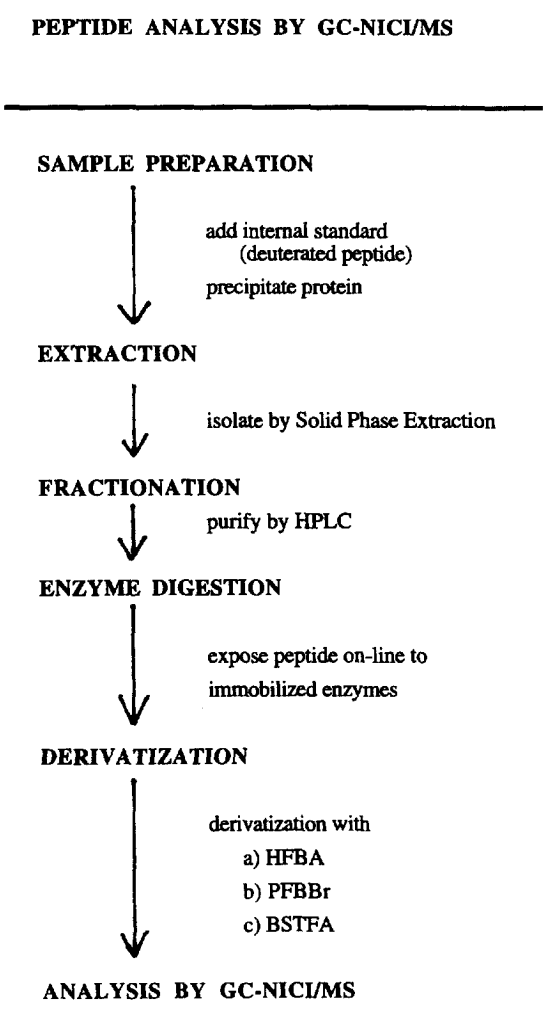


Fig. 2. Schematic diagram for the analysis of the synthetic peptides that have been added to and extracted from biological fluids.

the effluent was passed through a pneumatically operated six-port switching valve, which was programmed through the HPLC controller to transport effluent either to waste or onto the immobilized enzyme column. At this point, post column addition of buffer (25 m*M* TEA–formate buffer, pH 10.4) onto the enzyme column at a flow of 1.0 ml/min was initiated through the use of a switching valve and a low-pressure pump. LC fractions containing the peptide of interest were collected from the enzyme column during a 5 to 6 min period at a reduced flow of 0.15 ml/min applied during the collection period only. Decreasing the LC flow reduced the concentration of organic effluent through the enzyme column, thereby minimizing enzyme inactivation. Following enzymatic digestion, peptide fragments were collected into silanized conical glass test tubes and then prepared for GC–NICI-MS analysis. This method was evaluated using the model heptapeptide and Met–enkephalin–Arg–Gly–Leu in plasma. Because chromatographic peak retention times may shift during the course of a sample run, β-casmorphin (Tyr–Pro–Phe–Pro– Gly–Pro–Ile; 0.1 μg/μl) dissolved in mobile phase was used as an external retention time maker.

2.7.1. Fractionation

Chromatographic separations were carried out on a C_{18} column at a flow-rate of 0.3 ml/min. The mobile phase utilized for the separation of the heptapeptide was A: 0.1% TFA and B: CH_3CN–TFA (40:0.1), with a gradient employed from 20 to 60% B in 20 min. The mobile phase utilized for the separation of Met–enkephalin–Arg–Gly–Leu was A: 0.1% formic acid and B: CH_3CN–formic acid (60:0.1), with a gradient from 20 to 60% B in 25 min.

2.7.2. Enzyme digestion

A variety of commercially available enzyme preparations including trypsin bound to either DITC glass, polyacrylamide, or acrylic beads and chymotrypsin attached to either beaded agarose, carboxymethylcellulose beads, or acrylic beads was evaluated. The enzymes bound to the various forms of solid support were separately suspended into 0.2 *M* Na_2SO_4, pH 8.0, and poured into a 2 mm glass column [8]. Of the enzymes tested, trypsin immobilized on DITC glass was available with the highest specific activity of 10 000 units/g of solid support, and was most effectively interfaced with the HPLC system. Trypsin and chymotrypsin were evaluated for the time required for complete enzymatic cleavage of the model heptapeptide (10 μg). The sample reaction time on the enzyme column was altered by stopping the buffer flow through the column for either 0, 2, 5, 10, or 15 min; thereby allowing the peptide to interact with the immobilized enzyme. The enzyme buffer flow-rate through the immobil-

