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ANALYSIS OF INSULIN PREPARATIONS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

L. F. LLOYD* and P. H. CORRAN
Chemistry Section, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB (Great Britain)
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SUMMARY

A reversed-phase high-performance liquid chromatographic system is described for the rapid and complete separation of bovine and porcine insulin from their readily formed monodesamido derivatives under isocratic conditions in the presence of the ion-pairing agent cetrimide. The system is suitable for the direct analysis of formulations of insulins of mixed bovine and porcine origin, and gives satisfactory results with a number of readily available commercial packings. Human insulin is not resolved from porcine in this system, but an alternative system allows the complete separation of all three insulins and their monodesamido derivatives, although acceptable peak shapes were obtained only on a limited number of packings.

INTRODUCTION

A wide variety of insulin preparations, differing in formulation, duration of action and purity, is available for diabetic therapy. At present all materials used in licensed preparations are derived from bovine and porcine pancreases. Although total chemical syntheses of insulin by classical fragment condensation and by stepwise solid-phase methods have been reported[1–4], neither approach has apparently proved commercially attractive, though both have been widely used for the preparation of analogues for bioactivity studies. Two recent methods for the large-scale production of human sequence insulins, one by semi-synthetic modification of the closely related porcine sequence[5] and the other in genetically modified micro-organisms (either producing A and B chains separately for subsequent combination or via proinsulin)[6], appear to be economically feasible, and insulins synthesised by both routes are at present under clinical trial in a number of countries.

The amino acid sequences of all three insulins are very similar (Table I), with variations occurring in only three positions and the maximum difference being three substitutions between bovine and human.

Although insulin preparations may often be required to be labelled with the species of origin, there is at present no officially recognised procedure for satisfac-

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TABLE I

SPECIES-SPECIFIC AMINO ACID RESIDUES IN VARIOUS INSULINS

Species	*Position*			*Composition*	
	A chain		*B chain*	*Thr*	*Ala*
	8	*10*	*30*		
Ox	Ala	Val	Ala	1	3
Pig	Thr	Ile	Ala	2	2
Human	Thr	Ile	Thr	3	1

torily verifying this information where the insulin is of mixed origin. The *British Pharmacopoeia* (1980)[7] includes in the monograph for Insulin a test for species of origin in which the identity is deduced from the content of alanine and threonine following hydrolysis and amino acid analysis. This procedure is not sufficiently sensitive to be applied to the determination of mixtures. However, this information could be obtained for a mixture of porcine and bovine insulin if the A and B chains were separated before the amino acid determination, since bovine A chain contains no threonine and porcine A chain no alanine, but this procedure is both complex and time-consuming.

Several reversed-phase high-performance liquid chromatographic (HPLC) systems for the chromatography of insulins have been described[8–17,24,25], some of which separate native porcine insulin from native bovine. Only two systems[16,24] have been shown to resolve bovine and porcine insulins and their readily formed degradation products, the A_{21} monodesamido derivatives which are found to some extent in most preparations. These forms of insulin possess similar biological potency to the native hormone and consequently no limit is set upon their content in pharmaceutical preparations. Most of the chromatographic systems mentioned above fail to resolve adequately bovine monodesamido-insulin from native porcine, and clearly this may give rise to inaccuracy in results for mixtures in which significant deamidation has occurred. It is desirable that any analytical method for an official specification should be shown to perform satisfactorily on packing materials from different commercial manufacturers. In each case[16,24] where resolution of a mixture of bovine and porcine native and monodesamido-insulins has been published, the separation has been demonstrated only on a single commercial packing.

We describe here a simple isocratic system which achieves this separation, is suitable for the analysis of formulations and gives satisfactory results on a wide range of commercial packings. The introduction of human sequence insulin preparations onto the market means that future systems should be capable additionally of resolving human native and monodesamido-insulin. In our system native human insulin is not resolved from native porcine, and experience suggests that most of the isocratic systems quoted above are unlikely to resolve human insulin from bovine and porcine, particularly when deamidation has occurred, because of inadequate selectivity or efficiency or both. We describe another simple system which resolves all these species and forms of insulin but which gives satisfactory results only on a limited number of the commercial packings examined. A preliminary account of the first system is in press[18].

