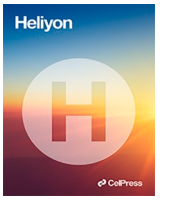
Heliyon 10 (2024) e39809

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

A novel technique for trehalose and sucrose determination in therapeutic monoclonal antibodies using a high-performance liquid chromatography–evaporative light scattering detector

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ARTICLE INFO

Keywords:
Therapeutic monoclonal antibodies
High-performance liquid chromatography
Evaporative light scattering detector
Trehalose
Sucrose

ABSTRACT

Sucrose and trehalose are commonly used excipients in therapeutic monoclonal antibodies that play a pivotal role in ensuring the safety and stability of drugs. Though it is necessary to control the concentrations of these substances during the quality control of their release, there is currently no comprehensive method for simultaneously determining sucrose and trehalose concentrations. Herein, we established a high-performance liquid chromatography–evaporative light scattering detector (HPLC-ELSD) method and validated it in accordance with the International Council for Harmonization Q2 guidelines. This method utilized the Poroshell 120 HILIC-Z chromatographic column and effectively separated sucrose and trehalose with a detection limit of 0.001 mg/mL. The accuracy recovery rate was within a range of 90%–110 %, and the precision relative standard deviations were all less than 5.0 % (n = 6). The method thus demonstrated good repeatability and linearity, making it suitable for determining the sucrose and trehalose concentrations in therapeutic monoclonal antibodies.

1. Introduction

Therapeutic monoclonal antibodies have the advantages of strong targeting, clear efficacy, and few adverse reactions [1]. They have been rapidly developed in recent years, and their market share has been increasing annually. To date, hundreds of therapeutic monoclonal antibodies have been approved for sale in the market by the United States Food and Drug Administration [2]. Therefore, enhancing and refining quality-control methods for monoclonal antibodies is of great importance for their release and supervision.

The active ingredients of therapeutic monoclonal antibodies are proteins, which are characterized by their large relative molecular mass and complex molecular composition [3]. Various excipients are typically added as pH adjusters and solubilizers during the development of formulations to ensure the activity and stability of the active ingredients [4–6]. Trehalose and sucrose are commonly used as excipients in therapeutic monoclonal antibodies; indeed, one or both of these excipients are used in over half of currently marketed therapeutic monoclonal antibody drugs. Both trehalose and sucrose are non-reducing disaccharide protectants that possess

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https://doi.org/10.1016/j.heliyon.2024.e39809
Received 10 March 2024; Received in revised form 23 October 2024; Accepted 23 October 2024
Available online 24 October 2024

high glass transition temperatures [3,7]. Trehalose is composed of two glucose molecules linked by a 1,1-glycosidic bond, exhibits low hygroscopicity, and is non-reducing. Trehalose can form a unique protective film on the cell surface under extreme conditions, such as high temperatures, extreme cold, and drought, preventing the loss of membrane protein activity and thereby maintaining cell viability [8]. Sucrose, which is composed of one glucose molecule and one fructose molecule, primarily functions by inhibiting changes in the secondary structure of proteins. It also prevents protein drug inactivation and denaturation due to aggregation during freeze-drying and storage [9,10]. Variations in the content of these excipients can greatly affect the quality and stability of drugs [11]. Therefore, to ensure the safety of therapeutic monoclonal antibodies, the amount of sucrose and trehalose added must be strictly controlled as part of their full life-cycle management and quality control. Current quantitative analysis methods for these substances include colorimetry, enzymatic methods, and high-performance liquid chromatography (HPLC) [12–15]. Colorimetry is based on the color reaction of anthrone with carbohydrates [11], but it lacks specificity. Enzymatic methods indirectly quantify sugars by detecting the glucose produced by sugar hydrolysis, but they suffer from errors due to their indirect nature. HPLC is a commonly used method in drug quantitative analysis, and it is characterized by its simplicity, speed, and accuracy.

