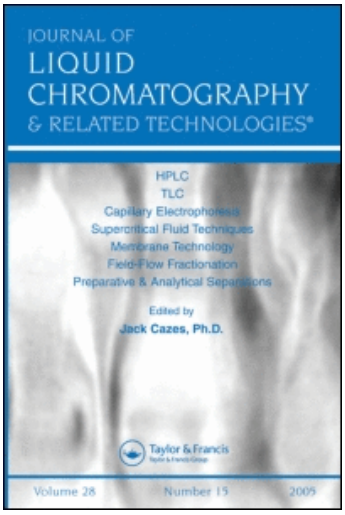

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Insulin Evaluation in Pharmaceuticals: Variables in RP-HPLC and Method Validation

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INSULIN EVALUATION IN PHARMACEUTICALS: VARIABLES IN RP-HPLC AND METHOD VALIDATION

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ABSTRACT

In this paper different factors, including solvent composition, pH, flow rate, temperature and organic modifier volatility, were examined for their effects on the resolution of Insulin by Reversed Phase High-Performance Liquid Chromatography (RP-HPLC). At the same time several insulin formulations from different pharmaceutical companies have been analyzed for the existence of degradation products. Method validation and reproducibility have been determined by means of two different chromatographic systems. Variation of key parameters demonstrated that a decrease in pH (<3.8) provides a good peak shape, at the same time enhances the insulin retention. An increase in temperature, decreased retention time and enhanced resolution. This information has been applied to insulin formulation assay, where no degradation products were found.

INTRODUCTION

In the last decade, HPLC analysis has become the most commonly used method in drugs analysis, being one of the most accurate, precise and reproducible methods available. However, this method has been found to have significant limitations when applied to polypeptides and proteins analysis due to their

special structural characteristics. These limitations have been most evident in the study and evaluation of protein- or peptide-based drugs, especially with regard to the method's resolution and recovery capacity (1).

In recent years a large number of reports have been published describing the application of insulin to a variety of reversed high-performance liquid chromatography systems. In the majority of these reports, insulin has been used as one among several polypeptides and proteins in order to characterize the separation capacity of the system (2-12). However, few publications have dealt with the quantification of impurities and derivatives in insulin formulations (13). We felt the need to optimize an analytical insulin determination method that was quick, precise, reproducible and which would, at the same time, allow us to identify and quantify possible degradation products in insulin formulations.

In this paper, a reversed phase HPLC technique is described, and the different factors capable of influencing chromatographic resolution, such as solvent composition, organic modifier volatility, flow rate, pH, and temperature were examined. The information provided in this section is to be apply to the practical problem of insulin formulation's assay.

MATERIALS AND METHODS

Apparatus

The analysis has been carried out using two different chromatographic systems: a Waters Associates Inc.(Milford, MA,

USA) HPLC equipped with an M-45 solvent delivery system, an 440 UV-Visible detector operating at 280 nm. Detector outputs were monitored by a model 740 Data Module plotter/integrator. Injections were made by a Rheodyne (Berkeley, CA, USA) injector loop (20 µl); and a Konik KNK-500 (Barcelona, Spain) HPLC system, KNK-500 pump, G-gradient quaternary with microprocessor, KNK-200 UV-Vis detector, Hewlett-Packard HP-3394-A integrator, 20 µl Rheodyne Inc. injector. In both systems a Waters Columns µbondapak C_{18} (10 µm, 3.9 mm x 30 mm), Waters division of Millipore Co. (Milford, MA, USA) were used.

Samples and solvents were clarified by filtration through porous membranes, Millex-GV (0.22 and 0.45 µm), Durapore 0.45 µm, Waters division of Millipore Co. (Milford, MA, USA).

Reagents

Monocomponent human insulin crystals, were provided by Novo Research Institute (Bagsvaerd, Denmark). Insulin standards were provided by The National Institute for Biological Standards and Control, England.

Insulin injections (porcine and bovine) and human monocomponent (r-DNA and semisynthesis) from two pharmaceutical companies, Novo Nordisk A/S (Bagsvaerd, Denmark) and Eli Lilly Company (Indianapolis, Indiana, USA).

