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Syntheses of deuterated leu-enkephalins and their use as internal standards for the quantification of leu-enkephalin by fast atom bombardment mass spectrometry

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SUMMARY

We have developed a synthetic method for the preparation of di- and pentadeuterated leu-enkephalin (LE), Tyr-Gly-Gly-Phe-Leu, by proton-deuterium exchange using CF_3COO^2H. Four to six deuterium atoms are introduced using a reaction temperature of 120°C and if 5% of ^2H_2O is added the di-deuterated LE is obtained. These deuterated compounds are used as internal standards to plot calibration curves of LE using fast atom bombardment mass spectrometry.

Key words: deuterated leu-enkephalin; deuterium labelling; fast atom bombardment; quantitative analysis; neuropeptides; calibration curves.

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INTRODUCTION

Fast atom bombardment mass spectrometry (FAB-MS) has become a valuable technique for studying peptides. FAB mass spectra of many peptides have been clarified and this method is useful for elucidating the chemical structures of unknown peptides. Quantitative analysis of peptides by FAB has not been developed as much as qualitative analysis and in fact only a few quantitative studies have employed this technique (1,2).

Our interest was focussed on leu-enkephalin (LE), Tyr-Gly-Gly-Phe-Leu, quantification. This endogenous neuropeptide has important biological activities. Neuropeptides play significant roles as neurotransmitters in brain and spinal cord. Quantification of LE, and of other opioid peptides, has been studied by several different approaches in the last few years. Many laboratories use radio-immunoassay (RIA) (3), however this method does not seem to be as specific as required (4). Recently MS has been employed for the quantitative analysis of peptides, and was first used for the quantitative measurement of endogenous peptides in the form of FD (field desorption)-MS (5-6). FAB-MS with its simpler method and its higher sensitivity superseded FD-MS in the quantification of peptides (7).

One of the main points of interest is the choice of internal standard (IS) since quantitative MS analysis requires a suitable IS. This may be a commercially available unnatural analog of the compound of interest (8-9) but this kind of IS presents the disadvantages of different chemical behaviour. Since the IS should preferably be added to the biological extract before purification for analysis, the different behaviour can reduce the accuracy of the quantitative data. Furthermore, the analysis itself can be influenced by differences in structure between the IS and the compound to be measured.

One of the advantages of MS quantitative analysis is that a stable isotope-labelled analog of the compound can be used as IS. Quantification of enkephalin with FD-MS utilized [^{18}O]-labelled enkephalin as IS (10). Syntheses of deuterated enkephalins such as [$^{2}H_{8}$-Phe]-Met-enkephalin (11) or deuterated LE through platinum calalyzed exchange (12) were also developed.

This paper describes the fast and easy synthesis of deuterated analogs of LE by exchange of aromatic protons with deuterated trifluoroacetic acid. These compounds were tested for quantitative analysis of LE by FAB. Comparison with the use of a synthetic analog as IS confirmed that results were better with isotope labelled analogs.

MATERIALS AND METHODS

Products. Leu-enkephalin and DADLE (Tyr-DAla-Gly-Phe-DLeu) were purchased from Bachem (Torrance, CA, USA). Deuterated trifluoroacetic acid (99.5 atom % ^{2}H) and deuterated water (99.95 atom % ^{2}H) came from Merck (Darmstadt, F.R.G.).

Synthesis of pentadeutero LE. One mg of LE was dissolved in 200 µl of $CF_3COO^{2}H$ in a 0.5 ml screw cap vial. The vial was placed in an oven and heated at 120°C for 4 hours,

then the acid was evaporated under a nitrogen stream. This process was repeated another time. To hydrolyze the the resulting acetylated LE the reaction mixture was dissolved in 50 µl of NH_4OH 25% and left at room temperature for 2 hours.

Synthesis of dideutero LE. One mg of LE was dissolved in 200 µl of CF_3COO^2H, containing 5% of ^2H_2O, in a 0.5 ml screw cap vial. The vial was placed in an oven and heated at 100°C for 4 hours, then the acid was evaporated under a nitrogen stream. This process was repeated second time.

FAB mass spectrometry. All analyses were done on a VG 70-250 mass spectrometer with its standard FAB source and target. The source was at room temperature. Xenon was used as bombarding gas and accelerated at 8 kV. Quantitative measurements were done in the negative ionization mode, acquiring spectra from m/z 450 to m/z 700, at a scan time of 4 sec/decade.

