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# Analysis of human β-endorphin 28–31 (melanotropin potentiating factor) and analogues by high-performance liquid chromatography of their 9-fluorenylmethoxycarbonyl derivatives

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ABSTRACT

A method is described for the determination of sub-picomole amounts of Lys-Lys-Gly-Glu [the C-terminal tetrapeptide sequence of human β-endorphin, refered to as melanotropin potentiating factor (MPF)], a putative neurotrophic agent. Attempts to raise antibodies to the peptide were not successful and we have therefore developed a method based on the fluorescence of its 9-fluorenylmethyl chloroformate (FMOC) derivative which provides a sensitivity comparable to that of radioimmunoassay. Standard solutions, cerebrospinal fluid or central nervous tissue extracts are first treated with FMOC-Cl. The resulting mixture of FMOC-peptides is then subjected to high-performance liquid chromatography (HPLC) and quantified using a fluorescence monitor. By this procedure, MPF and related peptides can be analysed from one sample in a single HPLC run. The method was also applied to determine the rate of release into a phosphate-buffered saline medium of a metabolically stable analogue of MPF from a slow-release formulation of the compound.

## INTRODUCTION

The tetrapeptide Lys-Lys-Gly-Glu is the C-terminal portion of human β-endorphin (β-$E_h$), referred to as MPF (melanotropin potentiating factor accords with the peptide's first recorded property [1], other descriptive names being human β-lipotropin 88–91 and β-endorphin 28–31). Both MPF and β-$E_h$ elicit a number of physiological responses which are not attributable to the N-terminal opioid region of β-E and are not naloxone-reversible. For example, structure–activity relationships in the *Urodele* limb regeneration assay [2] and the stimulation of dopamine output from cultured adrenal medullary cells [3] show that MPF is the smallest active sequence of β-$E_h$. Evidence for MPF receptor binding has been shown in its ability to displace tritiated β-$E_h$ from non-opioid binding sites in NG108-15 cells in the presence of the δ-opiate receptor agonist DSLET [4], although receptor binding has not been directly demonstrated using radiolabelled MPF.

For further understanding of the physiological role of the peptide, a sensitive assay was required. The possibility of using a radioimmunoassay was explored. However, when applied to MPF, the methodology successfully developed for the enkephalins [5] failed, despite the use of MPF conjugates with a variety of large carrier proteins [6]. Attempts to raise antibodies by monoclonal techniques were also unsuccessful.

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In devising an alternative method of analysis, derivatisation of MPF with a fluorescent moiety, 9-fluorenyl chloroformate (FMOC-Cl) followed by separation of the FMOC-MPF derivative by high-performance liquid chromatography (HPLC), was considered potentially capable of achieving the required sensitivity and of resolving MPF from other similar peptides of interest as well as interfering compounds.

Of the many fluorescent labels described for amino acid HPLC analysis, those most often used and which exhibit maximal fluorescence yields include dansyl chloride, fluoresceine isothiocyanate, *o*-phthalaldehyde (OPA), fluorescamine and FMOC-Cl. Some difficulties are associated with the use of these reagents. For example derivatisation with dansyl chloride is difficult to perform when the analytes are present at low concentration in a complex biological matrix, and OPA derivatives are not stable [7]. The label chosen was FMOC-Cl because it reacts rapidly and in high yield with primary and secondary amino acids under mild conditions producing highly fluorescent and stable derivatives [8] and the reaction is easily reversible. These properties have led to the application of FMOC-Cl as a pre-column fluorescence label for HPLC analysis of amino acids [9] and certain drugs containing an amino group [10]. It is also applicable for the derivatisation and analysis of peptide hormones [11]. The reaction is as shown in Fig. 1.