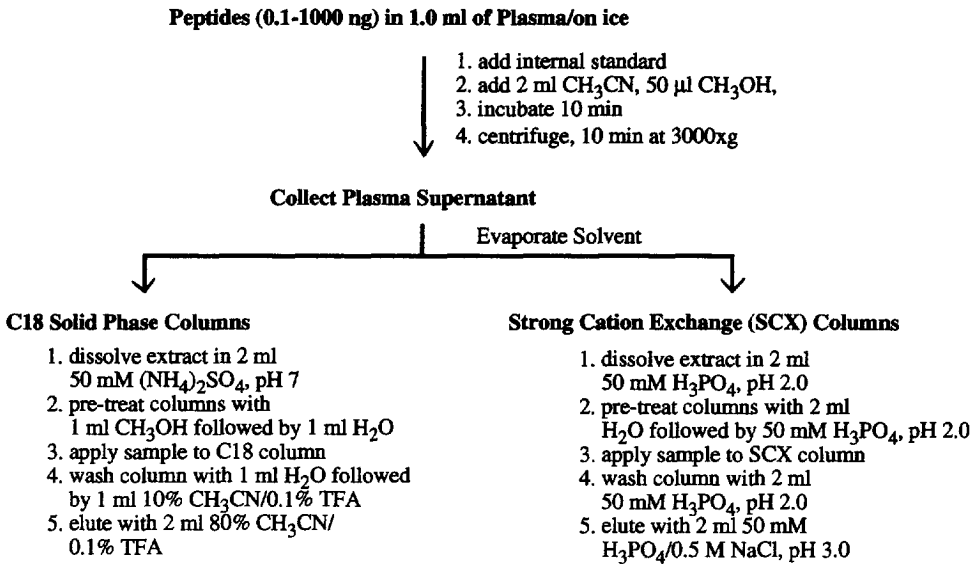


Fig. 3. Isolation of the synthetic peptides that have been added to and extracted from plasma by SPE.

ized enzyme column was evaluated at flows of 0.5, 0.8, 1, 1.5, or 2.0 ml/min, using 10 m*M* Na_2SO_4, pH 8.0. After use, the immobilized enzyme column was stored between use at 4°C after rinsing with 10 m*M* Na_2SO_4, pH 8.0. The stability of the immobilized enzyme columns was also assessed, and is discussed in Section 3.

2.8. Derivatization

2.8.1. HFB/PFB/TMS derivatives

The derivatization was performed in 6-ml conical glass tubes, which were previously silanized using 10% dimethyldichlorosilane in toluene. Peptide fragments were collected into the silanized tubes and

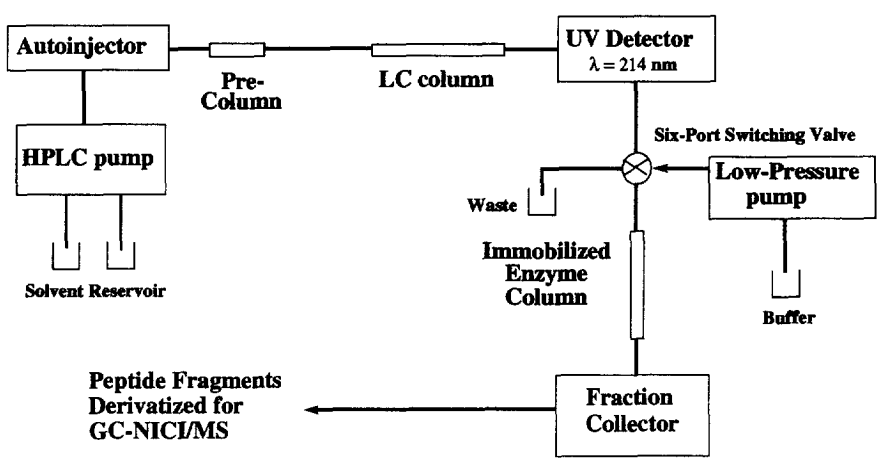


Fig. 4. Schematic diagram of the apparatus for isolation of peptides prior to GC–NICI-MS analysis.

taken to dryness using a stream of dry N_2. Each sample was reacted with 50 μl of HFBA–CH_3CN (1:5, v/v) for 5 min at room temperature, followed by evaporation with a stream of dry N_2. The dried residue was treated with 100 μl of a mixture consisting of CH_3CN–diisopropylethylamine–PFBBr (1:0.1:0.01, v/v/v) and reacted for 15 min at 70°C. The sample was cooled to room temperature, solvent was evaporated using a stream of dry N_2, and then the sample was treated with 25 μl of BSTFA, containing 1% TMCS for 5 min at room temperature. Excess reagent was evaporated using dry N_2; then the residue was dissolved in 25 μl of ethyl acetate and analyzed by GC–NICI-MS with SIM. Peptide fragments generated from Met–enkephalin–Arg–Gly–Leu digestion with trypsin do not contain hydroxylated amino acid residues and were, therefore, not treated with silylating reagents.