Acetonitrile LiChrosolv^R, orthophosphoric acid, and reactive grade monobasic sodium phosphate all were purchased from Merck Co. (Darmstadt, Germany); hydrochloric acid from Quimon (Barcelona, Spain). Reagent HPLC grade water, produced using a Milli-Q system (Millipore Co. Milford, MA, USA).

METHODS

The insulin standards were prepared for analysis according to USP XXII (14), dissolving them in 0.01 M hydrochloric acid to obtain a final concentration of 40 U/ml. Solution and suspension- type injections were prepared according to USP XXII. To avoid deamination reactions due to low pH, all samples were prepared a few minutes before the assay.

Two types of buffer system were investigated: Acetonitrile (AcN): water: phosphoric acid and phosphate buffer: Acetonitrile.

All solvents were prepared underwent the same treatment before use, being degassed (vacuum/ ultrasound) and filtered through a porous membranes (0.45μm).

RESULTS AND DISCUSSION

System suitability criteria based upon the separation of insulin and its monodesamido derivative were used to ensure the quality of HPLC separations. The resolution factor (R) between the insulin and the monodesamido insulin peaks is to be not less than 1.8. $R = 1.18\ (V_{Rb}-V_{Ra})\ /\ W_{ha}+W_{hb}$ where V_{Rb} and V_{Ra} = distance along baseline between the point of injection and perpendiculars dropped from the maxima of two adjacent peaks, W_{hb} and W_{ha} = the respective peak widths measured at half peak height.

Isocratic elution of bovine: porcine insulin mixture using 40:60 AcN: water: 0.1% phosphoric acid, resulted unsatisfactory, since a very poor peaks shape were obtained with a relatively low retention time. Modifications made to chromatographic conditions

(flow-rate and temperature) led to no improvement in resolution and only marginally affected the retention time. Due to the above- mentioned non- ideal peak shape obtained using system 1, correct estimations of resolution could not be performed, therefore it was discounted. The use of a mixture consisting of 73 volumes mixture of 0.1 M monobasic sodium phosphate, adjusted with orthophosphoric acid to a pH of 2.0, and about 27 volumes of acetonitrile, seemed to promise some improvement in the resolution of the different components of the sample. Several modification were made to achieve satisfactory resolution. First, the concentration of the organic component was fine-tuned, checking how the percentages of AcN used presented a critical point in the preparation (15). Very slight changes in the concentration of AcN produced drastic alterations in the retention times (figure 1). In view of this, a 70:30 concentration of phosphate buffer: AcN was chosen. Under these conditions, with a flow-rate of 1.6 ml/min, a relatively short retention time was obtained, as well as a good chromatographic separation, resolution factor of 1.98 for insulin/ monodesamido was obtained (figure 2). Theoretical plates number was calculated according to the formula $N=16(t/W)^2$ where t was the retention time for insulin and w the peak width measured at the peak base. For human insulin N was = 8400/m. Estimations of resolution and plate number were performed ten times or more.

It was observed that the same sample, analyzed several times throughout the day, showed an increasing retention time, with a linear relationship existing between the retention times of the different components and the duration of the analysis, due to the evaporation of the AcN. This problem was solved by connecting the mobile phase reservoir to a second reservoir with

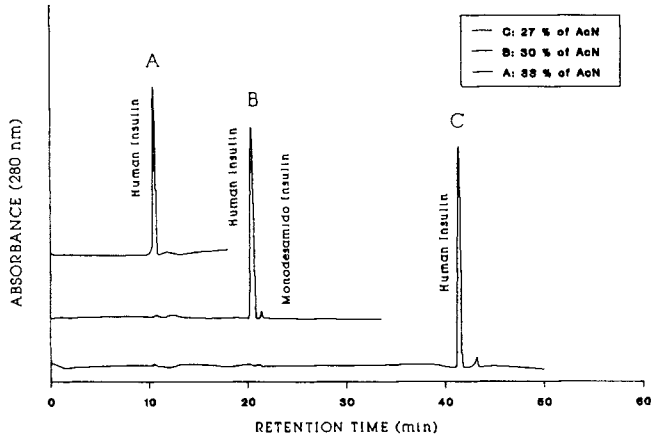

FIGURE 1. Effect of acetonitrile concentration on retention times.