Quantification. Varying amounts of LE were added to a constant amount of IS dissolved in H_2O/glycerol (2:1). Two µl of the solutions were transferred to the surface of the FAB probe. The water was removed in the vacuum block of the mass spectrometer before introducing the FAB probe into the ion source. Glycerol remained on the probe as matrix. Scans (15-20) were acquired, and average values of absolute peak intensity were used to plot the calibration curve.
This procedure was followed to obtain two different calibration curves with dideutero LE as IS: the first was plotted using six solutions at different LE concentrations: 5, 7.5, 10, 12.5, 15, 20 ng/µl, with a constant IS concentration of 5 ng/µl. The second was plotted with four solutions at LE concentrations of 25, 75, 125, 250 ng/µl and 50 ng/µl of IS. A single calibration curve was plotted with pentadeutero LE as IS; six solutions were employed at LE concentrations of 20, 30, 40, 75, 100, 200 ng/µl and 250 ng/µl of IS. The same procedure was followed with DADLE as IS. In this case the IS concentration was 25 ng/µl and four solutions at LE concentrations of 5, 10, 25 and 50 ng/µl were prepared.

RESULTS

Chemical syntheses

To obtain deuterated LE with CF_3COO^2H two different conditions were studied: 1) addition of pure CF_3COO^2H and hydrolysis of the obtained trifluoroacetylated LE or 2) addition of 5% ^2H_2O to the reaction mixture to prevent the LE acylation by the CF_3COO^2H. The second procedure led to a smaller proton-deuterium exchange, but the acylation was avoided. In this case we obtained the ion at m/z 556, corresponding to the dideutero LE, as the most important peak in the LE molecular weight region. The FAB spectrum of this compound is shown in Fig. 1. The undeuterated and monodeuterated species do not appear. The ratio between peak intensity at m/z 556 and m/z 557 is 100

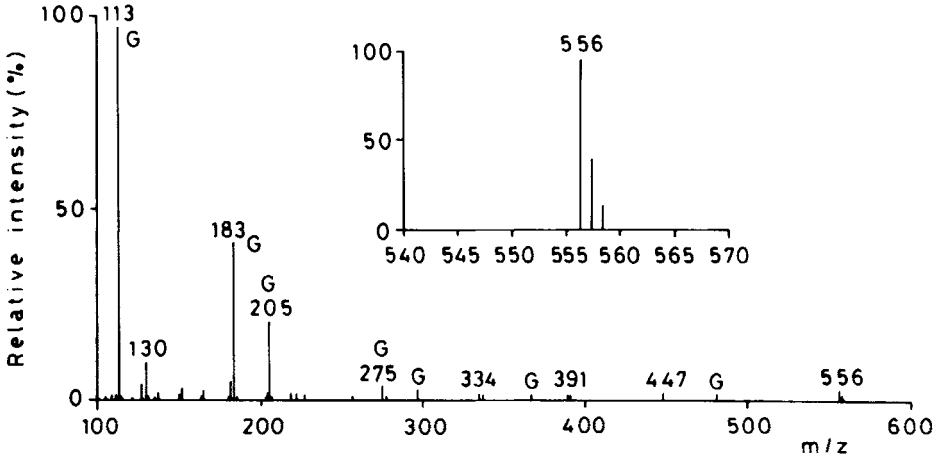

FIG. 1. Negative FAB mass spectrum of deuterated LE with CF_3COO^2H containing 5% ^2H_2O at 100°C for 8h. Glycerol was used as liquid matrix, and gave peaks marked as G.

