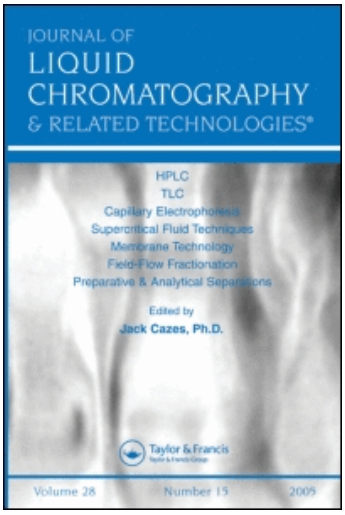
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Quantitative Determination of Polypeptides by Gradient Elution High Pressure Liquid Chromatography

M. E. F. Biemond[a]; W. A. Sipman[b]; J. Olivié[c]
[a] Corporate Research Department, Akzo Research Arnhem, Arnhem, The Netherlands [b] Diosynth B.V. (Akzo Pharma), Pilot Plant, Oss, The Netherlands [c] Research Development, Biochemistry Department, Organon, Serifontaine, France

To cite this Article Biemond, M. E. F. , Sipman, W. A. and Olivié, J.(1979) 'Quantitative Determination of Polypeptides by Gradient Elution High Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 2: 9, 1407 — 1435
To link to this Article: DOI: 10.1080/01483917908060146
URL: http://dx.doi.org/10.1080/01483917908060146

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 2(9), 1407-1435 (1979)

QUANTITATIVE DETERMINATION OF POLYPEPTIDES BY GRADIENT ELUTION HIGH PRESSURE LIQUID CHROMATOGRAPHY

M.E.F. Biemond*
Akzo Research Arnhem, Corporate Research Department,
P.O. Box 60, Arnhem (The Netherlands)

W.A. Sipman
Diosynth B.V. (Akzo Pharma), Pilot Plant,
P.O. Box 20, Oss (The Netherlands)

J. Olivié
Organon, Research Development, Biochemistry Department,
Eragny sur Epte, P.O. Box 6, 60590 Serifontaine (France)

Downloaded At: 19:27 24 January 2011

ABSTRACT

High pressure liquid chromatographic (HPLC) methods are described for the quantitative determination of polypeptides in pharmaceutical dosage forms or in synthetic and natural products.

Owing to the addition of an electrolyte for the inactivation of the residual silanol groups present on the modified silica surface, hydrophobic stationary phases were found which proved to be very suitable for the analyses of polypeptides.

Excellent results were obtained with tetramethylammonium containing buffer systems at pH 3 in a gradient of water-methanol. Thus the polypeptides arginine-, lysine vasopressin, oxytocin, ACTH (1-24), ACTH,

---

* to whom all correspondence should be addressed.

Copyright © 1979 by Marcel Dekker, Inc. All Rights Reserved. Neither this work nor any part may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, microfilming, and recording, or by any information storage and retrieval system, without permission in writing from the publisher.

β-endorphin, glucagon and porcine- and bovine insulin could be determined with coefficients of variation below 2%.

INTRODUCTION

In the past few years high pressure liquid chromatography (HPLC) has become increasingly important in the characterization of pharmaceutical substances. Especially the introduction of chemically bonded reversed phase materials has led to a tremendous increase in the applicability of this technique for the separation of amino acids and polypeptides.
The purity of polypeptides and the formation of by-products can be determined by different analytical techniques, like electrophoresis, isotachophoresis, ion exchange-, gel filtration- or thin layer chromatography[1] and recently by HPLC[2].

The purpose of this study was to develop a uniform HPLC-method for the characterization of the polypeptides arginine-, lysine vasopressin, oxytocin, ACTH (1-24), ACTH, β-endorphin[4], glucagon[5], bovine and porcine insulin[5], their by- and degradation products.

The use of chemically bonded phases on a silicagel matrix in high pressure liquid chromatography is well known for the characterization of pharmaceutical products[2,6]. The most widely used types are the reversed phase materials. A disadvantage of these materials, however, is their instability against higher (> 7.5) and lower (< 2) pH-values. A considerable decomposition can be observed for buffers of pH-values higher than 7 [8]. The support dissolves very slowly under these conditions, which is confirmed by the work of Wehrli[9].

Downloaded At: 19:27 24 January 2011

From the literature[10,11,12] it is known that RP-modified silica still contains some acidic silanol groups. Besides an ion-pair phenomenon, owing to certain additives (hydrophobic or hydrophilic modifiers), also an ion exchange effect can participate in the separation mechanism[13]. A sufficient deactivation of the unreacted silanol groups may be achieved when an electrolyte is added to the solvent as a modifier. The degree of deactivation depends on the concentration and the nature of the cation.

The use of ammonium-, alkylated ammonium-, potassium or sodium salts is described in the literature[8,9,15,16,17,18,27].
As shown by Buytenhuys et al.[14] the $TMA^{\oplus}$ cation is extremely effective in deactivating unreacted silanol groups in reversed-phase chromatography with chemically modified silicas. This system is applied by Van der Maeden et al.[12].

In this paper the performance of the tetramethylammonium ion as a modifier of RP-silica for the separation of polypeptides is described.
Earlier studies[12,19] have shown that gradient elution HPLC can excellently be used for accurate and rapid analyses.
Quantitative gradient-elution HPLC methods have been developed for arginine-, lysine vasopressin, oxytocin, ACTH (1-24), ACTH, β-endorphin, glucagon, porcine and bovine insulin.

EXPERIMENTAL

Apparatus

The gradient elution high performance liquid chromatograph consisted of two Waters 6000 pumps and a

Downloaded At: 19:27 24 January 2011

solvent programmer model 660 in conjunction with a Varian (Techtron 635), or Cecil 272 variable multiwavelength detector (set at 210 or 280 nm).

The samples were introduced by a Valco 7000 psi injection valve.

A computing integrator system (SP 4000, Spectra-Physics) was used for integration and data treatment.

During the investigation a 30-cm μBondapak C 18 column (Waters Assoc., Milford, Mass., USA) or two 30-cm Lichrosorb RP 18 (Merck, Darmstadt, Germany, 10 μm) columns in series, packed by a balanced-density slurry technique [14,20,21], and various gradients of water-methanol mixtures were used.

Downloaded At: 19:27 24 January 2011

Chemicals and Materials

In all experiments distilled water and reagent grade methanol and orthophosphoric acid (Baker, Deventer, The Netherlands) were used.
Tetramethylammonium hydroxide was applied as a 20% solution in methanol (Aldrich, Milwaukee, Wisc., USA).
In order to demonstrate the separation power of the HPLC-system, the polypeptides investigated were taken from Diosynth's production in various stages of the end purification.
The external standards were obtained by extra purification via chromatography, gel filtration or in case of tetracosactide, counter current distribution.
The β-endorphin originates from the Organon Organic Chemical Research Department.

Procedures

The gradient elution procedures were in all cases standardized as follows:

after running the appropriate gradient, followed by a reverse program of 5 min to initial conditions, the column was re-equilibrated for 10 min. A column temperature of 20°C was applied and the flow rate was 1 ml/min (pressure between 100-150 bar). Prior to use the solvents were degassed by vacuum with simultaneous stirring.

The samples were dissolved in the initital mobile phase or in aqueous acetic acid.

