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Separation and quantitation of serum proinsulin and proinsulin intermediates in humans

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ABSTRACT

Two reversed-phase high-performance liquid chromatographic (RP-HPLC) systems were developed for the separation of human insulin, proinsulin and the major proinsulin intermediates. The individual components were quantified using two enzyme-linked immunosorbent assays for insulin and proinsulin immunore-active material (PIM) after (passive) evaporation of the organic modifier. Serum samples from normal subjects and patients with non-insulin-dependent diabetes mellitus were immunopurified and analysed in one of the RP-HPLC systems. The proportion of PIM relative to insulin immunoreactive material was higher in the diabetic patient compared with that in the normal subject. In both, PIM was heterogeneous, consisting of intact proinsulin and des-proinsulin intermediates.

INTRODUCTION

The insulin precursor proinsulin is converted by specific enzymes in the β-cell secretory granules to insulin via several intermediate forms [1]: two trypsin-like enzymes cleave next to the paired basic amino acids in positions 31–32 and 64–65, respectively [2], followed by removal of the basic residues by carboxypeptidase H [3].

Elevated amounts of proinsulin immunoreactive material (PIM) in serum have been described in various conditions and diseases [4–7], including non-insulin-dependent diabetes mellitus (NIDDM) [8–10], and could be an indication of altered β-cell function. The extent to which the proinsulin intermediate forms exist together with intact proinsulin in serum is not known.

In order to determine the composition of PIM in serum samples, a method for the specific determination of proinsulin and all the individual conversion intermediates is required. As it is difficult to develop site-specific immunoassays that can distinguish between the small structural differences in the various proinsulin conversion products (Fig. 1), the aim of this study was to develop reversed-phase high-performance liquid chromatographic (RP-HPLC) methods capable of separating insulin,

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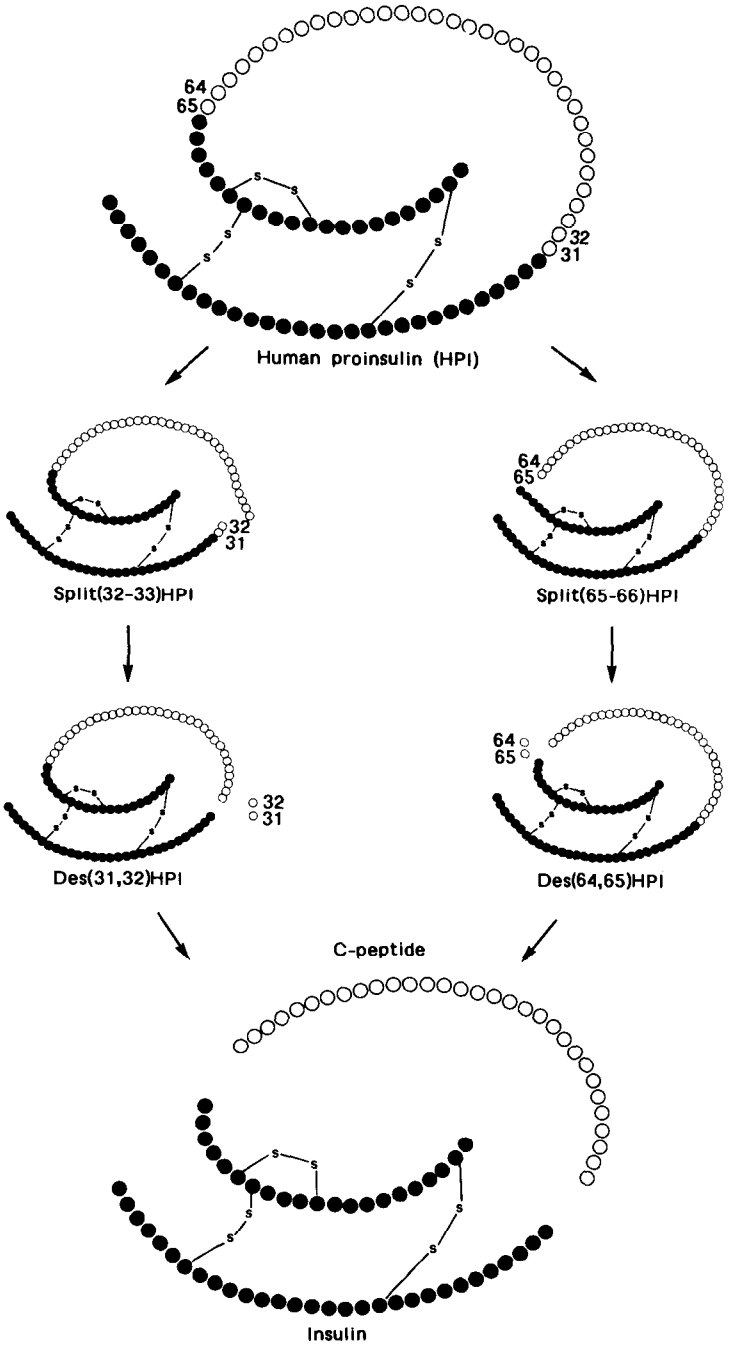

