Waters™

アプリケーションノート

Leveraging the Alliance™ iS Bio HPLC System as a Modern HPLC for Peptide Drug Substances Analysis in QC Environments

Duanduan Han, Robert E. Birdsall, Karen Nyholm

Waters Corporation

Abstract

The Alliance iS Bio HPLC System with MaxPeak™ High Performance Surfaces (HPS) Technology is a bio-inert liquid chromatography (LC) system designed to reduce surface/analyte interaction for biopharmaceutical manufacturing environments. As a modern HPLC instrument, the Alliance iS Bio HPLC System is engineered with features including Waters innovative MaxPeak HPS, lower dispersion volume, and a large volume mixer. Together with MaxPeak Premier columns, these features enable manufacturing environments to modernize methods, save resources, and increase throughput for biopharmaceuticals. In this study, we evaluate the Alliance iS Bio HPLC System in a routine QC testing environment for peptide-size biopharmaceuticals.

Insulin, insulin analogs, glucagon, and GLP-1 peptides were chosen as test cases given their relevance as classic and new treatment of diabetes with benefits of weight management. The study results demonstrate the Alliance iS Bio HPLC System is capable of running legacy compendial methods while allowing users the flexibility to take advantage of modern column chemistries to reduce operating costs. When compared against a legacy HPLC system, results demonstrate the Alliance iS Bio HPLC System is well suited for QC environments in the analysis and routine testing of biopharmaceuticals.

Benefits

- The Alliance iS Bio HPLC System delivers consistent performance for QC environments enabled by low

dispersion volume and improved mixing behavior

- Modernization of compendial methods reduces operating costs and analysis time

Introduction

Peptides are defined as a short chain of less than 40 amino acids connected by peptide bonds. Larger intact proteins can be enzymatically treated to cleave at the primary sequence at specific amino acid residues to produce peptide fragments that can be analyzed in routine assays for impurity monitoring, identity, and product efficacy. Alternatively, peptide-based therapies can be produced either through chemical synthesis or recombinant technology. As a drug therapy, peptides exhibit high affinity and specificity toward cell surface receptors with relatively lower immunogenic response compared to more complex biologics. These characteristics in combination with lower production costs have established peptide drugs as a viable alternative to more traditional drug therapies.

In this context, regulatory agencies expect comprehensive control strategies for manufacturing peptide drugs to ensure adherence to safety and compliance standards. Amino acid residue side chains can undergo process and/or product related modifications (e.g. deamidation, isomerization, N-terminal cyclization, and oxidation) that result in a change not native to the target sequence. These changes can impact the overall safety and efficacy of the drug products, thus requiring methods to monitor them to ensure manufacturing processes are controlled and drug products are safe for use. As part of qualification and validation activity, methods are often optimized to increase robustness and throughput to reduce costs and allow for easier deployment in QC labs. The Alliance iS Bio HPLC System is engineered as a modern HPLC to help ease the burden of QC labs in the manufacturing of biologics and peptide drugs as part of QC environments.

This study aims to assess the benefits of migrating and modernizing reversed-phase liquid chromatography (RPLC) methods with the Alliance iS Bio HPLC System with test cases of insulin analogs and glucagon-like peptide (GLP-1) drug substances.

Experimental

Systems

Alliance iS Bio HPLC System performance was compared to the legacy HPLC system.

Chemical

Reference standards (RSs) of insulin human, insulin lispro, insulin glargine, and glucagon were purchased from USP. Dulaglutide, exenatide, glucagon, tirzepatide, liraglutide, and semaglutide (acetate) were purchased from vendors. Hydrochloric acid, trifluoracetic acid, formic acid, sulfuric acid, and phosphoric acid were purchased from Fisher Chemical. Ammonium formate, ammonium sulfate, and dimethyl sulfoxide (DMSO) were purchased from Sigma. Ammonium hydroxide and anhydrous sodium sulfate were purchased from Honeywell. Monobasic potassium phosphate was purchased from Acros Organics. Glu-C enzyme was purchased from Promega. LC-MS grade water and acetonitrile were purchased from Honeywell.