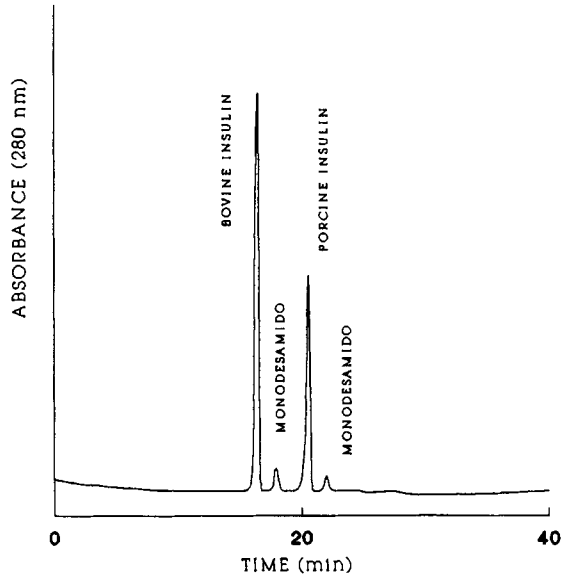

FIGURE 2. Chromatographic separation of a mixture of purified bovine and porcine insulins. Column: μBondapak C_{18}, AcN: phosphate buffer.

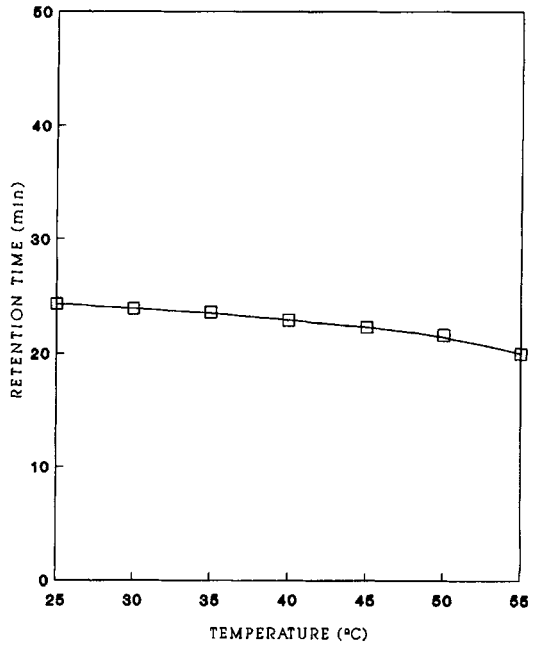

FIGURE 3. Dependence of retention times on temperature.

Acetonitrile by means of a Teflon tube, thus maintaining constant vapour pressure of acetonitrile in the main reservoir. In this way variation in the retention times throughout the analysis was reduced to a minimum (retention time C.V % = 0,98 for n = 67 samples).

It was decided to examine the effect of temperature on retention time and resolution since protein separation could be enhanced by elevated column temperatures. As is shown in figure 3, there is a slight decrease in the retention time for insulin and its derivatives with an increase in column temperature. At the same time, it has been observed that the resolution factor

(R) was found to increase slightly with a change of temperature from 25 to 55 ºC. Temperature of 45°C was chosen as the most appropriate for our analysis, thus protecting the protein from the possibility of degradation as a result of high temperatures.

A decrease in pH produces an increase in the retention times and improved resolution probably due to a necessity to minimise ionic and absorptive interactions with the reversed-phase support and to suppress ionisation of ampholyte acidic moeities which can cause peak doubling as different charged entities are formed from single peptide at high pH-values (16). We have, therefore, examined the behaviour of human insulin over a range of pH values (3.8 - 2) while maintaining a total phosphate concentration of 0.1 M. As is shown in figure 4, a decrease in pH from 3.8 to 2 caused an increase in the retention time, and it was noted that lowering the pH increased the resolution up to 2.1 . A pH of 2.2 was chosen to prevent deterioration of the columns and reduce the formation of the monodesamido derivative of insulin to a minimum.

