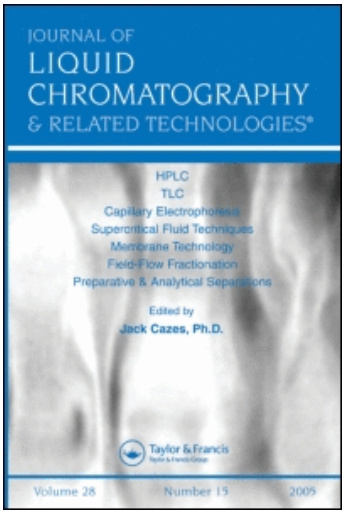

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THE USE OF A NON-POROUS REVERSED PHASE COLUMN FOR RESOLUTION OF PORCINE INSULIN FROM LOW MOLECULAR WEIGHT AMIDES IN THE SAME MATRIX

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ABSTRACT

A rapid, reversed phase, high performance liquid chromatography (HPLC) method for the quantitation of porcine insulin has been developed and validated at Emisphere Technologies. Insulin is assayed with a C4 silica based column in a matrix containing a patented Proteinoid Oral Drug Delivery System (PODDS™) for the oral delivery of protein and non-protein drugs. This technology is based on low molecular weight, thermally condensed and acylated amino acids (proteinoids) forming pH dependent microspheres to encapsulate the insulin. Chemical modification of the proteinoids were shown to enhance drug delivery but interfered with the HPLC assay by co-eluting with the insulin. This resulted in distortions to the insulin peak shape reduceing the accuracy and precision of the quantitative data. This method was then re-developed and the interferences removed by employing a unique, non-porous, poly(styrenedivinylbenzene) (PSDVB) packing material. The absence of pores eliminates the column efficiency (**N**) and resolution (**R**) values important for the separation of small molecules which conform to the principals of partition chromatography. Consequently the small proteinoid molecules elute at the column void while large proteins

such as insulin, which conform to the principals of partition chromatography, elute at a later time. The result is complete resolution of large proteins from peptides and low molecular weight compounds present in the same matrix.

INTRODUCTION

A rapid, reversed phase, high performance liquid chromatographic (HPLC) method for the routine quantitative analysis of porcine insulin was currently developed and validated in our laboratory. Briefly, it employs a C4 silica based column and gradient elution with a ten minute run time (1). The matrix used for validating this method contains a very concentrated solution of various low molecular weight, thermally condensed (2) or acylated (3) amino acids synthesized at Emisphere Technologies. These protein-like compounds (proteinoids) are components of a unique Proteinoid Oral Drug Delivery System (PODDS™) technology used to encapsulate and orally deliver insulin, and other proteins.

Once the insulin is encapsulated the excess is removed by centrifugation, as reported by Milstein et al. (4). The HPLC method referred to above is then employed to quantitate the percent of encapsulated insulin. As part of an on-going research program, further chemical modifications to the proteinoids have improved their drug delivery attributes. Often these modifications also alter their physicochemical properties which frequently causes partial overlap of elution times with insulin in this assay. This overlap distorts the insulin peak shape and reduces the accuracy and precision of the quantitative data sometimes to the point of invalidating the results. Consequently, this quantitative insulin HPLC method required re-development study to regain the accuracy and precision needed to quantitate the percent of insulin encapsulated.

This report summarizes this new insulin HPLC method, the chromatographic problems encountered with the chemically modified

proteinoids and the steps taken to overcome them. They include altering the mobile phase composition, gradient profiles and use of two column packing with different selectivity. One packing consists of a diphenyl group chemically bonded to silica and the other consists of a non-porous, poly(styrenedivinylbenzene) (PSDVB).

MATERIALS

The HPLC pump (model 250), autosampler (model ISS 200), variable wavelength UV detector (model LC-95) and Turbochrom data acquisition software (version 3.3) used in this research were purchased from the Perkin-Elmer Corp. The C4 column was a Brownlee, 30 x 4.6 mm, 7 mcn, 300 Angstrom, Aquapore from Applied Biosystems. The diphenyl column was a Brownlee, 30 x 4.6 mm, 10 mcn, 300 Angstrom from Applied Biosystems. The non-porus PSDVB column was a PRP-Infinity, 30 x 4.1 mm, 5 mcn from Hamilton. Acetonitrile (ACN) and tetrahydrofuran (THF) were HPLC grade, purchased from EM Science. Potassium phosphate and porcine insulin were purchased from Sigma Chemical Co . The proteinoids used in the matrix were produced by Emisphere Technologies, Inc. Their concentration was 40 mg/ml in all cases. All water was deionized by a filtration system from Millipore.