Stock and Sample

Insulin human RS, insulin lispro RS, and insulin glargine RS stock were prepared in 0.01 N HCl at 2 mg/mL. Dulaglutide and glucagon stock were prepared with DMSO at 1 mg/mL. Liraglutide and tirzepatide stock were prepared with DMSO at 0.5 mg/mL. Exenatide and semaglutide stock were prepared with ammonium formate buffer at 0.5 mg/mL (10 mM ammonium formate in water, adjust with ammonium hydroxide to a pH of 8.5). The GLP-1 panel mixture was prepared at 0.05 mg/mL for each peptide using diluent (0.5% trifluoracetic acid, 1% formic acid acetonitrile, 98.5% water). Enzyme digestion is performed according to USP monograph <121.1>.[2]

For Insulin Identification

LC system:	Legacy HPLC System
Detection:	TUV, λ = 214 nm
Column:	XSelect™ CSH™ C_{18} Column, 130Å, 5 µm, 4.6 mm x 150 mm, p/n: 186005290
Column temperature:	40 °C
Sample temperature:	10 °C
Injection volume:	20 µL
Flow rate:	1 mL/min isocratic, A:B 74:26
Mobile phase:	A: 0.2M anhydrous sodium sulfate in water, 0.27%

	phosphoric acid, pH 2.3 B: acetonitrile
LC system:	Alliance iS Bio HPLC System
Detection:	TUV, λ = 214 nm
Column:	XSelect CSH C_{18} Column, 130Å, 5 µm, 4.6 mm x 150 mm, p/n: 186005290
Column temperature:	40 °C
Sample temperature:	10 °C
Injection volume:	20 µL
Flow rate:	1 mL/min isocratic, A:B 74:26
Mobile phase:	A: 0.2M anhydrous sodium sulfate in water, 0.27% phosphoric acid, pH 2.3 B: acetonitrile

For Insulin Human and Analog Peptide Mapping

LC system:	Alliance iS Bio HPLC System
Detection:	TUV, λ = 214 nm
Column:	XSelect Peptide CSH C_{18} Column, 130Å, 5 µm, 4.6 x 100 mm, p/n: 186005289 XSelect Premier Peptide CSH C_{18} Column, 130Å, 2.5 µm, 4.6 x 50 mm, p/n: 186009907 (+eConnect™ p/n: 186009907RF)

Column temperature:	40 °C
Sample temperature:	10 °C
Injection volume:	50 µL for 5 µm, 4.6 x 100 mm Column 25 µL for 2.5 µm, 4.6 x 50 mm Column
Flow rate:	1 mL/min for 5 µm column 2 mL/min for 2.5 µm column
Mobile phase:	A: 700:100:200 H_2O:ACN:$(NH_4)_2SO_4$ buffer B: 400:400:200 H_2O:ACN:$(NH_4)_2SO_4$ buffer

Gradient Table for 5 µm, 4.6 x 100 mm Column

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	1.00	95.0	5.0	initial
3.00	1.00	95.0	5.0	6
30.00	1.00	41.0	59.0	6
35.00	1.00	20.0	80.0	6
40.00	1.00	95.0	5.0	6
50.00	1.00	95.0	5.0	6

Gradient Table for 2.5 µm, 4.6 x 50 mm Column

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	2.00	95.0	5.0	initial
0.75	2.00	95.0	5.0	6
7.50	2.00	41.0	59.0	6
8.75	2.00	20.0	80.0	6
10.00	2.00	95.0	5.0	6
12.50	2.00	95.0	5.0	6

For GLP-1 Panel

LC system:	Legacy HPLC System
Detection:	TUV, λ = 214 nm
Column:	XSelect Peptide CSH C_{18} Column, 130Å, 2.5 µm, 4.6 mm x 150 mm, p/n: 186007038
Column temperature:	60 °C
Sample temperature:	25 °C
Injection volume:	10 µL
Flow rate:	0.96 mL/min
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
LC system:	Alliance iS Bio HPLC System

