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Note

Separation of the monoiodinated isomers of insulin by high-performance liquid chromatography

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A number of reports describing the use of high-performance liquid chromatography (HPLC) for the separation of insulin and insulin derivatives have been published in recent years[1–19]. Using reversed-phase (RP) HPLC it has been possible to separate insulins from different species (porcine, bovine and human) as well as insulin, desamidoinsulin and proinsulin from the same species[1–16]. The application of gel permeation chromatography (GPC) in aqueous solvents has allowed the separation of crystalline insulin into the b-fraction (molecular weight 9000–12,000), the c-fraction (molecular weight 6000) and degradation products of insulin[17,18].

Especially RP-HPLC using ion-pairing compounds has proved to be a powerful analytical technique for the characterization of insulin: conventional crystalline insulin can be resolved into its main components, insulin peptide + 15–20 contaminants (desamidoinsulin, arginineinsulin, intermediary insulin, proinsulin, insulin dimer, etc.)[19].

Recently a method has been described for the preparation and purification of the four radioiodinated insulin isomers in which an iodine atom is attached to one of the four tyrosine residues (A14, A19, B16 and B26)[20]. This technique requires purification of the iodination mixture by gel electrophoresis and ion-exchange chromatography. Using RP-HPLC it has been possible to separate the A14 and the A19 isomer but with some contamination by unlabelled insulin in the A19 isomer peak[21].

This paper describes the separation of all four monoiodinated insulin isomers and non-iodinated insulin using RP-HPLC.

EXPERIMENTAL

Iodinated insulin

Highly purified porcine insulin was iodinated in urea-containing buffer, and the four derivatives of insulin (monoiodinated in tyrosine position A14, A19, B16 and

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B26) were separated from each other by gel electrophoresis and ion-exchange chromatography as described previously[20].

HPLC

A 250 × 4 mm I.D. LiChrosorb RP-18 (7 μm) equipped with a pre-filter of Perisorb RP-18 (30–40 μm) was eluted at 0.35 or 0.70 ml/min using two Altex Model 110 pumps controlled by an Altex Model 240 microprocessor. UV detection of the eluate was effected continuously at 210 nm with a Cecil Model 2112 instrument. The eluate was collected in a LKB Radirac fraction collector (1.0 min per fraction). The radioactivity in each fraction was measured in an automatic gamma-counter (Palle Medico technique).

Triethylammonium phosphate buffer (0.25 *M*) was prepared by titrating phosphoric acid to pH 3.00 with triethylamine. Acetonitrile volumes were measured by weight assuming a density of 0.782 g/ml. All reagents were of analytical-reagent grade.

RESULTS AND DISCUSSION

The retention times for the four monoiodinated insulin isomers and non-iodinated insulin were determined isocratically at a variety of acetonitrile concentrations and the results are shown in Fig. 1. The order of elution of the four isomers and insulin was A19, insulin, B26, B16 and A14. The same order of elution was found at all acetonitrile concentrations tested. From the curves shown in Fig. 1, approximately 27% acetonitrile was chosen for further isocratic separations.

Fig. 2 shows the separation achieved with an unfractionated iodination mixture. The initial peak is unreacted iodide ions followed by the four isomers plus insulin. As can be seen, separation of the four monoiodinated insulin isomers is achieved under the conditions used. The resolution between the B26 and B16 isomers

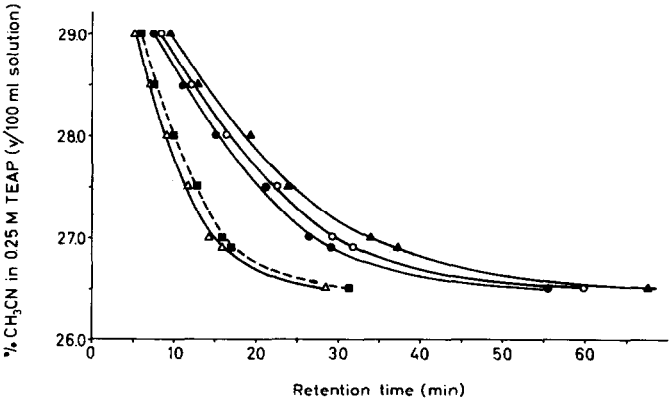

Fig. 1. Variation of retention time of monoiodinated isomers and insulin. Volumes of 20 μl of each of the four isomers containing 4 μg of insulin were separated isocratically at the indicated percentage of acetonitrile; flow-rate, 0.7 ml/min. Insulin elution was monitored at 210 nm and the ^{125}I-labelled monoiodoinsulin isomers were counted in the collected fractions (1.0 min per fraction). ■ = Insulin; ▲ = A14 iodoinsulin; △ = A19 iodoinsulin; ○ = B16 iodoinsulin; ● = B26 iodoinsulin.

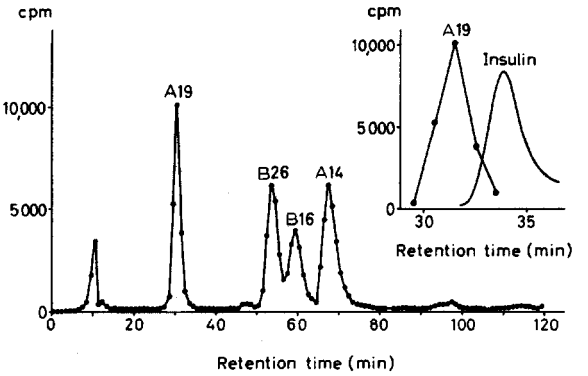

Fig. 2. Isocratic RP-HPLC separation of 20 μl of diluted iodination mixture (containing the four monoiodinated insulin isomers, a small amount of diiodoinsulin plus unlabelled insulin) using 0.25 *M* triethylammonium phosphate (pH 3.00) containing 26.9% (v/v) of acetonitrile at a flow-rate of 0.35 ml/min. Inset: separation of a mixture ofthe A19 isomer and 2 μg of insulin. UV registration, collection and counting as in Fig. 1.

is not satisfactory; however, their retention times differ by 6 min. As shown in the inset to Fig. 2, the A19 isomer is separated by 2.6 min from the insulin peak. Approximately 75% of the A19 isomer is eluted before there is any measurable elution of insulin. The small peak of radioactive material eluted at 97.5 min is diiodinated insulin derivatives.

The use of triethylammonium phosphate buffers in RP-HPLC for the separation of peptides and proteins was introduced by Rivier[8]. This system has been very useful for the separation of crystalline insulin[19]. In the present investigation it has also been found to achieve very good separations between insulin and the iodinated insulin derivatives. Other workers[21] used Tris–phosphate–EDTA buffer containing ethanol to achieve separation of the A14 and A19 monoiodinated isomers but with some insulin contamination of the A19 isomer. The present system, therefore, seems more effective in providing a reproducible system for the separation of all four monoiodinated isomers of insulin.

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