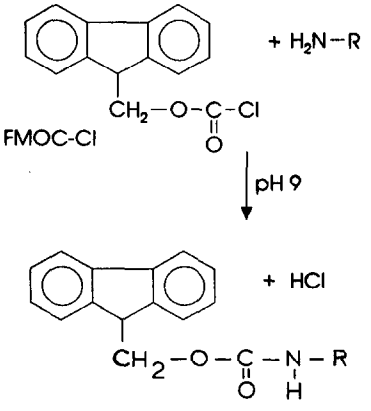


Fig. 1. Derivatisation reaction of amino acids with 9-fluorenyl methylchloroformate.

The present study describes the successful application of FMOC derivatisation in the development of an assay for MPF and related peptides. Conditions for the quantitative formation of the tris-FMOC derivative of MPF were established. The use of the assay in exploring the *in vitro* rate of release of Ac-Lys-D-Lys-Sar-Glu, a metabolically stable analogue of MPF [2], from a slow-release formulation of the peptide is described, together with a preliminary study to determine if MPF is either present in human cerebrospinal fluid (CSF) or its C-terminal Gln analogue in rat central nervous system tissue.

## EXPERIMENTAL

### *Reagents*

HPLC solvents were water and far-UV-grade acetonitrile (both HiPerSolv for HPLC, from BDH, Poole, UK) and trifluoroacetic acid (TFA) (protein sequencing grade) (Sigma, Poole, UK). Solvents were filtered and vacuum-degassed using a 0.2-μm Nylon-66 filter (Anachem, Luton, UK). FMOC-Cl was purchased from Fluka (Glossop, UK) and other reagents were of analytical grade. Accurel polypropylene tube, Type Q 3/2, was a gift from Akzo (Enka, Obernburg, Germany). Peptides were obtained from the following sources: MPF (Lys-Lys-Gly-Glu) was from Bachem (Saffron Walden, UK), Gly-Gln and Gly-Glu from Peninsula Labs. (Merseyside, UK). Ac-Lys-D-Lys-Sar-Glu, Tyr-Lys-Lys-Gly-Glu and Lys-Lys-Gly-Gln were synthesized as previously described [1,2].

### *HPLC conditions*

The HPLC system consisted of a 421 controller and two 112 pumps (both from Beckman, High Wycombe, UK) and an autosampler Type 9090 fitted with either a 10-μl or a 200-μl sample loop (Varian Assoc., Warrington, UK). The separation column was a reversed-phase Spherisorb 5-μm $C_6$ (150 mm × 4.6 mm I.D.) (Phase-Separations, Queensferry, UK). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. Gradient elution was carried out at ambient temperature, at a constant flow-rate of

1.0 ml/min. After injection of the sample (10–80 μl), the concentration of solvent B was increased from 45 to 75% (15 min) and then held at 75% B for 10 min, followed by a return to 45% B over 2 min. After a re-equilibration time of 10 min the column was ready for a further injection. Detection was achieved using a Type F1000 dual monochromator fluorescence monitor (Merck-Hitachi, Merck, Pool, UK) fitted with a 12-μl flow cell; the excitation wavelength was 260 nm and the emission wavelength 315 nm. The chromatograms were recorded on a chart recorder and integrated using a PC-based JCL6000 16-bit integrator (Jones Chromatography, Hengoed, UK).

*Tissue preparation*

Male Wistar rats weighing 200–250 g were killed by cervical dislocation. The brains were removed, frozen and stored at −20°C. Immediately prior to use, 50–100 mg of the thawed tissue was placed in a polypropylene tube, ice-cooled acetic (0.5 ml, 2 *M*) was added, and the mixture was homogenised for 30 s using an IKA Ultraturrax miniature turret probe (IKA, Staufen, Germany) at high speed (setting 7). The probe was washed with acetic acid (2 × 0.5 ml, 2 *M*), and the washes and the homogenate were combined. The combined mixture was centrifuged at 15 000 *g* for 30 min, and the supernatant was removed, freeze-dried and stored at −20°C. The freeze-dried tissue extract was resuspended in 1 ml of buffer (0.1 *M*, $Li_2CO_3$–$NaHCO_3$, pH 9.5) and a portion subjected to size-exclusion chromatography on a column of Sephadex G-25 (10 cm × 1 cm I.D.) using the same buffer. Fractions were collected and derivatised immediately.