Detection:	TUV, λ = 214 nm
Column:	XSelect Premier Peptide CSH C_{18} Column, 130Å, 2.5 µm, 4.6 x 150 mm, p/n : 186009909 (+eConnect p/n: 186009909RF)
Column temperature:	60 °C
Sample temperature:	25 °C
Injection volume:	10 µL
Flow rate:	0.96 mL/min
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile

Gradient Table for Both the Legacy HPLC System and Alliance iS Bio HPLC System

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	0.96	99.5	0.5	initial
2.00	0.96	99.5	0.5	6
22.00	0.96	45.0	55.0	6
25.00	0.96	5.0	95.0	6
26.00	0.96	5.0	95.0	6
28.00	0.96	99.5	0.5	6
40.00	0.96	99.5	0.5	6

For Glucagon and Impurity Profiling

LC system:	Alliance iS Bio HPLC System

Detection:	TUV, λ = 214 nm
Column:	XSelect Premier Peptide CSH C_{18} Column, 130Å, 2.5 µm, 4.6 x 150 mm, p/n: 186009909 (+eConnect p/n: 186009909RF)
Column temperature:	45 °C
Sample temperature:	10 °C
Injection volume:	10 µL
Flow rate:	1 mL/min isocratic, A:B 60:40
Mobile phase:	A: 0.12M monobasic potassium phosphate in water: ACN 8:2, 0.14% phosphoric acid, pH 2.7 B: water: acetonitrile 6:4

Chromatography Software

Legacy HPLC system:	Empower™ 3, FR4
Alliance iS bio HPLC system:	Empower 3.8

Results and Discussion

Intact Insulin Compendial Method Compliance

As a smaller biologic, insulin serves as an ideal molecule to initially evaluate the Alliance iS Bio HPLC System in its ability to support legacy compendial methods. Insulin monograph uses a L1 packing or C_{18} stationary phase for the identity test, and the mobile phase is comprised of a phosphate buffer where elution of the insulin is achieved using acetonitrile at isocratic conditions.[1] For intact insulin human, using the compendial method, both the legacy HPLC system and Alliance iS Bio HPLC System are able to meet the three system suitability

criteria listed in the monograph. As shown in Figure 1A, the criteria: 1) resolving the insulin human peak from the A-21 desamido impurity (resolution NLT 2.0); 2) insulin human main species peak with tailing NMT 1.8, and 3) the insulin main peak area consistency %RSD NMT 1.6% were met in both systems using five replicate injections for system suitability. While area was conserved across the platforms as demonstrated by the comparable %RSD for area, the Alliance iS Bio HPLC System was able to deliver a higher degree of accuracy in the composition of the mobile phase as shown in the lower %RSD of the retention time due to its overall design including a 680 µL mixer. This improved %RSD of the retention time translates into more robust methods for QC environments.

As shown in Figure 1B, the Alliance iS Bio HPLC System is fully compatible with the Empower CDS software allowing users to utilize the limits and reporting functionality of Empower to monitor assay acceptance criteria. In this example, the system suitability solution is required to stand at room temperature for NLT 3 days to obtain a solution containing NLT 5% of A-21 desamido insulin human. As shown in the report, results outside of specification are flagged in red for easy interpretation and reduced error in data analysis.