2.9. Analysis by GC–NICI-MS

A 1-μl aliquot of sample was analyzed by either GC-FID or GC–NICI-MS with selected ion monitoring. Peak-area measurements were obtained using selected ion monitoring for the heptapeptide fragments (d_0/d_3-AlaThr; *m/z* 367/370 or d_0/d_3-AlaPhe; *m/z* 431/434), Met– enkephalin–Arg–Gly–Leu fragment (d_0-GlyLeu; *m/z* 383) and Met–enkephalin–Arg–(d_{10}-Leu)–Gly fragment (d_{10}-LeuGly; *m/z* 393). Standard curves were obtained to evaluate the entire method for peptide analysis. Quantitative measurements were obtained from 0.01–50 ng/ml for the heptapeptide and from 0.1–1000 ng/ml for Met–enkephalin–Arg–Gly–Leu. The internal standard was added at 10 ng/ml for the heptapeptide and at 100 ng/ml for Met–enkephalin–Arg– (d_{10}-Leu)–Gly. The fit of each standard curve was assessed by means of either a weighted fit of $1/x$ or $1/x^2$ as well as a non-weighted linear fit.

3. Results and discussion

3.1. Stability in plasma

Stability studies of the model heptapeptide, Leu–enkephalin and Met–enkephalin–Arg–Gly–Leu in human plasma showed that they were relatively unstable, with half-lives of 2, 4 and 10 min, respectively. On the other hand, DSLET exhibited a half-life of 2 h, respectively. In other studies, Powell et al. [9] compared the stability of peptides in human serum, plasma and synovial fluid and reported that there were no differences in the rate of peptide degradation among the different biological matrices. In order to increase the stability of peptides in plasma, the model heptapeptide was also examined in the presence of the enzyme inhibitors leupeptin, EDTA, benzamidine, EGTA, TLCK and TPCK. Because these inhibitors were unable to retard the degradation of the model heptapeptide, they were not utilized for subsequent experiments.

3.2. Immobilized enzyme activity

The ability of TPCK-treated trypsin on DITC glass and TLCK-treated chymotrypsin on agarose gel to hydrolyze the model heptapeptide was assessed, using a buffer of 10 m*M* Na_2SO_4, pH 8. Enzyme activity was determined through the disappearance of the parent peptide and the formation of peptide fragments as monitored by HPLC with UV absorbance detection. The peptide fragments generated by trypsin digestion were Ala–Thr and Ala–Tyr–Ala–Phe–Lys; chymotrypsin yielded Ala–Phe, Ala–Tyr and Lys–Ala–Thr. Approximately 96% of the heptapeptide was cleaved by trypsin attached to DITC glass, whereas only 85% was hydrolyzed by chymotrypsin bound to agarose. Studies were also conducted to determine how rapidly the peptide was cleaved; it was found that the heptapeptide was cleaved almost instantaneously by both enzymes. It was also found that peptide cleavage was reduced at flow-rates greater than 1.0 ml/min of 10 m*M* Na_2SO_4, pH 8.

3.3. Extraction efficiency

The isolation of exogenous peptides that have been added to and extracted from plasma was evaluated with C_{18} and SCX columns. Recovery of [^{3}H]DSLET from plasma ($n=3$) was 85±3% for C_{18} and 70±2% for SCX. There are two reasons C_{18} columns were selected for future studies: (a) extraction efficiency was higher for C_{18} as compared to SCX and (b) the high salt content required for

elution from SCX columns was not conducive for HPLC analysis. Recovery of unlabeled Met–enkephalin–Arg–Gly–Leu and deuterium labeled Met–enkephalin–Arg–Leu–Gly from plasma using C_{18} SPE was 65 and 90%, respectively.