MATERIALS

Urea AR, ammonium sulphate AR, sodium dihydrogen orthophosphate and cetrimide (cetyltrimethylammonium bromide) were purchased from BDH (Poole, Great Britain), L(+)-tartaric acid from Sigma (Poole, Great Britain). Acetonitrile was obtained from Fisons (Loughborough, Great Britain), or Rathburn Chemicals (Walkerburn, Great Britain), far-UV or 'S' grade. Iodoacetic acid (BDH) was re-crystallised from petroleum ether before use. HPLC packings were: Nucleosil 5 μm C_{18} (Camlab, Cambridge, Great Britain); LiChrosorb RP-18 (BDH); Hypersil ODS (Shandon Southern, Runcorn, Great Britain); Spherisorb ODS (Phase Separations Queensferry, Clwyd, Great Britain); Ultrasphere ODS was purchased prepacked from Anachem (Luton, Great Britain) and Zorbax TMS prepacked from Dupont (Hitchin, Great Britain); Sephadex G-25 fine and G-50 superfine were obtained from Pharmacia (Hounslow, Great Britain). Crystalline highly purified native porcine, bovine and semi-synthetic human insulins were a gift of Novo Industries, Copenhagen, Denmark. Monodesamido-insulins were obtained by chromatography of de-amidated insulin samples by a modification of published procedures[19]. The purity of insulin samples was evaluated by polyacrylamide gel electrophoresis (PAGE) at pH 8.3 (ref. 26). The European Pharmacopoeia first insulin biological reference preparation (established 1976) was obtained from the European Pharmacopoeia Secretariat, Strasbourg. The fourth international standard for insulin for bioassay NIBSC 58/6 is held at this Institute as is the first international reference preparation of human insulin for bioassay. Purified bovine and porcine proinsulins were purchased from Novo Industries.

APPARATUS

HPLC apparatus was assembled form the following components: Altex 110, Cecil 201 or LDC 720 pumps, Cecil 272, 212 or Hewlett-Packard 1030B variable-wavelength UV monitors, and Shandon or home-made septum injectors or Rheodyne 7125 valve injectors. Columns (10, 15, or 25 cm × 5 mm I.D.) were slurry-packed in propan-2-ol. Amino acid analyses were carried out on an LKB 4102 amino acid analyser.

METHODS

HPLC

System 1. The mobile phase was prepared by mixing 75 volumes of 5 m*M* L-(+)-tartaric acid (or acetic acid)–0.1 *M* ammonium sulphate with 25 volumes of acetonitrile. Sufficient solid cetrimide was then added to give a final concentration of 14 μ*M*. Samples were dissolved in 5 m*M* tartaric acid–14 μ*M* cetrimide or, in the case of neutral formulations, acidified by the addition of 5% v/v acetic acid. Formulations with pH less than 4 (*e.g.* soluble insulin BP) were injected direct. Septum injections were normally carried out with the flow stopped. The column effluent was monitored at either 280 or 225 nm. Columns were stored in mobile phase.

System 2. The column eluant was prepared from 0.1 *M* sodium dihydrogen orthophosphate, adjusted to pH 2 with phosphoric acid, 70 volumes, and acetonitrile,

30 volumes. The column was thermostatted at 45°C with a circulating waterbath. Crystalline samples were dissolved in 50 m*M* HCl, and formulations were treated as described above. Detection was carried out at 210 or 280 nm. Columns were washed with methanol–water (1:1 v/v) after use.

Peak identification. Peaks were collected, neutralised, desalted on a column (20 × 2 cm I.D.) of Sephadex G25 equilibrated with 5 m*M* NH_4HCO_3 lyophilised and rechromatographed by HPLC. Identity was confirmed by co-chromatography with suitable reference compounds or, to confirm that particular peaks were native or desamido-insulin, by PAGE at pH 8.3.