METHODS

The origional method, developed and validated for the quantitative analysis of insulin used a C4 column and a linear gradient between two mobile phases (A and B) at a flow rate of 2.5 ml/min. Mobile phase A was composed of 10% THF and 90% aqueous 10 mM K_2HPO_4, pH 7.0. Mobile phase B was composed of ACN/THF(40:10) and 50% aqueous 10 mM K_2HPO_4, pH 7.0. The gradient conditions were from 40-70% B in 7 minutes

with a 3 minute hold. Detection was at 220 nm and the run time was 10 minutes. After it was discovered that the chemically modified proteinoids all had overlapping elution times with the insulin, variations in mobile phase composition were made and tested to eliminate this interference. These variations included changing the buffer strength to 5, 25, 50 and 100 mM K_2HPO_4, decreasing its pH to 3 and varying the percentage of ACN and THF in the gradient profiles. All of these attempts failed to completely resolve the insulin from the various proteinoids tested, resulting in this method no longer being valid.

The column selectivity was then changed to a diphenyl stationary phase using the first mobile phase composition and gradient profile. This attempt to adequately resolve insulin from all the proteinoids tested was unsuccessful. All of the successive mobile phase variations as described above also proved unsuccessful. The conditions found to provide the best, but still unacceptable, resolution were a two step linear gradient between the first mobile phase A and B compositions. The first step consisted of a 4 minute linear gradient from 10-35% B. The second step was a 3 minute linear gradient from 35-60% B with a 3 minute hold. The flow was 2.5 ml/min, detection was at 220 nm and the run time was 10 minutes.

Further attempts to adequately resolve the insulin from the proteinoids used a non-porous PSDVB column. The first mobile phase tried was the A and B compositions first described above. A two step linear gradient was successful in completely resolving the insulin from all proteinoids tested. The first step was a linear gradient from 10-70% B in 3 minutes followed by a 2 minute linear gradient from 70-85% B with a 5 minute hold. The flow was 2.5 ml/min, detection was at 220 nm and the run time was 10 minutes.

Precision and accuracy were determined for each column of different selectivity and the mobile phase gradient conditions adjusted to give the best

insulin resolution (**R**). A quantitative value for **R** was given in the USP, where $\mathbf{R} = 2(t_2-t_1)/W_2 + W_1$ (5). The criteria used for determining acceptable precision and accuracy limits for the data was taken from a recently published report on methods validation (6). These guidelines suggest no more than 15% coefficient of variation for precision and no more than 15% deviation from the nominal value for accuracy.

RESULTS

The partial over lap in elution times of the modified proteinoids with the insulin produced by the method employing the C4 column is shown in Figure 1. These elution effects cause the precision and accuracy values of a quality control standard to fall outside the accepted limits (Table 1). Modifying the mobile phase composition or its percentage of organic solvents is only partially successful in completely resolving the insulin from all the proteinoids tested. Changing the selectivity by the use of a phenyl bonded phase along with the use of various mobile phase compositions and gradient profiles is also unsuccessful in most cases (Figure 2). A non-porous PSDVB column, however, completely resolves insulin from all modified proteinoids tested using the original mobile phase composition and a rapid gradient profile (Figure 3). This non-porous support also provides the necessary resolution for acceptable precision and accuracy, even in high concentrations of proteinoid in the matrix.

DISCUSSION

The principles of partition chromatography demonstrate that increasing silica based column efficiency (**N**) results in an increased resolution (**R**) value. This is illustrated in Equation 1 which relates **R** to the column selectivity factor (a), capacity factor (k') and **N** (7,8).