3.4. Fractionation/enzyme digestion

Plasma samples purified by SPE were subjected to LC fractionation and enzymatic cleavage. There were several factors found to be important when utilizing the immobilized enzyme column on-line with the eluent from the analytical LC fractionation. First, it is advisable to maintain a low organic content in the analytical LC mobile phase in order to minimize enzyme degradation. Following an examination of various solvent systems (40, 60 and 80% CH_3CN–0.1% TFA as well as CH_3OH–0.1% TFA), it was found that 40% CH_3CN–0.1% TFA was able to provide good chromatographic separation of the heptapeptide without deleterious effects of the enzyme activity in the immobilized enzyme column. Second, decreasing the analytical LC flow-rate from 0.3 to 0.15 ml/min during the elution of the desired peptide onto the immobilized enzyme column minimized peptide hydrolysis. Third, increasing the enzyme column buffer (25 m*M* TEA–formate) flow to 1.0 ml/min resulted in an enhancement in enzymatic activity. Finally, the acidic pH of the analytical LC mobile phase was also found to negatively influence the enzyme activity. Compensation for the acidic pH was done by increasing the pH of the enzyme buffer added after the analytical column. Combining the acidic LC buffer to the basic enzyme column buffer resulted in a pH on the enzyme column from 8 to 9, a range where maximum enzyme activity was obtained. The integrity of the enzyme column was tested with the model heptapeptide prior to and following each experiment. Enzyme columns were discarded if maximum enzyme cleavage was not obtained for the heptapeptide.

The recovery of hydrolysis products from the analytical LC column/immobilized enzyme column/fraction collector system was assessed using an HPLC purified [^{3}H]Phe (100 000 dpm per LC injection; $n=3$). This amino acid was selected because it was readily available and would not be subjected to enzymatic degradation. The recovery of [^{3}H]Phe was found to be 93±0.3% for the analytical LC column, but was 84±1% for the combined analytical/immobilized enzyme column/fraction collector due to incomplete peak recovery through the switching valve.

Fig. 5 shows chromatograms for blank plasma and for 10 μg of the heptapeptide added to plasma analyzed by HPLC with UV absorbance detection. The heptapeptide and deuterated analog co-eluted with good peak shape (data not shown). Met–en-

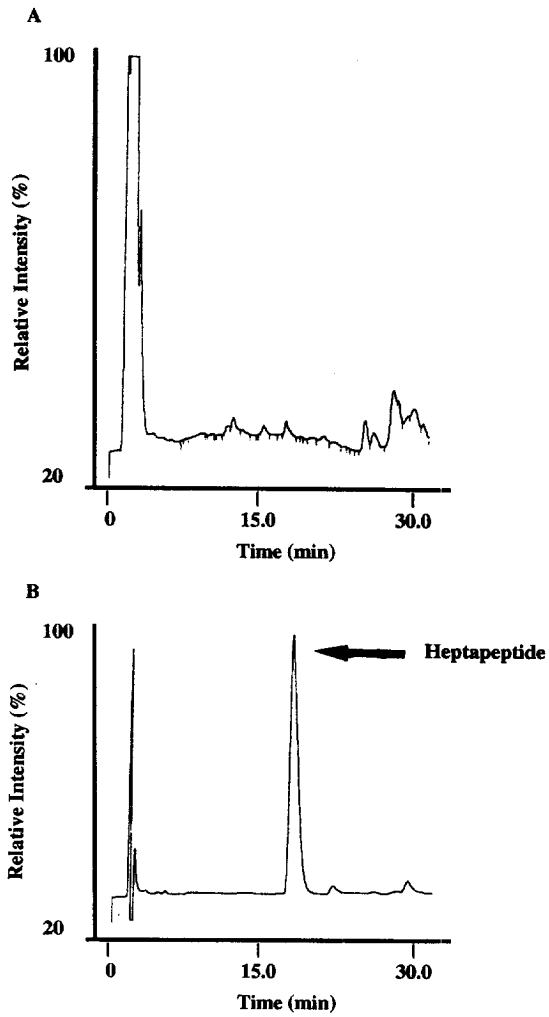


Fig. 5. LC chromatogram of blank plasma (A) and the heptapeptide (10 μg) in plasma (B) obtained with UV absorbance detection at 214 nm. The mobile phases utilized were A: 0.1% TFA and B: CH_3CN–TFA (40:0.1). The gradient was 20 to 60% B in 20 min.

kephalin–Arg–Gly–Leu-d_0 appeared to be slightly more lipophilic and eluted later than Met–enkephalin–Arg–Leu–Gly-d_{10}, the internal standard (12.4 and 10.9 min, respectively). Because Met–enkephalin–Arg–Gly–Leu and the internal standard did not co-elute, a sufficiently wider LC window was directed into the enzyme column to ensure complete recovery of the deuterated and non-deuterated peptides. Also, to minimize peak broadening, the CH_3CN content of the mobile phase B was increased from 40 to 60% CH_3CN; the increase in organic content did not detrimentally affect the enzyme activity of the immobilized trypsin column.

