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Note

Analysis of bovine, porcine and human insulins in pharmaceutical dosage forms and drug delivery systems

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Diabetes mellitus in man is well documented, having been recorded in ancient Egypt, Rome and Greece. However, the role of insulin in diabetes was not fully appreciated until the early part of this century. The first insulins available commercially consisted mainly of contaminated substances[1] but insulins now available are relatively pure. The monograph for insulin in the British Pharmacopoeia now includes a test for (a) identity and (b) other insulins. The latter test uses a reversed-phase high-performance liquid chromatographic (HPLC) method with an acetonitrile–phosphate buffer mobile phase. In addition, polyacrylamide gel electrophoresis is used for the related proteins and peptides test[2]. This, however, has been a recent addition and, prior to this, manufacturers used other methods, in addition to those tests laid down in the pharmacopoeias, to assay the insulin content and to monitor the levels of mono- and diarginine insulin, desamido insulin, intermediary insulin, proinsulin, dimer/polymer insulin etc.

The chromatographic techniques used fall into three major catagories: size-exclusion, ion-exchange and reversed-phase chromatography. The latter has become increasingly popular in recent years and many methods have been published. Some of these methods allow the analyst to determine species differences and also to determine the levels of impurities and breakdown products. Since these methods must be able to resolve upwards of ten such entities, they require long analysis times and, therefore, large volumes of mobile phase are used[3–11]. The elution time for insulin in these methods is usually in the region of 20 min and the resulting peak is poorly resolved and suffers from lack of sensitivity. A few methods have been developed that separate different insulins and the preservatives used in these formulations but again suffer from lengthy analysis times[12–15]. One paper describes a rapid method of analysis using a C_2 alkyl chain bonded silica column, which separates methylhydroxybenzoate, insulin and its breakdown products in 2 min[16]. However, it is not suitable for analysing mixtures of different insulins or one containing two or more preservatives. None of the systems were suitable for measuring very small quantities of insulin in solution (10–40 units/l) or of separating insulin from buffering agents present in some of the solutions tested. This paper describes two HPLC methods developed to measure the insulin content in two different situations. The first method was required to determine the stability of insulins stored in syringes and any interactions that may occur on mixing different types of insulin (soluble and protamine

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insulins and soluble and zinc insulins) together. This required a method that was rapid and capable of resolving insulin from its preservative and any breakdown products. The second method was developed to measure low doses of insulin which were added to intravenous and continuous ambulatory peritoneal dialysis (CAPD) solutions in clinical doses of 10–100 units per litre of solution.

MATERIALS AND METHODS

Chromatography

All assays were performed using an ACS Model 740 pump (ACS, Luton, U.K.), a Perkin-Elmer LC75 variable-wavelength detector (Perkin-Elmer, Beaconsfield, U.K.) and a Hewlett-Packard 3900A integrator (Helwett-Packard, Winnerish, U.K.). Injection was via a Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.).

Reagents

Porcine and bovine insulins (23 units/mg) were obtained from Sigma (St. Louis, MO, U.S.A.). Commercial insulin preparations were purchased from Nordisk UK (Epsom, U.K.), Novo Labs. (Basingstoke, U.K.) and Eli Lilly and Co. (Basingstoke, U.K.). HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, U.K.) and acetonitrile Far UV, sodium octanesulphonate, trifluoroacetic acid and glacial acetic acid were purchased from BDH (Poole, U.K.). Cetrimide was purchased from ICI (Macclesfield, U.K.). Dianeal 137 CAPD solution was purchased from Travenol Labs. (Norfolk, U.K.).