Fig. 1. Conversion of human proinsulin to (●) insulin and (○) C-peptide via split- and des-proinsulin intermediates. Amino acids 31, 32 and 65 are arginine and 64 is lysine.

proinsulin and the four major proinsulin intermediates. The individual fractions obtained after RP-HPLC were analysed in two enzyme-linked immunosorbent assays (ELISAs) for the determination of insulin [11] and PIM [12], and this paper reports such analyses of serum samples from normal subjects and NIDDM patients using one of the optimized RP-HPLC systems.

EXPERIMENTAL

Reagents

Trifluoroacetic acid (TFA) (peptide synthesis grade) was obtained from Applied Biosystems, phosphoric acid (analytical-reagent grade) from Merck, ammonium sulphate (Aristar) from BDH, triethylamine (99%) from Janssen Chimica and acetonitrile (HPLC grade S) from Rathburn Chemicals. All other chemicals were of analytical-reagent grade. Distilled water was obtained from a Millipore Milli-Q system, and all buffers were filtered (0.45 μm, Millipore) and vacuum/ultrasound degassed before use.

HPLC equipment

The HPLC system consisted of two Waters Assoc. Model M510 pumps, a Model 660 solvent programmer, a Model U6K injector, a Linear UVIS 200 detector and an LKB Model 2210 one-channel recorder.

HPLC columns

Nucleosil 100-5C_{18} (5 μm), 250 × 4.0 mm I.D., and 300-5C_4 (5 μm), 250 x 4.0 mm I.D. and 30 x 4.0 mm I.D., columns were obtained from Macherey-Nagel & Co., a LiChrosorb RP-18 (5 μm), 250 × 4.0 mm I.D., column from Merck and a Zorbax Protein Plus (6 μm), 250 x 4.6 mm I.D., column from DuPont.

HPLC conditions

The following mobile phases were used: 0.1% TFA (pH 2.0), 0.125 *M* triethylammonium phosphate (TEAP) (pH 4.0) and 0.125 *M* ammonium sulphate (AS) (pH 4.0), all in acetonitrile (ACN). The columns were eluted at ambient temperature at 1.0 ml/min with shallow linear acetonitrile gradients ranging from 1.2% to 6% during 60 min. The column eluate was monitored at 210 nm and collected in 0.5-min fractions in a FRAC 300 fraction collector (Pharmacia).

ELISA analyses

To avoid non-specific adsorption to the tubes, 50 μl of 0.04 *M* sodium phosphate (pH 7.4) containing 6% of human serum albumin (HSA) and 0.1% Tween 20 were added to each tube prior to the collection of fractions. After (passive) evaporation of acetonitrile overnight at room temperature, the fractions were compatible with the ELISA assays performed as described [11,12]. The standard operating range was 0–400 pmol/l for insulin and 0–80 pmol/l for proinsulin. Detection limits were 2.5 and 1.2 pmol/l, respectively.

