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SEPARATION, ISOLATION AND CHARACTERIZATION OF THE FOUR MONOIODINATED INSULIN TRACERS USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Baseline separation between insulin and insulin monoiodinated in Tyr A14, A19, B16 and B26 can be obtained using isocratic elution from a C_{18} column with triethylammonium trifluoroacetate–acetonitrile and the iodinated insulin derivatives can be isolated by lyophilization. Compared with similar tracers purified and isolated by disc electrophoresis/ion-exchange chromatography, the reversed-phase high-performance liquid chromatographically purified tracers are more homogeneous but show reduced binding affinity to adipocytes.

INTRODUCTION

Insulin labelled with radioactive iodine has found widespread use in receptor studies and radioimmunoassay. Depending on the choice of iodination procedure, all four tyrosyl residues in insulin (A14, A19, B16 and B26) can be mono- or disubstituted, and a fractionation procedure is needed to obtain a monoiodinated insulin tracer. This fractionation can be performed using disc electrophoresis[1] (leading to insulin monoiodinated in A14 or A19) or disc electrophoresis followed by ion-exchange chromatography[2], allowing the isolation of all four monoiodinated insulin derivatives.

Several reports have substantiated the applicability of high-performance liquid chromatography (HPLC) for the characterization of insulin and insulin derivatives[3–26], including reversed-phase HPLC (RC-HPLC) for the separation of unsubstituted insulin, A19 and A14 monoiodinated insulin[24,25] as well as the partial separation of unsubstituted insulin and the four monoiodinated insulin tracers[26]. This paper describes the RP-HPLC separation of unsubstituted insulin and insulin monoiodinated in A14, A19, B16 or B26 and compares the purity and binding affinity *in vitro* of all four insulin tracers prepared by RP-HPLC with those of similar tracers purified by a combination of disc electrophoresis and ion-exchange chromatography.

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EXPERIMENTAL

Insulins

Crystalline sodium insulin (batch G-63) and pancreatic crystalline human insulin were obtained from Nordisk Gentofte (Gentofte, Denmark). Both insulins were further purified by low-pressure ion-exchange chromatography and gel chromatography and contained more than 99.5% insulin peptide as shown by RP-HPLC[21].

Iodinated insulins

Insulin was iodinated in phosphate buffer containing 6 *M* urea using the lactoperoxidase method and the four monoiodinated insulin isomers were isolated as described earlier[2]. To remove lactoperoxidase and iodide, an aliquot of the iodination mixture was applied to a 60 × 1.6 cm I.D. Sephadex G-50 superfine column eluted with 0.1 *M* ammonium hydrogen carbonate solution (pH 8.0) containing 0.1 % HSA (human serum albumin; Behringwerke). The fractions containing the iodinated insulin derivatives were pooled, lyophilized and stored at −20°C in aliquots.

Diiodoinsulin derivatives were isolated from an iodination mixture using disc electrophoresis and further purified by additional disc electrophoresis of the diiodoinsulin gel slices[27]. The eluted diiodoinsulin mixture was freeze-dried and stored in aliquots at −20°C.

HPLC

Two Waters M45 pumps controlled by a Waters 660 gradient controller were used for the elution of 100 × 4.0 mm I.D. and 150 × 4.0 mm I.D. Spherisorb ODS-2 (3 μm) columns at 1.0 ml/min. The analytical columns were protected by a precolumn (40 × 4.0 mm I.D.) packed with the same material. A Waters U6K injector was used for sample application and the UV absorbance of the eluate was measured continuously at 230 nm in a Pye Unicam UV detector. The eluate was collected in 1-min fractions (Pharmacia, FRAC 300 fraction collector) and the radioactivity was measured in a 16-channel γ-counter (Hydrogamma 16). All HPLC separations were carried out at room temperature. A 1 % solution of triethylammonium trifluoroacetate was prepared by adjusting a 1% aqueous solution of trifluoroacetic acid (Fluka, glass distilled before use) to pH 3.00 with triethylamine (Janssen). This buffer was used as A, buffer B being 50 % A–50 % acetonitrile.