Procedure

System 1. A 3-μm ODS column, (3 cm × 5.2 mm I.D.) was used (Perkin-Elmer, Beaconsfield, U.K.). Two mobile phases were used. For human and porcine insulins the mobile phase was buffer–acetonitrile (78:27.5). The buffer consisted of 0.1 *M*–ammonium sulphate 0.005 *M* tartaric acid, with the final pH adjusted to 3.5 with sulphuric acid. Cetrimide was added to the mobile phase to give a final concentration of 0.002%. For bovine insulin, the running solvent was modified to buffer–acetonitrile (78:22), with the cetrimide concentration remaining at 0.002%. Peak height responses were linear for solutions containing between 0 and 80 units/ml ($r = 0.98$). All insulins were diluted before analysis; soluble insulins were diluted with water for injection and insoluble insulins were dissolved in and diluted with 0.1 *M* hydrochloric acid to give final solutions of 20 units/ml. Injection was via a 20-μl loop and the solvent flow was 2 ml/min. The wavelength of detection was 215 nm.

System 2. A 5-μm Spherisorb CN column (25 cm × 4 mm I.D.) was used (Hichrom, Reading, U.K.). For all insulins, the mobile phase was acetonitrile–water–trifluoroacetic acid–sodium octanesulphonic acid (70:30:0.1:0.1). Peak height and peak area responses were linear between 0 and 100 units/l in saline, 5% dextrose, Dianeal 137 CAPD solution and water ($r = 0.99$). Injection was via a 100-μl loop and the solvent flow was 2 ml/min. The area of detection was 10–100 units/l, the limit of detection being 0.1 unit/l. For very sensitive work in the range of 10–20 units/l the full scale deflection of the chart recorder was adjusted to 1 mV and the UV detector was used at its most sensitive setting. Acetonitrile Far UV was used to overcome problems of baseline noise at the low wavelength of detection (215 nm).

Both systems were initially developed using authentic bovine and porcine samples, each system then being adjusted to ensure that complete resolution between insulin and preservative was effected when used to determine insulin content.

RESULTS AND DISCUSSION

System 1

Fig. 1a shows a chromatogram of a porcine insulin preparation using the running solvent described in system 1, but using a 25-cm, 5-μm ODS column. As can be seen, the preservative and insulin are well resolved. However, altering the cetrimide and acetonitrile content, although giving more rapid elution, results in loss of separation, thus making the chromatography unsatisfactory. Using a 3-μm packing in a short (3 cm) column, with the same running solvent and insulin preparation, gives the chromatogram shown in Fig. 1b. The preservatives used in insulin preparations (methylhydroxybenzoate, phenol and *m*-cresol) elute after 2 min, and human and porcine insulins elute after 4.5 and 5 min, respectively. Bovine insulin preparations, using the same running solvent, give similar chromatograms, although the preservative and insulin peaks are not completely separated from each other. For the analysis

