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HPLC as a replacement for the animal response assays for insulin

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Abstract: The work describes the assay of the potency of insulin (drug substance) and its formulations by gradient elution reversed-phase high-performance liquid chromatography (HPLC). The methods are shown to differentiate between insulins of bovine, porcine and human sequence, and to be both reproducible and stability-indicating. The HPLC assay results show good agreement with those obtained by the mouse blood glucose assay. The advantages of the HPLC assays over the animal response assays are discussed. It is suggested that the animal response assays should now be replaced by the HPLC assays.

Keywords: *Reversed-phase high-performance liquid chromatography; insulin.*

Introduction

The official methods of assay for insulin and its formulations are still animal response assays [1–3], although the United States Pharmacopeia has begun to make the first official moves towards a physico-chemical assay [3–6]. Now that most of the insulin formulations available in the developed world are of the highly purified variety, the potency of the drug substance is much less variable and much more predictable. This simplifies the problem of predicting 'potency' by a physicochemical technique. Also there are increasing moral, scientific and commercial reasons for replacing the animal response assays.

Since 1978, descriptions have been published of HPLC separations of insulin (from various species) and related substances [7–29]. The present work seeks to demonstrate that it is now possible to replace the official animal response assays by HPLC assays, for both insulin and its formulations.

Experimental

The HPLC equipment comprised LDC Constametric IIG and Constametric I HPLC pumps, controlled by an LDC Gradient Master. Detection was by an LDC Spectromonitor III variable wavelength UV detector (Laboratory Data Control, Stone,

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Staffordshire, UK). Temperature control of the columns was achieved by the use of a brass column jacket (Magnus Scientific, Aylesbury, Buckinghamshire, UK) through which was circulated water from a constant-temperature circulator (Braun Thermomix 1420, from FT Scientific Instruments, Bredon, Gloucestershire, UK). Sample injection was with an M7100 Autosampler (Magnus Scientific). Data collection and integration was accomplished with a TriVector Trilab 2000 Multichannel Chromatography Data System (TriVector Systems International Ltd, Sandy, Bedfordshire, UK), which also controlled the LDC Gradient Master and Magnus M7100 Autosampler. The Brownlee Aquapore RP300 and Ultrapore RPSC HPLC columns were obtained from Analchem (Power Court, Luton, Bedfordshire, UK).

Water (HPLC grade), trifluoroacetic acid (sequencer grade), 2-methoxyethanol (glass distilled grade) and acetonitrile (HPLC "S" grade) were obtained from Rathburn Chemicals Ltd (Walkerburn, Peeblesshire, UK). Sodium dihydrogen orthophosphate (AnalaR grade) and phosphoric acid (AnalaR grade) were from BDH Chemicals (Poole, Dorset, UK).

Samples of insulin and insulin injections were from the Wellcome Foundation (Temple Hill, Dartford, Kent, UK). Samples of the proposed WHO reference standard for bovine insulin were from Dr A. Bristow (National Institute for Biological Standards and Control, London, UK).

Biological potencies of insulin were determined by the mouse blood glucose method of the British Pharmacopoeia [1].

Two HPLC methods were used, one for the drug substance and the other for insulin injections.

Method 1. HPLC method for insulin (drug substance)

The 250 × 4.6 mm i.d. column was packed with 10-μm Brownlee Aquapore RP300 and was kept at 45°C. Mobile phase A was 0.1 M sodium dihydrogen orthophosphate in 2-methoxyethanol–water (5:95, v/v), adjusted to an apparent pH of 2.0 with phosphoric acid. Mobile phase B was 2-methoxyethanol–acetonitrile (5:95, v/v). The gradient profile adopted was: 26.3% B isocratic for 13 min; a linear gradient from 26.3% to 36.3% B in 10 min; and 36.3% B isocratic for 10 min. The flow rate was 1.0 ml min^{-1}, and detection was at 210 nm with a range setting of 0.5 absorbance units full scale (a.u.f.s.). The percentage of mobile phase B was altered (±0.5%) to allow for day-to-day variation in retention time due to slight variations in the composition of the mobile phase; the target optimum retention time for beef insulin was 8.2 min. Samples were dissolved in 0.01 M hydrochloric acid to give a sample concentration of 1 mg ml^{-1}; 20 μl of this solution was injected on the column.