The following elution systems were used:

(i) for studying the influence of the pH and the buffer concentration of the mobile phase system: a linear gradient in 60 min from 20% B in A to 70% B in A and 30% B in A to 100% B for glucagon.

Solvent A was a 0.050 M solution of TMAH in water-methanol 50:50 (v/v) adjusted to a pH of 2-5 with orthophosphoric acid, and solvent B contained the same buffer at the same pH in water-methanol 20:80 (v/v).

For the experiments at pH <2 a linear gradient from 35% B in A to 100% B in 60 min was applied. Here the solvents were methanol-water 50:50 and 80:20 (v/v), respectively, adjusted to pH 0.5 and 2 with perchloric acid.

To study the influence of the buffer concentration, the same elution systems were used but with variable TMAH concentrations (0.005-0.05 M) and adjusted to pH 3 with orthophosphoric acid.

(ii) Quantitative Analysis

For the quantitative determination of the polypeptides linear gradients were applied with the solvent systems as given in Table 1.

Downloaded At: 19:27 24 January 2011

TABLE 1

polypeptide	gradient (v/v)	running time (min)
arginine vasopressin, lysine vasopressin, oxytocine	5% E in B → 70% E in B	30
tetracosactide, ACTH (1-24)	5% D in C → 70% D in C	45
porcine ACTH	50% D in C → 100% D in C	30
glucagon	50% A in D → 100% D	60
insulin (porcine and bovine)	20% A in D → 70% A in D	60
β-endorphin	30% D in C → 100% D	45

A = 50:50; B = 80:20; C = 75:25; D = 20:80;
E = 55:45 (v/v) water-methanol.

Downloaded At: 19:27 24 January 2011

In all solvents the TMAH-concentration had been made 0.050 M and the pH had been adjusted at pH 3 with orthophosphoric acid.

RESULTS AND DISCUSSION

In order to find the optimal conditions for the separation and quantitative determination of the polypeptides under investigation, a study was made of the effects on this separation of the pH, of the buffer (TMA-phosphate) concentration and of the stationary phase.

pH of the Mobile Phase

The pH of the mobile phase has a pronounced effect[8,9,11,13,22,23,24] upon the separation of the main- and by-products of polypeptides. Under basic conditions the reversed-phase materials and also some of the polypeptides mentioned are unstable.

On the other hand, insulin is insoluble near its isoelectric point (pI = 5.5). Hence we investigated the influence of the pH on the reversed-phase separation of polypeptides over the pH-range 0.5 - 5.

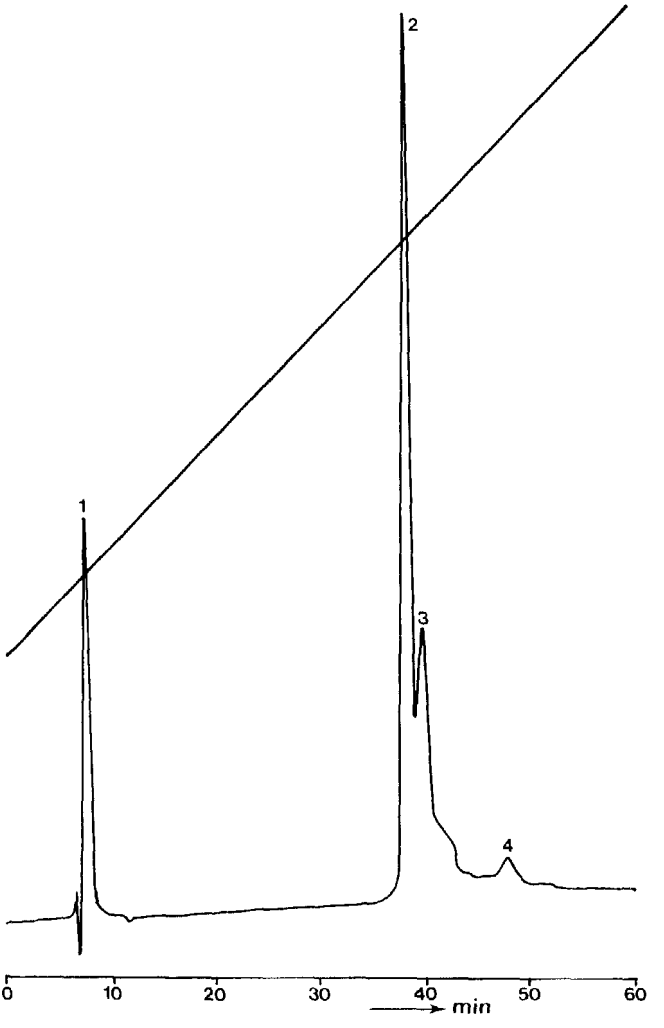
Under these acidic conditions the side-chain groups of lysine and arginine are protonated (pK_a ~ 10). At pH-values lower than about 2 all carboxylic acid groups (side chain and terminal) are undissociated.

At pH-values between 2 and 4 the β-carboxylic acid groups (side chain) are still undissociated, while the terminal carboxylic acid groups are dissociated[25,26]. The chromatograms in Fig. 1 show that this leads to a strong decrease in retention. Obviously, the hydrophilic character of the polypeptides increases from pH 2 to 4. The asymmetry and therefore the poor resolution between insulin and monodesamido insulin is probably due to the influence of the residual silanol groups present on the modified silica surface.

The replacement of perchloric acid by orthophosphoric acid has no influence on the retention (elution with the gradient at 63% (v/v) methanol). The better peak performance in the case of orthophosphoric acid is probably due to the higher acid concentration at pH 2 compared with that of perchloric acid.
The addition of acid to the eluent results in less interaction between the polypeptides and the remaining silanol groups, thus improving the peak shape[22].
The peak performance can be improved further by the addition of ammonium cations, especially TMA ions[12,14] which deactivate the remaining silanol groups of the RP-phase and perhaps also affect the carboxylic acid groups of the polypeptides, if working at pH > 2.

Downloaded At: 19:27 24 January 2011

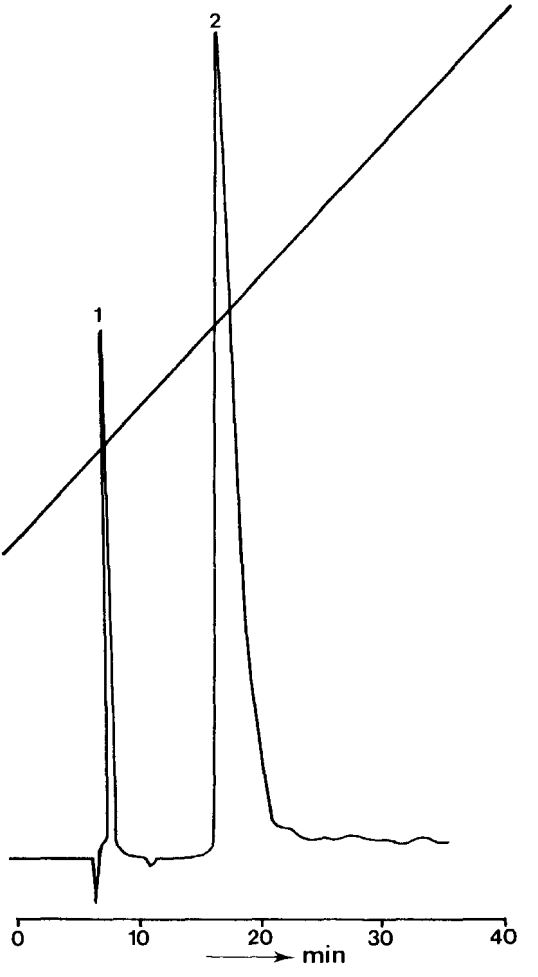
Downloaded At: 19:27 24 January 2011



(a)

The addition of the modifier tetramethylammonium phosphate to the eluent yields a better peak performance, but has no further influence upon the retention compared to the separation with the same solvent system without TMA-ions. Fig. 2.a, b shows this effect for insulin.

