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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HORMONAL PEPTIDES AND THEIR FLUORENYLMETHOXYCARBONYL DERIVATIVES

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SUMMARY

Reference methods for the quantitation of peptide and protein hormones in blood are urgently needed and high-performance liquid chromatography (HPLC) is potentially applicable. Owing to the low concentrations of these substances in body fluids, very low detection limits have to be achieved. This can be done on the one hand by reducing the diameter of the separation column and increasing the number of theoretical plates, and on the other by derivatization. Angiotensin II was chosen as a model compound. Reduction of the inner diameter from 4 to 2 mm increased the peak height by a factor of 3.4 (theoretical value 4.0). The peptide was derivatized with 9-fluorenyl methylchloroformate in lithium carbonate–sodium hydrogencarbonate or sodium borate buffer at different pH values. The precision (coefficient of variation) was 10.4%, the linear range 1:40 (r = 0.998) and the detection limit 500 fmol of derivative on-column. The volume injected was 2 μl. However, this is not sufficiently sensitive for the quantitation of most peptide hormones using an acceptable maximum specimen volume of 5 ml of serum or plasma.

INTRODUCTION

Peptide and protein hormones are determined in clinical chemistry almost exclusively by immunoassay methods such as radioimmunoassay. These methods show a sufficient performance in the terms of analytical criteria for routine use, but they do not fulfil the accuracy requirements for reference methods[1,2].

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Accuracy is always also a function of specificity, and the latter depends on the quality of the antibodies used. Cross-reaction of the antibodies with chemically similar compounds or immunologically still reactive degradation products is a major source of erroneous results in immunoassays. An antibody molecule detects only up to six neighbouring amino acids or sugar moieties[3] and not the whole molecule. Therefore, the accuracy of a result always remains questionable to a certain extent.

For the very accurate quantitation of steroid hormones, gas chromatography–mass fragmentography with isotope dilution is the method of choice. A more specific determination of peptide or protein hormones should be possible using high-performance liquid chromatography (HPLC) combined with a specific detection method such as fluorimetry or chemiluminescence. For this application, a very low detection limit is necessary owing to the low concentrations of these substances in body fluids. The range found in normal subjects varies from 1000 ng/ml for human placental lactogen to 0.001 ng/ml for antidiuretic hormone. For a typical specimen volume of 50–100 μl in immunoassays, the amounts to be detected lie in the lower picomole to attomole range.

Commonly, the sample volume used for a reference method should not exceed 5 ml. Therefore, the demands for the detection limit of such a method are very high and certainly not realizable at present for hormones with very low concentrations in blood. Lowering the detection limit in HPLC can be achieved on the one hand by reducing the separation column diameter, by increasing the number of theoretical plates and by optimizing the capacity factor, and on the other by preparing derivatives with large detection signals and useful chromatographic behaviour. Therefore, we used inner diameters of 2 mm with different stationary phase packing materials [normal (4–15 nm) and wide-pore (30–400 nm)] and prepared fluorescent derivatives of the hormones for detection. Our first results with angiotensin II are presented here.

EXPERIMENTAL

Experiments were performed in close collaboration with the research and development group of Merck (Darmstadt, F.R.G.). In both laboratories identical HPLC equipment was used.

Reagents

If not stated otherwise, all reagents were of analytical-reagent grade from Merck. Acetonitrile was of HPLC grade. Acetone was distilled over fluorenyl methylchloroformate (FMOC-Cl) (Fluka, Buchs, Switzerland) before use to remove interfering substances. ^{5}L-Ile-angiotensin II (MW 1046) was purchased from Serva (Heidelberg, F.R.G.). The radioactively labelled hormone ([3,5-$^{3}H_2$]tyrosyl; 1 Bq/mmol) was obtained from NEN Research Products (DuPont, Dreieich, F.R.G.).

Buffer A was sodium borate buffer (200 mmol/l) adjusted with 1 mol/l sodium hydroxide solution to pH 6–8. Buffer B was lithium carbonate–sodium hydrogencarbonate buffer (100 mmol/l), pH 8.5–10.

Equipment

A Model 1090 liquid chromatographic apparatus (Hewlett-Packard, Waldbronn, F.R.G.) was used.

Separations of derivatives were carried out with LiChrospher RP-2 (5 μm particle diameter) columns (125 × 2 mm I.D.) (Merck) under isocratic conditions at room temperature (23°C). The eluent was acetonitrile–10 mmol/l NaH_2PO_4 (pH 4.9) (54:46) at a flow-rate of 1.0 ml/min.

