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Reversed-phase high-performance liquid chromatographic characterization of acetic acid extracts of the normal and the diabetic human pancreas

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ABSTRACT

The combination of a divinylbenzene-based reversed-phase (RP) column and acetic acid gradients in water as mobile phase described in the accompanying paper was used for characterizing the extractable polypeptides from the normal and the diabetic human pancreas. The pancreas was lyophilized, minced and extracted three times in 3 *M* acetic acid. After mechanical clarification, the raw extracts were applied directly to the RP column. Alternatively, the extracts were lyophilized and subjected to size-exclusion chromatography on Sephadex G-50 in 3 *M* acetic acid. Two fractions with mol. wt. > 6000 dalton (Peak I) or with mol. wt. ≤ 6000 dalton (Peak II) were obtained.

The Sephadex G-50 size-exclusion chromatography and the RP-high-performance liquid chromatographic (HPLC) analyses of the crude extracts from a normal pancreas clearly demonstrated the weight distribution and differences between the exocrine pancreas (containing primarily the major digestive enzymes) and the endocrine pancreas (containing insulin, glucagon, etc.). RP-HPLC analyses of crude extracts from various normal pancreatic glands resulted in very similar UV profiles, whereas those from a number of individual diabetic glands differed. Chromatograms of acetic acid extracts from normal pancreata were similar when analysed before or after lyophilization, whereas lyophilization of acetic acid extracts of diabetic glands resulted in severely obscured chromatograms.

RP-HPLC analyses clearly demonstrated several differences between the diabetic and the normal pancreas. In the crude extracts, the extractable proteins from the diabetic pancreas were shifted towards lower molecular weight and/or hydrophobicity. Further, a peak co-eluting with authentic, human insulin could be demonstrated in the raw extract and in the peak II material from the normal pancreas, whereas virtually no mass signal was seen in the UV-profiles of similar materials from the diabetic gland. This finding was further verified by insulin radioimmunoassay (RIA) performed on the isolated fractions after RP-HPLC of a crude extract from a normal and a diabetic pancreas. The insulin content in the diabetic pancreas was found to be *ca.* 1% of that in the normal pancreas. When authentic glucagon was added to crude extracts from a diabetic pancreas, a single component was found after immediate analysis, but after several hours at room temperature the glucagon was found to be degraded. Added insulin was stable under these conditions.

Similar RP analyses were performed on a silica C_4 column eluted with an acetonitrile gradient in trifluoroacetic acid. Although the chromatograms were more complex than those obtained with acetic acid as mobile phase, they were comparable in outline, and the differences between the normal and the diabetic glands could again be demonstrated.

One of the reasons for the differences between the normal and the diabetic pancreas may be that the normal glands were removed and frozen immediately after death, whereas the diabetic glands were obtained after an ischaemia time of 6 h or more, leaving considerable time for post mortem enzymatic degradation.

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The RP-HPLC system is very suitable for these analyses. The acetic acid extracts can be applied directly to the RP column and, after lyophilization, the isolated fractions may be re-analysed [RIA or enzyme-linked immunosorbent assay (ELISA)], or subjected to a second (alternative) RP-HPLC. After such double RP-HPLC purification, almost all major individual components in the peak I and II material from a normal pancreas were sufficiently pure for microsequencing, and identification (based on 30 steps in the gas-phase sequencer and comparing the sequence information obtained with databases) again reflected the distribution of the principal components from the exocrine and the endocrine pancreas in the peak I and II materials.

INTRODUCTION

Extraction of biological samples (tissues, organs or whole organisms), often the first step in many isolation procedures for biopolymers, normally results in extremely complex mixtures of lipids, carbohydrates and proteins/peptides. One of these main groups may be excluded, or favoured, through a proper choice of solvent, *e.g.*, a very polar solvent will solubilize more protein material than lipid, but most often an aqueous extract will contain numerous related compounds in addition to those desired.

Characterization of such raw extracts is normally not performed with general analytical tools (*e.g.*, general protein or carbohydrate analysis) but with analyses specific for a single or a narrow group of compounds, such as biological activity (*e.g.*, enzymatic activity) or antigenicity [enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), etc.]. In the case of polypeptides, the development of analytical and preparative reversed-phase high-performance liquid chromatography (RP-HPLC) with sensitive, non-specific mass detection (UV, refractive index), or more specific post-column reactions, in the last decade has reached a level where the separation and determination of more than 100 polypeptides in a single chromatographic analysis can be achieved in less than 1 h. Consequently, this technique has been incorporated as a rapid downstream control analysis in the industrial scale extraction of biological materials, such as the production of insulin and growth hormone.

In this work we applied RP-HPLC for the characterization of crude extracts of the normal and the diabetic human pancreas. Acetic acid was chosen as solvent owing to the extremely good solubility of the majority of peptides and proteins from the endocrine pancreas in this solvent [1]. As we recently demonstrated that a polymeric phenyl-based RP column could be eluted with an acetic acid gradient without the use of organic modifiers [2], this system was used for the RP-HPLC characterization of the acetic acid extracts, thereby eliminating the need for solvent exchange or concentration/isolation procedures between extraction and characterization of the solubilized sample material. The UV profiles obtained were compared with those of similar separations using a silica-based RP column eluted with an acetonitrile gradient in trifluoroacetic acid (TFA). Several pancreatic proteins/peptides isolated after the acetic acid extraction and RP-HPLC in acetic acid gradients have been identified after amino acid sequencing, indicating the general applicability of this extraction/analysis system for identification purposes.

EXPERIMENTAL

The HPLC equipment, columns, samples and all analyses were performed as described in the accompanying paper [2]. Detailed descriptions of the stationary and mobile phases are given in the legends to the figures.