3.5. Derivatization

In order to efficiently derivatize peptide fragments that eluted from the immobilized enzyme column, it was necessary to optimize various aspects of the procedure. In particular, many derivatizing agents do not react optimally in the presence of buffers that are high in salt. However, a buffer is needed that will maintain maximum enzyme activity in the immobilized enzyme column. A variety of buffers was examined to determine which would be most suitable that included 0.1% TFA, 10 m*M* NH_4OAc, 20 m*M* NaOAc, 20 m*M* $NaHCO_3$, 100 m*M* Na_2CO_3, 25 m*M* pyridine–formate and 25 m*M* TEA–formate. Several of these buffers were selected on the basis of previous studies by Murayama et al. [10], in which it was shown that buffers such as Na_2CO_3, NaOAc and $NaHCO_3$ were compatible with the derivatizing agent PFPA. To evaluate the buffers, Phe (25 μg) and Ala–Thr (25 μg) were simultaneously spiked into each of the above solutions, the solvent evaporated to dryness, and the residue derivatized for analysis by GC-FID. The most compatible buffers were found to be either 25 m*M* pyridine–formate or 25 m*M* TEA–formate. TEA–formate was chosen for further work because it was capable of buffering at a more basic pH, thus compensating for the acidic pH of the analytical mobile phase, as discussed in Section 3.4.

Following enzymatic cleavage, peptide fragments were derivatized using HFBA, PFBBr and BSTFA. Previous studies done in our laboratory showed that these derivatizing reagents yield products that exhibit picomole detection levels of the derivative for analysis by GC–NICI-MS [11]. The derivatization method described in this work enabled the detection of tripeptides such as Ala–Leu–Ala, Gly–Gly–Phe and Ala–Gly–Gly.

3.6. Heptapeptide analysis

Following tryptic digestion of the mixture of deuterated and non-deuterated heptapeptides, the fragments formed were AlaThr-d_0 and AlaThr-d_3, whereas those produced by chymotryptic digestion were AlaPhe-d_0 and AlaPhe-d_3. Intense chromatographic peaks were observed through GC–NICI-MS analysis of the derivatives, with intense molecular ions detected at picomole levels. A representative NICI mass spectrum is shown in Fig. 6 for the AlaThr derivative, where the major ions at *m/z* 367 $(M-[PFB+TMSiOH]^-)$ and *m/z* 528 $(M-[HF+TMSiOH])^-$ can be seen, described previously by Márquez et al. [11]. In Fig. 7, selected ion retrieval for unlabeled and deuterated AlaThr produced by trypsin digestion of the heptapeptide (0.1 ng of d_0/10 ng of d_3) are shown. The corresponding traces for AlaPhe generated by chymotrypsin digestion of the heptapeptide (0.1 ng of d_0/10 ng of d_3) are shown in Fig. 8.

Evaluation of the methodology for isolation and derivatization of the heptapeptide in plasma (by C_{18} SPE, on-line LC interfaced to an immobilized trypsin column followed by derivatization for GC–MS analysis with NICI and SIM) revealed that standard curves were linear from 1–50 ng of heptapeptide-d_0 per ml of plasma. The data were best described using a non-weighted linear fit, and provided a correlation coefficient of 0.99 in the presence of 100 ng of the deuterated internal standard. The calculated concentrations, standard deviation and coefficient of variance (C.V.) averaged from three data sets are shown in Table 2. The observed concentrations showed a low C.V. of within 15% from 5–50 ng/ml, although much higher variability was observed below 5 ng/ml. When immobilized chymotrypsin bound to agarose was employed, it was not possible to obtain acceptable results at low concentrations in plasma, in part due to the lower enzyme activity of chymotrypsin bound to agarose. In addition, chymotrypsin bound to agarose was more susceptible to enzyme

