Application Note

Biopharma/Pharma

Modernizing the Insulin USP Monograph HPLC Method for Assay and Related Compounds

Analysis of insulin using Agilent InfinityLab Poroshell 120 columns and following the newly revised USP <621> guidelines

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Abstract

The original United States Pharmacopeia (USP) monograph HPLC methods for assay and related compounds analysis of insulin and human insulin were modernized with Agilent InfinityLab Poroshell 120 columns following the newly revised USP <621> guidelines. The original assay method uses an isocratic separation with a 4.6 × 150 mm, 5 µm column and requires 15 minutes for the analysis. When the method was transferred to the InfinityLab Poroshell 120 SB-C18 and EC-C18 columns (4.6 × 50 mm, 2.7 µm), the analysis time was reduced from 15 to 6 minutes (60% reduction in analysis time and solvent consumption), without method revalidation. The original method for related compounds uses a gradient separation with a 4.6 × 250 mm, 5 µm column. When the method was transferred to InfinityLab Poroshell 120 SB-C18 and EC-C18 columns (4.6 × 100 mm, 2.7 µm), the analysis time was reduced from 97 to 39 minutes (60% reduction in analysis time and solvent consumption) for insulin and from 73 to 29 minutes (60% reduction in analysis time and solvent consumption) for human insulin, without method revalidation. All system suitability requirements were met while achieving significant reductions in both analysis time and solvent consumption.

Introduction

In most USP monographs, there are HPLC methods for testing raw materials and formulated products. These methods have been the routine analysis techniques for generic pharmaceutical manufacturers. The methods mostly employ older column technology that includes conventional 5 µm particle columns. Due to the low efficiency of these types of columns, longer columns (e.g., 150 or 250 mm long) are often required, leading to long analysis times. USP only allows method transfer from conventional 5 µm columns to smaller-particle-size columns for isocratic methods, according to the previous USP <621> guidelines. The current USP <621> guidelines, revised in December 2022, now allow for the modernization of gradient methods both using totally porous particle (TPP, with smaller particle sizes) and superficially porous particle (SPP) columns.[1]

SPP has recently been applied to HPLC columns, providing higher efficiency than TPP columns. Agilent developed 2.7 µm InfinityLab Poroshell 120 columns that deliver similar efficiency to sub-2 µm TPP columns, with lower backpressure. The advantages of using the 2.7 µm SPP columns and a shorter column include run time and solvent consumption savings. These columns can be used with any HPLC and UHPLC system, because the columns use a standard 2 µm frit that can reduce the likelihood that particulates will plug the column. This feature extends column lifetime compared to sub-2 µm columns that use smaller frits.

In our previous work on insulin analysis, it was demonstrated that columns with a larger pore size (>100 Å) provided much higher efficiency and lower tailing factor, and that smaller particles provide higher efficiency. The 2.7 µm InfinityLab Poroshell 120 columns also provided the highest efficiency.[2] This result was also observed in previous work involving insulin and human insulin analysis according to the China Pharmacopoeia.[3,4]

In this application note, the original assay method and related compounds testing method for insulin and human insulin in the USP[5,6] were transferred to SPP columns within allowable adjustments under the current USP <621> guidelines, made official on 1 December 2022. The original methods were first run on 5 µm Agilent Pursuit C18 and Polaris C18-A columns, and the modernized methods were achieved on InfinityLab Poroshell 120 SB-C18 and EC-C18, 2.7 µm columns.

Experimental

Instruments and materials

An Agilent 1260 Infinity II LC was used with 0.17 mm tubing throughout this application. Table 1 shows the instrument configurations.

All reagents and solvents were HPLC grade. Acetonitrile, anhydrous sodium sulfate, phosphoric acid, ethanolamine, and insulin and human insulin standards were purchased from Anpel Laboratory Technologies (Shanghai, China). Water was purified using an Elga PURELAB Chorus system (High Wycombe, UK). The system suitability solution was prepared according to the USP monograph of insulin and human insulin.

The original method conditions and system suitability requirements are listed in Table 2. The gradient conditions with conventional columns and those with

Table 1. Instrument configurations.