Human CSF was collected in polypropylene tubes containing 20 μl of formic acid (90%) per tube and stored at −20°C in 2-ml aliquots until required. On thawing, a portion of the CSF (2 μl) was derivatised, and 80 μl were injected into the HPLC system.

*Derivatisation*

Stock solutions of MPF were made up in water at a concentration of 10 mg/ml and diluted 10- and 100-fold in water to provide solutions of 1.0 and 0.1 mg/ml. These were further diluted as necessary. Calibration standards were prepared by adding appropriate volumes (2–20 μl) of the appropriate concentration of stock or sub-stock solution to buffer to give a final volume of 100 μl. FMOC-Cl (4 m*M*) in acetone, acetonitrile or acetone–acetonitrile (1:1, v/v) (100 μl) was added to an equal volume of a solution of the standard peptide or unknown in buffer (0.1 *M* $Li_2CO_3$–$NaHCO_3$, pH 9.5), and the sample was mixed and allowed to stand for 10 min at room temperature. Water (100 μl) and ethyl acetate (2 ml) or *n*-pentane (1 ml) were then added, and the tube was vortex-mixed for 30 s. A portion of the aqueous layer (100 μl) was removed, and acetonitrile was added to a final concentration of 45% in the case when acetone was used as solvent. This ensured that all the FMOC-derivatised peptide remained in solution in the acetone-depleted aqueous layer, and that the composition of the sample to be injected was as close as possible to the initial HPLC solvent conditions. The derivatised peptide solution (10–80 μl) was then injected into the HPLC system.

*Mass spectrometry*

Peaks corresponding to FMOC-derivatised peptides that eluted from the HPLC system were collected, dried *in vacuo* and subjected to fast atom bombardment mass spectrometry (FAB-MS) (VG7070E, VG Analytical, Manchester, UK) using a thioglycerol matrix to obtain structural (molecular mass) confirmation of the peak identities.

*Slow-release formulations of Ac-Lys-D-Lys-Sar-Glu*

The MPF analogue Ac-Lys-D-Lys-Sar-Glu (69.9 mg) and poly(*dl*-lactide-co-glycolide) having a relative molecular mass (weight average) of 6000 [12] (1.33 g) were dissolved in anhydride-free glacial acetic acid (10 ml). The solution was frozen and freeze-dried for 18 h. The resulting residue was dried for 5 h *in vacuo* at 50°C and then compression-moulded at 60°C to provide the slow-release polymer formulation in the form

of a film of approximately 0.5 mm thickness. The theoretical level of incorporation of the analogue in the formulation on a w/w basis was 5%.

Five portions of the formulation were weighed and placed in polypropylene tubes with 1 ml of phosphate-buffered saline (0.1 *M* $Na_2HPO_4$–$NaH_2PO_4$, pH 7.4, and 0.14 *M* NaCl). The suspension was kept at 37°C, with occasional stirring, for fourteen days. Aliquots of the buffer were removed at intervals and replaced by an equal volume of fresh buffer. All aliquots were immediately frozen at −20°C prior to assay.

Accurel microporous polypropylene tubing has been shown to absorb MSH and experiments were conducted as described for MSH [13]. The tubing was first placed in absolute ethanol under reduced pressure (to remove air), and washed thoroughly with 0.9% saline. The washed tubing was then shaken for various time periods at ambient temperature with a solution (5 mg/ml) of the MPF analogue in 0.9% saline. Evidence for the absorption was sought by removing pieces of the tubing, stirring the pieces with fresh 0.9% saline (200 μl), and analysing aliquots by means of the assay.