All chemicals were of analytical-reagent grade. Distilled water was drawn from a Millipore Milli-Q plant and the buffers were Millipore-filtered (0.45 μm) and vacuum/ultrasound degassed before use.

HPLC samples

All samples were dissolved in 0.01 *M* hydrochloric acid and 50 μl were injected using a Hamilton syringe. After each sample application the syringe was rinsed three times with 0.01 *M* hydrochloric acid containing 0.1 % HSA and then rinsed three times with methanol–water (80:20) to avoid contamination. Each isocratic separation was followed by a column rinse using 5 % A–95 % B at 1.0 ml/min for 5 min to remove firmly bound impurities. Thereafter the column was reequilibrated for 30–60 min at 1.0 ml/min.

Once every week the columns were further rinsed by injecting 500 μl of 7 *M* urea–3 *M* acetic acid followed by re-equilibration for 60 min.

The binding affinity to isolated adipocytes was determined as described earlier[1,28].

RESULTS

The separation between unsubstituted insulin and insulin monoiodinated in A14, A19, B16 and B26 obtained by isocratic elution of Spherisorb ODS-2(3-μm)

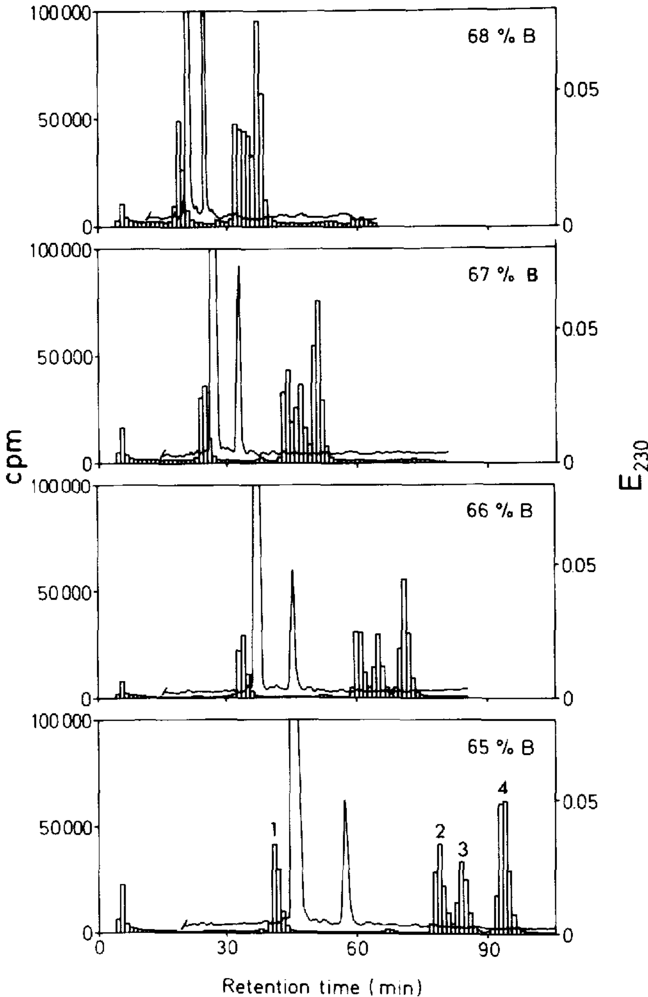

Fig. 1. Isocratic separation of 50 μl of iodination mixture (containing the four monoiodinated insulin isomers) plus 50 μg of added crystalline insulin using a Spherisorb ODS-2 column eluted at 1.0 ml/min with 1% triethylammonium trifluoroacetate (pH 3.00) containing (from top to bottom) 34, 33.5, 33 and 32.5% acetonitrile. The histogram represents the radioactivity in the collected fractions and the unbroken curve the UV absorbance of the eluate. By comparison with the individual monoiodinated insulin tracers isolated as described[2], the peaks numbered 1, 2, 3 and 4 showed the same retention times as A19, B26, B16 and A14 monoiodoinsulin, respectively. See Experimental for further details.

columns with 1% triethylammonium trifluoroacetate (pH 3.00) in varying acetonitrile concentrations is shown in Fig. 1. The bottom panel (32.5% acetonitrile) shows the separation of all four isomers from the unlabelled insulin within a reasonable time. If the acetonitrile concentration is lowered to 32%, A14 is eluted later than 3 h after injection. More than 33% acetonitrile in the buffer leads to a less satisfactory separation of B26, B16 and A14. The unsubstituted insulin is clearly separated from A19. The major impurity in the added unlabelled insulin (eluted at 57 min in the bottom panel) is desamido-insulin and the radioactivity eluted shortly after injection is $^{125}I^-$ formed in the iodination mixture during storage.