An evaporative light scattering detector (ELSD) is a universal type of detector. The column eluent enters the nebulizer and forms fine, uniform droplets under the action of the nebulizing gas, with larger, harder-to-nebulize droplets flowing out through a waste tube at the bottom. The droplets enter a heated drift tube, and as the mobile phase evaporates, sample molecules form aerosol particles suspended in the solvent vapor. Upon entering the detector, these molecules are irradiated by a laser diode, generating scattered light that is captured by a photodiode to produce an electrical signal. The intensity of this signal reflects the content of the compound. The response value of ELSD has an exponential and nonlinear relationship with the amount of measured substance, so it is necessary to fit it with a natural logarithm to ensure a good linear relationship between the peak area value and the sample size to obtain accurate results. The application of this fitting method enables ELSD to provide more accurate results when detecting low sample content. ELSD is the only detector that eliminates solvent peaks [15] and, compared with the refractive index detector (RID) and ultraviolet (UV) detection, it eliminates baseline drift due to temperature changes, offering better durability, ease of maintenance, and no special requirements for columns and reagents, thereby providing high-quality signals [16,17]. The use of HPLC-ELSD to determine the content of trehalose in cassava has been previously reported [8], though it was slightly different from the technique used for determining the contents of excipients in monoclonal antibodies.

Therapeutic monoclonal antibodies usually contain a variety of excipients. The general control strategy is to develop a method for one excipient, and independent methodological verification is required for each method. In this study, we developed an HPLC-ELSD method to simultaneously determine the contents of sucrose and trehalose, greatly reducing the time required for testing, and thereby providing convenience for the development and marketing of drugs. Methodological validation was then conducted in accordance with the International Council for Harmonization (ICH) Q2 guidelines. The results showed that this method had a detection limit of 0.1 mg/mL, with good specificity, accuracy, precision, linearity, and robustness. This method is suitable for determining sucrose and trehalose contents in the release of therapeutic monoclonal antibodies.

2. Materials and methods

2.1. Chemicals, reagents, sample, and the HPLC instrumentation

Chromatography pure grade acetonitrile and ammonium acetate were obtained from Honeywell International (Morris Township, NJ, USA). Ultrapure water was obtained from Millipore Milli-Q Ultrapure Water System (Burlington, MA, USA). Sucrose and trehalose were obtained from the United States Pharmacopeia (USP, Mr = 342.3 and Mr = 378, respectively). The therapeutic monoclonal antibodies S1 (containing trehalose at 20 mg/mL), S2 (containing sucrose at 20 mg/mL), and S3 (containing trehalose at 55 mg/mL) were conserved by our laboratory.

The 1290 Infinity II HPLC, ELSD detector, Poroshell 120 Hilic-Z column (3.0 × 100 mm, 2.7 μm), Poroshell Hilic Plus column (4.6 × 150 mm, 2.7 μm), and HILIC-OH5 column (4.6 × 100 mm, 2.7 μm) were purchased from Agilent (Santa Clara, CA, USA).

2.2. Sample and solution preparation

The mixed standard stock solution consisted of 150 mg each of trehalose and sucrose standard substances, which were precisely weighed and transferred into a 5-mL volumetric flask. A total of 2 mL of ultrapure water was added to completely dissolve the substances. The flask was then filled to the mark with ultrapure water, yielding a mixed control stock solution with a concentration of 30 mg/mL.

A total of 400 mg/mL of the trehalose/sucrose standard stock solution was prepared by precisely weighing 4000 mg of the trehalose standard substance in a 10-mL volumetric flask. A total of 5 mL of ultrapure water was added for complete dissolution, and then the flask was filled to the mark with ultrapure water. This resulted in a trehalose standard stock solution with a concentration of 400 mg/mL.

Mixed standard solutions were prepared by diluting the mixed standard stock solution with a 50 % acetonitrile aqueous solution to obtain concentrations of 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, and 3.0 mg/mL for standard curve preparation.

The specificity validation solution was prepared using four types of solutions, namely a blank solution (50 % acetonitrile water), a mobile phase solution (20 mM ammonium acetate: ACN = 20: 80), a preparation blank solution (water), and a 1.0 mg/mL standard solution. The 1.0 mg/mL standard solution was prepared using the 30 mg/mL mixed standard stock solution.

An accuracy and precision validation solution was prepared by preparing solutions of trehalose or sucrose at 50 %, 100 %, and 150 % concentration levels. We accurately transferred 10 μL of S1/S2 that had undergone deproteinization treatment (trehalose/sucrose concentration of 1.25 mg/mL) into a 2-mL centrifuge tube. We then added 250 μL, 500 μL, and 750 μL of the 400 mg/mL trehalose/sucrose standard stock solution, resulting in spiked trehalose/sucrose concentrations of approximately 0.625 mg/mL, 1.25 mg/mL, and 1.875 mg/mL, respectively. We then prepared six replicates at each concentration level for accuracy and precision validation. The solutions were diluted twofold using ultrapure water prior to injection, and the dilution factor was multiplied for the calculation.