Species identification by amino acid analysis

Formulations were precipitated with an equal volume of 20 m*M* zinc chloride and dried. Crystalline insulin samples (10 mg) were dissolved in 0.5 ml of 1.44 *M* Tris-HCl, pH 8.6. Then 0.6 g of urea and 10 µl of 0.135 *M* EDTA were added, and the insulin was reduced by the addition of 5 µl of 2-mercaptoethanol followed by 4 h incubation at 37°C. Then 67 mg of recrystallised iodoacetic acid in 0.25 ml of 1 *M* NaOH were added dropwise, and the solution was allowed to stand in the dark for 20 min. An equal volume of glacial acetic acid was added, the solution applied to a column (100 × 1 cm I.D.) of Sephadex G-50 superfine, equilibrated with 50% v/v acetic acid, and the peaks were eluted at 4 ml/h with the same solvent. Fractions of 1.5 ml were collected, and the tubes corresponding to the second peak of 280 nm absorption, containing the A chains, were pooled, diluted with water and lyophilised. The lyophilised solid was hydrolysed with 0.2 ml of 6 *M* HCl in a sealed, evacuated tube for 16 h at 110°C, the HCl was removed over NaOH pellets *in vacuo*, and a suitable aliquot (*ca.* 20 nmol) was applied to the amino acid analyser. The purity of the A chain preparation was confirmed by the absence of phenylalanine (present only in the B chains), and the ratio of porcine to bovine insulin was determined by the relative amounts of threonine and alanine.

RESULTS AND DISCUSSION

System 1

When mixtures of bovine and porcine native and monodesamido-insulins were chromatographed on reversed-phase columns with no ion-pairing agent such as cetrimide, bovine monodesamido-insulin was inadequately resolved from porcine native (α 1.06). At high concentrations (0.027 *M*) of cetrimide, porcine and bovine insulin were not resolved from each other, but were well separated from the two unresolved monodesamido derivatives. Over a range of cetrimide concentrations from 2.5 µ*M* to 25 m*M* all four of these compounds were completely resolved from each other (Fig. 1). Over the range 2.5–25 µ*M* cetrimide the absolute and relative retentions of the four peaks changed little, and a cetrimide concentration of 14 µ*M* was chosen for routine use. When reversed phase columns were conditioned with mobile phase containing 14 µ*M* cetrimide the time taken to establish stable retention times was excessive. Accordingly columns were preconditioned with mobile phase containing 0.027 *M* cetrimide. The delay in breakthrough of cetrimide, detected by its ability to extract the dye orange G into chloroform, corresponded to the absorption by the packing of *ca.* 150 mg/g of cetrimide (*i.e. ca.* 30 ml for a 15-cm column). Equilibration with

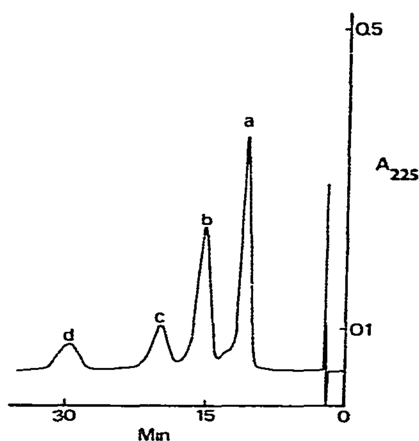

Fig. 1. Chromatogram of the Fourth international standard for insulin. Column, Hypersil ODS; mobile phase, 14 μM cetrimide in acetonitrile plus 0.1 M $AmSO_4$–5 mM tartaric acid pH 3.0 (25:75). Peaks: a, bovine native insulin; b, porcine native insulin; c, bovine monodesamido-insulin; d, porcine monodesamido-insulin.

mobile phase containing 14 μM cetrimide was then continued until retention times had stabilised. Little variation was found between batches of Hypersil ODS and satisfactory results were obtained with LiChrosorb RP-18 and Nucleosil C_{18}.

Reproducibility, recovery and detection limits

The peak height for either bovine or porcine insulin varied linearly with amount injected over the range 0.25–100 μg (correlation factor, 0.9980), and it was possible to detect less than 0.1 % of porcine monodesamido-insulin in a sample of bovine insulin. The detection limit for porcine insulin in bovine was about three times lower (100 μg was the maximum amount injected).

Use for formulations

Under the conditions described, formulations containing protamine, globin and preservatives such as methyl *p*-hydroxybenzoate and phenol may be analysed without special preparation. Protamine and globin are retained on the column indefinitely, and the use of a precolumn is advisable if more than the occasional sample of this type is to be analysed. When an acetonitrile concentration of 24 % was used, phenol and methyl *p*-hydroxybenzoate were eluted well before bovine insulin and did not interfere with the analysis, but in the absence of these preservatives the acetonitrile concentration could be raised to 25 % to allow a more rapid separation.