RESULTS

Fig. 1 (left) shows the elution profile obtained after Sephadex G-50 chromatography of lyophilized acetic acid extracts obtained after the first, second and third

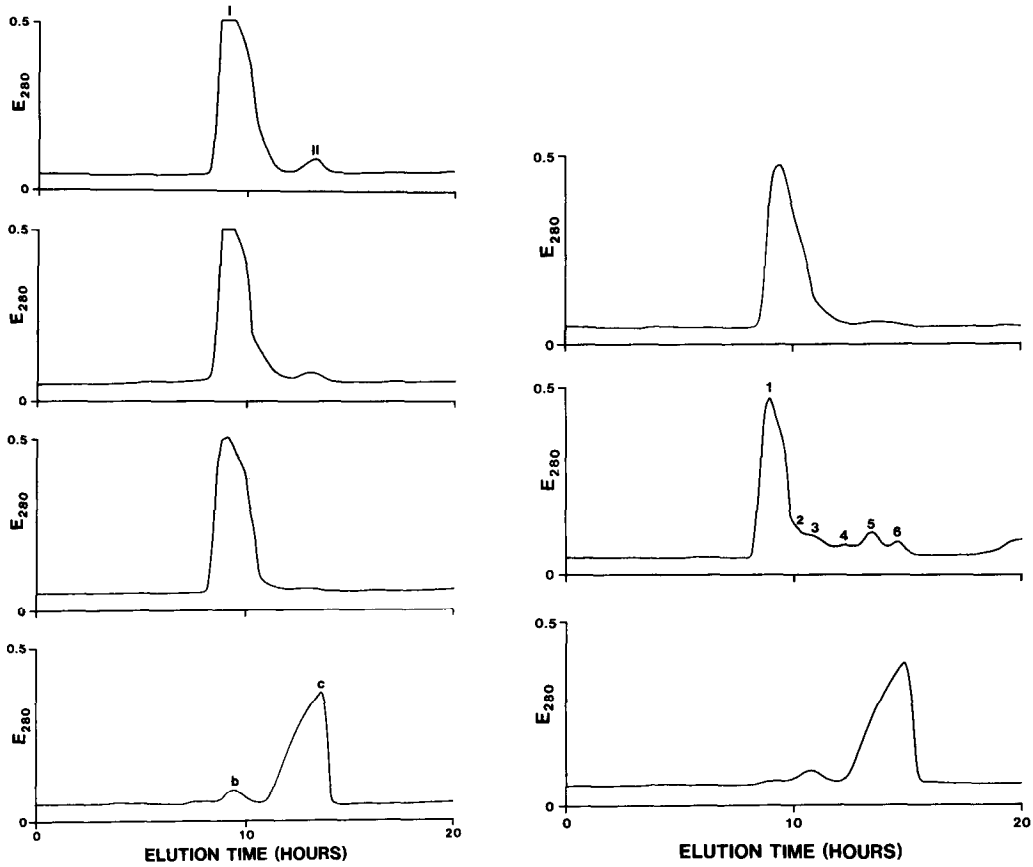

Fig. 1. Left: Sephadex G-50 chromatography of 200 mg of lyophilized material obtained from first, second and third extractions of a normal human pancreas. After lyophilization of the crude acetic acid extracts, the residues were dissolved in 3 *M* acetic acid, centrifuged and filtered. A 95 × 2.5 cm column was eluted at 4°C (*ca.* 20 ml/hour) with 3 *M* acetic acid. From top to bottom: first, second and third extractions. Peak I and II materials are marked "I" and "II", respectively. Bottom: 100 mg of crystalline, porcine insulin. The b- and c-components are marked accordingly. Right: Sephadex G-50 chromatography of 200 mg of lyophilized acetic acid extract (combined from the first, second and third extractions) of a normal human pancreas (top), a diabetic, human pancreas (middle) and 100 mg of crystalline, porcine insulin (bottom). The elution was performed as described for the left panel.

extractions of a normal human pancreas. In all three extracts, the majority of extractable polypeptides were eluted in the void volume fraction (peak I). A smaller fraction (peak II) with a similar elution volume to insulin peptide (compare to the bottom curve on the left) gradually decreased from the first to the third extraction. A comparison between the Sephadex G-50 profiles obtained from lyophilized acetic acid crude extracts of a normal and a diabetic pancreas is shown in Fig. 1, right. The diabetic extract has a similar distribution in mass between the void volume peak and the residual material with higher elution volume, but whereas only a single peak with molecular weight (MW) $\leqslant$ 6000 dalton was detected in the extract from a normal pancreas, this region contained two larger and three smaller peaks (marked 2–6), indicating a more heterogeneous distribution of the peptide region in the extract of the diabetic pancreas.

On average, 2–4 mg of peak II material and 50–80 mg of peak I material were obtained after Sephadex G-50 chromatography of 200 mg of lyophilized crude extract of a normal pancreas, indicating a considerable loss of material during the filtrations prior to gel chromatography. The protein concentrations in the extracts showed extremely wide variations, indicating the very heterogeneous sample material. The insulin contents in the crude extracts were equally scattered (Table I).

After sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of crude extracts of a normal and a diabetic pancreas, numerous proteins were distributed throughout the whole separation range (6000–300 000 dalton) in both extracts. SDS-PAGE of peaks I and II also verified that peak II material from a normal and a diabetic pancreas primarily contained peptides with MW similar to or lower than the effective MW fractionation range of the 8–25% gradient gel system (data not shown).

RP-HPLC analyses of the first extract from a normal and a diabetic pancreas, using a Dynosphere PD-102-RE column eluted with an acetic acid gradient, are shown in Fig. 2, upper, and those of the corresponding lyophilized extracts in Fig. 2, lower. For both the crude extracts and the lyophilized extracts, subsequent extrac-

TABLE I

CONTENTS OF PROTEIN AND INSULIN DETERMINED IN THE ACETIC ACID EXTRACTS OF THREE INDIVIDUAL NORMAL AND FIVE INDIVIDUAL DIABETIC HUMAN PANCREATA

The insulin and protein contents are calculated per gram of lyophilized pancreas.

Pancreas	Protein (mg/g)	Insulin (μg/g)
Normal	258	607
	161	463
	81	322
Diabetic	80	37
	59	38
	15	15
	41	19
	53	14