Standards

Human proinsulin (Lot A18-TS9-16) and the four major proinsulin intermedi-

ate standards, des(31,32)HPI (Lot A18-7W8-1), des(64,65)HPI (Lot A18-7W8-2), split(32–33)HPI (Lot A18-JC5-96) and split(65–66)HPI (Lot A18-JC5-92), were kindly donated by B. H. Frank (Lilly Research Labs.). Human insulin was obtained from Novo Nordisk. The standards were dissolved to give 0.1 mg/ml concentrations in 3 *M* acetic acid containing 0.1% HSA before the RP-HPLC analyses. Mono-[^{125}I-(TyrA14)]-porcine insulin obtained from Novo Nordisk and [^{3}H-Leu]proinsulin (rat) prepared as described [13] were used for recovery determinations.

Serum sample preparation

Owing to the low PIM concentration in serum, the samples (10 ml) from fasting normal humans (control) and fasting NIDDM patients were transferred to empty 10 × 1.5 cm I.D. siliconized Econo-Columns (Bio-Rad Labs.) followed by 200 μl of a guinea-pig anti-insulin immunobead slurry containing excess immunobinding capacity in relation to the sample. The columns were closed and rotated overnight at 4°C. The serum was then drained off, the immunobeads were washed with distilled water and subsequently insulin, proinsulin and intermediates were recovered after addition of 400 μl of 1 *M* acetic acid–30% ACN (15 min at 4°C), this procedure being repeated twice. The combined acetic acid–ACN solutions were lyophilized, redissolved in 200 μl of 3 *M* acetic acid–0.1% HSA and centrifuged prior to RP-HPLC analysis (corresponding to a 50-fold concentration).

RESULTS

A sample containing human insulin, proinsulin and the four major proinsulin intermediates (see Fig. 1) was analysed using different stationary–mobile phase combinations in order to obtain a satisfactory (baseline) separation of all the components. As mobile phases, TFA–ACN and also TEAP and AS in ACN, previously shown to be excellent for insulin–proinsulin separations [14], were applied. The results are summarized in Table I, from which it can be seen that only two systems were able to fulfil this demand. Representative chromatograms using LiChrosorb RP-18–AS–ACN and Nucleosil 300-5C4–TFA–ACN are shown in Figs. 2 and 3, respectively.

The ELISA determinations and the UV trace for the RP-HPLC-separated components in a sample containing 1 μg of insulin, proinsulin and each of the four intermediates using the Nucleosil–TFA–ACN system are shown in Fig. 4. Insulin ELISA was performed on fractions 1–45 and proinsulin ELISA on fractions 46–120.

The Nucleosil–TFA–ACN system was chosen for the separation of insulin and PIM in serum samples because guard columns packed with the same stationary phase are available and probably necessary in order to protect the separation column from harsh serum constituents and thus extend the column lifetime. Further, the deleterious effect of the corrosive salt used in the alternative system was avoided.

Examples of the application of this RP-HPLC analysis to serum samples from a normal subject (control) and a NIDDM patient are shown in Fig. 5. In Fig. 6 the proinsulin ELISA values are shown from a selected range of fractions with an extended concentration scale in order to reveal minor peaks.

To avoid cross-contamination from sample to sample and also from standards run between samples to verify the positions of the individual components by the UV signal, the Hamilton syringe used for injection of the sample was rinsed at least three

TABLE I

RP-HPLC SEPARATIONS OF INSULIN, PROINSULIN AND THE FOUR PROINSULIN INTERMEDIATES

Stationary phase	Mobile phase		
	0.1% TFA–ACN (pH 2.0)	0.125 *M* TEAP (pH 4.0)–ACN	0.125 *M* AS (pH 4.0)–ACN
LiChrosorb RP-18	Irreversible binding of insulin and proinsulin	One intermediate eluted with proinsulin	Baseline separation of all components
Nucleosil 100-5C_{18}	Irreversible binding of insulin and proinsulin	Two intermediates eluted with proinsulin	
Nucleosil 300-5C_4	Baseline separation of all components		
Zorbax Protein Plus	One des-proinsulin eluted with proinsulin		

times with 3 *M* acetic acid–0.1% HSA before each injection. ELISA of the fractions collected after injection of 200 µl of 3 *M* acetic acid–0.1% HSA after such a rinsing procedure showed no content of insulin and PIM.

As HSA and other hydrophobic proteins from the serum samples will bind to the stationary phase with the optimized shallow acetonitrile gradient used in this separation (maximum 32% acetonitrile), the column was eluted for 15 min with 60% acetonitrile after each analysis.