A repeatability validation solution was prepared using two types of repeatability validation solutions. One had six replicates of the 0.5 mg/mL sucrose or trehalose standard solution, and another consisted of samples S1, S2, and S3 prepared in line with the test sample solution method.

A linearity validation solution was prepared by diluting the mixed standard stock solution with 50 % acetonitrile water to obtain linearity validation solutions at concentrations of 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, and 3.5 mg/mL.

The sample solution was accurately transferred into 100 μL of S1 (containing trehalose at 20 mg/mL), S2 (containing sucrose at 20 mg/mL), and S3 (containing trehalose at 55 mg/mL) samples in a 2-mL centrifuge tube. Precisely 800 μL of the acetonitrile solution was added, and this was shaken for 2 min to remove proteins. We then accurately added 700 μL of the ultrapure water solution and shook the solution for another 2 min. This was then centrifuged at 12,000 rpm for 15 min, the supernatant was filtered through a 0.22-μm filter, after which the sample analysis was conducted. The sample was diluted 16-fold, and it needed to be multiplied by a dilution factor of 16 when calculating the concentration.

2.3. Optimization (experimental design)

The experimental design was established using a preliminary one-factor-at-a-time analysis using the chromatographic column, mobile phase system (which included the mobile phase system, the ammonium acetate ratio, and the salt concentration), elution mode, column oven temperature, injection volume, ELSD parameters, and protein removal method as the independent variables. The design space was defined according to the factor levels listed in Table 1. The aim was to ensure that the presumed optimum was within the factor range and that all of the experiments produced an evaluable response. According to the requirements of the quantitative method, the system suitability criteria were established as follows: the R^2 of the standard curve fitting equation should be greater than 0.99 and the theoretical plate number of the trehalose and sucrose peaks should be greater than 5000.

2.4. Method validation

We validated the method in terms of specificity, linearity, accuracy, precision, and repeatability in accordance with the ICH guideline Q2 (R2). The specificity of the method was verified using the specificity validation solution. The peak area log values of the mixed standard solution at the five levels of 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, and 3.0 mg/mL were plotted against the log values of the response, yielding the standard curve of the function. The function was used to verify the accuracy, precision, linearity, and repeatability of the method. The R^2 of the standard curve was calculated to establish the linearity, the recoveries of six injections of the accuracy and precision validation solution were calculated to obtain the accuracy, the RSDs of the six injections were calculated for the precision, and the recoveries and RSDs of the six injections of the standard and S1, S2, and S3 were calculated to validate the repeatability. The limit of quantitation and detection of sucrose and trehalose were determined using a mixed standard solution of 0.001 mg/mL and 0.025 mg/mL.

2.5. Sample detection

The S1, S2, and S3 monoclonal antibody samples were collected and deproteinized according to the method described in Section 2.2. (the sample solution). Six replicates of each sample were processed in parallel and then injected separately. The mean values and RSDs of the sucrose and trehalose concentrations were then calculated.

Table 1
Factors and levels for the response surface methodology optimization.

Factor	Unit	Level 1	Level 2	Level 3
chromatographic column	/	HILIC Plus	HILIC-z	HILIC-OH5
mobile phase system	/	triethylamine	ammonium acetate	/
salt concentration	mM	20	50	/
ammonium acetate ratio	%	15	20	25
elution mode	/	gradient elution	isocratic elution	/
column oven temperature	°C	30	50	70
injection volume	μL	5	2	1
atomization temperature	°C	55	65	75
evaporation temperature	°C	40	50	60
protein removal method	/	acetonitrile precipitation	ultrafiltration	/

3. Results

3.1. Optimization of the HPLC method

We optimized the method according to the design of the experiments in Section 2.3. When selecting the column, it was found that the three columns were able to separate sucrose, trehalose, and isomers of trehalose very well, though the retention of the Hilic Plus column was relatively weak. In addition, there was a slight difference between HILIC-z and HILIC-OH5; namely, the peak shape of the HILIC-Z column was sharp and symmetrical, while the peak shape of the HILIC-OH5 column was somewhat broad and required a higher concentration of salt to adjust. Therefore, the Poroshell 120 HILIC-Z column was chosen in this study (Fig. 1). When selecting the mobile phase, the baseline of the triethylamine system was much higher than that of the ammonium acetate system, which affected the linear range of the response. In addition, the ultimate noise was much greater than that of the ammonium acetate system; hence, the ammonium acetate system with less noise was selected as the mobile phase. When selecting the salt concentration of the mobile phase, there was no difference between the peak shape of the chromatographic peaks of the 50 mM and 20 mM ammonium acetate systems, but the noise of the 50 mM system was much higher; hence, 20 mM ammonium acetate was selected.