Modules and Software	Component Specifications
Agilent 1260 Infinity II Binary Pump (G7112B)	4-pos/10-port valve 600 bar (p/n 5067-4287)
Agilent 1260 Infinity II Multisampler (G7167A)	Vial, screw top, amber with write-on spot, certified, 2 mL, 100/pk (p/n 5182-0716) Cap, screw, blue, PTFE/red silicone septa, 100/pk (p/n 5182-0717)
Agilent 1260 Infinity II Multicolumn Thermostat (MCT, G7116A)	Standard flow heater (G7116-60015) Heater and column: Agilent InfinityLab Quick Connect assembly, 105 mm, 0.17 mm (p/n 5067-6166)
Agilent 1260 Infinity II Diode Array Detector WR (G7115A)	10 mm 13 µL flow cell (p/n G1315-60022) Long-life deuterium lamp (2.5 Hz/20 Hz)
Agilent OpenLab CDS, Version C.01.07	–

Table 2. Original LC method conditions for insulin and human insulin analysis.

Parameter	Value for Assay Method	Value for Related Compounds Analysis Method
Column	L1: 4.6 × 150 mm	L1: 4.6 × 250 mm
Mobile Phase	Dissolve 28.4 g anhydrous sodium sulfate in 1,000 mL water, pipet 2.7 mL phosphoric acid into the solution, and adjust with ethanolamine to a pH of 2.3 if necessary. Prepare a filtered and degassed mixture of this solution and acetonitrile (74:26).	Solvent: 0.2 mol/L sulfate (dissolve 28.4g anhydrous sodium sulfate in water, add 2.7 mL phosphoric acid, adjust with ethanolamine to a pH of 2.3, and add water to a volume of 1,000 mL) Solution A: prepare a filtered and degassed mixture of solvent and acetonitrile (82:18). Solution B: prepare a filtered and degassed mixture of solvent and acetonitrile (50:50).
Flow Rate	1.0 mL/min	1.0 mL/min
Column Temperature	40 °C	40 °C
Injection Volume	20 µL	20 µL
Detector	214 nm	214 nm
System Suitability Requirements	The relative standard deviation for replicate injections is not more than 1.6%; the resolution, R, between insulin or human insulin and A-21 desamido insulin is not less than 2.0; and the tailing factor for the insulin or human insulin peak is not more than 1.8	The resolution, R, between insulin or human insulin and A-21 desamido insulin is not less than 2.0; and the tailing factor for the insulin or human insulin peak is not more than 1.8

InfinityLab Poroshell 120 columns for related compounds analysis are shown in Table 3. The retention time of the main peak was adjusted by optimizing mobile phase composition on each column according to the retention time requirements of the main peak, as stated in the monographs.

Table 3. Gradient conditions for related compounds analysis of insulin and human insulin.

Column	Flow Rate (mL/min)	Gradient		Injection Volume (µL)	Multicolumn Thermostat (°C)	Diode Array Detector
		Insulin	Human Insulin			
Agilent Pursuit 200 Å C18, 4.6 × 250 mm, 5 µm (p/n A3000250X046)	1.0	Time (min) %B 0 28 60 28 85 67 91 67 92 28 97 28	Time (min) %B 0 28 36 28 61 67 67 67 68 28 73 28	20	40	214 nm, 2.5 Hz
Agilent Polaris C18-A, 4.6 × 250 mm, 5 µm (p/n A2000250X046)	1.0	Time (min) %B 0 28 60 28 85 67 91 67 92 28 97 28	Time (min) %B 0 28 36 28 61 67 67 67 68 28 73 28	20	40	214 nm, 2.5 Hz
Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm (p/n 685975–902)	1.0	Time (min) %B 0 30 24 30 34 67 36.40 67 36.80 30 38.80 30	Time (min) %B 0 30 14.40 30 24.40 67 26.80 67 27.20 30 29.20 30	8	40	214 nm, 40 Hz
Agilent InfinityLab Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-902)	1.0	Time (min) %B 0 30 24 30 34 67 36.40 67 36.80 30 38.80 30	Time (min) %B 0 30 14.40 30 24.40 67 26.80 67 27.20 30 29.20 30	8	40	214 nm, 40 Hz

Results and discussion

Both the assay method and related compound analysis method were transferred to InfinityLab Poroshell 120 columns. The chromatographic conditions were adjusted based on the newly revised USP <621> guidelines following the rules for isocratic and gradient methods, respectively.