Downloaded At: 19:27 24 January 2011

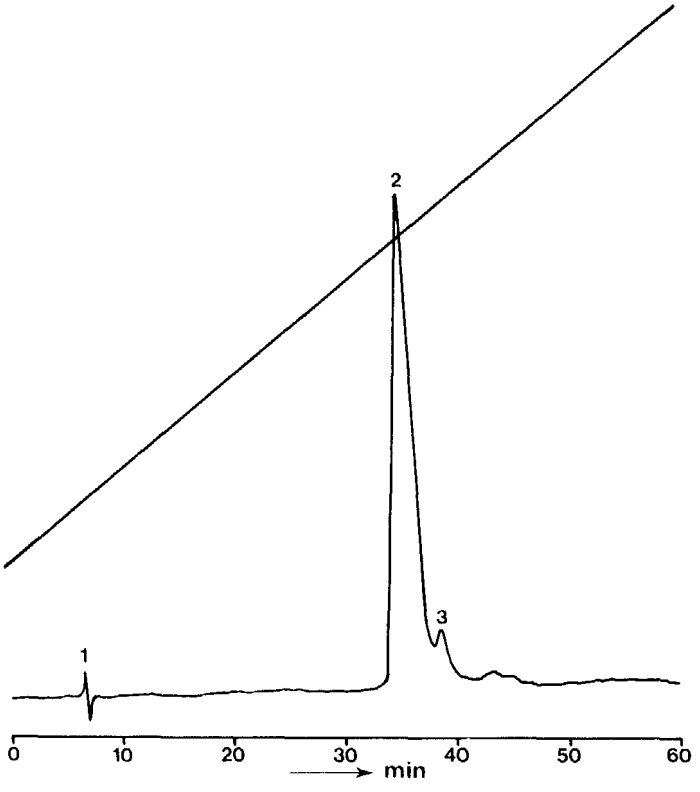


(b)

Figure 1: Gradient HPLC-separation of bovine insulin

a: with perchloric acid at pH 0.5,
b: perchloric acid at pH 2.

1 = solvent peak; 2 = bovine insulin;
3 = desamido insulin.



(a)

So the effect of tetramethylammonium cation can be twofold:

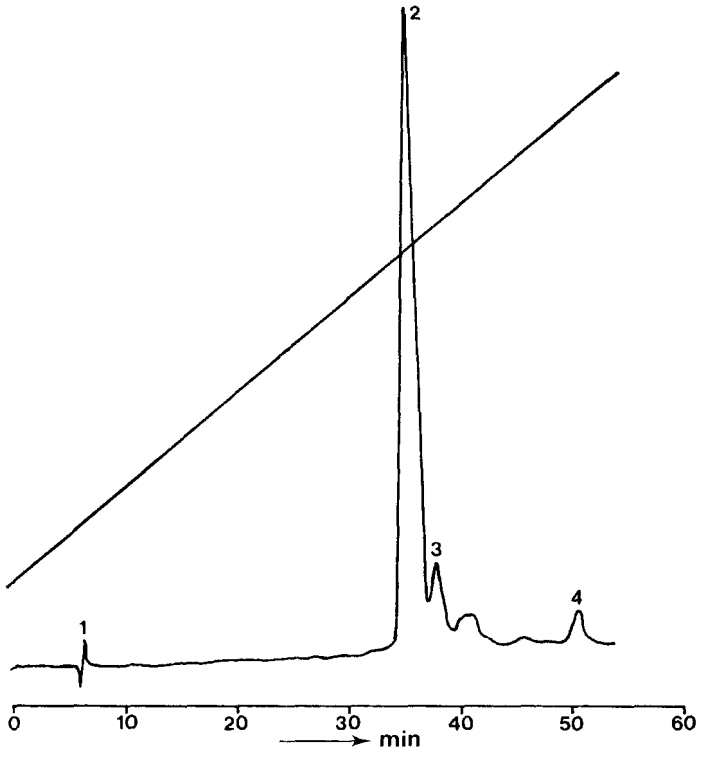
(i) reaction with the carboxylic acid groups present in polypeptides and

(ii) deactivation of the residual silanol groups on RP-modified silica surface.

The influence of the pH of the mobile phase on the resolution (R_s) of insulin-desamido insulin and glucagon-desamido glucagon is significant (Table 2).

In view of the life-time of the stationary phases and the possible degradation or fibrilization of poly-

Downloaded At: 19:27 24 January 2011



(b)

Figure 2: Gradient HPLC-separation of bovine insulin

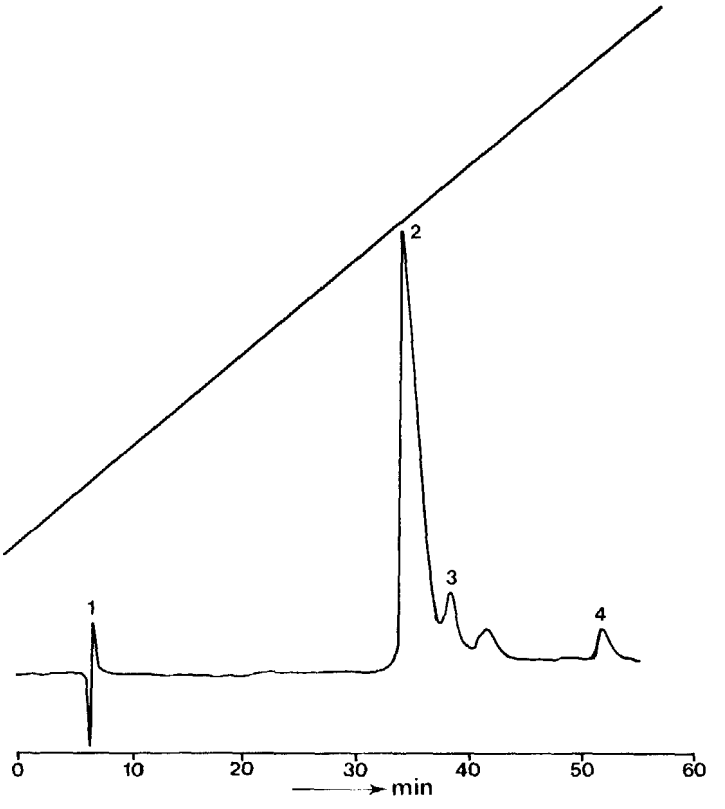
a: without TMAH; pH 2 (H_3PO_4),
b: 0.05 M TMA-phosphate (pH 2).

Further see Figure 1.