## RESULTS AND DISCUSSION

### *HPLC conditions*

Initial choices of elution solvents and type of column chemistry were influenced by previous work reported in the literature and a preference for volatile non-agressive solvents to simplify recovery and subsequent MS of the peptides. Variations in elution conditions and the use of different adsorbents resulted in large differences in separation and peak shape of the various FMOC-peptides and reaction by-product (FMOC-OH). Using an acetonitrile–water–TFA solvent system, silica, diol and $C_{18}$ columns proved unsuitable as they gave broad and overlapping peaks. Improved separation was seen with a Merck RP-8 matrix, but a Spherisorb $C_6$ material was finally chosen as giving the best separation of the columns tested. A typical HPLC elution profile of five derivatised peptides is shown in Fig. 2. The fluorescence peaks correspond to (1) Gly-Gln, (2) Gly-Glu, (3) FMOC-OH, (4) Ac-Lys-D-Lys-Sar-Glu, (5) Lys-Lys-Gly-Glu and (6) Tyr-Lys-Lys-Gly-Glu. Optimum detection was obtained at an excitation wavelength of 260 nm and 315 nm for emission.

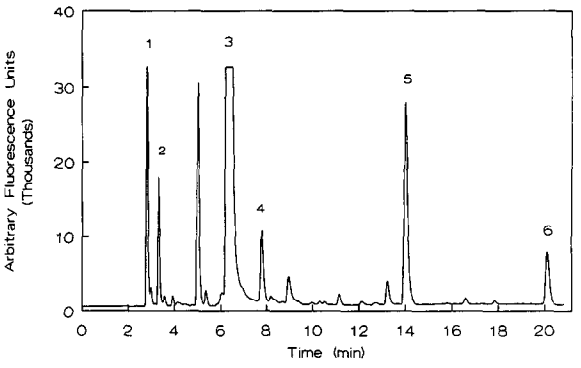


Fig. 2. Elution profile of five derivatised peptides: (1) Gly-Gln; (2) Gly-Glu; (3) FMOC-OH; (4) Ac-Lys-D-Lys-Sar-Glu; (5) Lys-Lys-Gly-Glu; (6) Tyr-Lys-Lys-Gly-Glu. The peptides were dissolved in buffer (0.1 *M* $Li_2CO_3$–$NaHCO_3$, pH 9.5) and the mixture was derivatised (see Experimental) with acetonitrile as the solvent for FMOC-Cl and *n*-pentane (1 ml) for the extraction. A 10-μl aliquot of the aqueous layer (equivalent to 2.5 ng of underivatised peptides) was injected into the HPLC system.

It has been shown that alterations in the time the sample spends in the sample loop of the HPLC injection system can cause non-reproducible sample losses [14]. Variability can also arise from differences in operator technique, for example syringe handling. To minimise these variations, an autoinjector with a 10-μl loop overfilled by a factor of 5 or a 200-μl loop filled to less than 100 μl was used.

### *Identification of peaks by mass spectrometry*

MPF has three amino groups all of which were expected to react with FMOC-Cl. It was important to establish that the reaction was reaching completion in a reproducible manner and to determine that the tris-FMOC peptide was formed. Only one peak corresponding to the derivatised peptide was identified on HPLC under optimum conditions. The peak area bore a linear relationship to the mass of peptide present before derivatization (Fig. 3). FAB-MS of this peak (following collection and evaporation to dryness) in a

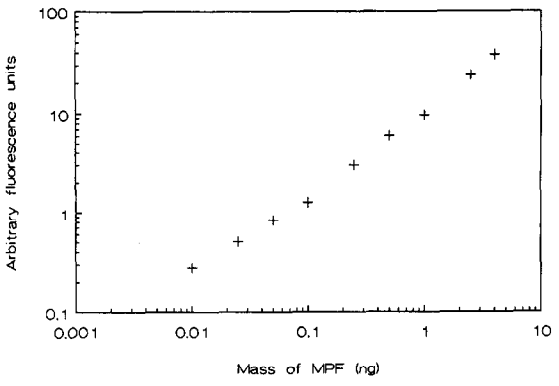


Fig. 3. Linear response of the HPLC–fluorescence system to the mass of MPF assayed. MPF was derivatised (see Experimental) with acetonitrile as the solvent for FMOC-Cl and *n*-pentane (1 ml) for the extraction. A 10-μl aliquot of the aqueous layer was injected into the HPLC system. Areas under the peaks were determined using a JCL6000 integrator.