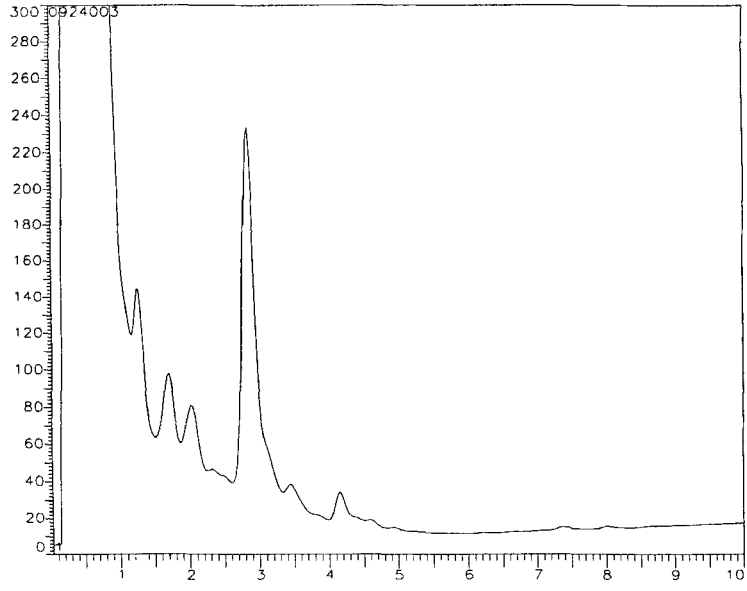

FIGURE 1. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.1 mm, 7 mcn, 300 Angstrom, C4 silica based column from Brownlee. Insulin retention time 4.1 minutes.

TABLE 1

Accuracy, Precision and Resolution values for Insulin were determined from repetitive injections of an aqueous standard consisting of 449 mcg/ml of insulin and 40 mg/ml of PODDS™ matrix E15.

HPLC Column	Accuracy Error (% deviation)	Precision (%CV)	Resolution (R)	n
C_4	84.7%	22.8%	0.66	2
Phenyl	3.9%	2.3%	1.20	4
Non-porous	1.0%	6.5%	2.85	4

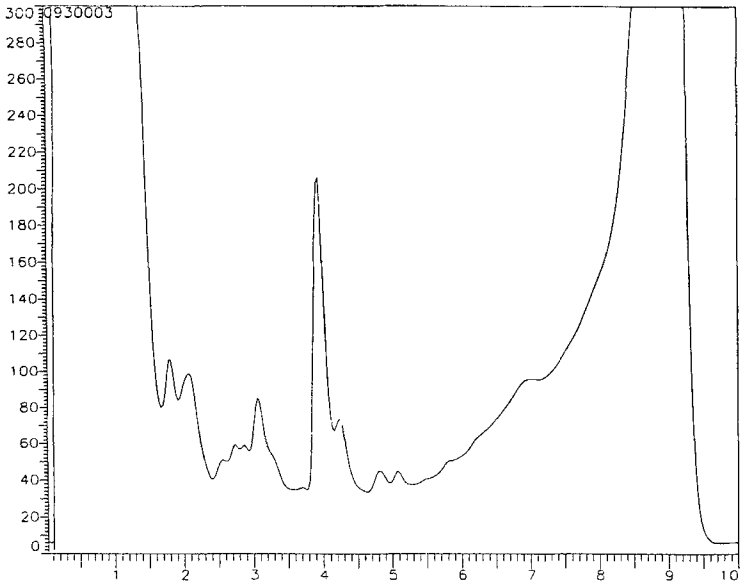

FIGURE 2. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.6 mm, 10 mcn, 300 Angstrom, diphenyl silica based column from Brownlee. Insulin retention time 5.0 minutes.

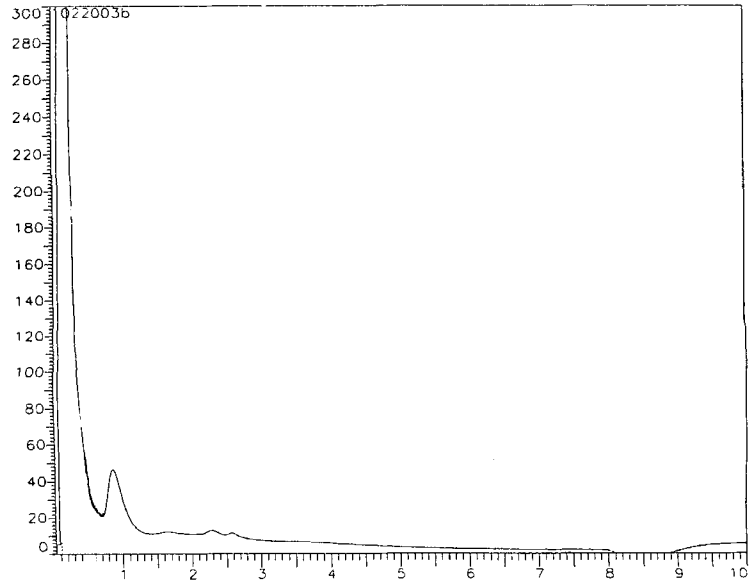

FIGURE 3. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.1 mm, 5 mcn, non-porous PSDVB based column from Hamilton. Insulin retention time 5.3 minutes.