It is very important that the pH in the injected sample is similar to that of the buffer. The retention times for the insulin tracers dissolved at neutral or slightly alkaline pH are considerably lower than those for the tracers dissolved in 0.01 *M* hydrochloric acid (data not shown).

Using disc electrophoresis it is possible to separate the diiodinated insulin derivatives from the monoiodoinsulins[27]. Fig. 2 shows the fractionation of the diiodinated insulin derivatives isolated by this procedure. Five major components are separated, three with retention times much longer than those of the monoiodinated insulins, one in front of but well separated from A19, and one close to B26 (see inset in Fig. 2).

Fig. 3 shows the separation of the monoiodinated porcine insulin tracers (top panel) and the analogous human derivatives isolated by disc electrophoresis/ion-exchange chromatography (bottom panel). The elution pattern after the $^{125}I^-$ peak is the same for the two species: A19–B26–B16 and A14, but the retention times for human insulin and the iodinated human insulin derivatives are lower than those of similar constituents of porcine origin.

Fig. 4 shows the purity of the four monoiodinated porcine insulin derivatives isolated by disc electrophoresis and ion-exchange chromatography[2]. Fig. 5 shows the purity of similar tracers isolated from RP-HPLC fractionation by freeze-drying. The HPLC-isolated tracers are purer than those isolated by disc electrophoresis/ion-exchange chromatography.

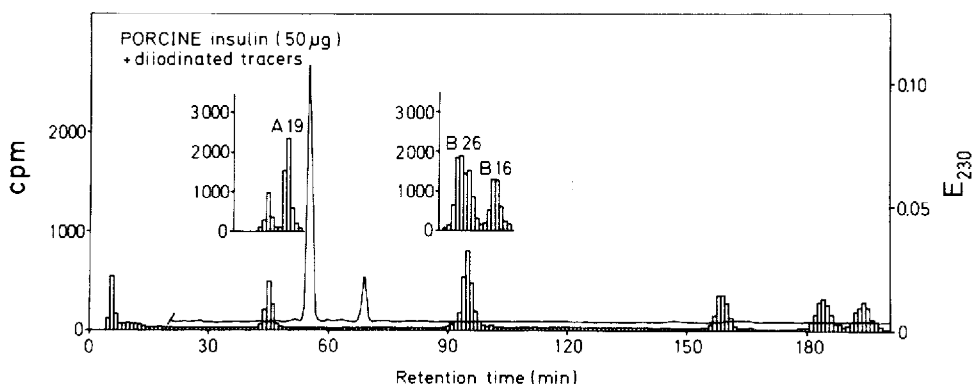

Fig. 2. Isocratic RP-HPLC separation of 50 µl of diiodoinsulin tracers plus added crystalline insulin (50 µg). Acetonitrile concentration, 32.5%; other conditions as in Fig. 1. The insets show the separation between the components in the diiodoinsulin mixture with retention times similar to A19 and B26 monoiodoinsulin. The amount of diiodoinsulins in the mixture applied is 8–9 times larger than that normally found in the iodination mixture.