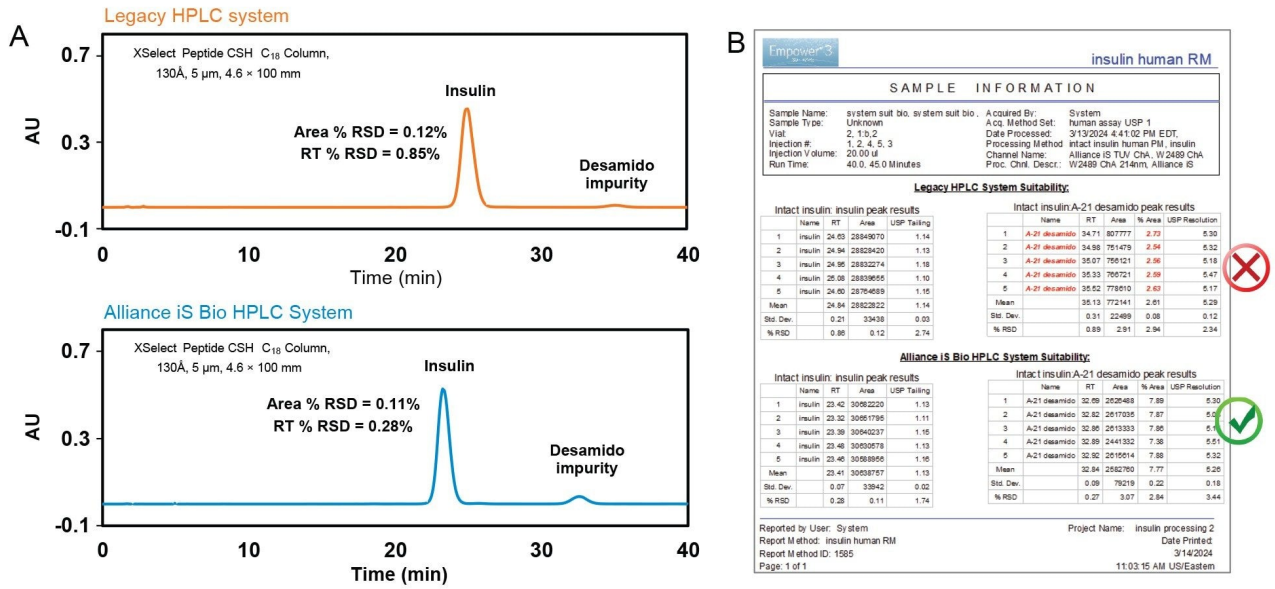


Figure 1. Intact insulin identification using compendial method. Both the legacy HPLC system and Alliance iS Bio HPLC System are able to (A) resolve the insulin human peak from the A-21 desamido impurity and (B) meet the three acceptance criteria of monographs. The Alliance iS Bio HPLC System was able to deliver a higher degree of accuracy in the composition of the mobile phase. In Empower report, results that failed to meet monograph acceptance criteria are flagged in red.

Insulin Peptide Mapping Compendial Method and Modernization

Continuing the study, the Alliance iS Bio HPLC System was evaluated for supporting compendial methods in insulin peptide mapping and fragment analysis. As shown in Figure 2, insulin human and two common analogs, insulin lispro and insulin glargine, each have four glutamic acids (Glu, E), and Glu-C enzyme specifically cleaves at the C-terminus of glutamic acid residues. Following digestion with Glu-C under non-reducing conditions, insulin produces four peptide fragments (as indicated by color) that can be used in identification assays. An important aspect of method migration is to demonstrate comparability in results between platforms. As shown in Figure 3A all four peptide fragments were well resolved when using the same method and column across LC platforms. More importantly, both area and retention time were conserved across platforms (Figure 3B) demonstrating that the Alliance iS Bio HPLC System is compatible with legacy methods.

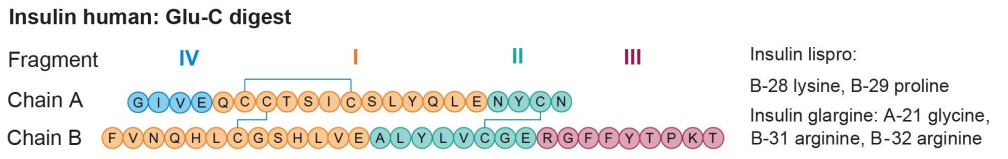


Figure 2. Insulin human produces four peptide fragments after Glu-C digestion. Insulin lispro and insulin glargine have slightly different amino acid sequence.