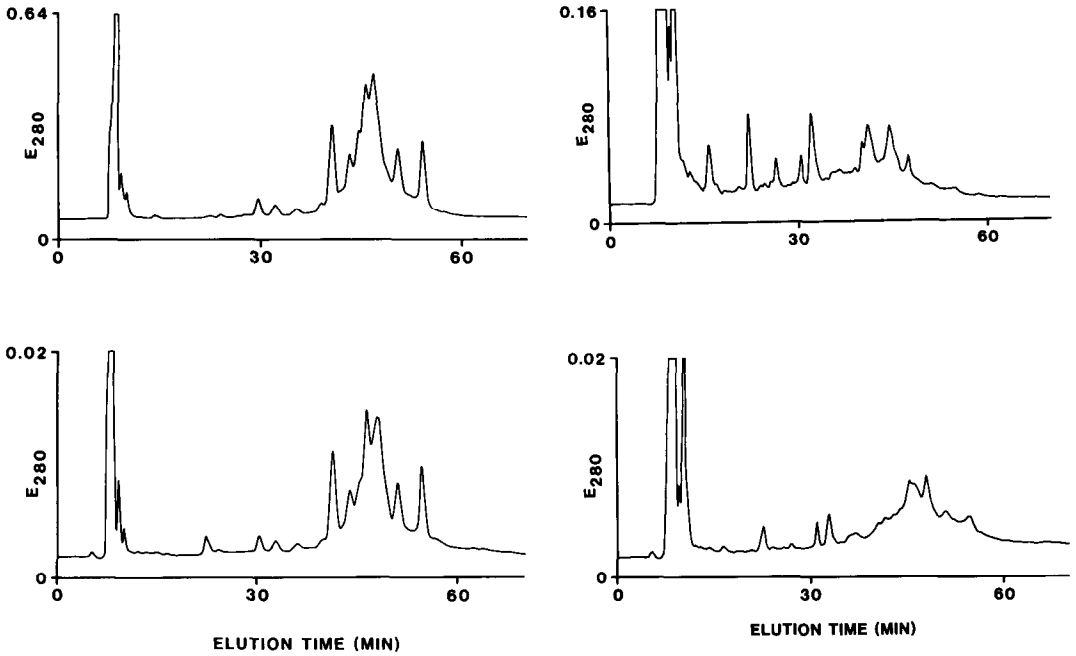

Fig. 2. RP-HPLC of 200 μl of crude acetic acid extract of a normal (upper left) and a diabetic human pancreas (upper right) and of the corresponding lyophilized acetic acid extracts solubilized to 1 mg/ml in 3 *M* acetic acid. A 200-μl volume was applied in the analysis of this material from a normal (lower left) and a diabetic pancreas (lower right). A 250 × 4.6 mm I.D. Dynosphere PD-102-RE column was eluted at 0.5 ml/min with an acetic acid gradient (37.5 → 90% acetic acid linearly during 60 min followed by 10 min isocratically at 90% acetic acid).

tions of both glands resulted in similar RP-HPLC profiles at reduced levels (data not shown). When the UV-profiles of normal glands (Fig. 2, left) are compared with those from a diabetic (Fig. 2, right), it can be seen that much less UV-absorbing material was present in the raw extract of the diabetic pancreas (note the different UV scales), and the RP-HPLC profile of the diabetic pancreas is very different from that of a normal pancreas. The diabetic protein distribution is shifted towards earlier elution, indicating lower hydrophobicity and/or molecular weight of the individual sample components. The dominant part in the RP-HPLC profile of the normal pancreas is a group of peaks eluted in the last third of the chromatogram, whereas in the extracts of the diabetic pancreas the impact of this group of peaks is strongly reduced, compared with the earlier eluting peaks, which now are the dominating sample constituents. In addition, lyophilization of the raw acetic acid extracts of a diabetic pancreas led to a considerable reduction in chromatographic separation efficiency (compare the upper and lower curves in Fig. 2, right). The last half of the chromatogram is seriously obscured, and the number of individual components in the whole chromatogram is reduced. In contrast, the RP-HPLC profile of the directly analysed crude extract from a normal pancreas was very similar to that obtained for the lyophilized extract (Fig. 2, left, upper and lower curves).

RP-HPLC analyses of peak I and II material from a normal and a diabetic pancreas are shown in Fig. 3, left and right, respectively. The sample constituents from the extract of the normal pancreas are clearly divided into two groups with different retention times, reflecting the separation according to MW after Sephadex G-50 chromatography, whereas the separation efficiency of the diabetic peak I material (Fig. 3, right, upper curve) was found to be reduced in a similar way to that above for the lyophilized total extract (Fig. 2, right, lower curve). However, the peptide-like peak II material derived from the diabetic pancreas was separated equally efficiently as that from the normal pancreatic extract (Fig. 3, lower curves). Similar results were obtained for other individual peak I and II materials [2], indicating that this reduction in separation capacity of the diabetic material is of a general nature.

Three different normal pancreatic glands (parts of the whole pancreas) were extracted, followed by RP-HPLC of the lyophilized crude acetic acid extracts (Fig. 4, left). It can be seen that the extraction–RP-HPLC analysis procedure resulted in comparable UV profiles: the majority of sample components were found to be present in each of the three different extracts, although limited variations in the significance of the individual peaks were noticed between the different samples. The sample components in the last part of the chromatogram were the most abundant, indicating that the majority of the extracted material consists of peak I material (compare with Fig. 3, left, upper curve). Comparable analyses were performed with three different diabetic pancreatic crude extracts (Fig. 4, right). The first half of the chromatograms differed more than that for similar extracts of normal glands, whereas the last parts of the chromatograms were comparable. However, these last parts of three RP-HPLC traces of the diabetic extracts were still significantly different from those of normal glands (compare with the left panel).

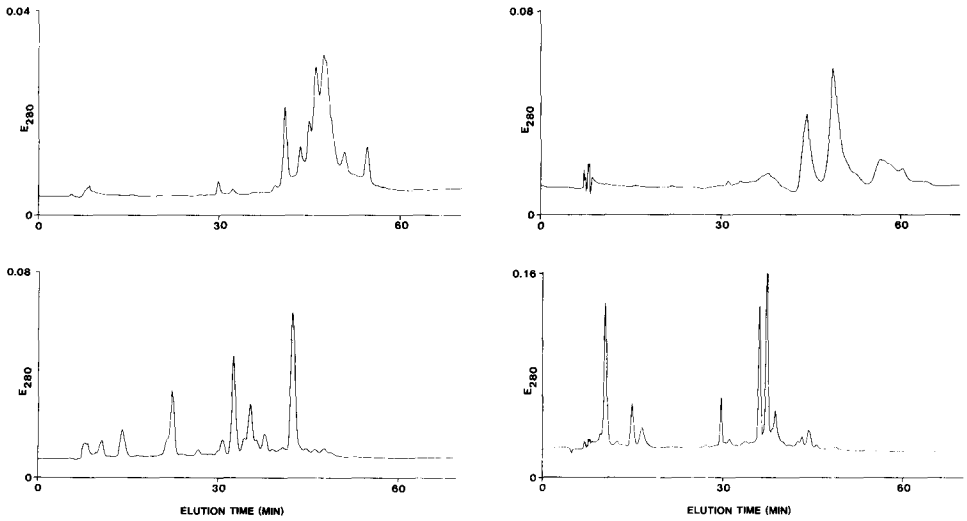

Fig. 3. RP-HPLC of peak I (upper curves) and peak II (lower curves) material (lyophilized crude acetic acid extract after Sephadex G-50 chromatography as described under Experimental), obtained from a normal human (left) and a diabetic human pancreas (right). The sample material was redissolved in 3 *M* acetic acid to 1 mg/ml, centrifuged and filtered (0.45 μm). 200 μl of both samples were applied. Stationary and mobile phases as in Fig. 2.