For detection, a Model F 1000 fluorescence spectrophotometer with a 12-μl flow cell was used (Merck-Hitachi, Darmstadt, F.R.G.). The excitation wavelength was 260 nm, the emission wavelength 310 nm and the band width 15 nm.

Method

A 5-μl volume of an aqueous solution of angiotensin II (0.5–20 nmol) was transferred into a conical polypropylene tube, dried with a stream of nitrogen and dissolved in 100 μl buffer A or buffer B as indicated in the next section, depending on the pH value desired. Then 100 μl of FMOC-Cl in acetone (15 mmol/l) were added and the reaction mixture was allowed to stand for 30 s at room temperature. FMOC-Cl, its hydrolysis product 9-hydroxymethylfluorene and acetone were extracted with five portions of 500 μl of *n*-pentane and the organic phases discarded. A 10-μl volume of the aqueous phase was diluted with 90 μl of NaH_2PO_4 solution (10 mmol/l) and 2 μl were injected into the HPLC column. Dilution was necessary as the vials of the automatic sampler had to be filled with at least 100 μl of sample.

RESULTS AND DISCUSSION

Column diameter and packing material

For a given amount of analyte, the smaller the peak width and consequently the higher the peak, the better is the detection sensitivity[4]. According to chromatographic theory[4], peak height is inversely proportional to the cross-sectional area. The height equivalent to a theoretical plate (HETP) has a smaller effect on the detection limit, and correlates inversely with the square root of the column length (eqn. 1)[4].

$$[c_i^{\mathrm{m}}]_{\mathrm{Lmax}} = \frac{Q_i}{\sqrt{2\pi}\,\varepsilon_{\mathrm{m}} A \sqrt{L H_i}\,(1 + \kappa_i)} \quad (1)$$

where $[c_i^{\mathrm{m}}]$ = maximum peak concentration of compound i in the detector; Q_i = amount of compound i injected; $\sqrt{2\pi}$ = shape factor of a Gaussian peak; A = column cross-sectional area; ε_{m} = fraction of column volume taken up by the mobile phase; H_i = HETP for a compound i; L = column length; κ_i = capacity factor of compound i.

Previously we tested various support materials concerning their properties for peptide separation. A mixture of underivatized non-hormonal oligopeptides was used to test the column performance and selectivity. They could be well separated within one run on narrow-pore Superspher RP-8 (Fig. 1). For angiotensin II, a reduction of the inner diameter from 4 to 2 mm led to an increase in the peak height, with a consequent reduction of the detection limit, by a factor of 3.4 (theoretical value 4.0 according to eqn. 1).