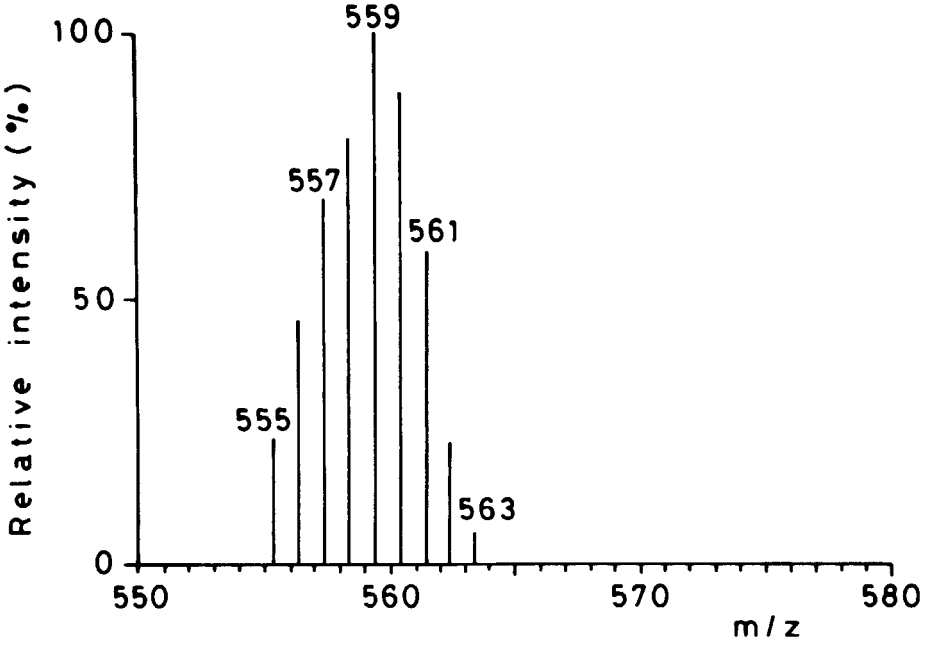

FIG. 2. Negative FAB mass spectrum of the molecular region of deuterated LE with CF_3COO^2H at 120°C for 8h after ammonia hydrolysis of the trifluoroacetic group.

to 41. The deuterium atoms are very probably introduced in the two ortho positions of the tyrosine aromatic ring according to the reactivity of the phenol. This is confirmed by the presence of ions at m/z 447 (M - CH_2-$C_6H_2{}^2H_2$0H), 391 (M - Tyr) and 334 (Gly-Phe-Leu).

The synthesis of deuterated LE at 120°C with deuterated trifluoroacetic acid as sole solvent gave the deuterated trifluoroacetylated LE as the most abundant product. In the negative FAB spectrum of LE after 8 hours at 120°C in CF_3COO^2H, the LE $(M-H)^-$ ion (m/z 554) was not observed. Peaks at higher m/z values (652-658) appeared corresponding to the $(M-H)^-$ of trifluoroacetylated LE, containing different numbers of deuteria. Acylated LE was treated with 25% NH_4OH to hydrolyze the trifluoroacetic group. After this treatment the FAB spectrum no longer showed the acylated LE peaks. There were several peaks in the LE molecular region, at m/z values of 555, 556, 557, 558, 559, 560, 561, 562, 563 with relative intensities of 24, 45, 69, 81, 100, 90, 59, 23, 6 (Fig. 2).

The same reaction, but at 70°C, produced a mixture of LE and trifluoroacetylated LE; after hydrolysis with ammonia 1, 2 and 3 deuteria were introduced. The ratios of the peaks 555, 556 and 557 were 40, 100, 36 (13).

Since acid treatment is common in the extractive procedure for neuropeptides, we treated deuterated LE with 10% HCl at room temperature for 60 min, but without obtaining any proton-deuterium exchange.

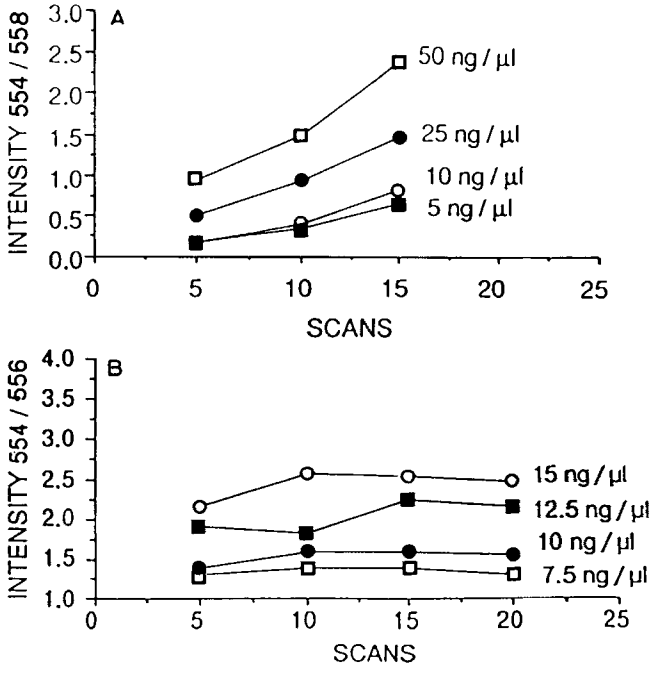

FIG. 3. A) ratios of the peak intensity between LE (varying amounts) and DADLE (25 ng/µl). B) ratios of the peak intensity between LE (varying amounts) and dideuterated LE (5ng/µl).