Method adjustments for assay analysis (adjustment for isocratic elution)

The original assay method is an isocratic method using a 1260 Infinity II LC system and a 4.6 × 150 mm, 5 µm C18 (L1) column. Based on a previous study, 5 µm columns with a larger pore size, including Pursuit C18 (200 Å) and Polaris C18-A (180 Å), were used for the original methods test. Chromatograms for system suitability are shown in Figure 1. Both columns show symmetrical peak shape and sufficient resolution between insulin and A-21 desamido insulin. The relative standard deviations for insulin and human insulin were all less than 0.50% for peak area, below the requirement for this test (1.6%, see Table 5).

Previously, under allowable adjustment guidelines in USP <621>, all columns and particle size adjustments could follow the L/dp rule. In that adjustment, the ratio of column length to particle size is kept constant within a range of –25% to +50%. Following the L/dp rule, an adjustment from a 5 µm, 150 mm column to a 2.7 µm, 50 mm column would not be an allowed adjustment, as the L/dp is lower than the allowed range. This rule still applies for TPP-to-TPP adjustments. However, when an adjustment that includes SPP is applied, adjustment follows the N rule.

The newly revised USP <621> allows changes from TTP to SPP columns as long as the plate number (N) is within –25% to +50% for an isocratic method. This requirement means that a direct comparison of N must be made using the measured compounds in both the original method and the final adjusted method when using SPP columns. InfinityLab Poroshell 120 SB-C18 and EC-C18 columns have been proven to contribute to successful insulin analysis. Therefore, choosing the column dimensions of the InfinityLab Poroshell 120 depends on the N value of insulin measured experimentally. Since the L/dp for a 50 mm, 2.7 µm column was just below the acceptable range for an allowed adjustment, this column was used to experimentally determine insulin efficiency. Columns with SPP have higher efficiency than those with similar size TPP. The N values achieved with 5 µm columns and 2.7 µm InfinityLab Poroshell 120 columns are shown in Figure 2.

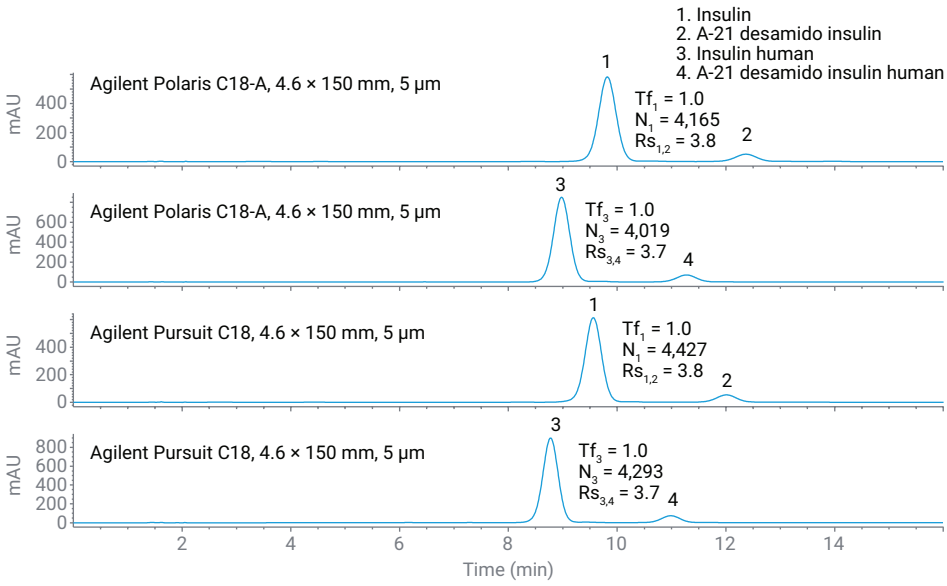

Figure 1. System suitability test for assay analysis using 4.6 × 150 mm, 5 µm columns.