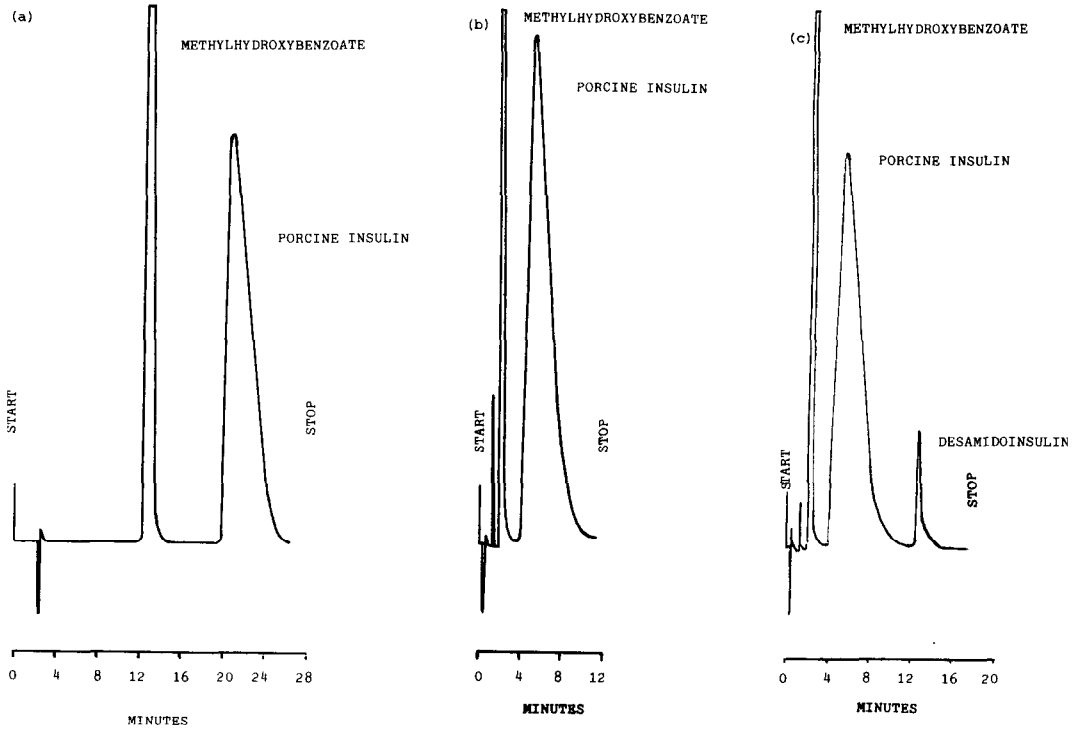
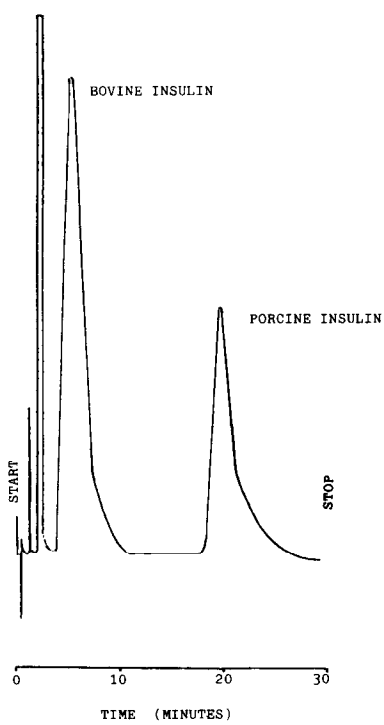


Fig. 1. (a) Chromatogram of porcine insulin (20 units/ml) and the preservative, methylhydroxybenzoate, using a conventional 25-cm, 5-μm ODS column and the mobile phase described for system 1 under Materials and methods. (b) Chromatogram of the same solution, using a 3-μm ODS packing in a 3-cm column and the same mobile phase. (c) Chromatogram of porcine insulin and desamidoinsulin after storage at 55°C.

of bovine insulin, a running solvent containing less acetonitrile was used, giving complete resolution. The preparation of the running solvent was critical and needed to be carried out with great care, using volumetric glassware throughout. Even variations in the order of 0.2% in either the buffer or acetonitrile content was sufficient to significantly alter the chromatography of the insulin preparations, necessitating the preparation of fresh running solvent. For the running solvents used, cetrimide content (0.002%) was critical in resolving the insulin from the preservative. The 3-μm column packing, compared with conventional 5-μm packings, provided higher separating efficiencies, faster separations and used less running solvent for the same analysis (approximately 20 ml as opposed to 60 ml).

This method of insulin analysis has been successfully used to measure the interactions that occur between soluble and zinc insulins (*i.e.* porcine soluble and porcine zinc, bovine soluble and bovine zinc and human soluble and human zinc), soluble and protamine insulins (*i.e.* porcine soluble and porcine protamine, bovine soluble and bovine protamine and human soluble and human protamine) and the stability of insulin preparations when stored in syringes at 4, 20, and 35°C (ref. 17). If, for example, porcine soluble and zinc insulins do interact, then any decrease in the soluble insulin content seen after centrifugation of the mixture would indicate that sol-



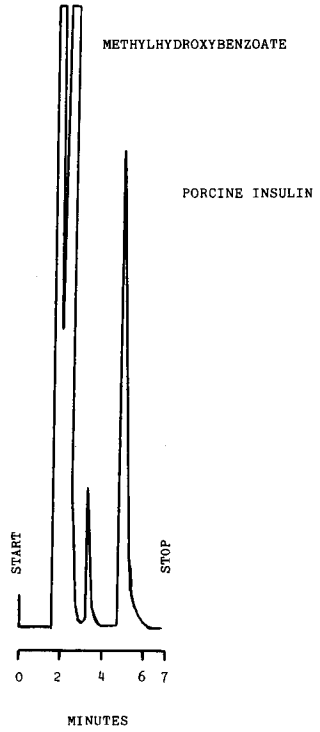


Fig. 2. Chromatogram of bovine and porcine insulin and methylhydroxybenzoate, using a 3-μm ODS packing in a 3-cm column and the mobile phase for bovine insulin decribed for system 1 under Materials and methods.