TABLE 2

pH	bovine insulin/ desamido insulin	glucagon/ desamido glucagon
2	1.11	1.80
3	1.11	1.80
4	0.98	1.33
5	0.98	1.31

Downloaded At: 19:27 24 January 2011



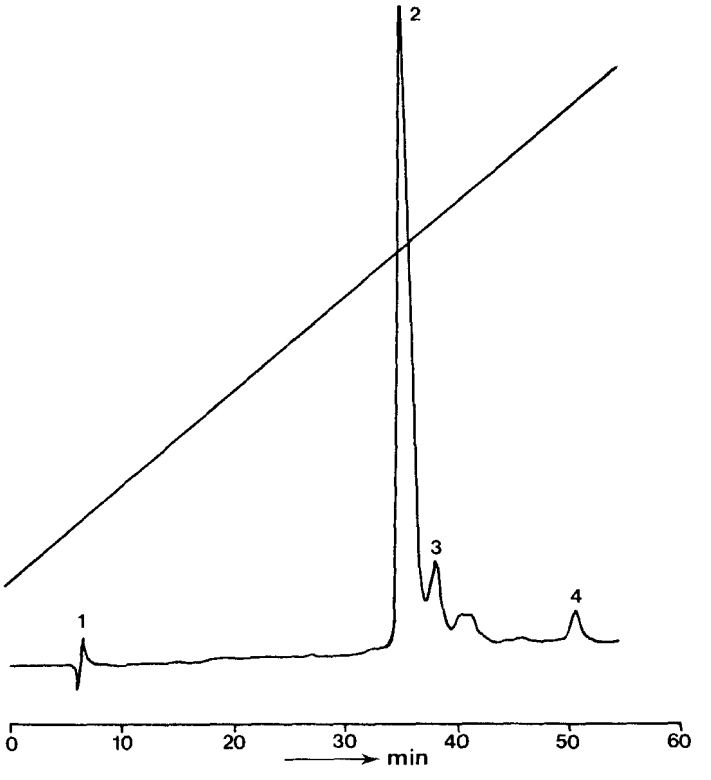
(a)

Downloaded At: 19:27 24 January 2011

peptides which can occur at lower pH's (< 2) a pH of 3 was chosen for the further investigations.

Concentration of the Buffer

Variation of the concentration of the tetramethylammonium phosphate buffer from 0.005 to 0.05 M at pH 3 has no influence upon the retention of insulin or glucagon. The peak performance improves with increasing buffer concentration up to 0.03 M (Figs. 3.a and 3.b). At higher buffer concentrations (0.03 - 0.05 M) no improvement is observed. So a further deactivation of



(b)

Figure 3: Gradient HPLC-separation of bovine insulin

a: with 0.005 M tetramethylammonium;
b: with 0.03 M tetramethylammonium.

Further see Figure 1.

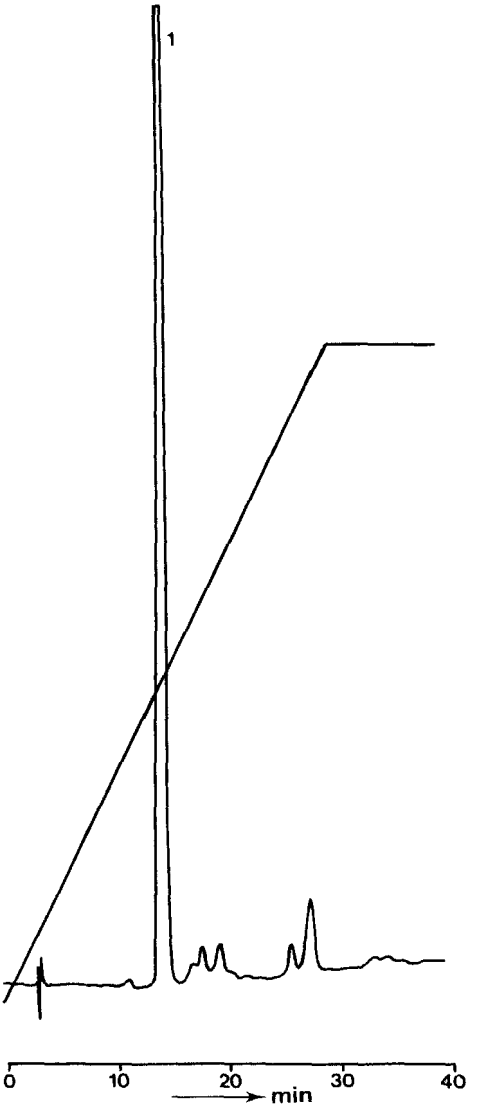
the residual silanol groups is achieved by applying tetramethylammonium phosphate buffer, while ion-exchange is improbable at these low pH-values ($\leq$ 3).

Stationary Phase

The choice of the stationary phase depends on the polypeptides which have to be characterized. Arginine-

Downloaded At: 19:27 24 January 2011

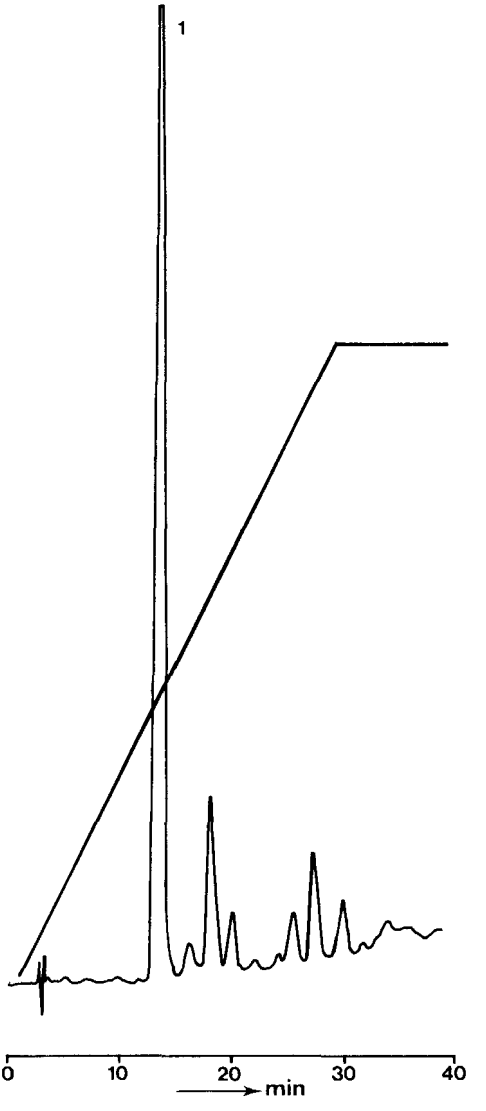
Downloaded At: 19:27 24 January 2011



(a)

and lysine vasopressin, oxytocin, ACTH (1-24), ACTH and β-endorphine can be excellently characterized on a μBondapak C 18 column (Fig. 4).