thioglycerol matrix confirmed its indentity as tris-FMOC-MPF. As shown in Fig. 4, it gave rise to a parent ion of *m/z* 1127.69 corresponding to the predicted M + 1 (1127.46) of the tris-FMOC peptide. The ion of *m/z* 906.15 corresponds to the molecular ion less one FMOC group (mass 223.2) presumably arising from the tris-FMOC peptide by cleavage of one FMOC group and a concomitant transfer of two hydrogens to the residual fragment containing two FMOC groups.

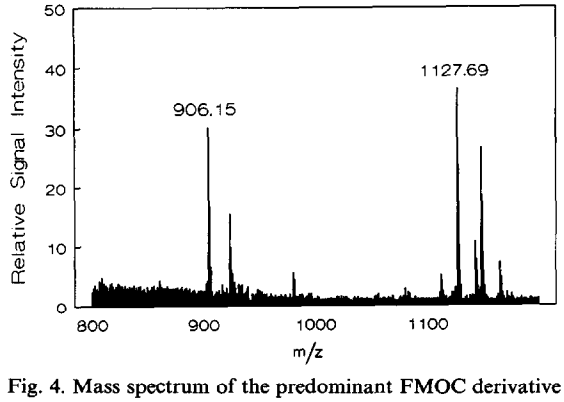


Fig. 4. Mass spectrum of the predominant FMOC derivative of MPF collected from HPLC. The ion of *m/z* 1127.69 corresponds to tris-FMOC-MPF. MPF was derivatised as described in the text and separated from FMOC-OH and other impurities by HPLC as described. The peak eluting at 14 min was collected, dried and subjected to FAB ionisation MS from a thioglycerol matrix as described.

*Effects of reaction time*

For amino acids the literature suggests a reaction time of up to 1 min for complete derivatisation of the sample [6,9] although longer reaction times have been used [15]. The reaction of angiotensin II with FMOC-Cl led to a fluorescence maximum of the main product after a reaction time of 30 s, which reduced to half the original value after 5 min [11]. For the series of peptides in this study, reaction times to achieve a single, stable fluorescence maximum differed from less than 1 min for Gly-Glu to 5 min for MPF.

*Effects of extraction solvent and FMOC-Cl solvent*

For a satisfactory analysis it was essential to remove FMOC-OH, produced by the reaction of FMOC-Cl with water, from the mixture. This highly fluorescent molecule elutes with a retention time of 6 min on the chosen HPLC system and unless removed it dominated the HPLC fluorescence trace, tailing and masking some derivatised peptide peaks. In most cases, for example with amino acids, efficient removal of FMOC-OH from the derivatised peptide mixture can be achieved by extraction with *n*-pentane or ethyl acetate. However, in the case of MPF, the tri-derivatised peptide is more hydrophobic and a significant proportion can be extracted in the organic layer. Therefore the effects of FMOC-Cl solvent, extraction solvent and pH on the partition between the organic and aqueous phases were studied to ensure a reproducible assay.

Four extraction solvents, ethyl acetate, *n*-pentane, chloroform and hexane, were evaluated. Chloroform and hexane were least effective at extracting only FMOC-OH. Ethyl acetate was highly efficient at removing the excess FMOC-OH. However, ethyl acetate extracted a significant proportion of the derivatised peptides into the organic layer, causing loss in reproducibility and sensitivity. These effects were compounded by the solubility of water and acetone in ethyl acetate, which resulted in a variable aqueous volume. Acetone depletion of the aqueous layer also caused some precipitation of the derivatised peptides, with further reduction in sensitivity.