Calibration curves

In our conditions negative FAB mode yielded a stable current of LE molecular ion and reduced noise caused by the liquid matrix. We therefore acquired spectra for calibration curves in the negative rather than the positive mode.

We tried DADLE, a LE analog from a commercial source, as IS but it was desorbed before LE, and this led to an unstable ratio of the peak intensity between IS and LE (Fig. 3A).

Both deutero LE obtained were used as IS to obtain calibration curves for LE quantification. The dideutero LE curves were plotted with different amounts of IS. This minimizes the contribution of the isotopic peak at m/z 556 from LE to the dideuterated LE molecular ion peak. This contribution produces a positive intercept in the calibration curves, whose value depends on the amount of deuterated LE added.

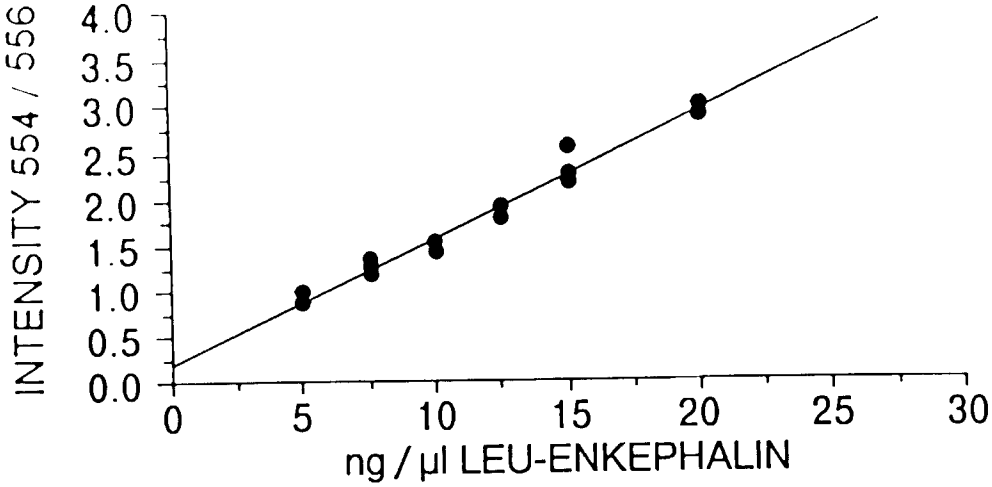

FIG. 4. Calibration curve for quantification of LE from 5 to 20 ng/µl with 5 ng/µl of dideuterated LE as IS.

With 5 ng/µl of dideutero LE as IS, the calibration curve for LE from 5 to 20 ng/µl was linear with a correlation coefficient of 0.985 (Fig. 4). In this case the ratio of the peak intensities between IS and LE was stable (Fig. 3B). The other curve with dideutero LE as IS, 50 ng/µl, was plotted from 25 to 250 ng/µl of LE and had a correlation coefficient of 0.999 (Fig. 5).

With the pentadeutero LE we used the peak at m/z 559 as IS reference peak. A single calibration curve was plotted with this IS with a wider range. In this case there were no contributions from the undeuterated LE isotopic peaks to the IS peak at m/z 559. With 150 ng/µl of IS, the calibration curve from 20 to 200 ng/µl presents a correlation coefficient of 0.998 (Fig. 6).