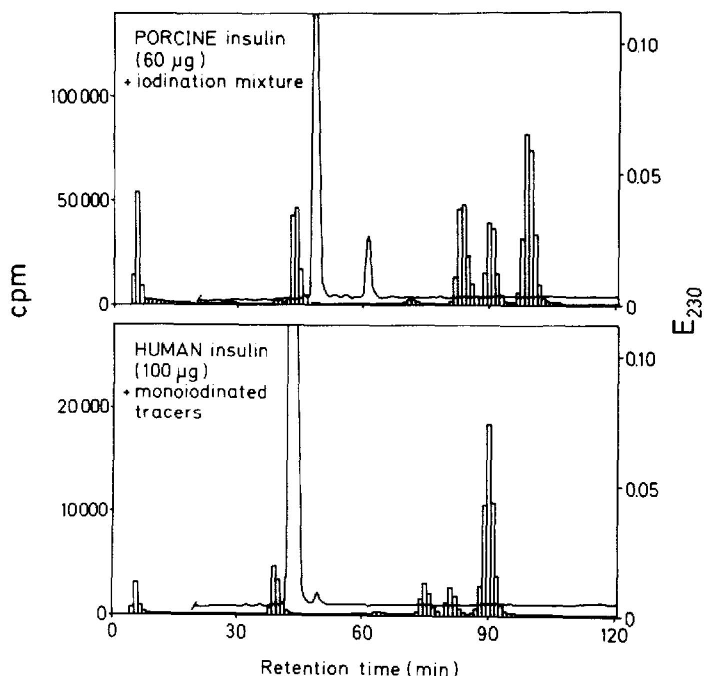

Fig. 3. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture (porcine) plus added porcine crystalline insulin (60 μg) (upper panel) and a mixture of the four monoiodinated human insulin tracers (isolated after disc electrophoresis and ion-exchange chromatography) plus added human insulin (100 μg) (lower panel). Acetonitrile concentration, 32.5%; other conditions as in Fig. 1.

When the monoiodinated insulin derivatives isolated after RP-HPLC fractionation were analysed for receptor-binding affinity to isolated rat adipocytes as described earlier[1,28], the HPLC-purified tracers showed reduced binding compared with that of similar tracers purified by low-pressure methods (Fig. 6). Removal of the buffer substances by Sep-Pak C_{18} (Waters) did not reconstitute the binding affinity to that of the tracers purified by conventional methods.

Lyophilization of the triethylammonium trifluoroacetate buffer leaves a small amount of an oily substance. Porcine insulin freeze-dried from the buffer shows the same disc electrophoresis pattern both before lyophilization and immediately after the lyophilization process as well as after 48 h of "incubation" in the non-volatile buffer residue. After each RP-HPLC separation some radioactivity is washed out of the column by the described rinsing procedure. This labelled material produces a blurred radioactivity zone over the total length of the running gel when isolated and subjected to disc electrophoresis and bears no relation to insulin or mono- or di-iodinated insulin derivatives. When large amounts of radioactivity were applied to the column, a thorough washing procedure (95% B, 7 *M* urea–3 *M* acetic acid, re-equilibration) always re-established the initial low background in radioactivity of the eluate.

The recovery based on radioactivity measurements in the collected fractions varies from 50 to 100% but the distribution of radioactivity among the four monoiodinated insulin isomers in the same iodination mixture is remarkably con-