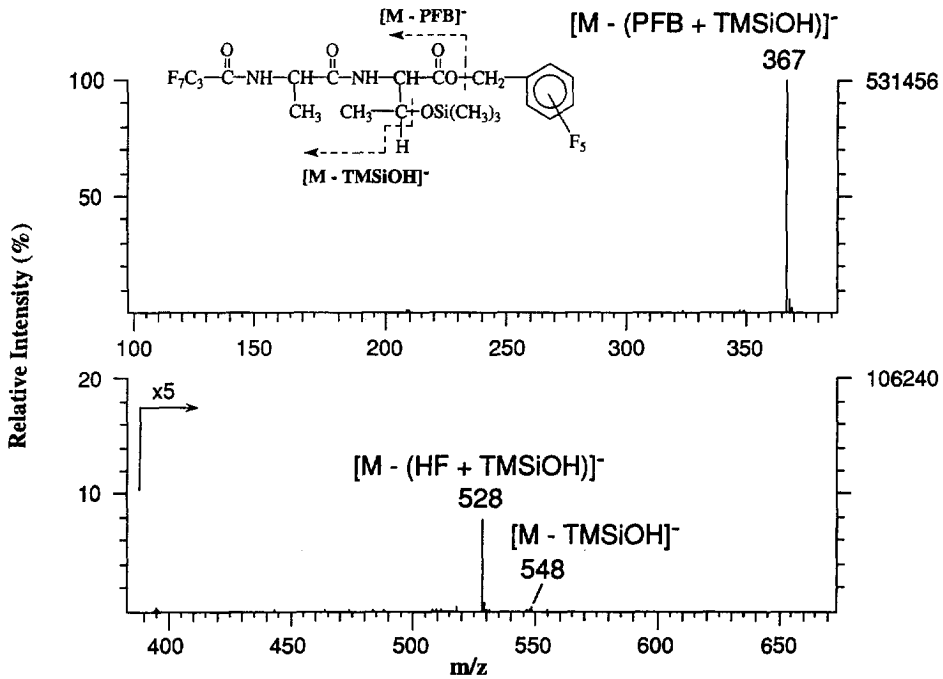


Fig. 6. NICI mass spectrum of the HFB/PFB/TMS-derivative of Ala–Thr.

degradation by the organic mobile phase than trypsin bound to glass; as a result, high background interferences were observed, which limited the detection of the heptapeptide. Although chymotrypsin attached to glass would most likely yield better results, this enzyme preparation is not commercially available; however, there are literature reports that describe the immobilization procedure [5,6,12].

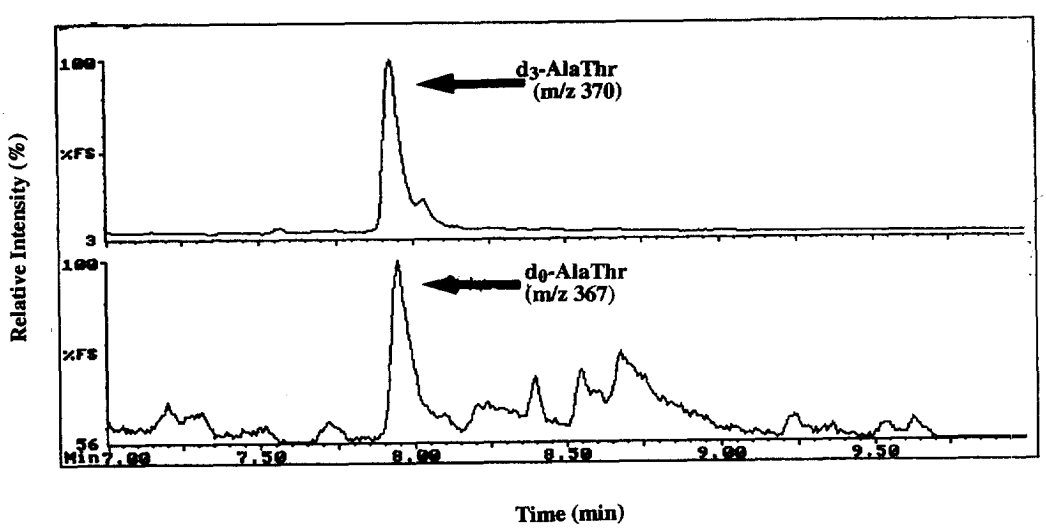


Fig. 7. Selected ion retrieval of the GC–NICI-MS analysis of dipeptides produced by trypsin digestion of the heptapeptide (0.1 ng of d_0/10 ng of d_3; per ml of plasma).