$$R = (1/4)(a-1)N^{0.5}\,[k'/(1+k')] \quad (1)$$

The direct consequence of Equation 1 is that **R** is proportional to the square root of **N**. This suggests that high **N** values should be indicative of high **R** values for any given column (7,8). A numerical value for **N** may be determined from the retention time (t_R) and peak width at half-height ($W_{1/2}$) from Equation 2 (5).

$$N = 5.54(t_R/W_{1/2})^2 \quad (2)$$

A report by Pearson et al. (9) however, suggested that a common misconception is that a column of high **N** value will necessarily indicate superior **R** for proteins. Their comparison of different brands of silica having different calculated values for **N** showed that the column with the highest calculated **N** produced the poorest **R** of proteins with more than 30 residues. Conversely, the column with the lowest calculated **N** produced the greatest **R** value. It was concluded that increasing the **N** or plate number of silica-based columns, decreases the chromatographic **R** of proteins which suggests that they do not conform well to the principals of partition chromatography.

The separation and **R** of large proteins do however, conform well to the principals of adsorption chromatography (10). Separations based on these principals rely on the equilibria of the solute between the solvent and the hydrophobic coating of the support. According to this model, a protein is resolved by adsorbing onto the hydrophobic surface from the mobile phase and remaining adsorbed until it is displaced by a sufficiently high

concentration of organic solvent. The **R** of a mixture of large proteins, therefore is entirely dependent upon their ability to be adsorbed versus their solubility in an organic solvent and is independent of column **N** or plate number.

Another factor that must be considered for optimal protein **R** is the contribution of the silica pore size. Smaller pore silicas (<120 Angstroms) are reported to be inferior to large-pore-diameter supports for resolution and recovery of proteins. Pore sizes of 300 Angstroms offered the best resolution and recovery but was not indicative of success in every case (9). The principals of partition chromatography suggest that inefficient recovery and uneven pore size distribution may contribute to ghosting and band broadening sometimes produced by large proteins when chromaographed on small pore silica columns (7,8).

These principals and factors have led to the development and use of columns with lower calculated **N** values and large pore size for the chromatography of large proteins. These features allow uniform elution for proteins while decreasing the **R** of low molecular weight compounds causing them to elute closer to the column void. Many large pore silicas however, maintain enough **R** and uneven pore size distribution to adequately retain some small peptides and low molecular weight compounds. This may increase their elution time sometimes to the point of interfereing with the elution of large proteins. This was apparently the case in the methods using the 300 angstrom pore size C4 and diphenyl columns.

A PRP-Infinity, non-porous column was developed to eliminate all pore diffusion effects by reducing the value of **N** to zero which consequently reduces the value of **R** to zero in Equation 1. Since there is essentially zero **R**, those compounds which separate according to the principals of partition chromatography will elute at the void. Those compounds which separate by the principals of adsorption chromatography will be retained until they are

eluted by the appropriate amount of organic solvent. The method using the non-porous PRP-Infinity column described herein illustrates how these principles may be combined for the successful separation of a large protein from an abundance of low molecular weight compounds.

Since the PRP-Infinity column reduces the value of **N** to zero in Equation 1, the calculated **R** values between each of the columns has no value. This makes it difficult to represent the resolving ability of these columns in a numerical context. An appropriate solution is determining **R** values using the equation $R = 2(t_2-t_1)/W_2 + W_1$ (4). For each column, the retention time and peak width of the proteinoid peak eluting closest to the insulin peak may approximate t_1 and W_1. The elution time and peak width of the insulin peak then may be t_2 and W_2.

This non-porous technology proves very useful in this case for separating a large protein from a complex mixture of low molecular weight compounds with different physicochemical properties. This type of chromatography may be used to develop a method with no residual diffusion or ghosting effects in minimal development time. The result is a rugged, quantitative method capable of excellent separations in minimal run time.

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