It has been shown that variability in the yield of lysine can be reduced by using acetone–acetonitrile (1:1, v/v) as the solvent for FMOC-Cl in the derivatisation reaction [14]. This was also effective in counter-acting the variable loss of MPF due to precipitation. However, the aqueous volume still varied. The use of acetonitrile alone had a further advantage in that it is almost completely immiscible with *n*-pentane. This resulted in a constant aqueous volume after extraction. Table I shows the variability in the assays of a mixture of MPF and Ac-Lys-D-Lys-Sar-Glu, with *n*-pentane and ethyl acetate extractions and the use of alternative solvents for the FMOC-Cl reaction. It is apparent that extraction with *n*-pentane provided much better recovery and sensitivity than ethyl acetate, as well as reducing the variability in the assays. This was due to the fact that the volume of the aqueous layer was no longer variable. Similar findings were observed with acetonitrile as solvent for FMOC-Cl.

*Effects of temperature*

We investigated the effect of temperature on HPLC fluorescence yield, since it has been shown [16] in some cases to be highly temperature-dependent. We held the cell at 0 or 50°C by running the column at 0 and at 50°C with minimum tube length between column and cell, and found no changes in the sensitivity of the assay.

*Limits of detection of the assay*

Limits of detection of 25 fmol have been claimed for α-amino acids using FMOC derivatisation with on-column fluorescence detection and capillary columns [14], and limits of 26–61 fmol for secondary amines under normal HPLC conditions [17]. Application of the technique in hormone analysis has provided higher limits of detection (500 fmol). For the MPF assay, the smallest peak of pure derivatised material that could be reliably integrated at a signal-to-noise ratio of 5:1 corresponded to 25 fmol of peptide. The limits of detection for Lys-Lys-Gly-Gln and Ac-Lys-D-Lys-Sar-Glu were the same. Thus, for the peptides under study, which carry more than one FMOC-derivatisable group, the limit of detection is the same or higher than that found for amino acids. It should be noted that these limits of detection are not limits of quantitation of MPF from a biological matrix as obtained by an assay validation procedure involving determination of the precision and accuracy of the assay at a number of concentrations spiked into the sample matrix. The limits of detection are the quantities of peptide as injected onto the column. It

TABLE I

INTRA-ASSAY VARIABILITY

The generalised conditions for derivatisation (see Experimental) were applied to a mixture of MPF (50 ng) and Ac-Lys-D-Lys-Sar-Glu (50 ng). After extraction, 10 μl of the aqueous layer (equivalent to 2.5 ng of each peptide) were injected into the HPLC system, and the peak corresponding to each peptide was identified and integrated. Peak area is expressed as integrated fluorescence (area under the curve, arbitrary units), and the recovery is normalised to that obtained using acetonitrile–acetone (1:1, v/v) as solvent and *n*-pentane for the extraction (= 1.00). Values in parentheses are coefficients of variation.

| Extraction solvent, reaction solvent | *n* | Ac-Lys-D-Lys-Sar-Glu | | Lys-Lys-Gly-Glu | |
|---|---|---|---|---|---|
| | | Peak area | Normalised recovery | Peak area | Normalised recovery |
| Ethyl acetate, acetone | 5 | 114 060 ± 17 347 (15.2%) | 0.58 | 47 517 ± 12 720 (26.8%) | 0.131 |
| *n*-Pentane, acetonitrile–acetone (1:1, v/v) | 6 | 195 777 ± 3967 (2.0%) | 1.00 | 361 615 ± 5386 (1.5%) | 1.00 |
| *n*-Pentane, acetonitrile | 5 | 129 950 ± 1093 (0.8%) | 0.66 | 323 625 ± 6601 (2.0%) | 0.895 |

would be expected that the limit of quantitation from a biological matrix of a validated assay based on this method would be at least five-to ten-fold higher.

*Inter- and intra-assay variability*

Table I shows the normalised response of the assay system to a mixture of MPF and Ac-Lys-D-Lys-Sar-Glu using three combinations of extraction and derivatisation solvent.