Comparison with existing methods

The proportions of porcine and bovine insulins present in three insulin samples of mixed origin were determined by the HPLC system described and by separation of A and B chains followed by amino acid analysis as outlined in the Methods section. All three preparations contained substantial amounts of monodesamido-insulins, and the proportions of each species were determined by summing the appropriate peak areas (measured at 280 nm). The results of this comparison are listed in Table II. The

TABLE II

DETERMINATION OF THE PROPORTIONS OF BOVINE AND PORCINE INSULINS IN THREE SAMPLES BY HPLC AND BY ANALYSIS OF A CHAINS

Preparation	*Species*	*Expected content*	*Amino acid analysis*	*HPLC (system 1)*		*Total*	*HPLC (system 2)*		*Total*
				Native insulin	*Monodesamido-insulin*		*Native insulin*	*Monodesamido-insulin*	
First EP biological reference preparation	Bovine	72	80	71.0	5.0	76	68.2	7.5	75.7
	Porcine	22	20	22.7	1.3	24	22.6	1.7	24.3
Fourth international standard	Bovine	55	55	47.1	7.9	55	45.8	10.1	55.9
	Porcine	45	45	38.4	6.6	45	35.9	8.2	44.1
A commercial biphasic preparation	Bovine	70	74	63.0	5.0	68	Same sample		
	Porcine	30	26	26.6	5.4	32	not available		

agreement between results obtained by HPLC and by amino acid analysis of A chain preparations is satisfactory, particularly as conventional amino acid analysis typically has a coefficient of variation of *ca.* 5.0%, and small losses of threonine relative to alanine may be expected during hydrolysis. The precision of the HPLC technique seems adequate for a pharmacopoeial method, and the detection of better than 0.1% for one species in another, whether native or desamido, is adequate for all practical purposes.

The chief disadvantage of this chromatographic system is its inability to distinguish human from porcine insulin (α 1.08). Since formulations of human insulin may become generally available in the near future this is a serious weakness and necessitated the development of an alternative system (2) capable of differentiating between porcine and human insulins. For complete resolution of all six compounds a 25-cm length column was employed and an operating temperature of 45°C was necessary to improve the selectivity of the system. The separation of all six compounds: bovine, human, porcine native and monodesamido-insulins is illustrated in Fig. 2. In contrast to the cetrimide system, the order of elution of porcine native and bovine monodesamido-insulin is reversed and porcine insulin is separated from human. The influence of temperature on capacity factor (k') is shown in Fig. 3. Elevation of column temperature caused a corresponding increase in k' values, giving at 45°C almost baseline separation of the peaks. In addition the decrease in viscosity of the eluant reduced the high back-pressure obtained with a 25-cm column and 5-μm packings. Under the conditions used, optimal column performance was always a prerequisite for separation of all six insulins. Some C_{18} packings examined (LiChrosorb RP-18, Spherisorb ODS) gave poor insulin peak shapes despite good column

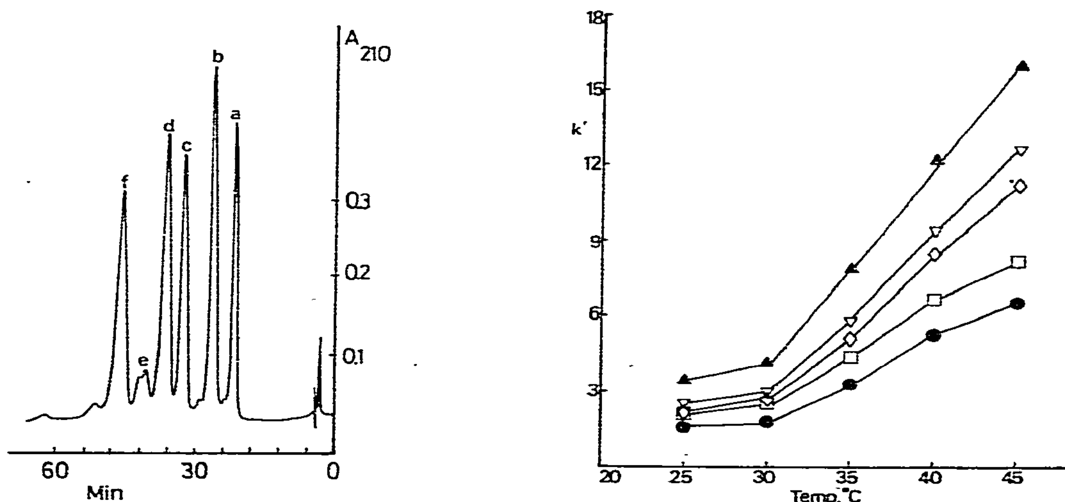

Fig. 2. Separation of bovine, human and porcine native and monodesamido-insulins. Column, Ultrasphere ODS; mobile phase, acetonitrile–sodium phosphate buffer pH 2 (30:70). Peaks: a, bovine native insulin; b, bovine monodesamido-insulin; c, human native insulin; d, porcine native insulin; e, human monodesamido-insulin; f, porcine monodesamido-insulin.