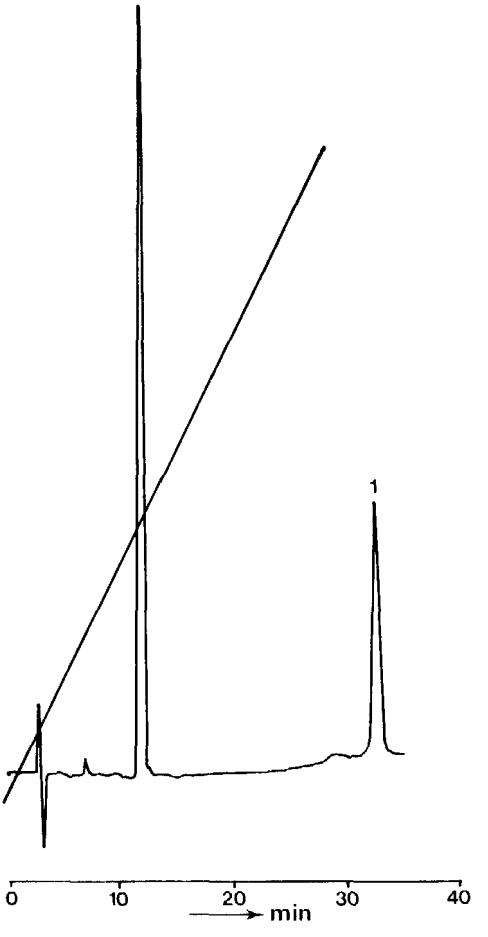
Downloaded At: 19:27 24 January 2011



(b)

The selectivity of this material (even with two columns in series) is insufficient for the separation of insulin-monodesamido insulin and glucagon-monodes-

Downloaded At: 19:27 24 January 2011

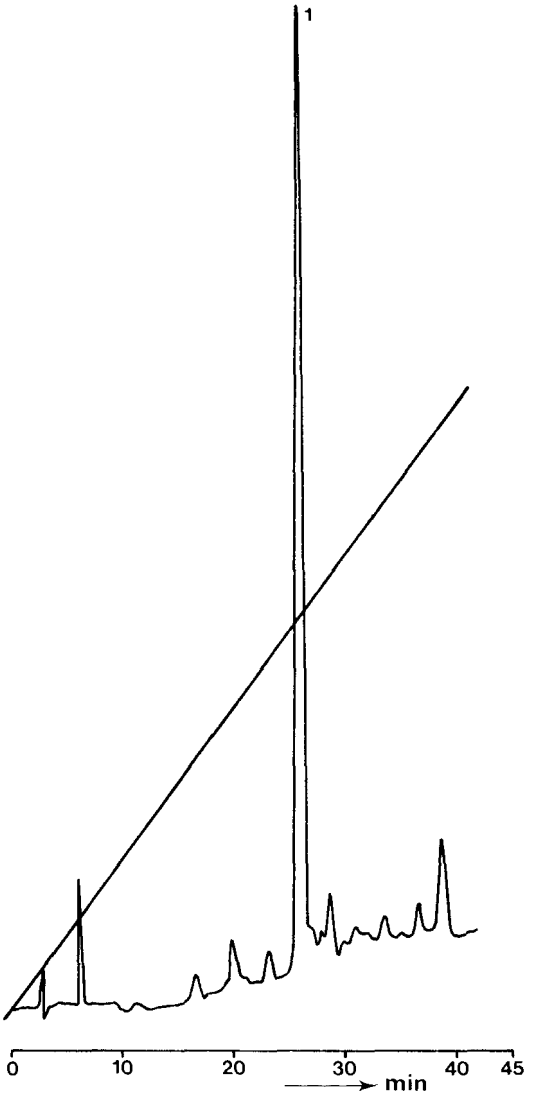


(c)

amidoglucagon owing to its relatively low hydrophobic character. The best separation of the desamido compounds from their main products is achieved on two home-made 30 cm Lichrosorb RP 18 columns in series.

In our experience, this is the most hydrophobic stationary phase with the best selectivity tested for polypeptides.

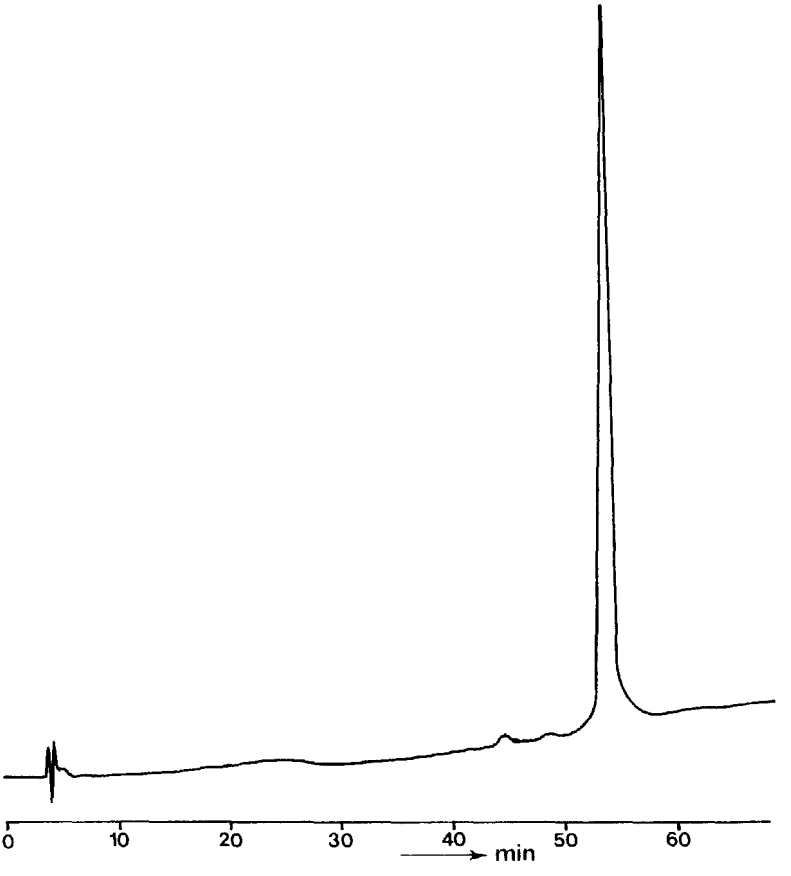
Downloaded At: 19:27 24 January 2011



(d)

The separating power of the Lichrosorb RP 18 system is demonstrated in Fig. 5, showing the complete separation of bovine- and porcine insulin.

Downloaded At: 19:27 24 January 2011



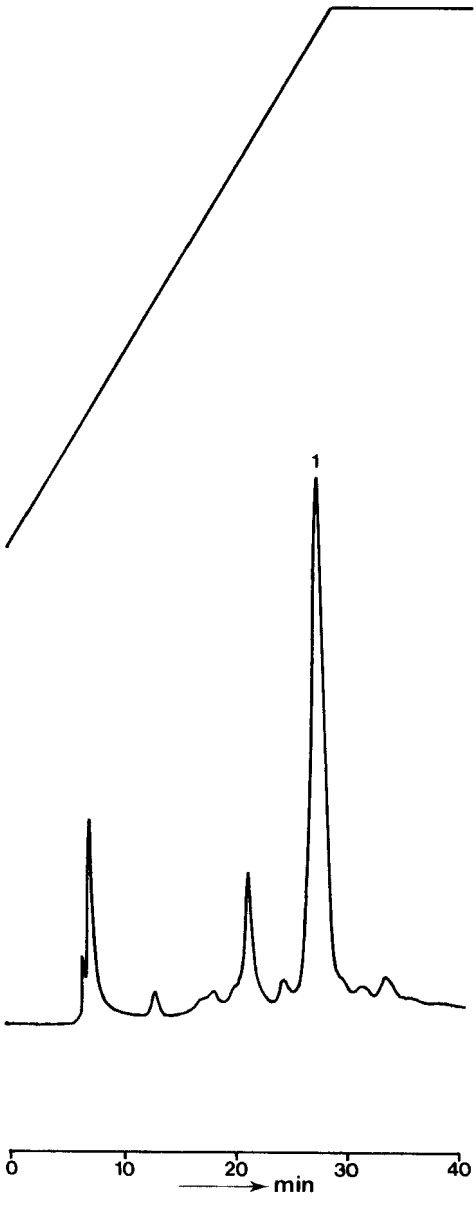
(c)

The differences between these two compounds are the amino acids in the positions A8, 10 (alanine and valine for bovine insulin and thryptophan and isoleucine for porcine insulin).