Method Validation

With the temperature, pH, composition and flow-rate conditions for the mobile phase fixed, analysis was carried out to determine the validity of the chromatographic assay, as well as the concentrations of insulin and derivatives. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)(2)] (14) require that test methods, which are used for assessing compliance of pharmaceutical products with established specifications, must meet proper standards of accuracy and reliability. Various solutions of Human insulin standard were

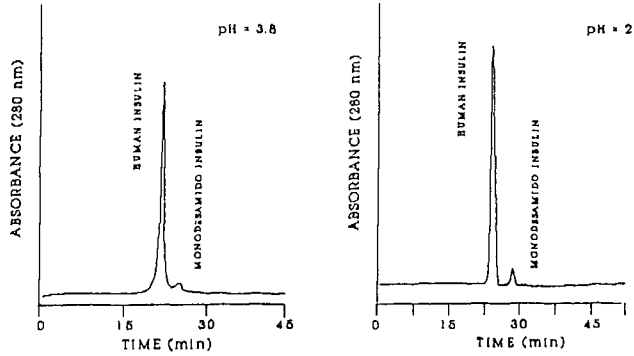

FIGURE 4. Effect of pH on retention times and resolution.

chromatographed individually to determine the linearity of the response, five replicate injections were run for each sample, the relative standard deviation for replicate injections were 0.09% The calibration curve was realized with an excellent correlation coefficient of 0.9997 for a range of concentration from 2 to 60 U/ml (figure 5). The precision of the HPLC assay, was performed over a 3-day period, calculating the standard deviation and variation coefficient for 5 determinations per day of two different solutions of a 40 U/ml sample of human insulin, the data obtained were, standard deviation S = ± 0.110 U. and C.V. = 0.28 % . The accuracy was evaluated by calculating the recovery for three samples of insulin spanning 20% , 50% and 100% of the expected content. 99.24%, 100.22% and 99.71% were obtained as a result of recovery. Limit of detection and limit of quantitation were 0.03 U/ml and 0.098 U/ml.

In order to check the reproducibility of the method, a series of trials were carried out using a Konik KNK-500 HPLC system. Identical results were obtained when mobile phase

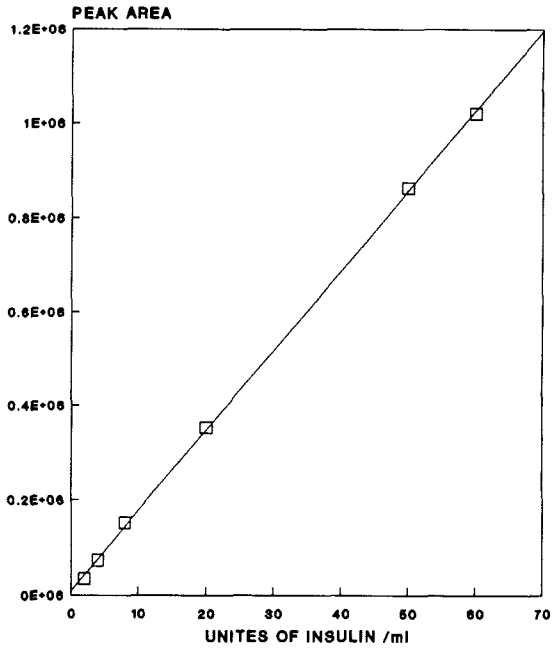

FIGURE 5. Calibration curve showing relationship between peak area and concentration of insulin, coefficient of linear correlation = 0.9997.

component were mixed outside the HPLC system (variation coefficient 1.4% n = 20). However, when the system was programmed to produce a 70:30 mixture of phosphate buffer: AcN, under the same conditions and using the same column, there was no separation of the peaks corresponding to the monodesamido derivatives of insulin. This could be due to a failure of the system's microprocessor.

Monodesamido derivatives would be due to the acid medium in which the extraction was carried out (13). In order to check this, both the original injections and the samples of these analyzed were stored at 4 - 6°C for a month. At the end of this

period, an increase in the concentration of the monodesamido derivative was observed to have taken place in the samples already analyzed. Yet there were no changes to the original injections. This is a symptom of the degradation of the active principal in an acid medium.

The information provided in the previous sections was applied to insulin formulation's assay, where no degradation products were found.

CONCLUSIONS

The technique described is capable of separating molecules which, according to Brange (13), only differ by one amino acid and at the same time is capable of separating molecules which have virtually the same molecular weight, such as insulin and the monodesamido derivatives. This technique is precise and reproducible, and can therefore be relied upon to produce the results expected.

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