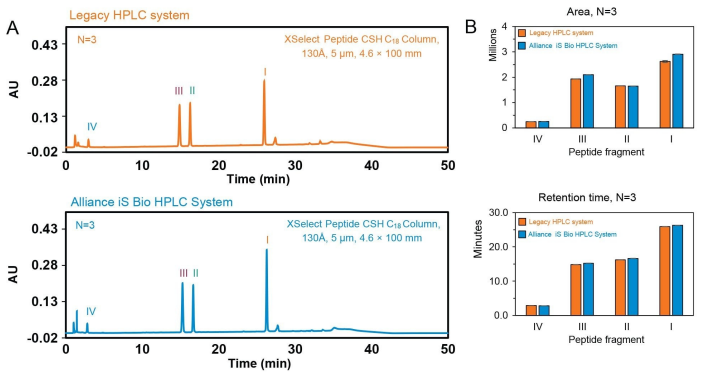


Figure 3. Insulin human produces four peptide fragments after Glu-C digestion. Using compendial method, both systems (A) meet monograph criteria of resolving the peak of fragment II and III and (B) show peak area and retention time repeatability of four peptide fragment peaks.

For insulin, compendial suitability requirements focus on fragment II and III as they represent a critical pair that can be impacted by resolution and peak tailing.[2] As shown in Figure 4A, the Alliance iS Bio HPLC System was able to resolve insulin human, lispro, and glargine critical pairs (fragment II and III) without issues and meet compendial requirements for resolution (R_S) ⩾3.4 and peak tailing (T_f) ⩽1.5, further demonstrating the Alliance iS Bio HPLC System as a LC platform with broad compatibility with legacy methods. One distinct advantage of the Alliance iS Bio HPLC System over traditional HPLC platforms is the ability to run at higher pressure. This feature allows users to take advantage of the separation efficiency gains of smaller particles. To demonstrate this, the compendial method was scaled using the Waters Columns Calculator following guidance in USP General Chapter <621>[3] to run on a 50 mm column package with 2.5 µm sized particle stationary phase. As shown in the blue chromatogram (Figure 4B), the selectivity of the separation was preserved allowing compendial requirements for resolution and peak tailing of fragment II and III to be met while simultaneously reducing the solvent consumption, sample use, and analysis time by 50%, 50%, and 75%, respectively. These results demonstrate, the Alliance iS Bio HPLC System is capable of supporting modern column technology while reducing operating costs and allowing users to fully leverage the system’ s capabilities as a modern HPLC platform.

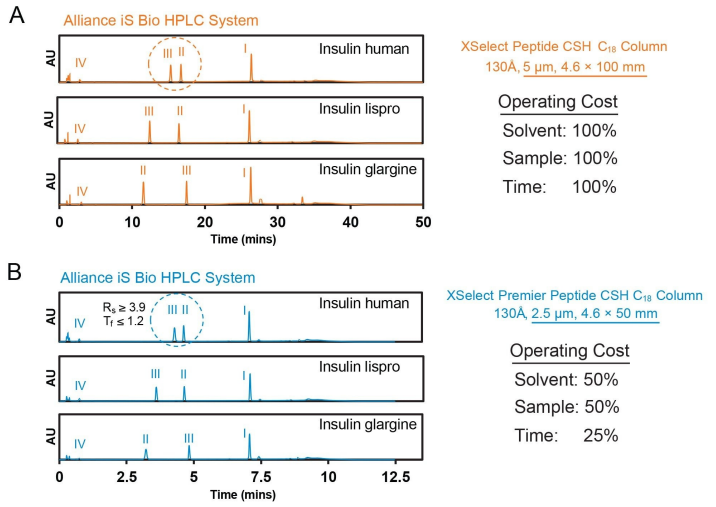


Figure 4. Peptide fragments of insulin analogs are resolved using (A) compendial method and (B) USP <621> compliant scaled method aided by Waters Columns Calculator, and the scaled method reduces operation costs.

New Peptide Drug Modalities Method Development

A panel of peptide drugs including glucagon and GLP-1 peptides were used to further explore the Alliance iS Bio HPLC System and its capability to utilize more efficient chemistry as part of method modernization. An XSelect Premier Peptide CSH C_{18} Column with 2.5 µm particle size was used to develop a new method that meets USP acceptance criteria. As per the monograph, the first desamido glucagon impurities must be resolved (resolution NLT 1.5), and all four desamido impurities must be clearly visible.[4] A sample is required to contain NMT 14% total of all four desamido glucagons and NMT 31% total impurities. As shown in the Figure 5A inset, the forced degradation study under acid condition confirmed the desamido glucagon peaks as part of the system suitability, and these desamido impurities intensity increased over time (stored at ambient temperature for 5 days). The vendor glucagon sample was tested as received (Figure 5B) and it was not able to meet the impurity thresholds outlined in the USP monograph compared to the reference standard. This observation demonstrates that the Alliance iS Bio HPLC System meets the impurity analysis requirement of current monographs.