Application of Gradients

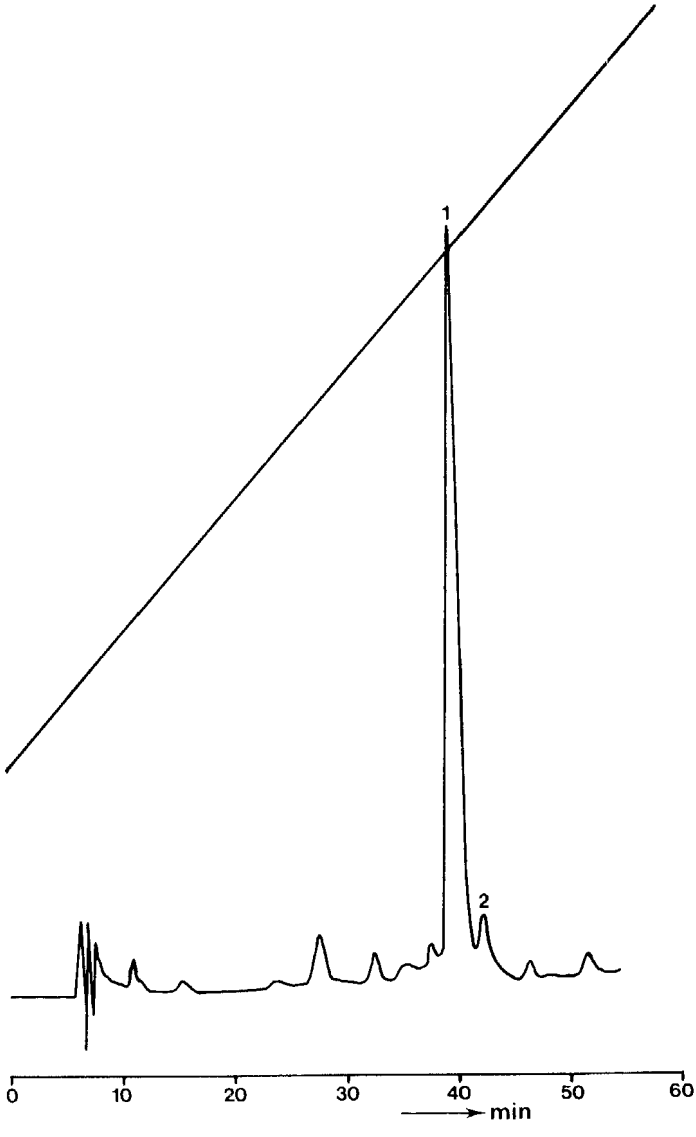
The application of isocratic elution has also been investigated. However, it gives less information

Downloaded At: 19:27 24 January 2011



(f)

Downloaded At: 19:27 24 January 2011



(g)

about the possible impurities present in the sample: some by-products can be retained to such an extent or irreversibly bonded that no reproducible quantitative results can be obtained. These by-products will elute from the column in narrow bands in applying a gradient

Undesirable mixing effects of the solvents used for the mobile phase have been minimized by optimizing the water-methanol mixtures in such a way that the two pumps deliver solvent mixtures, that differ in composition as little as possible.

Quantitative Analysis

The proportionality of the peak area to the amount of oxytocin injected was measured in the range 5-100 µg. The calibration graph proved to be linear in this range (correlation factor 0.9995).
Similar results were obtained for lysine- and arginine vasopressin, ACTH (1-24) and ACTH.
The quantitative determination of β-endorphin, glucagon and the two insulins was carried out according to a 100% analysis.

Downloaded At: 19:27 24 January 2011

Figure 4: Gradient HPLC-separation of polypeptides of various purities

a: arginine vasopressin	6.6	mg/ml
b: lysine vasopressin	11.5	mg/ml
c: oxytocin-acetate solution	5	mg/ml
d: ACTH (1-24)	6	mg/ml
e: β-endorphin	0.2	mg/ml (210 nm)
f: porcine ACTH 69 I.U-/mg	6	mg/ml
g: glucagon	0.3	mg/ml (210 nm)

Injection: 20 µl

Downloaded At: 19:27 24 January 2011

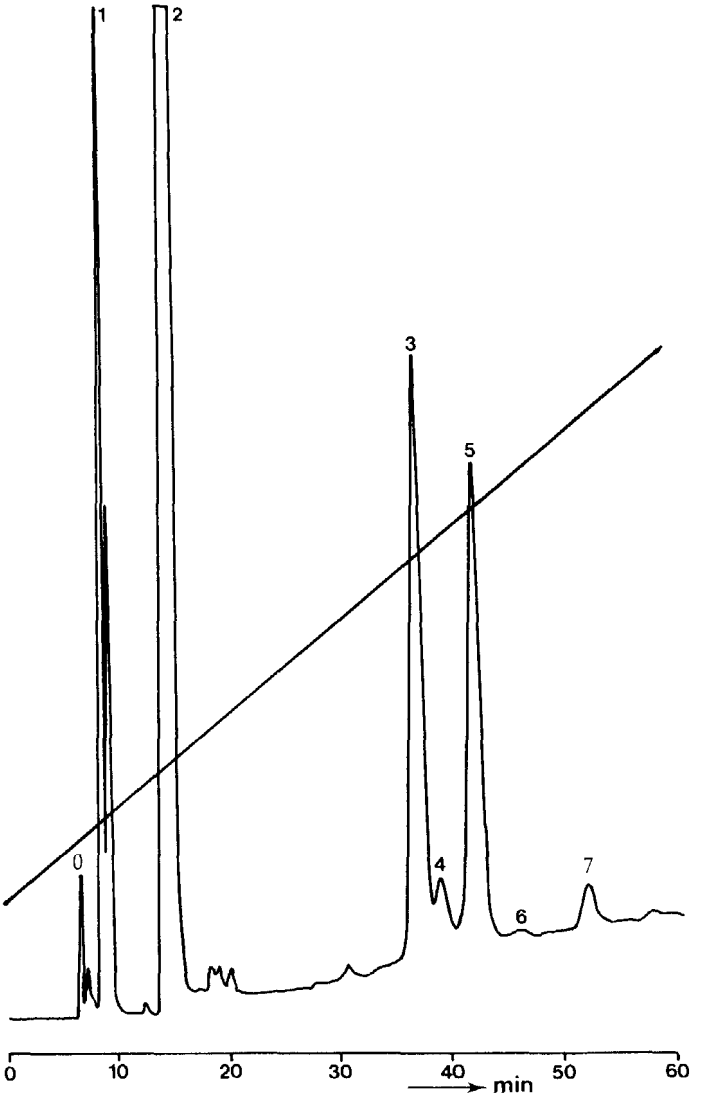


Figure 5: Gradient HPLC-separation of bovine and porcine insulin

0: solvent peak; 1: p-hydroxybenzoic acid; 2: p-hydroxymethyl benzoate; 3: bovine insulin; 4: monodesamido bovine insulin; 5: porcine insulin; 6: monodesamido porcine insulin; 7: proinsulin (?).