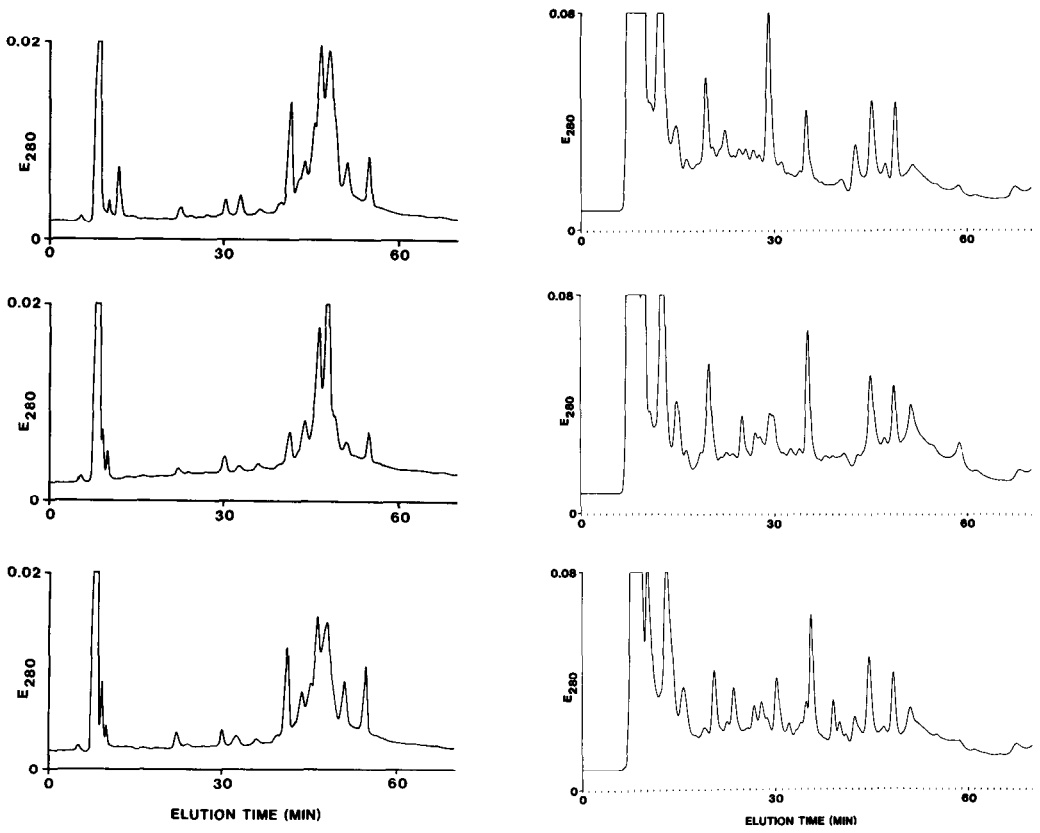

Fig. 4. Left panel: RP-HPLC of lyophilized crude acetic acid extracts of three different normal human pancreatic glands. The lyophilization residues were redissolved to 1 mg/ml in 3 *M* acetic acid, centrifuged and filtered (0.45 μm). 200 μl were applied in all analyses. Stationary and mobile phases essentially as in Fig. 2. Right panel: RP-HPLC of 200 μl crude acetic acid extracts of three different diabetic, human pancreatic glands. Stationary and mobile phases as in Fig. 2.

In order to compare the separation capacity of the Dynosphere column eluted with an acetic acid gradient with that of a commonly used RP-HPLC system, the crude extracts of a normal and diabetic pancreas were separated on a Nucleosil 300 Å C_4 column eluted with an acetonitrile gradient in TFA (Fig. 5). The chromatograms of extracts from a normal pancreas were remarkably similar in the two different systems, again demonstrating the predominant position of the sample components eluted in the last third of the chromatogram (compare Fig. 5, upper, with Fig. 2, left, upper curve). The HPLC profile of the extract from the diabetic pancreas (Fig. 5, lower) was more detailed than obtained after elution of the Dynosphere column with an acetic acid gradient (Fig. 2, right, upper curve) with respect to the components eluted in the first half of the chromatogram, again emphasizing the shift in hydrophobicity and molecular weight compared with the late-eluting main components in extracts of the normal pancreas.

The elution positions of insulin, proinsulin and glucagon in the Dynosphere–

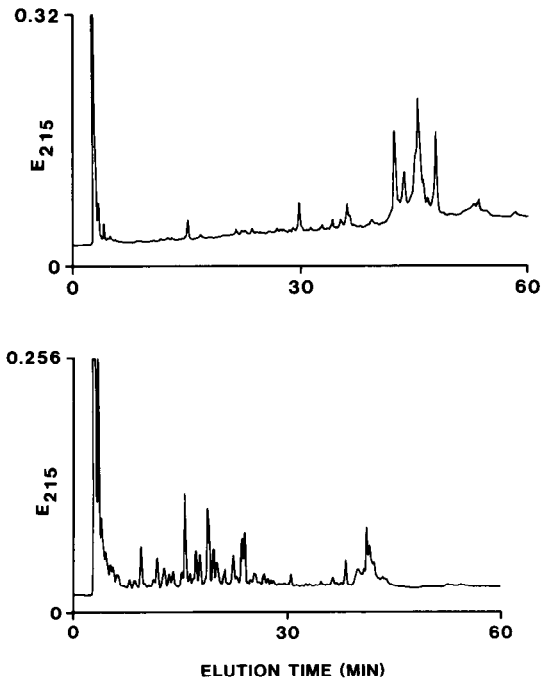

Fig. 5. RP-HPLC of 10 μl of the crude acetic acid extract of a normal human (upper) and a diabetic human pancreas (lower) using a 250 × 4.0 mm I.D. Nucleosil 300 Å C_4 column eluted at 1.0 ml/min with an acetonitrile gradient (12 → 57% acetonitrile linearly during 60 min) in 0.1% TFA. Separation temperature: 45°C.

acetic acid RP system were mapped in the normal and the diabetic crude extracts after spiking with small amounts of the authentic hormones (Fig. 6). Insulin and glucagon co-eluted in this system, and the retention times of these two hormones were similar to that of an already existing peak in the extract from the normal pancreas (upper curve), whereas very little UV-absorbing material was present at those positions in the chromatogram of diabetic crude extract where insulin, glucagon and proinsulin were eluted (lower curve).