Fig. 3. Chromatogram of porcine insulin in 5% dextrose (40 units/l) and methylhydroxybenzoate, using a 5-μm 25-cm nitrile column and the mobile phase described for system 2 under Materials and methods.

uble insulin has been lost as a result of an interaction with excess zinc and/or adsorption onto crystalline or amorphous insulin. The method also distinguishes between insulin and its deamidation product, desamidoinsulin, when subjected to accelerated degradation by storage at high temperatures (Fig. 1c). This is seen as an additional peak eluting after insulin. The coefficient of variation of ten replicate standard injections of 40 units/l of porcine, bovine and human insulin preparations were 0.9, 0.7, and 0.7%, respectively, using peak area determinations.

Although insulins from different species are not mixed for clinical use, available standards (*e.g.* insulin EPBRP) contain insulins from bovine and porcine sources. The method described for the analysis of bovine insulin (system 1) in the methodology can be used to successfully resolve bovine and porcine mixtures (Fig. 2) and bovine and human mixtures. Using this system, bovine, human and porcine insulins elute after 5, 16, and 20 min respectively.

System 2

Fig. 3 shows a chromatogram of a porcine insulin preparation diluted in 5% dextrose, using system 2 described under Materials and methods. The preservatives in the preparations studied (methylhydroxybenzoate and *m*-cresol) elute after 2 min. There was no difference in retention time (5 min) between porcine, bovine and human insulins, using this method. The peak eluting at 3 min was found in all solutions containing dextrose and was unaffected by the addition of insulin. All insulin standards were prepared in the solution appropriate to the delivery system being studied, since peak height and shape were found to vary according to the dextrose and electrolyte content. The preparation of the running solvent was again found to be important, in this instance, trifluoroacetic acid and sodium octanesulphonate were the critical components. Coefficient of variation, using peak height values for porcine, bovine and human insulins were 0.8, 1.1 and 0.9%, respectively.

The method is very sensitive, especially when acetonitrile Far UV is used, allowing the chart recorder and detector to be used at their most sensitive settings. This method has been used to determine the extent to which insulin undergoes adsorption onto the surface of CAPD bags and intravenous infusion systems[18]. Insulin is added to dextrose, saline and CAPD solutions in amounts varying from 10 to 100 units/litre, depending upon clinical usage, thus requiring a degree of sensitivity not claimed by previous published methods. It has the advantage that insulin is measured directly unlike other methods of insulin analysis which involve the use of radiolabelled insulin (radiotracer and radioimmunoassay methods).

Without the addition of an ion-pairing agent to both solvent systems, the insulin remained on the column indefinitely. For system 1, the addition of cetrimide to the running solvent had a dramatic effect upon the retention of all insulins. Increasing cetrimide concentrations rapidly decreased the retention time; 0.002% was the concentration at which the insulins were best resolved from any preservatives present. For system 2, the addition of trifluoroacetic acid and sodium octanesulphonate reduced the retention time of insulin (5.5 min) allowing rapid analysis, good resolution and increased sensitivity. The increased acetonitrile content allowed the preservatives to be rapidly eluted, well before the insulin.

CONCLUSIONS

Two HPLC methods for the analysis of insulin are described. Both have advantages over existing methods. Method 1 gives rapid analysis, distinguishes between different insulins and is stability indicating. Method 2 gives rapid analysis and is capable of measuring very low levels of insulin in solution but can not be used to separated insulins from different species. Both systems are economical, using low volumes of solvents.

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