By repeated injection of the same polypeptide solution the coefficients of variation of the chromatographic procedures were found to be < 1.2%.
The coefficients of variation for the total analyses (including sampling and weighing) are ≤ 2%.

Stability of Polypeptides

The stability of the polypeptides in the chromatographic system depends on the pH and the nature of the solvent[25,26].
Therefore the stability of polypeptides was investigated in different solvents and at various pH-values.
In solution at room temperature insulin (and other polypeptides which can convert into a desamido compound e.g. glucagon) degrades upon standing.

The formation of the primary degradation product monodesamido insulin as a function of pH and TMA-phosphate was followed for 2 weeks by means of quantitative HPLC. The results are shown in Fig. 6.

For insulin and glucagon the decrease in the content found is negligible when the polypeptide solution (TMA-phosphate or aqueous acetic acid) is analysed on the day of preparation and stored at lower temperature (4°C).

Taking these precautions, no detectable amount of insulin is converted into the desamido-form even when very pure insulin (pharmaceutical preparation of porcine insulin) is injected (Fig. 7).

CONCLUSIONS

- Gradient elution HPLC on RP-modified silica is an excellent method for both the quantitative and

Downloaded At: 19:27 24 January 2011

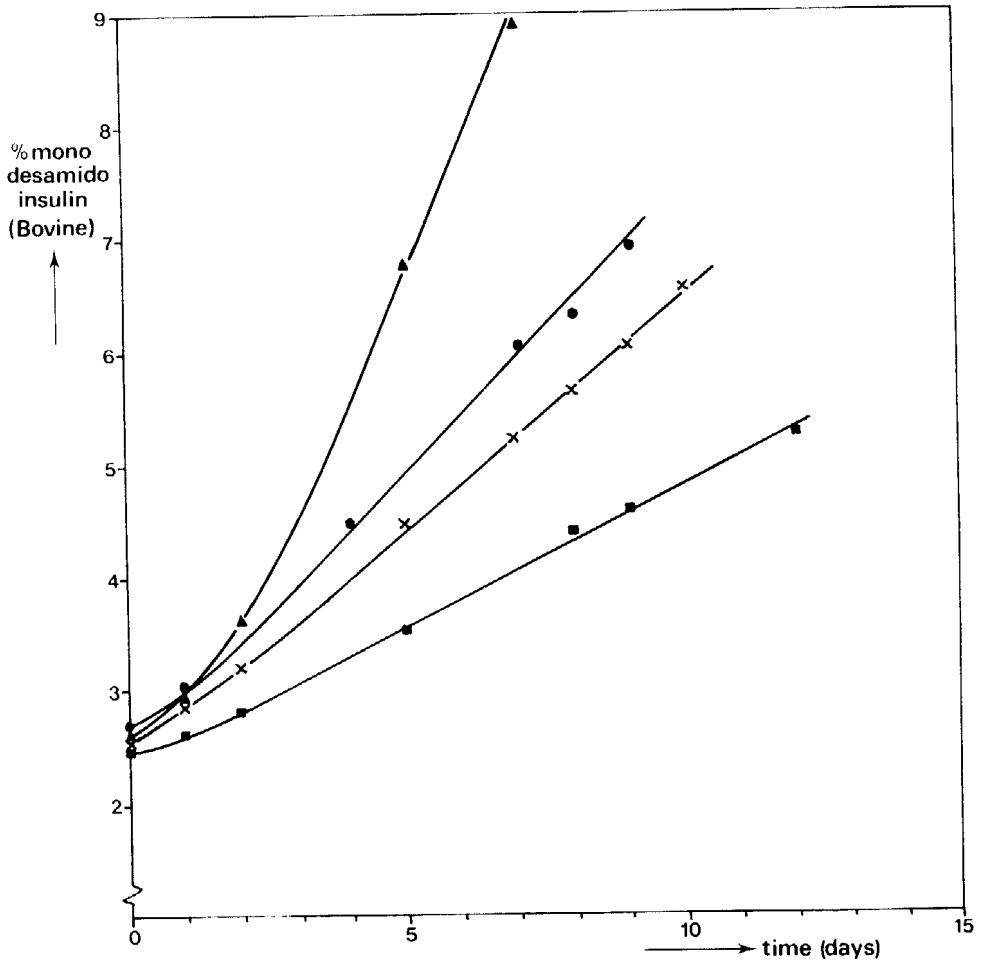


Figure 6: Influence of the pH and the nature of the solvent on the primary degradation product of bovine insulin

■ = aqueous acetic acid, pH 4
× = aqueous acetic acid, pH 3
● = aqueous acetic acid, pH 1.4
▲ = methanol/water 50:50 (v/v), 0.05 M tetramethylammonium phosphate, pH 3

Downloaded At: 19:27 24 January 2011

Downloaded At: 19:27 24 January 2011

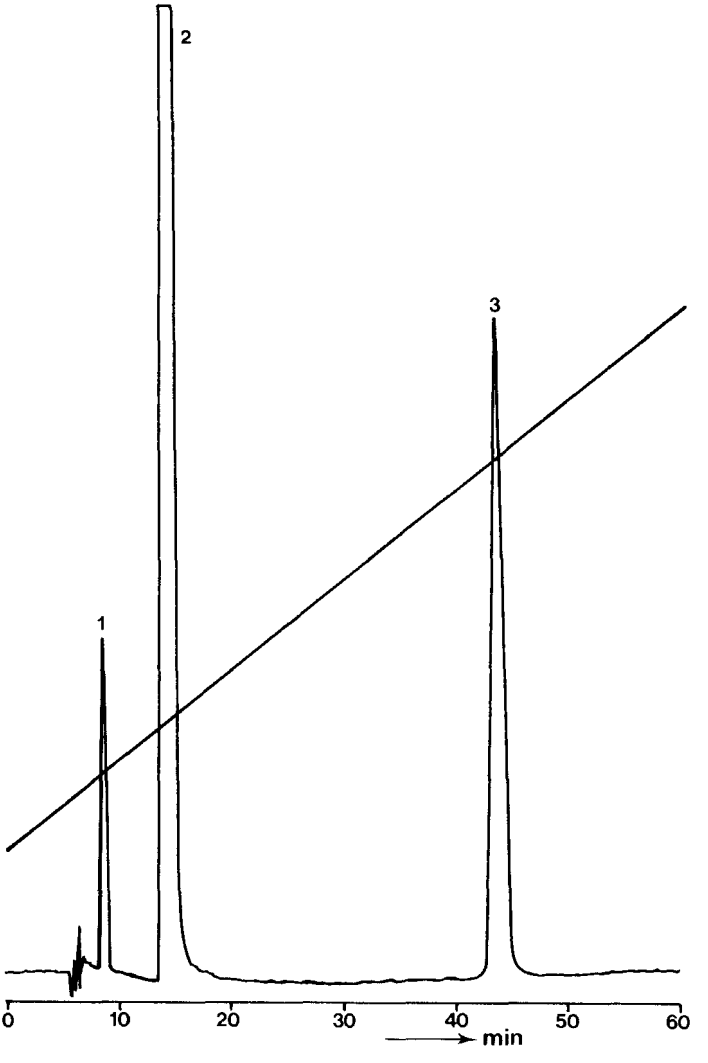


Figure 7: Gradient HPLC-separation of a pharmaceutical preparation (NOVO-monocomponent) of porcine insulin (40 I.U./ml)