In order to confirm the retention times of the serum PIM, a standard containing insulin and HPI was analysed before and after each serum sample. Further, a mixture of the four intermediates was analysed within each batch of column and mobile phase.

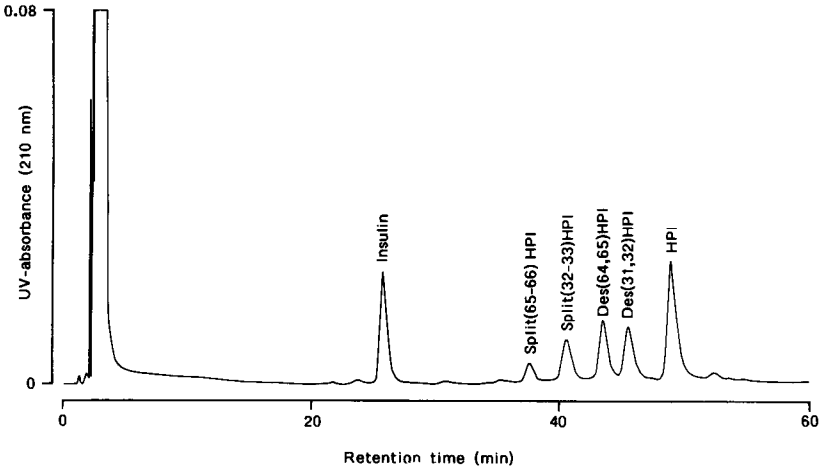

Fig. 2. RP-HPLC separation of human insulin (*ca.* 1 µg), proinsulin (HPI, *ca.* 2 µg) and the four proinsulin intermediates (*ca.* 1 µg of each) using a LiChrosorb RP-18 column eluted at 1.0 ml/min with a linear acetonitrile gradient (29.4 to 33.6%) in 0.125 *M* ammonium sulphate (AS) (pH 4.0) during 60 min.

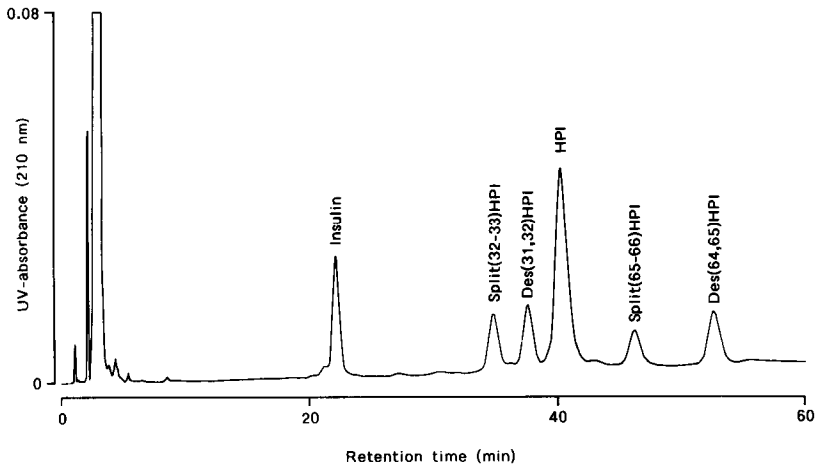

Fig. 3. RP-HPLC separation of human insulin (*ca.* 1 μg), proinsulin (*ca.* 3 μg) and the four proinsulin intermediates (*ca.* 1 μg of each) using a Nucleosil 300-5C_4 column eluted at 1.0 ml/min with a linear acetonitrile gradient (29.4 to 30.6%) in 0.1% TFA during 60 min.