Method 2. HPLC method for insulin injections

The 75 × 4.6 mm i.d. column was packed with Ultrapore RPSC at ambient temperature. Mobile phase A was 0.05% (v/v) trifluoroacetic acid in water. Mobile phase B was 0.05% trifluoroacetic acid in acetonitrile–water (60:40, v/v). The gradient was linear from 40% B to 44% B in 30 min. The flow rate was 2 ml min^{-1} and detection was at 214 nm with a range setting of 0.2 a.u.f.s. Samples were prepared by adding 1.0 ml of 0.02 M hydrochloric acid to 1 ml of the insulin injection; 20 μl of this solution was injected into the column.

Estimates of potency were made against a reference standard of known potency; allowance was made for the potency of desamido insulin being 0.9 times that of insulin

[3]. Two reference standards were used, batch P46 from the Wellcome Foundation and, later, the proposed WHO reference standard bovine insulin. The former had a potency, determined by the mouse blood glucose assay, of 26.3 units/mg (27.1 units/mg on an anhydrous basis) whereas the latter was found to have a potency of 24.8 units/mg.

The proposed WHO reference standard was found to be a much better analytical standard since it was much purer than batch P46; the use of the WHO standard simplified the assignment of peaks and the "potency" calculation.

Results

Insulin (drug substance)

The HPLC method used for the drug substance resolves bovine, porcine and human insulins (Fig. 1) and the retention times are given in Table 1. Figures 2 and 3 show the chromatograms of the European Pharmacopoeia reference substance and 4th International Standard respectively. Both standards are of mixed bovine/porcine origin, but are also shown to contain bovine and porcine desamido insulins and insulin oligomers; these latter compounds are known decomposition products of insulin [30]. Clearly these

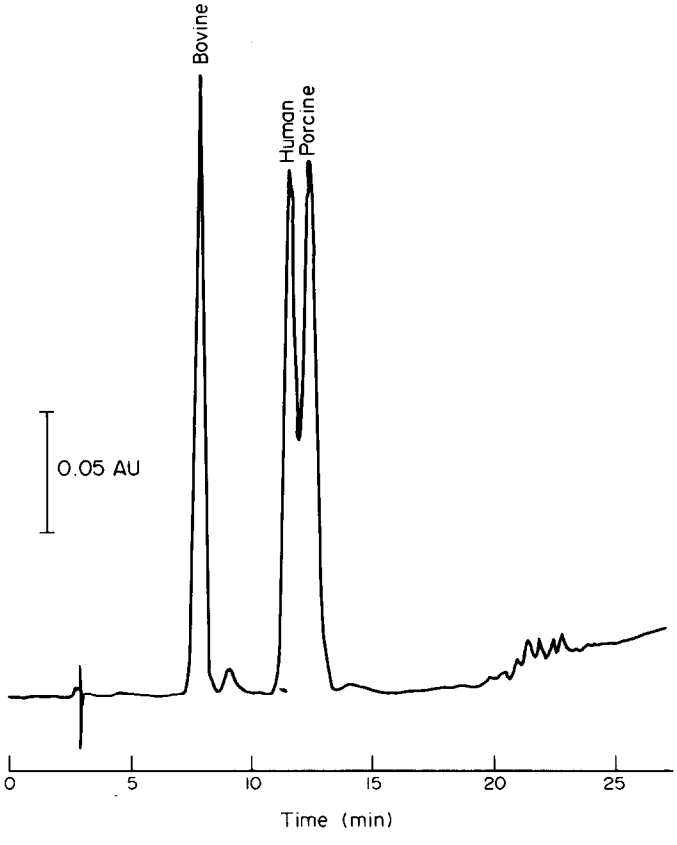

Figure 1
Chromatogram of a mixture of a bovine, human and porcine insulins, separated by HPLC method 1.

Table 1
Retention times of insulins and related substances on the HPLC system used for the drug substance

Time (min)	Insulin
7.11	Bovine proinsulin
8.21	Bovine insulin
9.54	Bovine desamido insulin
11.64	Human insulin
12.52	Porcine insulin