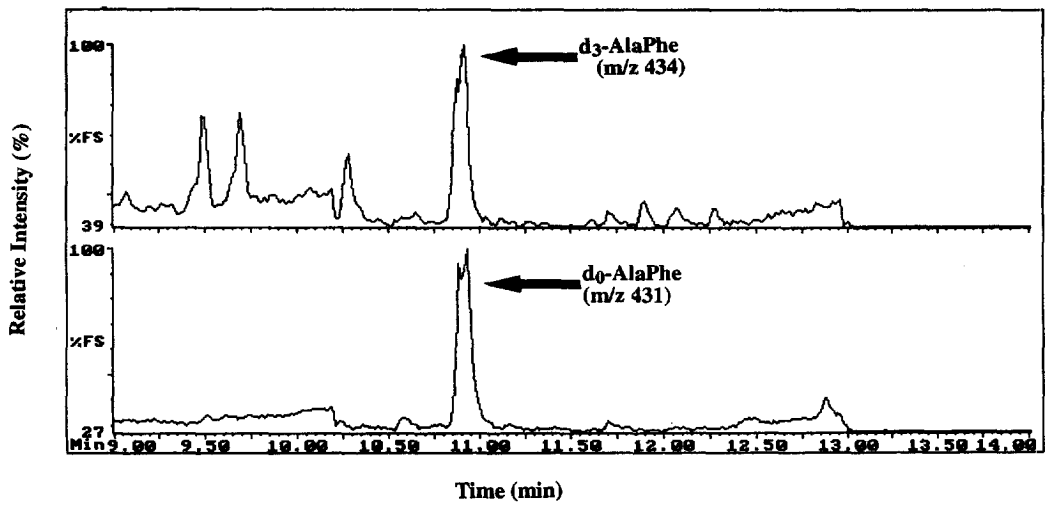


Fig. 8. Selected ion retrieval of the GC–NICI-MS analysis of dipeptides produced by chymotrypsin digestion of the heptapeptide (0.1 ng of d_0/10 ng of d_3; per ml of plasma).

3.7. Met–enkephalin–Arg–Gly–Leu analysis

Peptide fragments selected for monitoring after trypsin digestion were the dipeptide Gly–Leu-d_0 (*m/z* 383) for Met–enkephalin–Arg–Gly–Leu and d_{10}-LeuGly (*m/z* 393) for the internal standard, Met–enkephalin–Arg–(d_{10}-Leu)–Gly. Analysis by GC–NICI-MS resulted in good chromatographic separation with intense ions formed that could be detected at picomole levels. In Fig. 9, selected ion retrieval for Met–enkephalin–Arg–Gly–Leu-d_0 and Met–enkephalin–Arg–(d_{10}-Leu)–Gly (10 ng of d_0/100 ng of d_{10} per ml of plasma) are shown. Linearity was observed using a non-weighted linear fit from 50–1000 ng of Met–enkephalin–Arg–Gly–Leu-d_0 per ml of plasma in the presence of 100 ng of the deuterated internal standard. Table 3 lists results for three separate analyses of Met–enkephalin–Arg–Gly–Leu. The observed concentrations showed relatively good C.V. within 15% from 10–1000 ng/ml and a correlation coefficient of 0.99.

Table 2
Quantitative analysis of the synthetic heptapeptide (Ala–Tyr–Ala–Phe–Lys–Ala–Thr) that has been added to and extracted from plasma

Concentration added (ng/ml)	Concentration[a] measured (mean±S.D.) (ng/ml)	Coefficient of variation (%)
1.0	0.8±0.3	34.1
5.0	5.2±0.7	14.1
10.0	9.8±0.9	9.1
50.0	50.0±0.2	0.5

[a] The values represent the mean±S.D. for 3 determinants. Samples were isolated by C_{18} SPE, fractionated by HPLC, digested on-line by immobilized trypsin, derivatized to produce AlaThr/HFB/PFB/TMS, and analyzed by GC–NICI-MS with selected ion monitoring.

4. Conclusions

The analytical method described here has been shown to provide sensitive and selective quantification of derivatized peptide fragments. Even though the model heptapeptide selected to evaluate the analytical method was unstable in plasma, reasonable linearity was obtained following isolation from plasma by C_{18} SPE and digestion by immobilized trypsin. In general, the method for peptide analysis appears to be more reproducible at the higher end of the curve and is most amenable to immobilized trypsin bound to DITC glass. The advantages of peptide analysis by means of the on-line enzymatic cleavage method combined with the derivatization method for GC–MS analysis include faster sample analysis, minimal sample loss and controlled en-