A 15%–25 % 20-mM ammonium acetate/acetonitrile concentration could achieve the separation of the target peak from impurities. Therefore, the 20 % ammonium acetate–80 % acetonitrile system was selected as the final mobile phase by considering the analysis time and resolution. The sample was treated using the precipitated protein method, so there were no obvious impurity peaks except for the target peak. In addition, because the gradient elution time was long, the isocratic elution method was selected. This method could be completely eluted in 5 min, so a run time of 5 min was selected. With an increase in temperature, the half-peak width of the chromatographic peak decreased, and the peak shape symmetry was better. Additionally, the pressure of the column decreased, so a temperature higher than 70 °C was used.

When the injection volume was 5 μL, there was obvious solvent affection. This resulted in serious deformation of the chromatographic peak and affected the calculation of the peak area. When the injection volume was 1 μL, the response was low. Therefore, the injection volume was set at 2 μL. For the ELSD parameters, according to the principle of the highest signal-to-noise ratio, the drift tube temperature was selected to be 75 °C, the atomizer temperature was selected to be 40 °C, and the gas flow rate was selected to be 1.8 SLM. Regarding the protein removal method, the ultrafiltration method had a better effect on protein removal, but the cost was higher and the repeatability was not as good as that of the protein precipitation method. Therefore, the organic solvent protein precipitation method was used in this experiment. The final parameters were as follows: the column was a Poroshell 120 HILIC-Z (3.0 × 100 mm, 2.7 μm; PN: 685975-324); the column temperature was 70 °C; the injection volume was 2 μL; the mobile phase was 20 mM ammonium acetate: acetonitrile = 20:80; the flow rate was 0.8 mL/min; and the ELSD parameters were evaporation = 75 °C, nebulizer = 40 °C, gas flow = 1.8 SLM, data rate = 80 Hz, and smoothing = 30 s.

3.2. Validation of the HPLC method

3.2.1. System suitability

The following validation experiments were conducted under conditions that met the system suitability requirements, namely, that all of the fitted curves had an R^2greater than 0.99 and the theoretical plate numbers for the trehalose and sucrose peaks were both greater than 5000.

3.2.2. Specificity

The chromatograms of the specificity validation solutions are shown in Fig. 2. The results showed that the retention time of sucrose

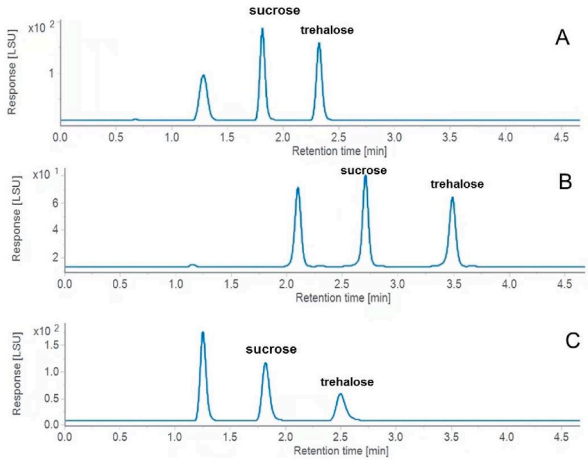

Fig. 1. Chromatographic column screening results (A: Hilic-Z; B: Hilic-OH5; C: Hilic Plus).

was 1.777 min and that of trehalose was 2.270 min, except for the 1.0 mg/mL standard solution, where no interference peaks were detected in blank solution, mobile phase solution and preparation blank solution. These results indicated that other substances in the specificity validation solutions did not interfere with the detection of sucrose and trehalose, and the specificity of the method was good.

3.2.3. Linearity

Linearity refers to the extent to which the measured response is proportional to the concentration of sucrose or trehalose in the sample within a range from 0.25 mg/mL to 3.5 mg/mL. General rule 9101 of the Chinese Pharmacopeia stipulates that regression equations, correlation coefficients, and the fitting curve should be listed. USP 1225 specifies that the correlation coefficient, Y-axis intercept, regression line slope, and residual sum of squares should be submitted. We verified the linearity of the method. The linearity validation solution was injected, and the peak areas were recorded, with the log values of sucrose or trehalose concentration as the abscissa and the peak area log values as the ordinate, fitting the standard curve. The fitting function of this method in the concentration range of 0.25–3.5 mg/mL was as follows: for sucrose, $\log(y) = 1.2763 \log(x) + 3.1796$, with $R^2 = 0.999$; for trehalose, $\log(y) = 1.3018 \log(x) + 3.1499$, with $R^2 = 0.999$. The fitting curve is shown in Fig. 3.