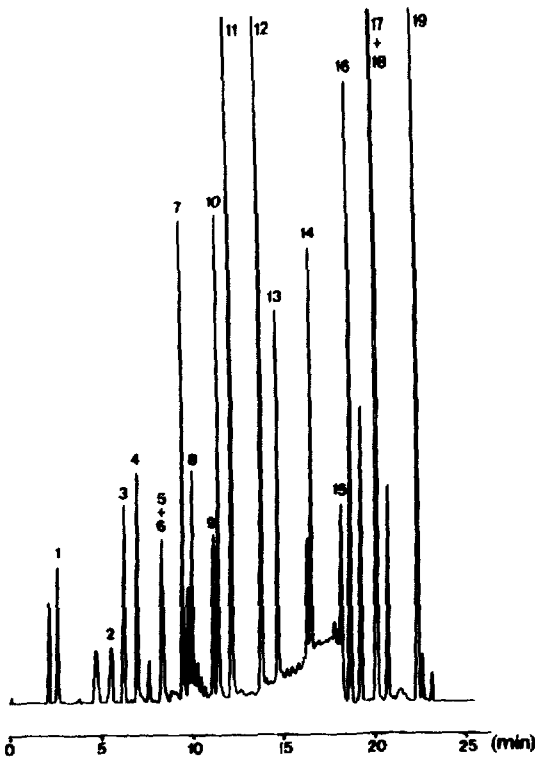

Fig. 1. Separation of 19 underivatized peptides on Superspher RP-8, 3 μm (250 × 4 mm I.D.). Flow-rate, 1 ml/min; room temperature; UV detection (210 nm); 100% C to 50% D within 20 min. C = 0.35 mol/l perchloric acid (pH 0.5); D = acetonitrile. Compounds: 1 = Gly-Gly-Gly-Gly; 2 = Ala-Ala-Ala; 3 = Ala-Ala-Ala-Ala; 4 = Ala-Ala-Ala-Ala-Ala; 5 = Leu-Gly-Gly; 6 = Glu-Thr-Tyr-Ser-Lys; 7 = Glu-Thr-Tyr; 8 = Ala-Ala-Tyr-Ala-Ala; 9 = Gly-Gly-Leu; 10 = Gly-Phe-Gly; 11 = Gly-Gly-Phe; 12 = Tyr-Tyr-Tyr; 13 = Tetravaline; 14= Glu-His-Phe-Arg-Trp-Gly · HCL; 15 = Leu-Leu-Leu; 18 = Z-Pro-Leu-Gly-NH_2; 17 = Phe-Phe-Phe; 18 = Leu-Trp-Met-Arg-Phe; 19 = Phe-Phe-Phe-Phe.

Derivatization

Choice of the derivatization reagent. A variety of fluorescent labels have been described; in a recent review[5] 64 derivatizing reagents are listed. For amino acid HPLC analysis, fluorescamine, *o*-phthalaldehyde (OPA), phenylisothiocyanate (PITC), dansyl chloride, FMOC-Cl, 4-fluoro- and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole have been the most extensively used compounds. To our knowledge, however, there has been little work on the quantitation of fluorescent peptide derivatives by HPLC[6,7].

The derivatization reagent should be generally applicable for our purpose. Therefore, our interest was focused on reagents that derivatize amino and possibly even hydroxy groups. The intrinsic fluorescence sensitivity of the derivative should be as high as possible for the reasons outlined above. Therefore, only a few fluorescent derivatizing reagents were taken into consideration, namely OPA, dansyl chloride, fluorescamine, fluoresceine isothiocyanate (FITC) and 9-fluorenyl methylchloroformate (FMOC-Cl).

The last two reagents are known to have high quantum yields[5]. We chose

FMOC-Cl, which has been used for the HPLC analysis of amino acids by Einarsson *et al.*[8]. FMOC-Cl reacts with primary and secondary amines and, depending on the reaction conditions chosen, hydroxy functions are also reactive. Reaction of amino groups with FMOC-Cl results in the formation of the corresponding formyl amides. With hydroxy groups the corresponding formyl esters are formed. The derivatives show sufficient stability[5,8]. A disadvantage is that the excitation wavelength is in the UV range and the Stokes' shift is only about 50 nm.

Dependence of derivative formation on reaction time. The reaction of angiotensin II with FMOC-Cl is very rapid (Fig. 2). The highest peak area of the main product with a retention time of 5.2 min is reached after only 30 s. Subsequently the yield falls to half of the maximum. A second peak after 8.5 min shows no time dependence. Fig. 3 shows a chromatogram of the angiotensin derivative.

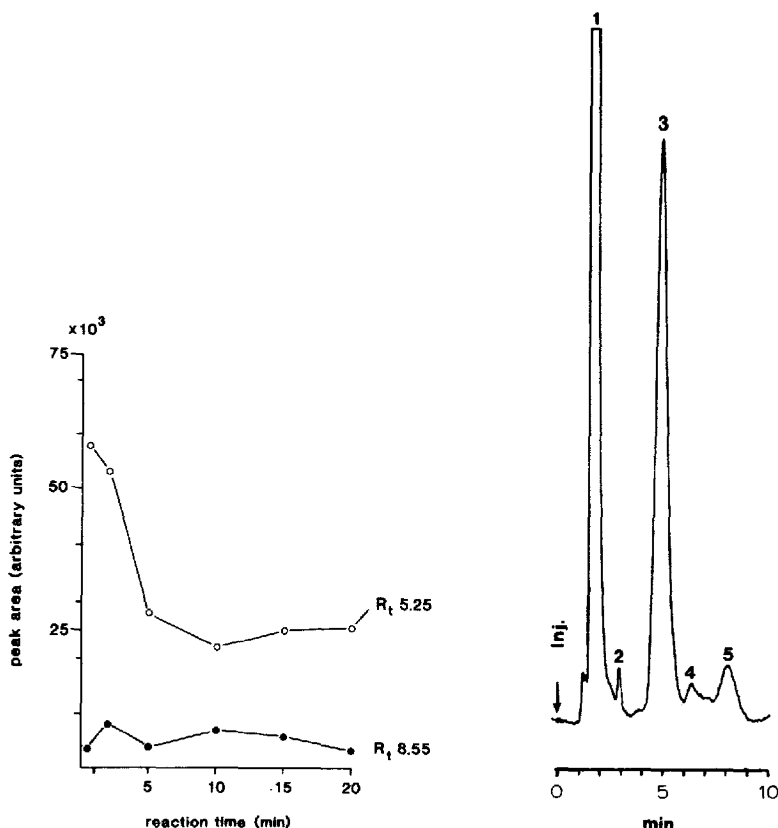

Fig. 2. Dependence of the reaction of angiotensin II with FMOC-Cl on reaction time (R_t = retention time).