During these spiking experiments, it was noted that when glucagon was added to the diabetic crude extract, the resulting chromatogram depended on the time spent in the autosampler before the actual column separation: when the glucagon-spiked extract was analysed immediately, a single glucagon-peak was observed (Fig. 7, left, middle curve), but after 15 h, two additional major peaks with shorter retention times than glucagon was observed (Fig. 7, left, lower curve). This was not an effect of instability of the diabetic acetic crude extract as such, since the UV profile of an immediately analysed crude extract was found to be similar to that obtained from the same extract stored at room temperature for 48 h (data not shown).

A similar experiment was performed after addition of crystalline glucagon plus trace amounts of [^{125}I]glucagon (Fig. 7, right). Immediate analysis of the tracer alone or added to the diabetic crude extract resulted in a single UV glucagon peak and a radioactivity peak with a slightly reduced retention time (plus a UV peak correspond-

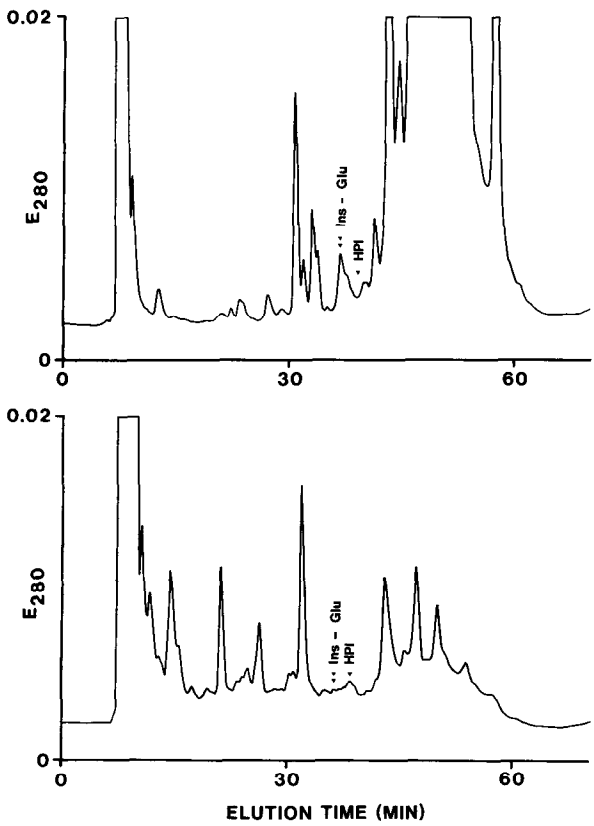

Fig. 6. RP-HPLC of 25 μl of crude acetic acid extract from a normal human (upper) and a diabetic human pancreas (lower). The elution positions of human insulin (Ins), porcine glucagon (Glu) and human proinsulin (HPI) are marked with filled triangles. Stationary and mobile phases as in Fig. 2.

ing to the human serum albumin added to the tracer preparation; Fig. 7, right, upper and middle cruves). However, after 48 h at room temperature, the crude extract with added glucagon again showed two major UV peaks with markedly reduced retention times, and all the radioactivity was eluted in the first major peak (Fig. 7, right, lower curve). This degradation of glucagon could only be demonstrated in the case of the diabetic crude extract. If glucagon was incubated in crude extract of a normal, human pancreas for 17 h in a WISP autosampler, a single UV glucagon peak was obtained after RP-HPLC. Further, under the conditions causing degradation of added glucagon, insulin added to the diabetic and the normal extracts was found to be totally stable (data not shown).

Although no distinct UV peak with a similar retention time to that of added human insulin could be demonstrated in crude extracts of a diabetic pancreas, a small UV peak, co-eluting with added insulin, was observed when peak II material (assumed to be enriched with polypeptides with MW $\leqslant$ 6000 dalton) from a diabetic pancreas was analysed. As many insulin dependent diabetes mellitus (IDDM) pa-