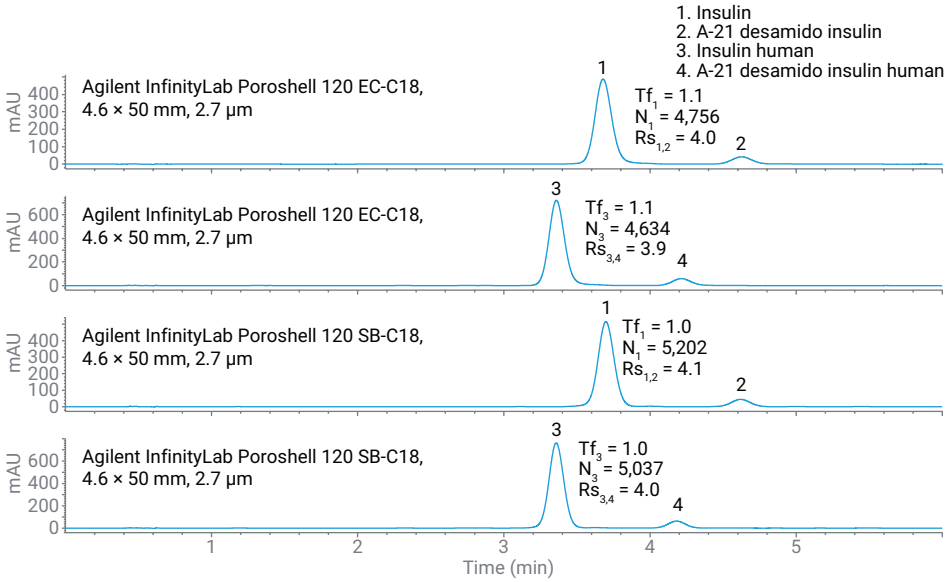

Figure 2. System suitability test for assay analysis using Agilent InfinityLab Poroshell 120 4.6 × 50 mm, 2.7 µm columns.

The N values of the adjusted method on 50 mm, 2.7 µm columns are both within the allowable range shown in Table 4. This result means that the assay method can be transferred to the 50 mm, 2.7 µm columns without additional method validation, as long as it meets the requirements for system suitability (Table 5).

The allowed adjustment to injection volume was calculated using Equation 1.

Equation 1.

$$V_2 = V_1 \times (L_2 \times dc_2^2)/ (L_1 \times dc_1^2)$$

Where V_2 is the injection volume of the adjusted method; V_1 is the injection volume of the original column; L_2 is the adjusted column length; L_1 is the original column length; dc_2 refers to the adjusted column internal diameter; and dc_1 refers to the original column internal diameter.

Therefore, the injection volume was proportionally reduced from 20 to 6.7 µL.

The allowed adjustment to flow rate was calculated using Equation 2.

Equation 2.

$$F_2 = F_1 \times [(dp_1 \times dc_2^2)/(dp_2 \times dc_1^2)]$$

Where F_2 is the flow rate of the adjusted method; F_1 is the flow rate of the original method; dp_2 is the adjusted column particle size; dp_1 is the original column particle size; dc_2 refers to the adjusted column internal diameter; and dc_1 refers to the original column internal diameter.

Table 4. Column dimension change for assay analysis.

Column	N of Insulin	Acceptable Range (–25% to +50%)	N of Human Insulin	Acceptable Range (–25% to +50%)
Agilent Polaris C18-A, 4.6 × 150 mm, 5 µm	4,165	3,124 to 6,048	4,019	3,014 to 6,029
Agilent Pursuit C18, 4.6 × 150 mm, 5 µm	4,427	3,320 to 6,641	4,293	3,220 to 6,440
Agilent InfinityLab Poroshell 120 EC-C18 4.6 × 50 mm, 2.7 µm	4,756	Within range	4,634	Within range
Agilent InfinityLab Poroshell 120 SB-C18 4.6 × 50 mm, 2.7 µm	5,205	Within range	5,037	Within range

Table 5. System suitability summary for assay analysis.

Column	Tailing Factor		Resolution		RSD of Area (%) for Five Injections	
	Insulin	Human Insulin	Insulin	Human Insulin	Insulin	Human Insulin
Agilent Polaris C18-A, 4.6 × 150 mm, 5 µm	1.0	1.0	3.8	3.7	0.4	0.07
Agilent Pursuit C18, 4.6 × 150 mm, 5 µm	1.0	1.0	3.8	3.7	0.08	0.3
Agilent InfinityLab Poroshell 120 EC-C18 4.6 × 50 mm, 2.7 µm	1.1	1.1	4.0	3.9	0.2	0.05
Agilent InfinityLab Poroshell 120 SB-C18 4.6 × 50 mm, 2.7 µm	1.0	1.0	4.1	4.0	0.1	0.09
USP System Suitability Requirements	≤1.8		≥2.0		≤1.6%	

In this case, the calculated flow rate is 1.85 mL/min. Based on the revised <621> guidelines, an additional change in flow rate of ±50% is permitted, after an adjustment due to a change in column dimensions. The flow rate can be adjusted within the range of 0.9 to 2.8 mL/min, provided the system suitability requirements are met. For compounds with larger molecular weights, such as insulin, increasing the flow rate did not proportionally reduce the elution time of the peak, so a flow rate of 1 mL/min is still used for the adjusted methods.