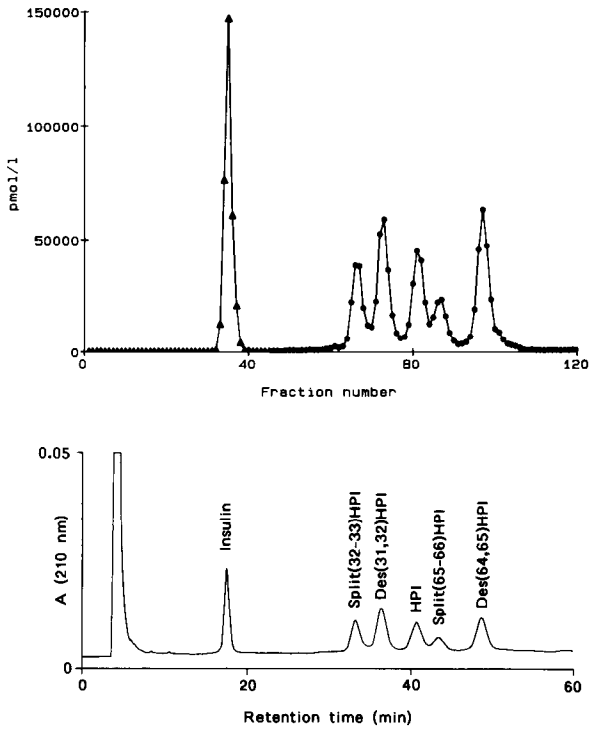

Fig. 4. RP-HPLC separation of human insulin, proinsulin and the four proinsulin intermediates (1 μg of each) eassentially as in Fig. 3, but using another batch of Nucleosil stationary phase protected by a guard column. 0.5-min fractions were collected followed by (▲) insulin ELISA of fractions 1–45 and (●) proinsulin ELISA of fractions 46–120.