1: p-hydroxybenzoic acid; 2: p-hydroxymethyl benzoate; 3: porcine insulin.

qualitative characterization of the polypeptides arginine-, lysine vasopressin, oxytocin, ACTH (1-24), ACTH, glucagon, β-endorphin, bovine- and porcine insulin.

- The pH of the mobile phase affects the retention and the peak performance of the polypeptides. pH-values of around 3 are to be preferred.

- In order to prevent undesirable retardation effects (e.g. ion-exchange) an electrolyte like tetramethylammonium phosphate has to be added to the solvent. Tetramethylammonium phosphate in several concentrations affects only the peak performance, not the peak retention.

Downloaded At: 19:27 24 January 2011

ACKNOWLEDGEMENT

The authors wish to thank Mr. F.A. Buytenhuys and Mr. F.P.B. van der Maeden for their helpful discussions and Mr. P.C.G.M. Janssen for the technical assistance of column packing.

REFERENCES

1. Schlichtkrull, J., Brange, J., Christiansen, A.H., Hallund, O., Heding, L.C., Jørgensen, K.H., Clinical Aspects of Insulin-Antigenicity, Diabetes 21, 649, 1971.

2. Calam, D.H., Applications of chromatography in the standardization and control of biological products, J. of Chromatogr., Proceedings of the 12th International Symposium on Chromatography Baden-Baden, 55, 1978.

3. Schröder, E. and Lübke, K., The Peptides, Vol. III, Academic Press, New York, 1966.

4. Conference on ACTH and related peptides: structure, regulation and action, Ann. N.Y. Acad. Sci., 297, 1977.

5. Geiger, R., Chemie des Insulins, Chemiker Zeitung 3, 111, 1976.

6. Karger, B.L., Gieze, R.W., Reversed-phase Liquid Chromatography and its application to Biochemistry, Anal. Chem. 50, 1978.

7. Rivier, J.E., Use of Trialkyl Ammonium Phosphate (TAAP) Buffer in Reverse Phase HPLC for High Resolution and High Recovery of Peptides and Proteins, J. of Liquid Chromatogr. 1, 343, 1978.

8. Krummen, K., Frei, R.W., The separation of nona peptides by reversed-phase high performance liquid chromatography, J. of Chromatogr., 132, 27, 1977.

9. Wehrli, A., Hildenbrand, J.C., Keller, H.P., Stampfli, R., Frei, R.W., Influence of organic bases on the stability and separation properties of reversed-phase chemically bonded silica gels, J. of Chromatogr. 149, 199, 1978.

10. Hansen, J.J., Greibrokk, T., Currie, B.L., Johansson, K.N.G., Folkers, K., High pressure liquid chromatography of peptides, J. of Chromatogr. 135, 155, 1977.

11. Lundanes, E., Greibrokk, T., Reversed-phase chromatography of peptides, 149, 241. 1978.

12. Van der Maeden, F.P.B., Van Rens, P.T., Buytenhuys, F.A., Quantitative analysis of d-Tubocurarine chloride in curare by column liquid chromatography, J. of Chromatogr. 142, 715, 1977.

13. Sugden, K., Cox, G.B., Loscombe, C.R., Chromatographic behaviour of basic amino compounds on silica and ODS-silica using aqueous methanol mobile phases, J. of Chromatogr. 149, 377, 1978.

14. Buytenhuys, F.A., Van der Maeden, F.P.B., Gel permeation chromatography on unmodified silica using aqueous solvents, J. of Chromatogr. 149, 489, 1978.

Downloaded At: 19:27 24 January 2011

15. Larsen, B., Viswanatha, V., Chang, S.Y., Hruby, V.J., Reverse phase high pressure liquid chromatography for the separation of peptide hormone diastereoisomers, J. of Chrom. Sci. 16, 207, 1978.

16. Burgus, R., Rivier, J., Use of high pressure liquid chromatography in the purification of peptides. Presented at the XIVth European Peptide Symposium in Wepion, 1976.

17. Tsuji, K., Robertson, J.H., Bach, J.A., Quantitative high pressure liquid chromatographic analysis of Bacitracin, a polypeptide antibiotic, J. of Chromatogr. 99, 597, 1974.

18. Krummen, K., Frei, R.W., Quantitative analysis of Nonapeptides in pharmaceutical dosage forms by high performance liquid chromatography, J. of Chromatogr. 132, 429, 1977.

19. Van der Maeden, F.P.B., Biemond, M.E.F., Janssen, P.C.G.M., Oligomer Separations by gradient elution high performance liquid chromatography, J. of Chromatogr. 149, 539, 1978.

20. Cassidy, R.M., Le Gay, D., Frei, R.W., Study of Packing Techniques for small-particle silica gels in High Speed Liquid Chromatography, Anal. Chem. 46, 340, 1974.

21. Linder, H.R., Keller, H.P., Frei, R.W., A new slurry packing Technique for columns in High Speed Liquid Chromatography, J. of Chrom. Sci. 14, 234, 1976.

22. Hancock, W.S., Bishop, C.A., Prestidge, R.L., Harding, D.R.K., Hearn, M.T.W., The use of phosphoric acid in the analysis of underivatised peptides by reversed-phase high pressure liquid chromatography, J. of Chromatogr. 153, 391, 1978.

23. Hancock, W.S., Bishop, C.A., Meyer, L.J., Harding, D.R.K., Hearn, M.T.W., Rapid analysis of peptides by high pressure liquid chromatography with hydrophobic ion-pairing of amino groups, J. of Chromatogr. 161, 291, 1978.

24. Molnár, I., Horváth, C., Separation of amino acids and peptides on non-polar stationary phases by high pressure liquid chromatography, J. of Chromatogr. 142, 623, 1977.

Downloaded At: 19:27 24 January 2011

25. Mahler, H.R., Cordes, E.H., Basic biological chemistry, Harper & Row, Publishers, third printing, New York, 1968, 32-40.

26. Lehninger, A.L., Biochemistry, Worth Publishers, 2nd edition, New York, 1970.

27. Hancock, W.S., Bishop, C.A., Battersby, J.W., Harding, D.R.K., Hearn, M.T.W., The use of cationic reagents for the analysis of peptides by high pressure liquid chromatography, J. of Chromatogr. 168, 377, 1979.

Downloaded At: 19:27 24 January 2011