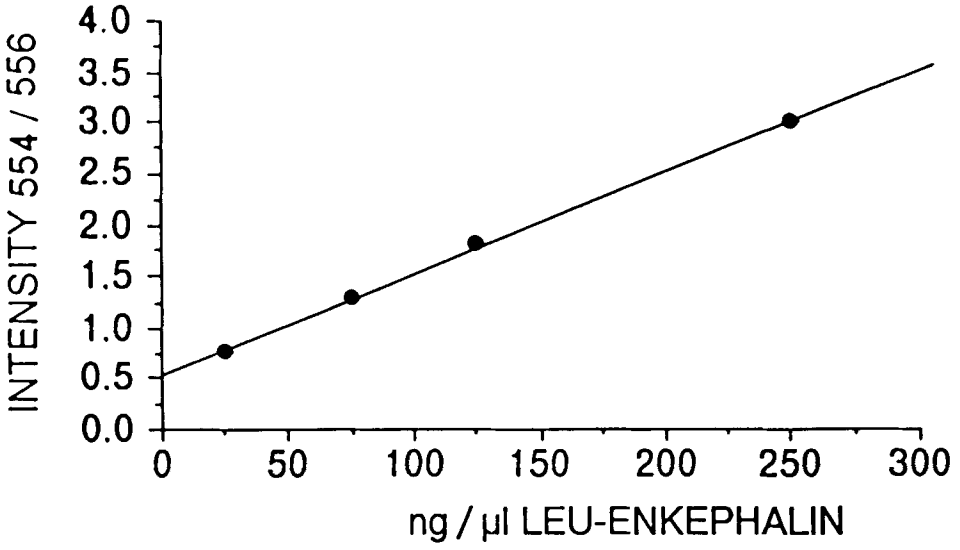

FIG. 5. Calibration curve for quantification of LE from 25 to 250 ng/µl with 50 ng/µl of dideuterated LE as IS.

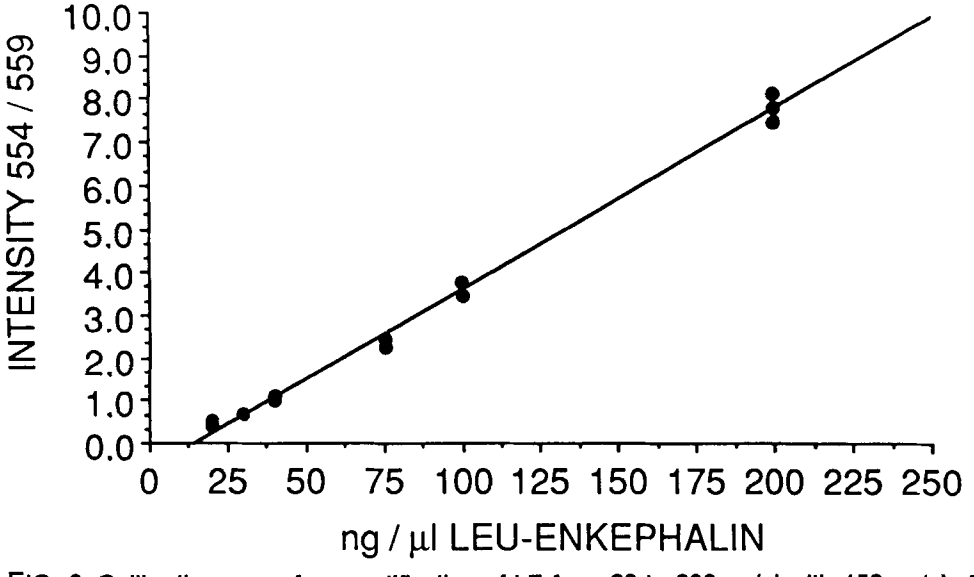

FIG. 6. Calibration curve for quantification of LE from 20 to 200 ng/µl with 150 ng/µl of pentadeutero LE as IS.

DISCUSSION AND CONCLUSIONS

In LE quantitative measurement by FAB-MS, two kinds of IS may be used, a chemical analog or a stable isotope-labelled LE. Although the analog is easy to obtain commercially, its chemical behaviour in extraction, purification and instrumental analysis may differ from the compound of interest. Indeed we noticed an unstable ratio of the

peaks corresponding to LE and the analog used.
By using the stable isotope-labelled LE we synthesized as IS these problems are overcome. Furthermore, its synthesis is fast, easy and cheap. This method is applicable to small-scale reactions and avoids complex synthetic chemistry. The procedure described could serve as a general method for synthetizing deuterated IS useful for the quantification of a large number of endogenous neuropeptides. In fact all these molecules contain a tyrosine residue in their aminoacid chain. Another possible application is for studies involving NMR.
We present the synthesis of two deuterated LE with different number of deuteria. In MS the choice between them as IS should be made considering that the dideuterated LE gives a sharper distribution of the peaks in the molecular region, and the contribution of the 556 ion from natural LE must be taken into account. The other LE has the disadvantage of a wide distribution of deuteria into the molecule, but its molecular peaks are well separated from those of natural LE.

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