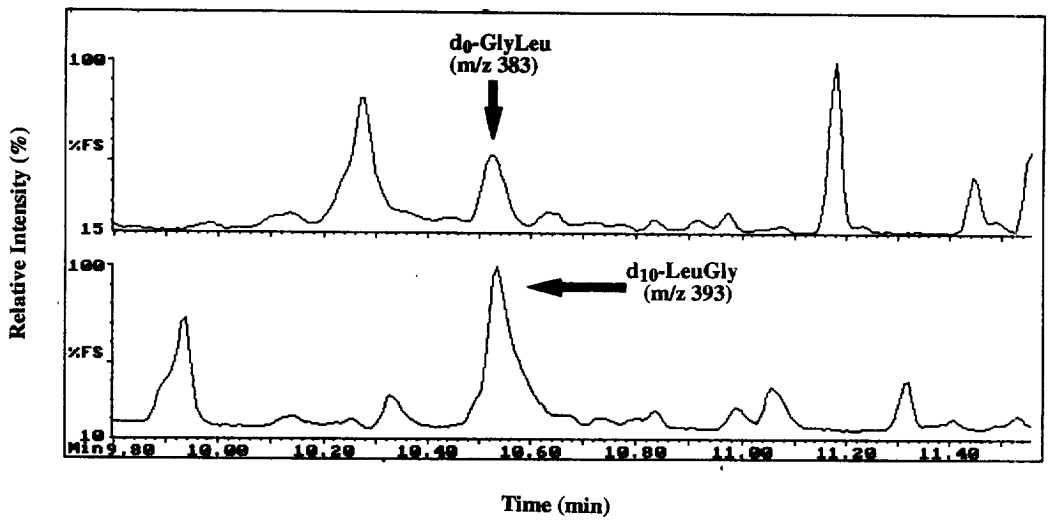


Fig. 9. Selected ion retrieval of the GC–NICI-MS analysis of dipeptides produced by trypsin digestion of Met–enkephalin–Arg–Gly–Leu-d_0 and Met–enkephalin–Arg–Leu–Gly-d_{10} (10 ng of d_0/100 ng of d_{10}; per ml of plasma).

Table 3
Quantitative analysis of the synthetic Met–enkephalin–Arg–Gly–Leu that has been added to and extracted from plasma

Concentration added (ng/ml)	Concentration measured[a] (ng/ml)		
	Day 1	Day 2	Day 3
10.0	19.9±1.2 (6.0)	38.8±11.7 (30.0)	7.9 (n.a.)[b]
50.0	62.4±3.6 (5.8)	64.9±5.7 (8.3)	47.3±9.8 (21.0)
100.0	81.5±8.3 (10.0)	85.6±10.0 (12.0)	116.0±4.5 (21.0)
1000.0	1010.0±44.1 (4.4)	1060.0 (n.a.)[b]	959.0 (n.a.)[b]

[a] The values represent the mean±S.D. for 2 determinants with the percent coefficient of variation given in parenthesis. Samples were isolated by C_{18} SPE, fractionated by HPLC, digested on-line by immobilized trypsin, derivatized to produce GlyLeu/HFB/PFB, and analyzed by GC–NICI-MS with selected ion monitoring.
[b] n.a., not available; only individual analyses were performed for these samples.

zymatic reactions with less autolysis than with enzymes in solution.

In previous studies, Caprioli et al. [3] and Liberato et al. [1] demonstrated high background interferences for peptide analysis when peptide cleavage was accomplished by either enzymes in solution or by total hydrolysis with acid. In contrast, the method described here shows that implementing the use of immobilized enzymes to digest peptides minimizes the high background problems experienced after total hydrolysis and thus, results in lower detection limits and selectivity of the peptide, which are essential for pharmacokinetic and disposition studies. The current method has the potential of being extended to a wide variety of peptides through the use of other immobilized enzymes like carboxypeptidase Y, thermolysin, and V8 protease. A future goal of these investigators is to characterize a series of biologically active peptides in a biological matrix for pharmacokinetic studies using this analytical method.

Acknowledgments

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References

[1] D.J. Liberato, E.P. Heimer, E.K. Fududa, M. Ahmad and W.A. Garland, in Proceedings of Am. Soc. Mass Spectrometry, San Francisco, CA, June 5–10, 1988, p. 433.
[2] T. Hayashi, M. Shimamura, F. Matsuda, Y. Minatogawa, H. Naruse, J. Chromatogr. 383 (1986) 259.
[3] R.M. Caprioli, W.E. Seifert Jr., D.E. Sutherland, Biochem. Biophys. Res. Commun. 55 (1973) 67.
[4] J. Crowther, V. Adusumalli, T. Mukherjee, K. Jordan, P. Abuaf, N. Corkum, G. Goldstein, J. Tolan, Anal. Chem. 66 (1994) 2356.
[5] H.Y. Kim, D. Pilosof, D.F. Dyckes, M.L. Vestal, J. Am. Chem. Soc. 106 (1984) 7304.
[6] R.D. Voyksner, D.C. Chen, H.E. Swaisgood, Anal. Chem. 188 (1990) 72.
[7] H. Fouda, M. Nocerini, R. Schneider, C. Gedutis, J. Am. Soc. Mass Spectrom. 2 (1991) 164.
[8] K.A. Cobb, M. Novotny, Anal. Chem. 61 (1989) 2226.
[9] M.F. Powell, H. Grey, F. Gaeta, A. Sette, S. Colon, J. Pharm. Sci. 81 (1992) 731.
[10] K. Murayama, N. Shindo, R. Mineki, K. Ohta, Biomed. Mass Spectrom. 8 (1981) 165.
[11] C.D. Márquez, S.T. Weintraub, P.C. Smith, J. Chromatogr. B 658 (1994) 213.
[12] R.B. Van Breemenm, R.G. Davis, Anal. Chem. 64 (1991) 2233.