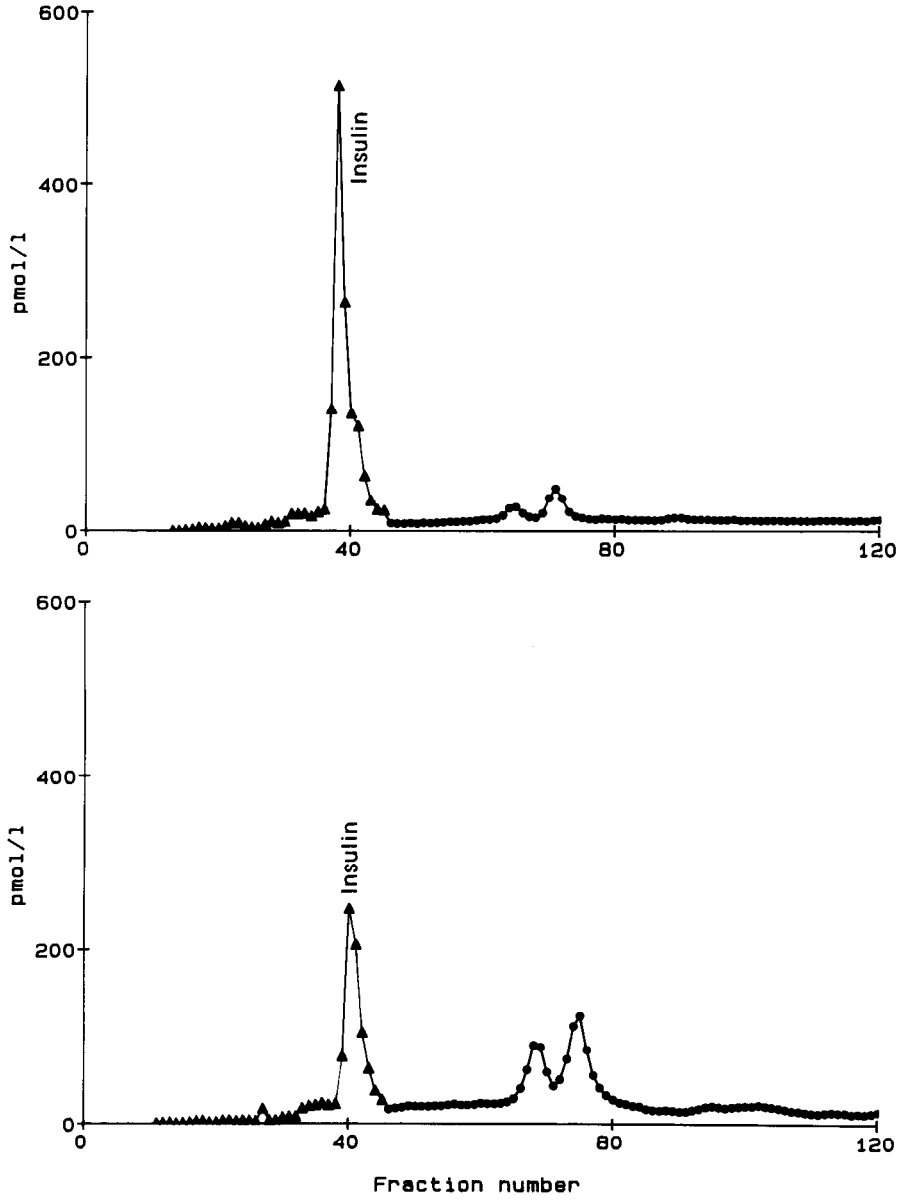

Fig. 5. RP-HPLC of immunoaffinity-purified serum from a normal human (control) and a NIDDM patient (upper and lower curve, respectively) using a Nucleosil 300-5C_4 column as described in Fig. 3. Insulin and proinsulin ELISA as in Fig. 4. The proinsulin ELISA results for fractions 46–100 are shown with an extended scale in Fig. 6.

The recovery of insulin and proinsulin after RP-HPLC of picogram to nanogram amounts was evaluated using radioactively labelled insulin (mono-[^{125}I-(TyrA14]-insulin) and proinsulin ([^{3}H-Leu]proinsulin) as samples. The recoveries (amount of radioactivity collected relative to the radioactivity injected) were found to be in the range 99–105%.

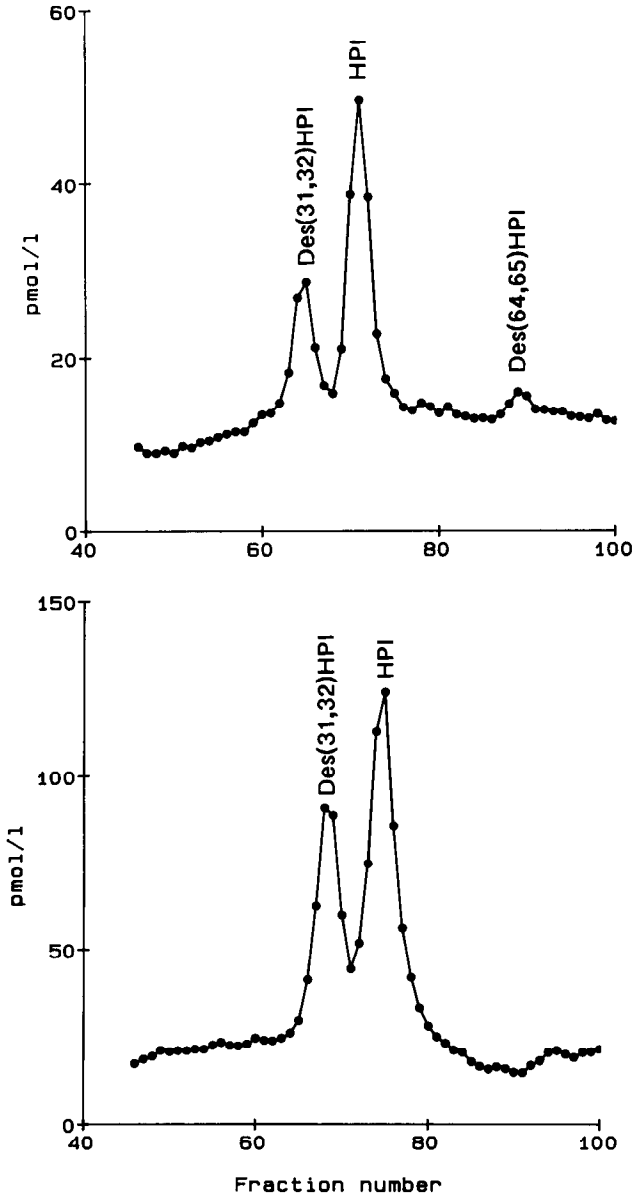

Fig. 6. Proinsulin ELISA of fractions 46–100 (see Fig. 5) corresponding to serum samples from a normal human (control) (upper curve) and a NIDDM patient (lower curve).