Using a single extraction with ethyl acetate (2 ml), and acetone as the solvent for FMOC-Cl, the intra-assay variability for the peptides was in the range 15–30%. An improvement resulted when acetonitrile–acetone (1:1, v/v) or acetonitrile alone were used as solvent, and the extraction was done with *n*-pentane (variability 1.5–2 and 0.8–2%, respectively). The effective recovery in the case of acetonitrile–acetone (1:1, v/v) was somewhat higher than that in the case of acetonitrile alone, because acetone is soluble in *n*-pentane. This reduces the volume of the aqueous layer and concentrates the sample.

Table II shows the inter- and intra-assay variability for the same two peptides using acetonitrile as the derivatisation solvent and 2 ml of *n*-pentane for the extraction. The intra-assay variability showed a maximum of 3.5% (mean 2.3%) for Ac-Lys-D-Lys-Sar-Glu and a maximum of 4.2% (mean 2.4%) for Lys-Lys-Gly-Glu. This fits well with the results for the amino acids obtained by Einarsson *et al.* [14] (average 6.6% variability based on peak height) (7.1% for lysine), and for the octapeptide angiotensin II [10].

TABLE II

INTER- AND INTRA-ASSAY VARIABILITY

A stock solution of MPF (1 ng/μl) and Ac-Lys-D-Lys-Sar-Glu (1 ng/μl) in 3 ml of $Li_2CO_3$–$NaHCO_3$, pH 9.5, was divided into a total of twenty-five aliquots of 100 μl each and frozen at −20°C. On successive days, five aliquots were thawed and treated as described in Table I using pure acetonitrile as solvent for FMOC-Cl and *n*-pentane (2 ml) for the extraction. Each injection, 10 μl from the aqueous layer, was equivalent to 2.5 ng of each peptide. Values in parentheses are coefficients of variation.

| Assay No. | Ac-Lys-D-Lys-Sar-Glu | Lys-Lys-Gly-Glu |
|---|---|---|
| 1 | 138 073 ± 4324 (3.1%) | 366 882 ± 15 471 (4.2%) |
| 2 | 149 009 ± 5246 (3.5%) | 383 321 ± 12 399 (3.2%) |
| 3 | 139 739 ± 3157 (2.3%) | 374 074 ± 5104 (1.4%) |
| 4 | 134 439 ± 2076 (1.5%) | 357 570 ± 3834 (1.1%) |
| 5 | 129 610 ± 1372 (1.1%) | 327 487 ± 6406 (2.0%) |
| Overall | 138 174 ± 6437 (4.7%) | 361 867 ± 19 157 (5.3%) |

It has not been necessary to use an internal standard in this assay in order to obtain these coefficients of variation. This is perhaps surprising in view of the need for an extraction, but has not been found necessary by others who have used the method for amino acids. It would be possible to use MPF itself as an internal standard when the stable analogue were being assayed in non-human tissue, and *vice versa.*

*Application of the assay to slow-release formulations*

Slow-release formulations of Ac-Lys-D-Lys-Sar-Glu were required to investigate the effect of chronic administration of this peptide *in vivo.* Accurel tubing was unable to adsorb and release any of the peptide under conditions previously employed successfully with MSH. Consequently, a copolymer slow-release formulation was developed. Fig. 5 shows the rate of release of the stable analogue from the copolymer formulation, corresponding to 0.015–0.02 mg/h during days 3–10 and the release was complete after thirteen days. This corresponds to a total release of 4.1% (w/w) of the stable MPF analogue, compared with a 5% (w/w) theoretical incorporation.