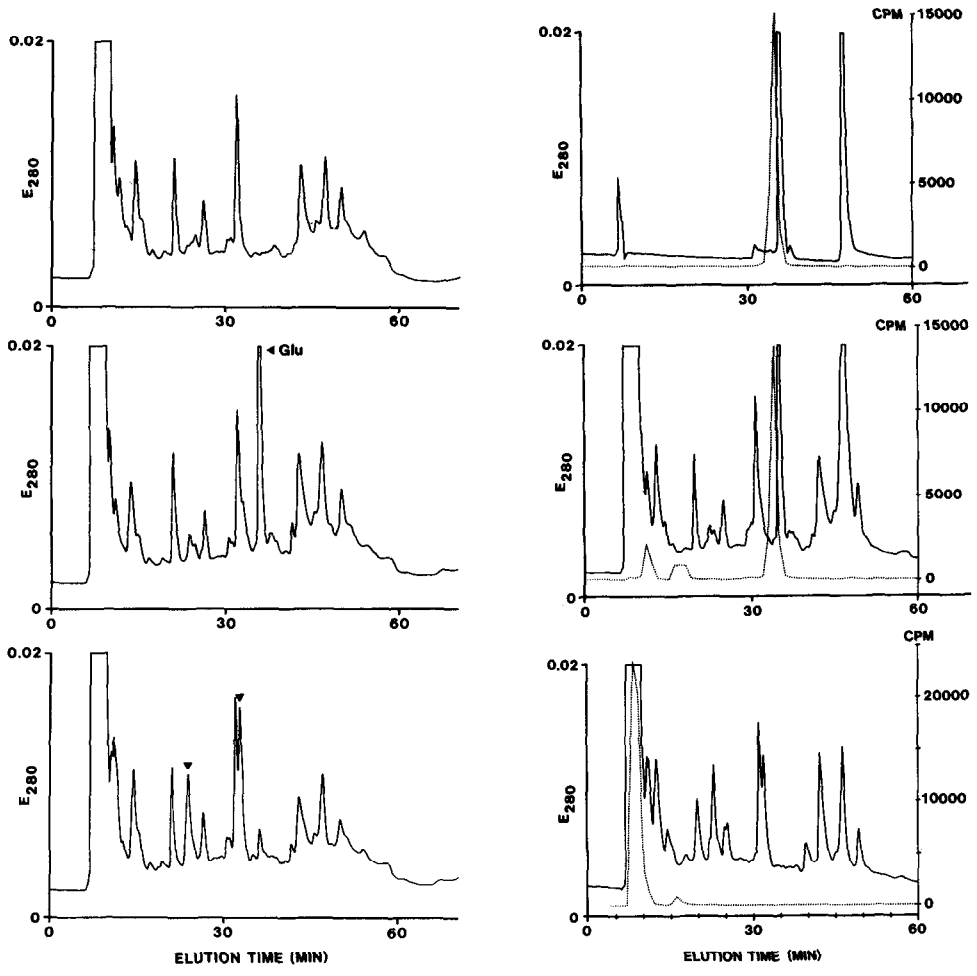

Fig. 7. Left: RP-HPLC of 25 μl of crude acetic acid extract from a diabetic human pancreas (upper) and the same extract spiked with 10 μg of crystalline, porcine glucagon (Glu) and injected immediately (middle curve) or after 15 h in a WISP autosampler (lower curve). The two major additional peaks formed from the added glucagon are marked with filled triangles. Right: RP-HPLC of 10 μg of crystalline, porcine glucagon + *ca.* 35 000 cpm of [^{125}I]glucagon (upper curve), of 35 μl of crude acetic acid from a diabetic, human pancreas spiked with 10 μg of crystalline, porcine glucagon + *ca.* 35 000 cpm of [^{125}I]glucagon, analysed immediately (middle curve) or after storage at room temperature for 48 h (lower curve). Solid curves, UV; dotted curves, radioactivity. The large peak in the last part of the chromatograms represents serum albumin added to the [^{125}I]glucagon tracer preparation. Stationary and mobile phases as in Fig. 2.

tients maintain small amounts of β-cell function after the clinical onset of diabetes mellitus, the nature of this peak was investigated further. RP-HPLC of crude extracts from a normal and a diabetic pancreas was followed by insulin RIA of the isolated fractions, and it could be clearly demonstrated, that small amounts of immunological reactive insulin were present in the diabetic pancreas (Fig. 8). Approximately 1% of the amount of insulin extracted from the normal pancreas could be demonstrated

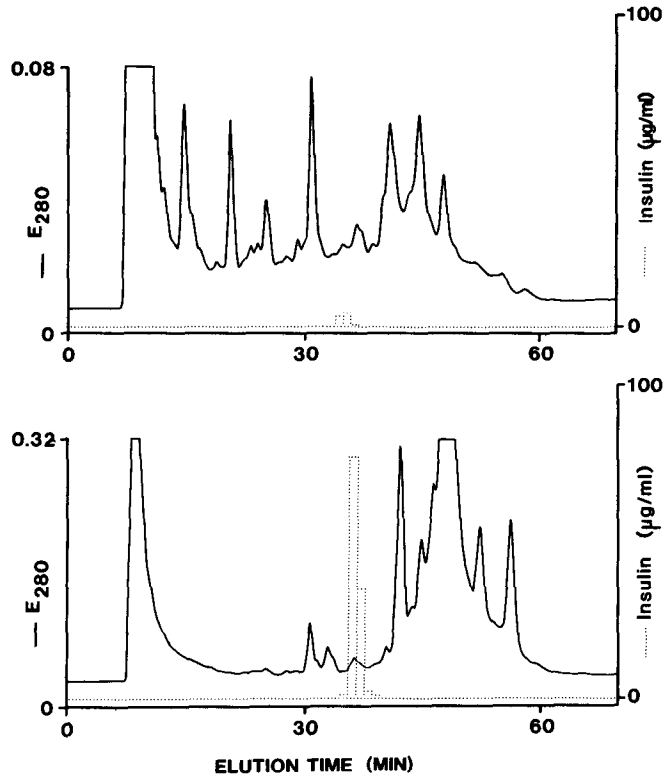

Fig. 8. RP-HPLC of 200 μl of raw acetic acid extract of a diabetic human pancreas (upper) and a normal human pancreas (lower). Fractions (1 min) were collected and lyophilized in a Speed Vac concentrator, redissolved and analysed for insulin content (RIA) as described under Experimental. Stationary and mobile phases as in Fig. 2.

after RP-HPLC of the extract of the diabetic gland. The minor content of insulin in the diabetic pancreata was also directly confirmed by insulin RIA of the crude extracts (Table I).

The present acetic acid RP-HPLC system should, in theory, be suitable as an isolation–identification system, owing to the volatile mobile phase. As it is obvious, that the number of sample components demonstrated in the UV profiles of the crude extracts was less than the number of components demonstrated after SDS-PAGE or after silica C_4–TFA–acetonitrile RP-HPLC, a second purification step, preferably involving a different stationary or mobile phase, would probably be necessary before any sequence analyses.

As a first attempt to make the above-described Dynosphere–acetic acid separations (all performed on a 250 × 4.6 mm I.D. column) a truly preparative isolation system, larger amounts of crude extracts were loaded on a 180 × 16.0 mm I.D. Dynosphere column and eluted with a similar acetic acid gradient at 4.0 ml/min. Almost identical UV profiles were obtained when the 16 mm I.D. column was loaded with up to 10 mg of lyophilized crude extract of a normal pancreas (data not shown). Approximately 5 mg of peak I and 3 mg of peak II material were then separated on

the 16 mm I.D. column, and the major peaks from both separations were collected manually. These individual fractions were then further purified using a Nucleosil 300 Å C_4 column eluted with an acetonitrile gradient in TFA or a TSK Phenyl 5PW RP+ column eluted with an acetonitrile gradient in 100 m*M* ammonium hydrogencarbonate. In the former instance, the acetic acid column eluate could be loaded directly