The USP assay method for insulin and human insulin were transferred from conventional 4.6 × 150 mm, 5 µm TPP columns to 4.6 × 50 mm, 2.7 µm SPP columns without additional method validation. The analysis time was reduced from 15 to 6 minutes with 60% reduction in analysis time and solvent consumption. System suitability criteria were evaluated and reached with both methods. It is obvious that laboratory productivity and sample throughput can be enhanced using the described approach.

Method adjustments for related compounds analysis (adjustment for gradient elution)

The original related compounds analysis using a gradient method was first tested using a 1260 Infinity II LC system and 4.6 × 250 mm, 5 µm Pursuit C18 (L1) and Polaris C18-A (L1) columns. Chromatograms for system suitability are shown in Figures 3 and 4. System suitability requirements are easily met (see Table 6). The Pursuit C18 column provided better resolution between the main insulin peak and an impurity eluting before it, which only appeared in the insulin system suitability solution.

According to the newly revised USP <621> guidelines, this gradient method can be transferred to a suitable UHPLC column to reduce both analysis time and solvent consumption. The revised USP <621> guidelines allow changes from TTP to SPP columns for gradient methods as long as the ratio $(t_R/W_h)^2$ is within −25% to +50% (where t_R is retention time, and W_h is peak widths at half-height). The original method for related compounds analysis was transferred to InfinityLab Poroshell 120, 4.6 × 100 mm, 2.7 µm columns and the ratio $(t_R/W_h)^2$ on both InfinityLab Poroshell 120 columns (shown in Table 7) was also well within an acceptable range. This result means that method revalidation is not required when transferring the original methods to the described InfinityLab Poroshell 120 columns.

Chromatograms for related compounds analysis achieved with InfinityLab Poroshell 120 columns are shown in Figures 5 and 6. The system suitability results are shown in Table 6. All system suitability values, including tailing factor, resolution, and peak area relative standard deviation measurements, met requirements. Both InfinityLab Poroshell 120 EC-C18 and SB-C18 columns

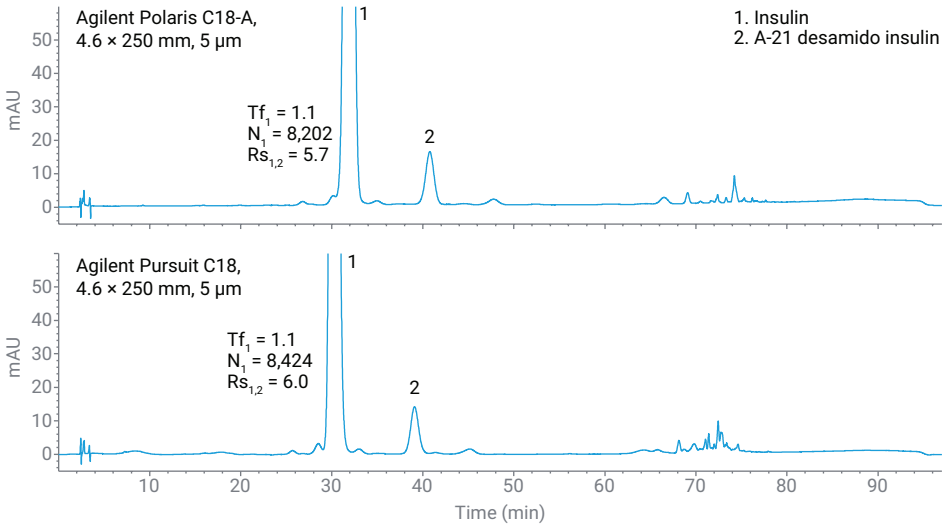

Figure 3. System suitability test for related compounds analysis of insulin using 4.6 × 250 mm, 5 µm columns.