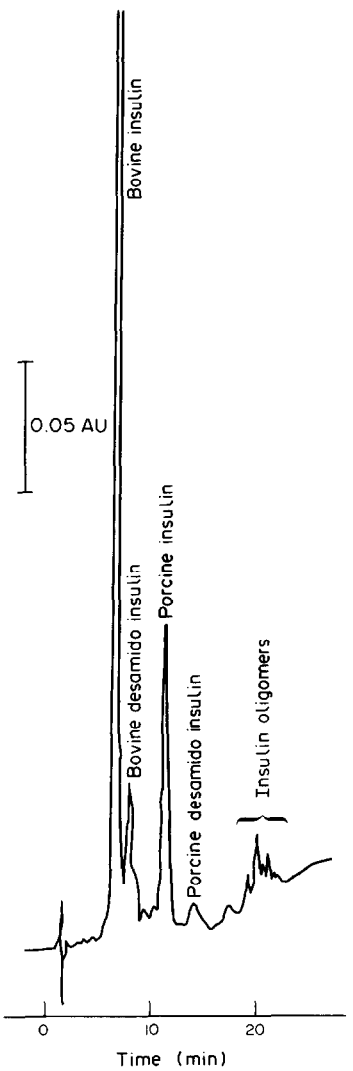

Figure 2
Chromatogram of the European Pharmacopoeia Reference Substance Insulin, analysed by HPLC method 1.

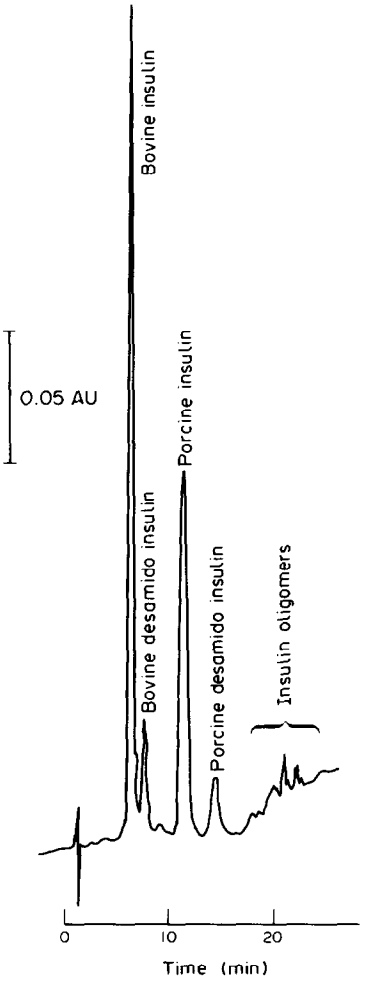

Figure 3
Chromatogram of the 4th International Standard Insulin, analysed by HPLC method 1.

two reference substances are unsuitable for calibrating an HPLC assay for insulin: hence efforts are being made by the WHO to provide pure, monocomponent standards for bovine, porcine and human insulins.

The method was shown to give a linear response over the concentration range 0.1–2.0 mg ml^{-1}, with a correlation coefficient of 0.9978 ($n = 5$, $p < 0.01$).

Two batches of bovine insulin were analysed by two operators on two occasions and gave the results shown in Table 2. These data were examined by the method of BS5497 [31] and analysis of variance (ANOVA) as described previously [32]. The data were acceptable by Dixon's Test, but with Cochran's test a straggler was detected in the results from sample P31 (the 26.81 units/mg result). With this straggler omitted, the data were acceptable by both tests and gave a mean repeatability of 0.31 unit/mg and a mean reproducibility of 0.91 unit/mg. The analysis of variance showed that the variance due to different occasions was not significant, but that the variance due to different operators was significant ($p < 0.05$). Although this variance was statistically significant, it was not considered chemically significant since on no occasion did the results differ by more than 0.86 unit/mg between operators.

Table 2
Results of HPLC potency determinations (in units/mg) for two batches of drug substance, analysed on two occasions by two operators

	Sample P29		Sample P31	
	Occasion 1		Occasion 2	
Operator	1	2	3	4
1	26.65	27.03	26.14	26.81
2	26.35	26.17	25.85	25.84

Six batches of bovine insulin were assayed by the HPLC method and by the mouse blood glucose method. The results (Table 3) show good agreement between the two methods.

Previous work [30] has shown that immunochemical determinations of potency do not detect all the decomposition which occurs when insulin (drug substance) is subjected to accelerated stability tests, and that chemical methods are the only way of detecting this decomposition. Table 4 shows a comparison of HPLC determinations of potency and immunochemical determinations of potency on samples of bovine insulin that had been stored for 3 months at various temperatures.

Table 3
Results of assaying six batches of bovine insulin by the HPLC method and the mouse blood glucose method

Batch	Assay by HPLC (units/mg)	Assay by MBG (units/mg)
29	26.9, 26.9, 26.6	27.0
31	26.3, 26.2	26.9
32	26.5, 26.8	26.5
33	26.5	26.8
36	25.7	26.6
37	25.7	26.4

Table 4
Comparison of HPLC potency determinations and immunochemical potency determinations on samples of bovine insulin that had been stored for 3 months at various temperatures

Temperature of storage (°C)	HPLC potency (units/mg)	Immunoactivity by RIA (units/mg)
−20	26.1	28.1
5	24.9	28.8
25	25.6	28.8
37	24.8	26.9
50	22.3	26.5
60	19.6	23.7

Insulin injections

A typical chromatogram from the assay of an insulin injection is shown in Fig. 4, with a typical retention time for bovine insulin of 9.2 min, and for bovine desamido insulin of 12.9 min.