DISCUSSION

There have been few reports of RP-HPLC separations of insulin, proinsulin and proinsulin intermediates [3,15], whereas several immunological methods for the determination of proinsulin and proinsulin intermediates in plasma and serum have

been published [10,16–19]. Various degrees of cross-reactivity confuse these results and all individual intermediates have never been determined directly.

We evaluated the use of several stationary–mobile phase combinations in order to obtain the desired separation. From Table I it can be concluded that TFA–ACN in combination with a Nucleosil C_4 column was able to separate all six components (Fig. 3). The two C_{18} columns showed irreversible binding of insulin and proinsulin with this mobile phase (as previously described in ref. 20) and were thus unsuitable. Neither of the two C_{18} stationary phases eluted with TEAP–ACN was able to separate all the components, whereas one of these columns gave a baseline separation of all six components when AS–ACN was used as the mobile phase (Fig. 2).

By comparison of the separation patterns shown in Figs. 2 and 3, it can be concluded that the selectivities of the two stationary–mobile phase combinations were very different, resulting in altered elution orders of the proinsulin and proinsulin intermediates. A change in selectivity was also observed using the same stationary phase (LiChrosorb RP-18) with AS–ACN at different pH values. At pH 4.0 all components were baseline separated, whereas the two split-proinsulins were found to co-elute and des(64,65)HPI eluted later than proinsulin at pH 3.0 (data not shown).

Further differences from the elution orders shown in Figs. 2 and 3 were found when an Ultrasphere C_{18} column was eluted with 100 m*M* phosphoric acid–12 m*M* triethylamine–50 m*M* sodium perchlorate (pH 3.0) in shallow acetonitrile gradients during 100 min [15] and when a LiChrosorb RP-18 column was eluted with 50 m*M* phosphoric acid–100 m*M* sodium perchlorate–10 m*M* heptanesulphonic acid (pH 3.0) in a shallow acetonitrile gradient [3]. These results clearly show the versatility of RP-HPLC and the need for careful optimization of both the stationary and mobile phases.

The insulin and proinsulin ELISA determinations in the fractions collected from the separation of a mixture of insulin, proinsulin and proinsulin intermediates using the preferred RP-HPLC system, Nucleosil–TFA–ACN, were comparable to the UV trace as shown in Fig. 4 (the UV trace in Fig. 3 was obtained with a different batch of Nucleosil stationary phase, showing that batch-to-batch variations might occur).

The system was subsequently used to measure the content of insulin and PIM in serum from controls and NIDDM patients. Representative examples are shown in Figs. 5 and 6. Compared with a control (Fig. 5, upper curve), the NIDDM serum (Fig. 5, lower curve) showed a higher proportion of PIM (the minor peaks eluted around fractions 60–80) relative to insulin immunoreactive material. The PIM was shown to be heterogeneous (Fig. 6) in both categories, consisting of intact proinsulin and des-proinsulin intermediates. Their identities were deduced by comparison with the retention times of insulin, HPI and the four intermediates, as analysed before and after the actual serum sample.

Proinsulin in human serum has previously been reported to be heterogeneous [16–19], although intact proinsulin infused intravenously in diabetic subjects reveals less than 1% proteolytic degradation and when infused subcutaneously revealed 4–11% processing [15], the main product being des(31,32)HPI. The primarily detected proinsulin intermediate in human serum is reported to be the split(32–33)HPI [10,19,21]. This does not necessarily conflict with our finding of the des-HPI forms in serum, as the immunological methods do not distinguish between the split- and des-

forms. The RP-HPLC analyses of the individual split-HPI standards showed no transformation to the corresponding des-HPI forms during chromatography (data not shown). Further investigations on this aspect are in progress.

The recovery of insulin and proinsulin after RP-HPLC was found to be quantitative. As radiolabelled proinsulin intermediates were not available, their recoveries could not be determined as such; however, as their hydrophobicities and molecular weights are very similar to those of proinsulin, their recoveries would be expected to be similar. The recovery in the immunoaffinity purification step has yet to be evaluated.

In conclusion, we have developed two rapid (60 min) RP-HPLC methods for the baselin separation of insulin, proinsulin and the four major proinsulin intermediates. One of the methods (with Nucleosil–TFA–ACN) will be used for future evaluations of the insulin and PIM composition in serum from different patient categories in order to obtain information on possible changes in β-cell function.

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