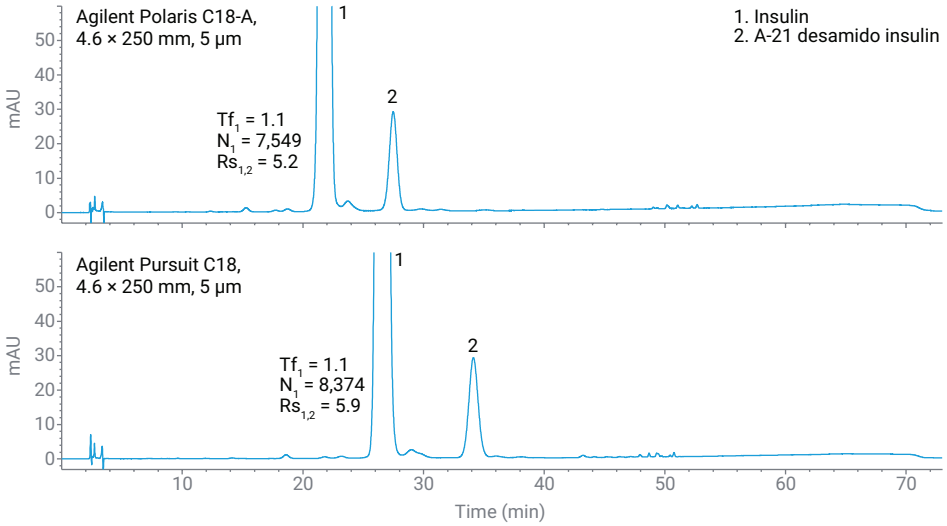

Figure 4. System suitability test for related compounds analysis of human insulin using 4.6 × 250 mm, 5 µm columns.

Table 6. System suitability summary for related compounds analysis.

Column	Tailing Factor		Resolution	
	Insulin	Human Insulin	Insulin	Human Insulin
Agilent Polaris C18-A, 4.6 × 250 mm, 5 µm	1.1	1.1	5.7	5.2
Agilent Pursuit C18, 4.6 × 250 mm, 5 µm	1.1	1.1	6.0	5.9
Agilent InfinityLab Poroshell 120 EC-C18 4.6 × 100 mm, 2.7 µm	1.0	1.0	6.0	5.6
Agilent InfinityLab Poroshell 120 SB-C18 4.6 × 100 mm, 2.7 µm	1.1	1.1	6.4	5.8
USP System Suitability Requirements	≤1.8		≥2.0	

Table 7. Column dimension change for related compounds analysis.

Column	t_R (min)		W_h (min)		$(t_R/W_h)^2$		Acceptable Range	
	Insulin	Human Insulin	Insulin	Human Insulin	Insulin	Human Insulin	Insulin	Human Insulin
Agilent Polaris C18-A, 4.6 × 250 mm, 5 µm	31.819	21.748	0.8369	0.5892	1,445.5	1,362.4	1,084 to 2,168	1,022 to 2,044
Agilent Pursuit C18, 4.6 × 250 mm, 5 µm	30.220	26.520	0.7751	0.6821	1,520.1	1,511.6	1,140 to 2,280	1,134 to 2,267
Agilent InfinityLab Poroshell 120 EC-C18 4.6 × 100 mm, 2.7 µm	13.440	9.248	0.3500	0.2428	1,474.6	1,450.8	Within range	Within range
Agilent InfinityLab Poroshell 120 SB-C18 4.6 × 100 mm, 2.7 µm	13.951	9.251	0.3431	0.2639	1,653.4	1,228.8	Within range	Within range

provided symmetrical peaks, while the InfinityLab Poroshell 120 SB-C18 column resolved an impurity before the insulin peak more effectively than the InfinityLab Poroshell 120 EC-C18 column. SB-C18 is a non-endcapping C18 phase that provides different selectivity to the end-capped EC-C18 phase, which provides more flexibility for method development.

The adjustment of injection volume and flow rate can also be carried out based on Equations 1 and 2. Similarly, the flow rate remains at 1 mL/min for 4.6 × 100 mm, 2.7 µm columns.

The gradient time was adjusted using Equation 3.

Equation 3.

$$t_{G2} = t_{G1} \times (F_1/F_2)\,[(L_2 \times dc_2^2)/(L_1 \times dc_1^2)]$$

Where t_{G2} is the gradient segment time for the adjusted method; t_{G1} is the gradient segment time for original method; F_2 is the flow rate for the adjusted method; F_1 is flow rate for original method; L_2 is the column length for the adjusted method; L_1 is the column length for the original method; dc_2 refers to the adjusted column internal diameter; and dc_1 refers to the original column internal diameter.