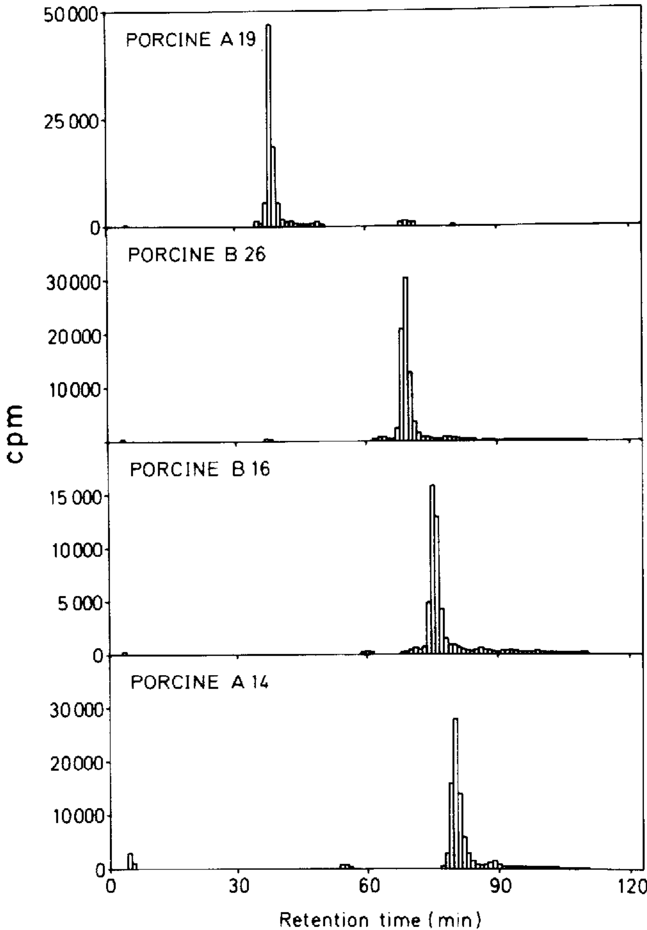

Fig. 4. Isocratic RP-HPLC separation of 50 μl of each of the four monoiodinated insulin tracers (porcine) isolated by disc electrophoresis and ion-exchange chromatography. Acetonitrile concentration, 32.5%; other conditions as in Fig. 1.

stant: A19, 19.2 $\pm$ 0.6%; B26, 23.3 $\pm$ 0.2%; B16, 18.4 $\pm$ 0.4%; and A14, 39.1 $\pm$ 0.4% (mean $\pm$ S.D., n = 8).

DISCUSSION

In binding studies and radioimmunoassays it is desirable to use homogeneous insulin tracers, as the four monoiodinated [^{125}I]insulin derivatives show different binding affinities to insulin receptors[2,29]. Recent papers have described the use of RP-HPLC for separating the A14 and A19 [^{125}I]insulin tracers[24,25] and for the partial separation of insulin and all four monosubstituted [^{125}I]insulin derivatives[26]. The present method allows the baseline separation of insulin, A14, A19, B16 and B26 [^{125}I]insulin and can be performed in less than 2 h. Diiodinated [^{125}I]insulins are well separated, except for one component which is eluted close to B26 (Fig. 2). However, if