Fig. 3. Chromatogram of angiotensin II derivative (20 pmol on-column). For conditions, see text. 1 = Hydroxymethylfluorene; 3 = angiotensin II derivative; 2, 4, 5 = unknown by-products.

Dependence of derivative formation on pH. The reaction of angiotensin II with FMOC-Cl shows a marked dependence on pH (Fig. 4). Three peaks appear that can be assigned to angiotensin derivatives. This could be explained by the different reactivities of the various functional groups of angiotensin. This peptide contains an α-

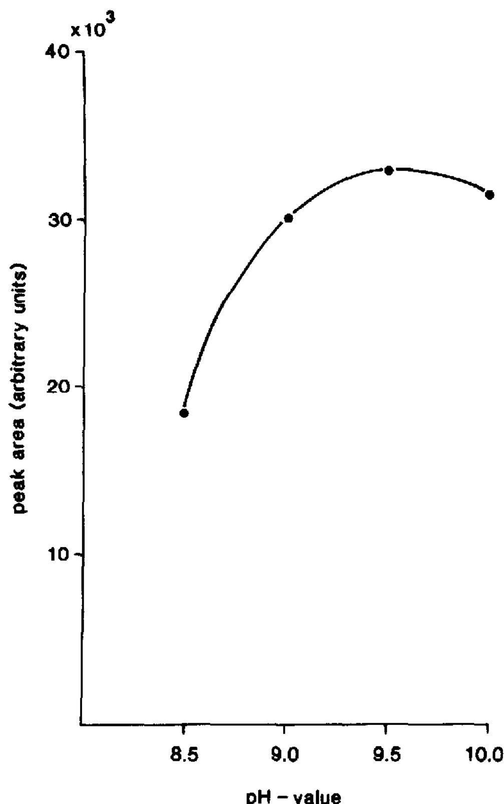

Fig. 4. Dependence of the reaction of angiotensin II with FMOC-Cl on pH. Conditions: 5 nmol of angiotensin II; sodium carbonate buffer; reaction time, 30 s; other conditions as in the text.

amino group at the N-terminal asparagine, one phenolic hydroxy group at the tyrosine moiety and one secondary amino group at the histidine moiety.

Because its buffer capacity is limited, it is not possible to use borate buffers above pH 8.5. Therefore, experiments were performed with a lithium carbonate buffer up to pH 10. A maximum yield was observed at pH 9.5, and subsequent experiments were carried out at this pH.

Extraction step

The extraction step was checked using radioactively labelled angiotensin II. After derivatization in each extraction step the transfer of radioactivity into the *n*-pentane phase was measured. As expected, the total loss was very small (only 2.8%). Probably this small amount does not correspond to the intact derivative, but rather to a small exchange of tritium under the reaction conditions.

Linearity

The relationship between amount of angiotensin II derivatized and the peak area is linear over a range of 1:40 (0.5–20 nmol) (r = 0.998). The excess of FMOC-Cl was 75- to 3000-fold relative to angiotensin II.

Repeatability

The coefficient of variation of the method is 10.4% (n = 9) at an angiotensin II concentration of 5 nmol per tube, corresponding to 10 pmol on-column.

Detection limit

The detection limit is 500 fmol of derivative injected with a signal-to-noise ratio of 3:1. Noise was measured as full amplitude. This is relatively high compared with the low femtomole range given for amino acids by Einarsson *et al.*[8]. This discrepancy cannot be explained at the moment.

CONCLUSIONS

These experiments with a reduced chromatographic column diameter and a consequent increase in the number of theoretical plates gave promising results. Derivatization with a strong fluorophore such as FMOC-Cl led to a decrease in the detection limit, but not to the extent needed in most cases for the proposed application. Therefore, we are pessimistic about reaching this goal by using normal fluorimetric detection. Perhaps the use of high-energy laser fluorimeters, although very expensive, could solve this problem.

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