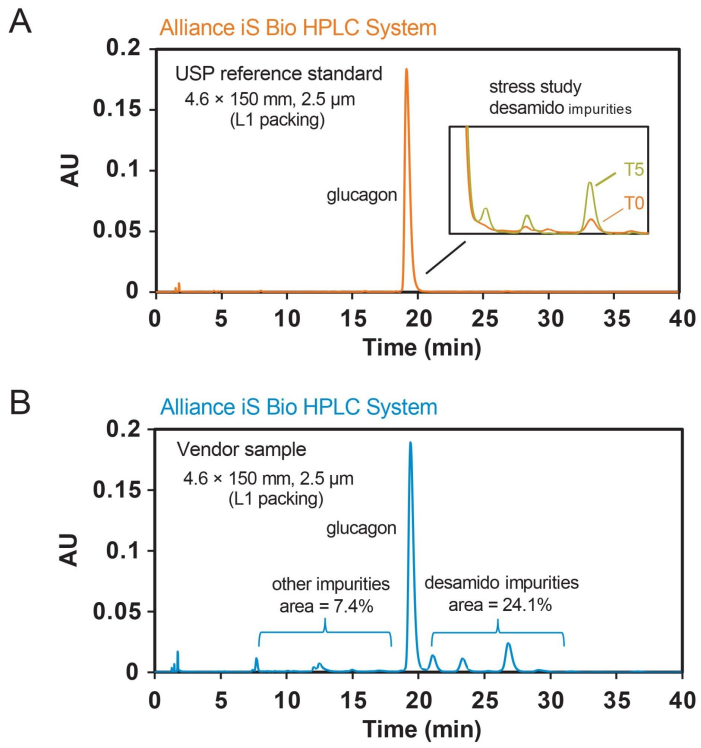
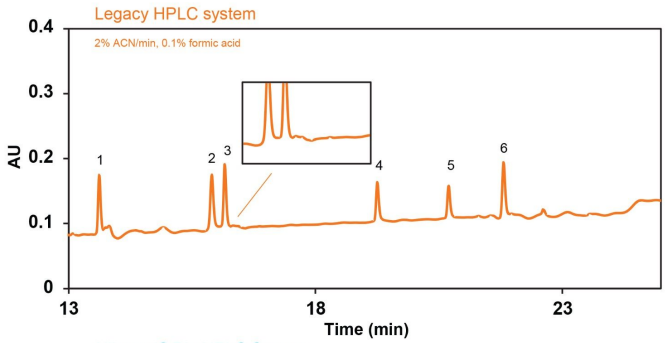


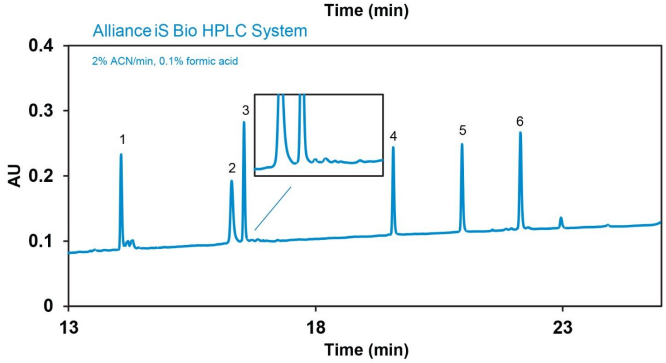
Figure 5. Glucagon impurities are detected and resolved per monograph requirements (A). Glucagon reference standard was degraded in acidic condition for five days at ambient temperature. The vendor sample failed to meet impurity threshold specified in the monograph (B).