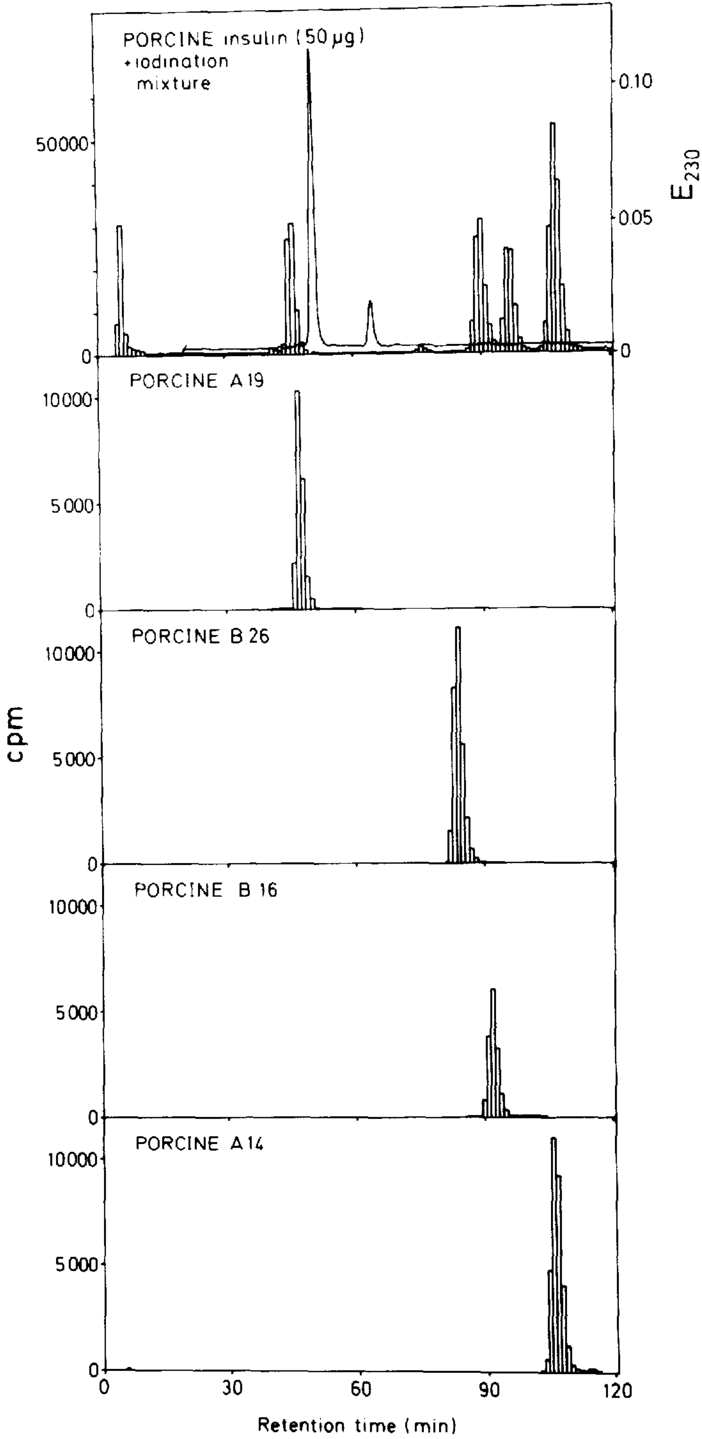

Fig. 5. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture plus added crystalline insulin (porcine, 50 μg) (upper panel) and the four monoiodinated insulin isomers isolated from RP-HPLC fractionation by lyophilization. Acetonitrile concentration, 32.5%; other conditions as in Fig. 1.

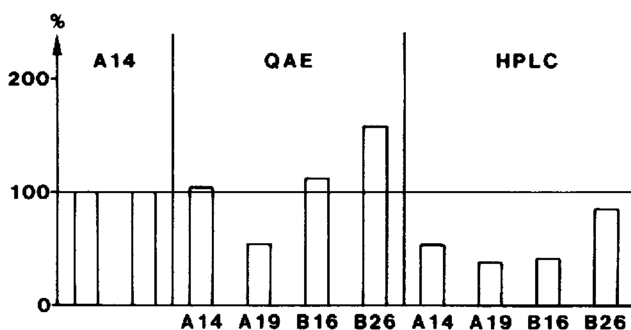

Fig. 6. Binding affinity to isolated adipocytes of monoiodinated insulin derivatives prepared by disc electrophoresis/ion-exchange chromatography[2] (QAE) or by RP-HPLC (HPLC) relative to A14 monoiodoinsulin prepared by conventional methods[28].

the iodination is performed with an iodination degree lower than 0.14, the total amount of diiodinated insulin derivatives is about 5 % of the monosubstituted derivative on a molar basis, and collection of the first 75 % of the B26 fraction will lead to a B26 tracer virtually free of diiodo-insulin.

The superiority of the present method over the partial separation of the B26 and B16 tracers obtained on LiChrosorb RP-18 (7 μm) eluted with triethylammonium phosphate (TEAP)[26] is probably due to the use of Spherisorb ODS-2 (3 μm), which offers a large number of theoretical plates per metre and (probably) a different selectivity towards insulin and insulin derivatives. The ion-pairing capacity of the fluoroacetic acid may be superior to that of TEAP, but as the primary effect of the ion-pairing agents is to mask the residual silanol groups that could lead to non-ideal peak shapes, and as insulin peptide can be eluted in almost perfectly symmetrical peaks in both systems[30], the improved separation on the 3-μm column is principally due to the reduced theoretical plate height.

The present system allows the isolation of very pure monoiodinated insulin derivatives but is very sensitive towards changes in temperature, acetonitrile content and pH in the applied sample. To obtain reproducible results it is recommended to thermostat the column, to weigh out the acetonitrile and to adjust the pH of the sample. Even then the absolute retention times for the four tracers will vary (Figs. 4 and 5) but the fractionation pattern is reasonably constant.

The fact that all four isolated insulin tracers showed reduced binding affinity to isolated adipocytes raises doubts about the general ability of polypeptides and proteins to preserve their biological activity when exposed to the extremely non-physiological conditions under which RP-HPLC is performed: high pressure, shear forces, exposure to poisonous organic solvents and the hydrophobic chromatographic matrix. Moreover, in the present buffer system it cannot be excluded that F_3CCOO^- will bind irreversibly to the arginine residues in insulin and iodinated insulin derivatives. The specific effect of these individual influences is at present under investigation in this laboratory, but it is strongly advised to compare the biological activity of any peptide/protein isolated after HPLC fractionation with the activity of

the same compound purified to a comparable degree under "physiological" conditions.