*Application of the assay to human cerebrospinal fluid and rat brain extracts*

Human CSF was diluted in buffer (0.1 *M* $Li_2CO_3$–$NaHCO_3$, pH 9.5) and derivatised as described above. Fig. 6a shows a typical elution profile of CSF and Fig. 6b shows a CSF sample spiked with 50 ng of MPF. As can be seen, there is a very small peak eluting at the same retention time as authentic MPF in the unspiked sample. We have found similar results when *post-mortem*

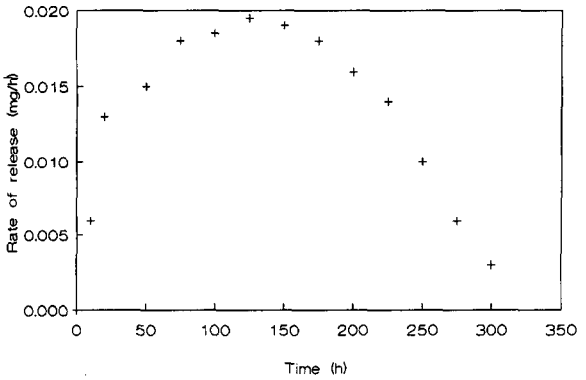


Fig. 5. Rate of release of Ac-Lys-D-Lys-Sar-Glu from its copolymer formulation. The peptide was incorporated in a slow-release copolymer which was then weighed and allowed to release into aqueous solution. Aliquots were removed and stored frozen until the end of the experiment. These aliquots were then derivatised as described in the text and analysed by HPLC. The release was calculated as rate of release per hour.

human brain tissue is derivatised in the same way, and we assume this material to be MPF. Experiments are currently under investigation in order to confirm this by MS, both in the case of human samples and in rat tissue extracts. In the rat brain experiments (data not shown), there is a peak eluting at the retention time of Lys-Lys-Gly-Glu which represents the equivalent sequence in the rat, and none where human MPF elutes.

## CONCLUSIONS

We have demonstrated that it is now possible to assay MPF and a series of structurally related peptides (Ac-Lys-D-Lys-Sar-Glu, Lys-Lys-Gly-Gln, Gly-Glu, Gly-Gln) by derivatisation with FMOC-Cl followed by reversed-phase HPLC separation of the reaction products with fluorescence detection. These peptides are of great interest because of their neurotrophic and other important actions. Other techniques, such as radioimmunoassay, proved not to be applicable due to the low immunogenicity of the peptide. The method is reproducible and has a limit of detection of 25 fmol injected peptide. We are currently using this assay to investigate both the tissue distribution and metabolism of these peptides

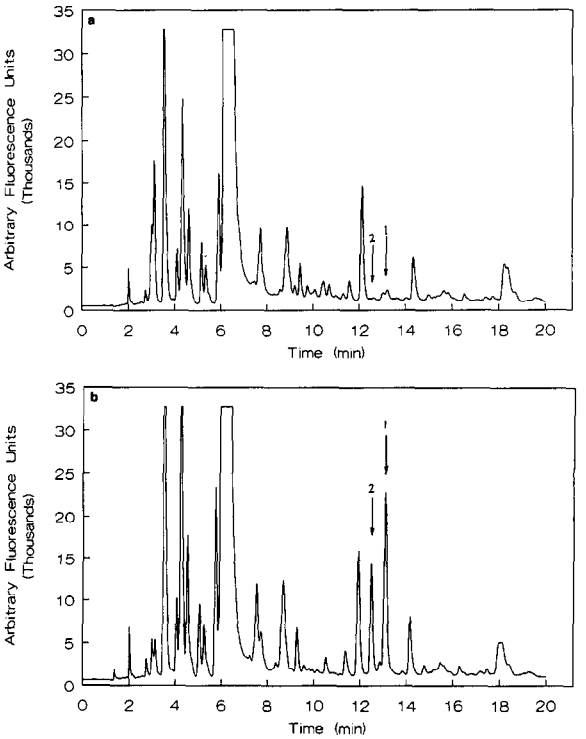


Fig. 6. (a) Elution profile of derivatised CSF. Human CSF (2 μl) was derivatised as described in the text. (b) A second aliquot was spiked with 50 ng each of MPF and Lys-Lys-Gly-Gln and derivatised. Each sample was run on HPLC. Peaks: 1 = MPF; 2 = Lys-Lys-Gly-Gln.

following their administration by various routes and also to determine the endogenous levels of MPF in the various regions of human CNS.

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