In the absence of regulatory accepted monographs, the Alliance iS Bio HPLC System is well suited for method scouting or optimization following regulatory guidance. As shown in Figure 6, a peptide assay was developed for glucagon and a panel of five GLP-1 peptide drug substances using conventional reversed phase liquid chromatography conditions with water and acetonitrile as mobile phase containing 0.1% formic acid additive.[5] The large mixing volume and low system dispersion of the Alliance iS Bio HPLC System (Figure 6B) was able to produce chromatograms with low baseline noise and improved peak shape for the peptide drug substances when compared to the legacy HPLC system (Figure 6A) resulting in improved integration accuracy as evident in the lower area % RSD (Figure 6C). The improved performance observed with the Alliance iS Bio HPLC System enables increased sensitivity in the detection of low abundant impurities (inset Figure 6A, 6B) that may play a critical role in new peptide drug approval.

A



B



C

Peak	Analyte	Legacy HPLC system (n=6)			Alliance iS Bio HPLC System (n=6)		
		Area	RSD%	Width (min)	Area	RSD%	Width (min)
1	Glucagon	355246	7.06%	0.06	340303	0.28%	0.03
2	Exenatide	366927	6.43%	0.08	393247	0.70%	0.06
3	Dulaglutide	389359	6.60%	0.06	425136	0.66%	0.03
4	Semaglutide	253344	5.05%	0.06	339947	1.57%	0.04
5	Liraglutide	229023	6.77%	0.06	345345	2.04%	0.04
6	Tirzepatide	374355	1.45%	0.06	423525	1.63%	0.04

*Width (min): peak width at 50% peak height

Figure 6. For a panel of glucagon and five GLP-1 peptides, the larger mixing volume and lower system dispersion of the Alliance iS Bio HPLC System (A) was able to produce chromatograms with lower baseline noise and improved peak shape for the peptides drugs when compared to the legacy HPLC system (B). This enables improved accuracy in the detection (C) and integration of low abundant impurities (inset A,B).

Conclusion

The Alliance iS Bio HPLC System is a modern HPLC platform well-suited to meet regulatory requirements in existing monographs or develop new methods for new drug substances that lack compendial guidance. For

classic drug substances where monographs are readily available, the Alliance iS Bio HPLC System has displayed improved peak area and retention time repeatability for intact insulin identification using the compendial method. Combined with a smaller particle stationary phase, it facilitates USP <621> compliant method scaling of insulin analogs peptide mapping with a 75% reduction in analysis time and 50% reduction in mobile phase and sample consumption. For novel peptide drug substances, the Alliance iS Bio HPLC System provides a robust platform to resolve impurities and screen vendor supplied glucagon samples for their qualification of monograph standards. For novel GLP-1 peptide drug substances where monographs are not yet available, the Alliance iS Bio HPLC System serves as a valuable tool for method scouting and optimization. It detects low abundance impurities and offers low baseline noise and improved peak shape leveraging its default large mixing volume and low system dispersion.

References

1. USP. Insulin human. In: USP-NF. Rockville, MD: USP; Dec 1, 2015.
2. USP. Physicochemical Analytical Procedures for Insulins <121.1>. In: USP-NF. Rockville, MD: USP; Dec 1, 2016.
3. USP. Chromatography <621>. In: USP-NF. Rockville, MD: USP; Dec 1, 2022.
4. USP. Glucagon for injection. In: USP-NF. Rockville, MD: USP; Feb 1, 2019.
5. Clements B, Rainville P. Development of Separation Methods for GLP-1 Synthetic Peptides Utilizing a Systematic Protocol and MaxPeak High Performance Surface Technology. Waters Application Note. 720008267. 2024 April.

Featured Products

Alliance iS Bio HPLC System <https://www.waters.com/waters/nav.htm?cid=135133140>

Empower Chromatography Data System <https://www.waters.com/10190669>

720008345, May 2024

© 2024 Waters Corporation. All Rights Reserved.
利用規約 プライバシーポリシー 商標 キャリア 法的通知およびプライバシー通知 Cookies
Cookie 環境設定