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REFERENCES

1 J. Gliemann, O. Sonne, S. Linde and B. Hansen, *Biochem. Biophys. Res. Commun.*, 87 (1979) 1183–1190.
2 S. Linde, O. Sonne, B. Hansen and J. Gliemann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 573–579.
3 M. E. F. Biemond, W. A. Sipman and J. Olivié, in D. Brandenburg (Editor), *Insulin —Chemistry, Structure, and Function of Insulin and Related Hormones*, Walter de Gruyter, Berlin, New York, 1980, pp. 201–206.
4 D. H. Calam, *J. Chromatogr.*, 167 (1978) 91–108.
5 U. Damgaard and J. Marcussen, *Horm. Metab. Res.*, 11 (1979) 580–581.
6 A. Dinner and L. Lorenz, *Anal. Chem.*, 51 (1979) 1982–1983.
7 M. T. W. Hearn, W. S. Hancock, J. G. R. Hurrell, R. J. Fleming and B. Kemp, *J. Liquid Chromatogr.*, 2 (1979) 919–933.
8 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415–418.
9 M. J. O'Hara and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209–226.
10 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343–366.
11 S. Terabe, R. Konaka and K. Inouye, *J. Chromatogr.*, 172 (1979) 163–173.
12 G. Vigh, Z. Varga-Puchony, J. Hlavay and E. Papp-Hites, *J. Chromatogr.*, 236 (1982) 51–59.
13 M. Gazdag and G. Szepesi, *J. Chromatogr.*, 218 (1981) 603–612.
14 G. Szepesi and M. Gazdag, *J. Chromatogr.*, 218 (1981) 597–602.
15 L. F. Lloyd and D. H. Calam, *J. Chromatogr.*, 237 (1982) 511–514.
16 J. K. Wilson and G. J. Hughes, *Chimia*, 35 (1981) 327–333.
17 L. F. Lloyd and P. H. Coran, *J. Chromatogr.*, 240 (1982) 445–454.
18 N. Asakawa, M. Tsuno, Y. Saeki, M. Matsuda, T. Hattori, M. Yeyama, A. Shinoda and Y. Miyake, *J. Pharm. Soc. Jap.*, 102 (1982) 43–48.
19 Y. Pocker and S. B. Biswas, *J. Liquid Chromatogr.*, 5 (1982) 1–14.
20 B. S. Welinder, *J. Liquid Chromatogr.*, 3 (1980) 1399–1416.
21 B. S. Welinder and F. H. Andresen, in J. Gueriguian (Editor), *Hormone Drugs. Proceedings of the FDA-USP Workshop on Drug and Reference Standards for Insulins, Somatropins, and Thyroid-axis Hormones*, United States Pharmacopeial Convention, Inc., Rockville, 1982, pp. 163–177.
22 F. E. Regnier, *Anal. Biochem.*, 126 (1982) 1–7.
23 A. U. Parman and I. M. Rideout, *J. Chromatogr.*, 256 (1983) 283–291.
24 J. Markussen and U. P. Larsen, in D. Brandenburg (Editor), *Insulin — Chemistry, Structure, and Function of Insulin and Related Hormones*, Walter de Gruyter, Berlin, New York, 1980, pp. 161–168.
25 F. B. Stentz, R. K. Wright and A. E. Kitabchi, *Diabetes*, 31 (1982) 1128–1131.
26 B. S. Welinder, S. Linde and J. S. Brush, *J. Chromatogr.*, 257 (1983) 162–165.
27 B. P. Maceda, S. Linde, O. Sonne and J. Gliemann, *Diabetes*, 31 (1982) 634–640.
28 S. Linde, B. Hansen, O. Sonne, J. J. Holst and J. Gliemann, *Diabetes*, 30 (1981) 1–8.
29 O. Sonne, S. Linde, T. R. Larsen and J. Gliemann, *Hoppe-Seyler's Z. Physiol. Chem.*, 364 (1983) 101–110.
30 B. S. Welinder, unpublished results.