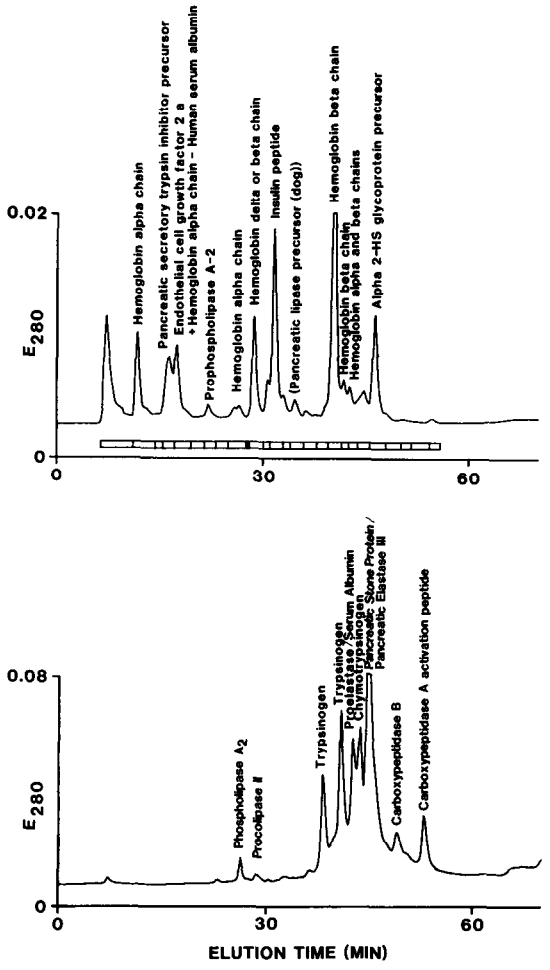

Fig. 9. Upper: RP-HPLC separation of peak II material [redissolved in 3 *M* acetic acid to 10 mg/ml, centrifuged and filtered (0.45 μm)] from a normal human pancreas using a 180 × 16.0 mm I.D. Dynosphere PD-102-RE column eluted at 4.0 ml/min with an acetic acid gradient (37.5 → 90% acetic acid linearly during 60 min, followed by 10 min isocratically at 90% acetic acid). 275 μl (= 2.75 mg of peak II material) were applied, and fractions were collected as indicated above the abscissa. After rechromatography of these fractions (Nucleosil C_4–TFA–acetonitrile) the major peaks were isolated and subjected to amino acid sequencing. For identification, the sequence information obtained after 30 degradation cycles was compared with published amino acid sequences in a database. Lower: RP-HPLC separation of peak I material from a normal human pancreas using the same stationary and mobile phase. 500 μl (= 5 mg of peak I material) were applied, and fractions corresponding to the main peaks were collected. Rechromatography, amino acid sequencing and identification as described for the upper panel.

onto the Nucleosil column (after dilution with one volume of distilled water), whereas lyophilization of the acetic acid column eluate was found to be necessary before analysis in the ammonium hydrogencarbonate system (if the acetic acid was neutralized with ammonia solution, the large amounts of ammonium acetate in the sample seriously disturbed the separation).

The major components obtained after the second purification steps were isolated by lyophilization and subjected to amino acid sequence analyses. Usually 30 degradation cycles were performed, and the sequence information obtained was compared with published sequences in databases for identification. The preliminary results of these analyses are shown in Fig. 9. It should be noted that the database identifications are shown in the figures, although only parts of the sequences have been identified in the actual RP-HPLC fractions. A complete sequence, or a mass spectrometric MW determination of the isolated compounds, might have identified which part of the sample molecule was actually present, but such analyses were not performed in these preliminary identification experiments.

DISCUSSION

The most frequently published procedure for the extraction of human and animal pancreatic glands utilizes acidified ethanol [3–19]. However, this procedure has been adapted from the industrial-scale extraction of insulin based on the pancreata of slaughterhouse animals, and it is highly specific for insulin and similar pancreatic peptides (glucagon, pancreatic polypeptide, somatostatin, etc.). The most common acidifier in these extractions is sulphuric acid, specially adapted for the porcine pancreas. However, when in the early 1970s we studied the efficiency of this extraction procedure for pancreatic glands from young cattle and humans, we observed a significantly lower yield compared with fresh porcine glands. In the case of young cattle, comparable amounts of insulin were extractable with ethanol acidified with phosphoric acid instead of sulphuric acid, but for human glands similar high yields were obtained only after extraction with 3 *M* acetic acid or 3 *M* acetic acid in 8 *M* urea. The last solvent was extremely efficient: after three successive extractions, virtually only the connective tissue remained in a non-solubilized state, but owing to the overwhelming amount of very different solubilized compounds and the absence of modern HPLC techniques, an isolation procedure for insulin was difficult to design [1].

Today, preparative HPLC is immediately involved in such considerations, but still the choice of the extraction medium for a "general" extraction of pancreatic glands (*i.e.*, extracting the majority of peptides and proteins present) must be carefully considered. Acidified ethanol or similar alcohols are certainly not an obvious choice, as the solubility of proteins is normally limited in this solvent, and the alcohol must necessarily be removed before the extraction medium can be applied to an RP column. A mixture of 3 *M* acetic acid in 8 *M* urea is very powerful solvent for most peptides and proteins, but large volumes of this solvent are hardly the most desirable in a normal RP binding–elution procedure.

The use of 3 *M* acetic acid remains an attractive possibility. The proteolytic enzymes present in large amounts in the exocrine pancreas are inhibited, the solubilizing properties for proteins are equal to that of acetic acid–urea, but the extraction medium is directly compatible with standard RP chromatographic systems. As we

recently have reported the elution of several proteins and peptides, including insulin and glucagon, from a polymeric phenyl column using an acetic acid gradient [2] (*i.e.*, without the use of common organic modifiers), it should be possible to perform the extraction and RP-HPLC separation in essentially the same liquid phase, thereby avoiding any sample manipulations between the extraction and the initial chromatographic purification.

Sephadex G-50 size-exclusion chromatography of the acetic acid pancreatic extract reflects the expected distribution between extractable "peptides" and "proteins": the endocrine part of the pancreas amounts to *ca.* 1–2% (on a weight basis) and the content of the major pancreatic hormone (insulin, MW 6000 dalton) is roughly 25% of that. Approximately 75% of the protein content of the exocrine pancreas consists of the major digestive enzymes (trypsin, chymotrypsin, lipase, amylase, elastase, carboxypeptidase A and B), which, together with serum albumin and haemoglobin (expected to be present in large amounts in extracts of vascularized tissue), contribute to the large void-volume peak I (Fig. 1).

When a normal human pancreas was extracted three times, the RP-HPLC profiles of the extracts were found to be very similar, with the exception of the amount of solubilized material. Lyophilization of these crude acetic acid extracts did not change the UV profiles obtained after RP-HPLC, in contrast to the findings for the diabetic human pancreas, where lyophilization of the crude acetic acid extracts resulted in deterioration of the separation pattern, especially in the last half of the chromatograms (Fig. 2). When the crude extract of a normal pancreas was compared with that of a diabetic pancreas, it was obvious that the diabetic extracts contained considerably less protein/peptide mass, and that the UV profile of solubilized proteins/peptides from a diabetic pancreas was displaced towards lower molecular weight and/or hydrophobicity. This displacement is clearly seen in all three diabetics (Fig. 4, right).