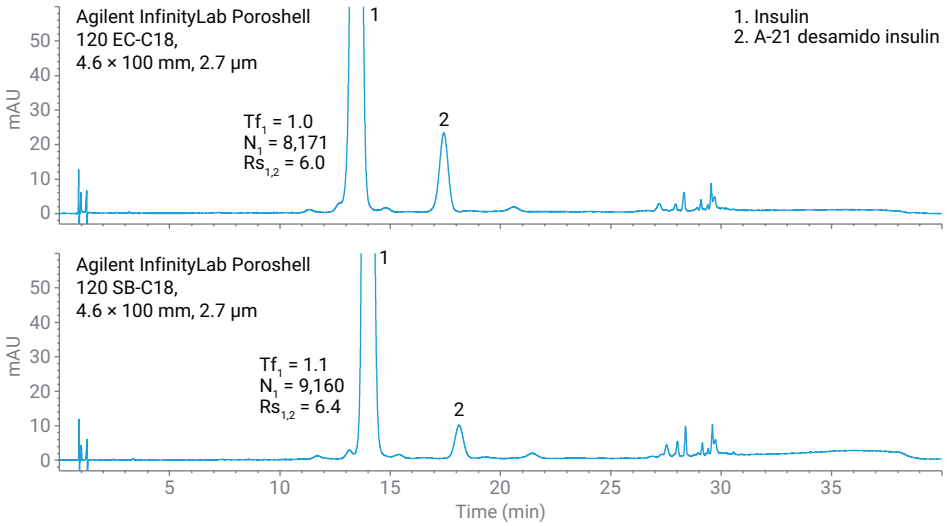

Figure 5. System suitability test for related compounds analysis in Insulin using Agilent InfinityLab Poroshell 120 4.6 × 100 mm, 2.7 µm columns.

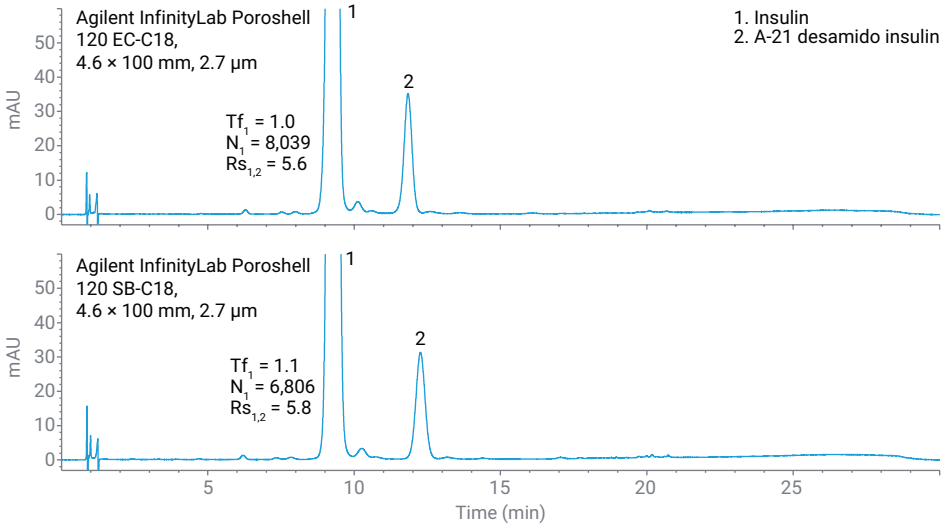

Figure 6. System suitability test for related compounds analysis in human insulin using Agilent InfinityLab Poroshell 120 4.6 × 100 mm, 2.7 µm columns.

In summary, by using the modernized USP method with InfinityLab Poroshell 120 columns, the overall run time and solvent use for related compounds analysis were significantly reduced. A total of 60% reduction in analysis time and solvent consumption was achieved when using InfinityLab Poroshell 120 columns. System suitability criteria were evaluated and reached with both methods. It is clear that laboratory productivity and sample throughput can be enhanced using the described approach.

Conclusion

USP methods of assay and related compounds analysis for insulin and human insulin using older column technology with conventional, 5 µm TPP columns can benefit from newer technology such as SPP columns. These methods can be modernized to feature Agilent InfinityLab Poroshell 120 columns, which provide similar or improved results while significantly reducing analysis times and mobile phase use. These method adjustments are allowable according to the newly revised USP <621> guidelines without additional method validation.

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