The method was shown to be linear over the concentration range 0.5–3.5 mg ml^{-1}, with a correlation coefficient of 0.9999 ($p < 0.01$, $n = 8$).

Replicate injections on the same occasion showed a relative standard deviation of 1.5% ($n = 65$), while repeat assays on five different occasions gave a relative standard deviation of 4%.

'Placebo' injections of insulin zinc suspension (mixed), neutral soluble insulin injection and isophane insulin injection showed no interfering peaks in their chromatograms.

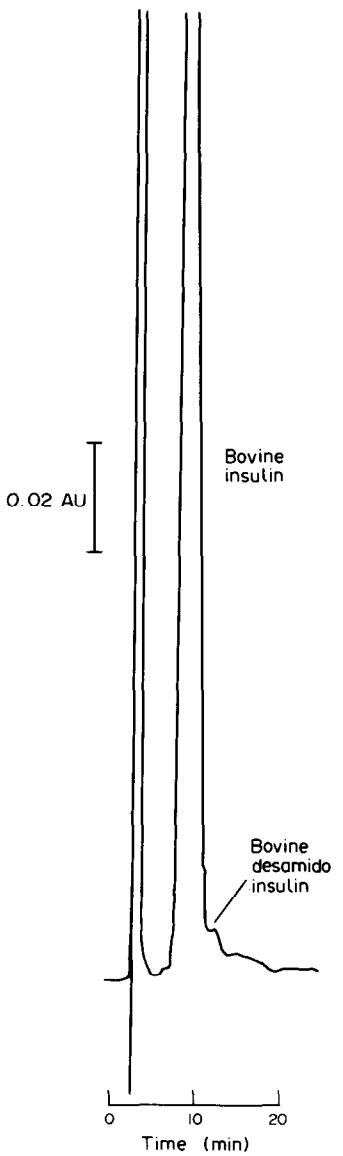

Figure 4
Typical chromatogram of an insulin injection analysed by HPLC method 2.

A comparison of results by the mouse blood glucose assay and potency determinations by HPLC is shown in Table 5. It is not possible to measure a classical correlation between the mouse blood glucose assay and the HPLC assay, due to the wide limits on the precision of the mouse blood glucose assay. However, it is noteworthy that, with one exception, the HPLC assays give results that are within the fiducial limits of the mouse blood glucose assays. The one exception, results for batch L1140, has defied all searches for an explanation.

Samples of insulin injections from accelerated stability tests were examined to see if the drop in potency determined by the mouse blood glucose assay was reflected in the HPLC assay. The results are shown in Table 6. The HPLC assay consistently gives lower results than those obtained by the mouse blood glucose assay, indicating that the HPLC assay detects greater decomposition. Decomposition that leaves the receptor binding site on the insulin molecule intact is not reflected in a drop in the mouse blood glucose assay. This is analogous to the situation with samples of the drug substance subjected to accelerated stability tests.

Discussion

In the last few years there has been an increase in the commercial availability of reversed-phase HPLC columns specially tailored for the protein chemist. These columns are generally based on a silica gel of large pore size (usually about 30 nm), to which is bonded alkyl chains of a stated carbon number (typically C_1, C_4, C_8 or C_{18}). Any remaining free silanol groups are then 'capped' with trimethyl silane, or a similar silane, to ensure a non-polar surface which will minimize any unpredictable, non-reversible adsorption to the silica. All the work described in this paper was carried out on such columns, since the resolution and peak symmetry with these columns were superior to those obtained on conventional reversed-phase columns. The retention of the peptides was found to be very sensitive to the content of organic modifier in the mobile phase and hence gradient elution over a narrow range of mobile phase composition gave excellent resolution of insulin from its related substances. These effects are in accord with known phenomena on the reversed-phase HPLC of proteins and peptides (Di Bussolo [33]).