Fig. 3. Effect of temperature on insulin separation. ●, Bovine native insulin; □, bovine monodesamido-insulin; ◇, human insulin; ▽, porcine native insulin; ▲, porcine monodesamido-insulin.

test efficiencies; the most satisfactory results were obtained with Ultrasphere ODS and Hypersil ODS. This HPLC method can also be used to determine the species of origin of crystalline insulins and formulations, unambiguous assignment of species in this case being made by spiking a standard mixture of insulins with the unknown samples.

Detection of insulin-related contaminants in preparations

Occasionally, unidentified minor components were observed in insulin preparations. The relative amounts of these increased with ageing. Samples of formulated and bulk insulins subjected to accelerated degradation at elevated temperatures showed in addition many late-eluting peaks, probably owing to the presence of aggregated forms of insulin. However, we did not observe significant amounts of these components in formulations which had been subjected to normal treatment and which were within the expiry date. Since one of the most important immunogenic contaminants in insulin preparations is proinsulin, we examined the behaviour of purified bovine and porcine proinsulins in both systems (human proinsulin was not available). Bovine proinsulin eluted slightly later than, but was incompletely resolved from, porcine native in system 1, and porcine proinsulin was not eluted from the column. In system 2 bovine proinsulin was eluted slightly earlier than bovine native insulin whereas porcine proinsulin was eluted after porcine monodesamido-insulin.

Prediction of elution order of closely related peptides

Several workers have attempted to establish methods of predicting the retention times and elution order of peptides from a knowledge of their composition[20–23]. While it is unreasonable to expect that these computational procedures may enable the calculation of absolute retention times for different chromatographic systems and packings, one might hope that the relative retentions of the homologous series of peptides provided by the insulins and their monodesamido-derivatives used in this work would be correctly predicted. In fact only one method predicted the elution order in system 2 at 45°C with any accuracy, that of the π values used by Pliška *et al.*[22] (Table III). None of these methods predicted the rather unexpected behaviour of

TABLE III

ELUTION ORDER OF INSULINS IN SYSTEM 2

Insulin	*Actual order*	*Predicted order**			
		Method 1	*Method 2*	*Method 3*	*Method 4*
Bovine native	1	4	2	1	6
Bovine desamido	2	6	5	2=	5
Human native	3	1	1	2=	2
Human desamido	5	3	4	5	1
Porcine native	4	2	3	4	4
Porcine desamido	6	5	6	6	3

* Method 1: computed from relative lipophilicities using Rekker constants[20]. Method 2: calculated from retention coefficients determined by reversed-phase HPLC with NaH_2PO_4 pH 2 mobile phase and acetonitrile gradient[21]. Method 3: calculated from π values as reported by Pliška *et al.*[22]. Method 4: calculated from Σf constants[23].

bovine proinsulin in this system. Other workers[14,24] have also observed that bovine proinsulin is eluted much earlier relative to insulin than might be predicted from amino acid compositions, and that the relative retentions of native and proinsulin vary considerably from system to system, perhaps owing to interactions with residual silanols[14].

That peptides of identical composition but different sequence, or diastereoisomeric peptides differing at one position only, can be separated by reversed-phase HPLC indicates that other, less well understood, structural factors may be involved in retention, and perhaps the more variable C peptide of proinsulin differs sufficiently markedly in conformation under chromatographic conditions to explain the difference in retention observed.

CONCLUSIONS

In analysis of insulins, HPLC has advantages over conventional techniques such as amino acid analysis in that it is fast and sensitive and no sample preparation is necessary. Both systems described can be used for determining the species of origin of formulations as well as bulk insulins. In addition, these systems may be used to assess the purity of insulin preparations, although neither system was found suitable for quantitation of the content of bovine or porcine proinsulins to the very low relative concentration (less than 0.1 %) required in the *British Pharmacopoeia*.

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