In outline, the extracts of three different normal pancreatic glands were similar (Fig. 4, left), whereas those of three diabetics were less comparable but clearly very different from a normal extract. The observed MW and/or hydrophobicity displacement for the diabetic extract compared with the normal extract was also clearly demonstrated when the RP-HPLC analyses were performed using a Nucleosil C_4 column eluted with the TFA–acetonitrile system (Fig. 5). Although a larger number of sample components were detected in the TFA–acetonitrile system (primarily owing to the use of UV registration at 215 nm instead of 280 nm in the acetic acid systems) [2], the use of these two highly different stationary–mobile phase combinations nevertheless resulted in surprisingly similar chromatograms. The MW and hydrophobicity differences between the extractable proteins and peptides from the normal and the diabetic pancreas could be demonstrated after Sephadex G-50 size-exclusion chromatography and also RP-HPLC with two different stationary–mobile phase combinations. This could reflect the difference in time between clinical death and removal/freezing of the two types of pancreatic tissue. If the pancreas is left at body temperature after death, most of the digestive enzymes present in the exocrine pancreas will be active for hours, resulting in marked cleavage of the pancreatic polypeptides. The finding that glucagon was degraded after incubation with extracts of the diabetic pancreas probably reflects that during the ischaemia time, the proteolytic enzymes in this pancreas were transformed to their active state, in contrast to the conditions in

the normal pancreas (removed and frozen shortly after death), where the majority of the enzymes would be expected to be present as inactive proenzymes.

It might seem surprising that insulin was not degraded under similar conditions. However, it was recently reported that acid saline extracts of the rat submaxillary gland (known to contain large amounts of glucagon-like material) were able to degrade [^{125}I]glucagon and [^{125}I]pancreatic polypeptide, whereas [^{125}I]insulin was left intact. The degradative effect could be inhibited by the addition of thiol proteinase inhibitors, whereas aprotinin had no effect [20]. Further, when pieces of surgically removed human pancreatic glands were left at room temperature for up to 12 h after the dissection, it was found that glucagon was much more susceptible to proteolytic enzymes than insulin and pancreatic polypeptide [21].

Further evidence for the proteolytic degradation is the finding of several serum albumin and globin chain sequences in the peak II material (Fig. 9 upper). Intact globin and human serum albumin would not be expected to appear in a size-exclusion fraction isolated according to a molecular weight of $\leqslant$ 6000 dalton.

The reason for the markedly reduced RP-HPLC separation efficiency of lyophilized sample material from the diabetic pancreas is not clear. During the extraction and isolation procedures it was noticed that the diabetic pancreas contained considerably more fatty substances than the normal pancreas. After centrifugation of the raw extract, three phases were obtained: pancreatic tissue as precipitate, a top layer consisting primarily of semi-liquid/semi-solid lipids and, in between, a fairly clear, aqueous layer containing the solubilized pancreatic sample material in 3 *M* acetic acid. It was difficult to decant this interphase completely from the lipids, especially when the lipid layer at the top was pronounced, and after lyophilization of the acetic acid crude extracts from the diabetic pancreas the lyophilization residue had an oily appearance, in contrast to those from a normal pancreas, which remained fairly dry after lyophilization. As the sample preparation before RP-HPLC was identical for crude extracts and lyophilized pancreatic extracts (high-speed centrifugation in Eppendorf tubes followed by 0.45-μm filtration), the harmful effect can probably be localized to the lyophilization in the presence of lipids.

The general usability of the Dynosphere–acetic acid system for succeeding analyses was illustrated by the insulin RIA analyses performed after lyophilization of collected fractions (Fig. 8) and also by the initial sequence results obtained after a secondary RP purification of the major components in peak I and II material from a normal pancreas. The major digestive enzymes from the exocrine pancreas and the major hormone from the endocrine pancreas (*i.e.*, insulin) were easily identified (Fig. 9). Further sequencing of minor fractions are in progress.

It is worth mentioning, that the extraction and the two RP separations were performed without any isolation steps (lyophilization, precipitation). This procedure should therefore be well suited for isolating components present in very small amounts, where isolation and redissolution may severely reduce the yield before the sample can be transferred to the amino acid sequencer. If the final RP-HPLC is performed in micro-columns, the isolated polypeptide may be collected directly from the outlet tube of the UV photometer onto a glass-fibre disc and transferred to the gas-phase sequencer. The peptide separation efficiency of the present acetic acid system is lower than that of an alkylsilica–TFA–acetonitrile system. However, the polymeric stationary phase utilized here is the first to be eluted with acetic acid gradients

in water, and other existing polymer-based RP columns or future developments in stationary phase design may well increase the selectivity and separation capacity in acetic acid gradients.

For the isolation of pure peptides/proteins from crude extracts containing numerous sample components, more than one RP-HPLC system is often needed. To obtain maximum resolution, it is essential to apply two RP-HPLC methods with different stationary phase and/or mobile phase compositions, and the initial one could well be a polymeric RP column eluted with an acetic acid gradient in water. The reduced sensitivity (due to the inapplicability of low-UV detection) is less inconvenient in the initial part of a purification procedure, and such a system may be cleaned up with strong alkaline and will tolerate a considerably higher number of crude samples than an alkylsilica column, which remains the obvious choice for the second-dimension RP-HPLC.

In conclusion, we have developed an extraction procedure for pancreatic glands and an RP-HPLC analysis system for the characterization of these extracts using the same solvent throughout, thereby allowing a direct characterization and identification (RIA, sequence determinations) of extracted compounds. The resulting UV profiles clearly reflect the different distributions of proteins and peptides in the exocrine and the endocrine pancreas.

The RP chromatograms obtained from a number of normal pancreatic glands were found to be very similar, whereas those from diabetic glands were mutually more different and deviated considerably from the composition of extracts of normal glands. The reason for this discrepancy is probably a much more pronounced post mortem enzymatic decomposition of the pancreatic polypeptides in the diabetic glands, which were removed several hours after clinical death.

Screening experiments with other commercially available polymer-based RP columns and application of the extraction and characterization procedure to the normal human pancreas with similar ischaemia times to the diabetic pancreas, to pancreatic glands obtained from experimental animals with chemically or immunologically induced insulin-dependent diabetes mellitus and to experimental animals with pancreatic tumours are in progress.

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