The results show that for insulin (drug substance) and for the insulin injections examined, the HPLC assay gives an estimate of the potency that is in good agreement with that obtained by the mouse blood glucose method. The HPLC methods give results that are more precise and more reproducible than those obtained by the mouse blood glucose method. The close agreement between the results by the HPLC assay and those by the animal response assay is in accord with similar findings by Smith *et al.* [29], who analysed the drug substance and injections of human, bovine and porcine origin. However, their HPLC method had an analysis time of 150 min, compared with the 30 and 33 min of the methods described in the present paper.

With insulin (drug substance) and insulin injections subjected to accelerated stability tests, the HPLC assay method detects decomposition that cannot be detected by either the mouse blood glucose assay or the immunochemical assay. These results confirm previous findings [30] that the HPLC method detects decomposition of the insulin that is not demonstrated by the immunochemical assay. Presumably this is because a drop in the immunochemical assay would be caused only by chemical decomposition of the antigenic determinant whereas decomposition of other parts of the insulin molecule might leave the antigenic determinant intact to react with the antibody. Since some of the

Table 5
A comparison of assay results for insulin injections determined by mouse blood glucose (MBG) assay and HPLC assay

Injection type	Batch no.	Potency by MBG (units/ml)	Fiducial limits ($p = 0.95$)	Potency by HPLC (units/ml)
Neutral soluble insulin injection 100 units/ml (Neusulin)	A1150	93.6	86.2–101.6	90.8
	A1214	96.5	87.5–106.4	103.4
	A1220	96.2	89.7–100.5	100.5
	A1224	101.3	90.0–114.1	95.6, 96.0
	A1226	95.2	84.1–107.8	96.1
	A1240	100.6	90.0–112.6	98.5, 98.7
	A1242	108.2	100.2–116.9	104.7
	A1268	107.4	96.4–119.8	105.7
	A1274	103.7	91.4–117.6	102.7
	A1276	107.3	98.5–117.0	102.1
	A1302	95.3	81.4–111.5	93.9
	A1308	101.8	93.5–110.7	101.0
Isophane insulin injection 100 units/ml (Neuphane)	N1234	95.8	87.5–104.9	88.0
	N1236	99.7	87.7–113.4	102.4, 95.5
	N1256	103.4	91.5–116.8	96.1
	N1258	107.2	96.0–119.7	100.6
	N1272	106.4	95.5–118.6	105.3, 99.1
	N1280	97.9	87.7–109.4	98.3
	N1292	101.8	92.3–112.3	105.3
	N1310	99.6	88.6–111.9	104.5
Insulin zinc suspension (mixed) 100 units/ml (Neulente)	L1126	101.9	88.4–117.5	97.1
	L1140	94.2	86.7–102.3	79.8
	L1192	105.3	96.9–114.4	98.4, 97.5
	L1230	93.8	82.5–106.7	92.4
	L1232	92.8	83.0–103.8	100.2
	L1260	96.5	88.4–105.3	100.4, 96.7
	L1262	103.8	90.1–119.5	99.5
	L1270	104.2	94.9–114.4	95.1

Table 6
Comparison of potencies determined by mouse blood glucose (MBG) assay and HPLC assay on insulin injections subjected to accelerated stability tests

Injection type	Storage temperature (°C)	Storage period (months)	Potency by MBG (units/ml)	Potency by HPLC (units/ml)
Insulin zinc suspension (mixed) (100 units/ml) (Neulente)	5	24	90.2	84.7
	5	12	96.6	78.0
	25	12	81.9	69.4
	25	12	85.2	72.0
Isophane insulin injection 100 units/ml (Neuphane)	15	12	75.0	70.4
	25	12	79.5	77.0
Insulin injection BP 100 units/ml	25	12	89.8	85.5

decomposition products still show hypoglycaemic activity, it is arguable that the mouse blood glucose result is the most meaningful in terms of diabetic therapy. Nevertheless, the indication of decompositon obtained from the HPLC result is still useful information to the pharmaceutical scientist.

Two different HPLC systems were found necessary since the requirements for the analysis of the drug substance and injections are slightly different. For the analysis of injections, all that is required is a method that separates insulins from desamido insulins and from any other related substances, so that the potency of the injection can be estimated. Hence a relatively quick method can be used. For the analysis of the drug substance it is much more useful to have a method that shows a full impurity profile and simultaneously gives an estimate of the potency.

The HPLC assay of insulin has many advantages over the animal response methods. The HPLC method is quick (less than 2 h sample), cheap and is easily automated for unattended, continuous analysis. The animal response methods are imprecise, expensive and time-consuming.

In these days of social pressure to move away from the unnecessary use of animals in the testing of pharmaceuticals, it would appear that there is now a very real possibility of removing from the pharmacopoeias the animal response assays for insulin.

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