3.2.4. Repeatability

The repeatability validation solutions were injected, and the mean values and RSDs of the peak areas of trehalose and sucrose were calculated for the six replicates. The results are shown in Table 2. The peak area RSDs were all less than 3.0 %, indicating that the method was reproducible.

3.2.5. Accuracy

Accuracy refers to the degree to which the result measured by the method is close to the true value or reference ratio. The accuracy and precision validation solutions were injected, and the peak area of each validation solution was recorded. The sucrose and trehalose concentrations were calculated using the standard curve regression, and the mean recoveries, standard deviations (SDs), and RSDs (n = 6) of each validation solution were calculated according to the actual added amount. The results are shown in Table 3. The recoveries of trehalose at the three concentration levels in S1 were in the range of 97.19 %–103.83 %. The recoveries of the three concentration levels of sucrose in S2 ranged from 98.64 % to 104.69 %. The average recoveries of the validation solutions at all of the concentration levels of trehalose and sucrose were in the range of 90 %–110 %, and the method's accuracy was good.

3.2.6. Precision

We evaluated the precision of the method by measuring three different precision validation solutions in the six tests. The precision results are shown in Table 4. The RSDs for the recoveries of the accuracy and precision validation solutions were all less than 5.0 % (n = 6). For sucrose, the minimum RSD was 1.75, and for trehalose the minimum RSD was 1.77. The results showed that the precision of the method was good.

3.2.7. Limit of quantitation

A 0.025 mg/mL mixed standard solution was prepared using the mixed standard stock solution and injected. The S/N ratios of sucrose and trehalose were 13.5 and 12.2, respectively, which were greater than the quantitative requirement of the S/N = 10. Therefore, the method was quantitatively limited to 0.025 mg/mL.

3.2.8. Limit of detection

The mixed standard stock solution was prepared into 0.001 mg/mL mixed standard solution and injected. The S/N values of sucrose and trehalose were 4.9 and 4.1, respectively, which were both higher than the requirement of S/N = 3. Therefore, the detection limit of this method is 0.001 mg/mL.

3.2.9. Sample detection

Six replicates of S1, S2, and S3 were prepared in parallel. The peak area of sucrose or trehalose in the samples was substituted into the fitting function to calculate the measured concentrations. This was multiplied by a dilution factor of 16 to obtain the actual sample

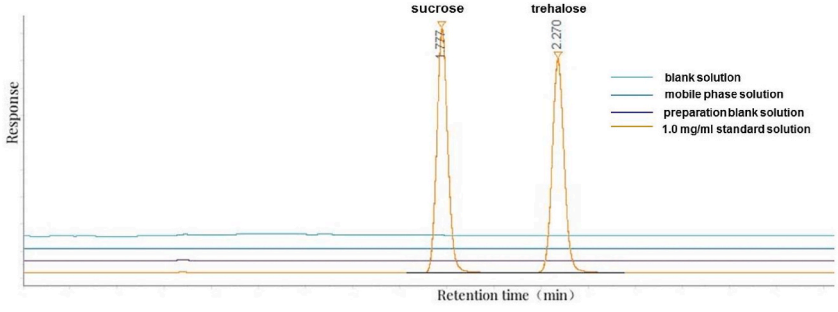

Fig. 2. Chromatograms of specificity validation solutions.

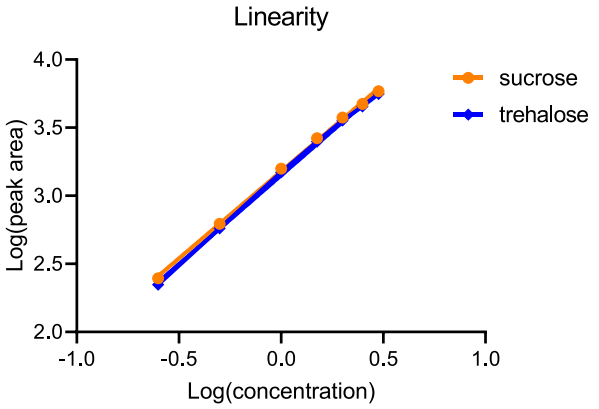

Fig. 3. Linearity fitting curve.

Table 2
Average area, SDs, and RSDs of the chromatographic peaks of the repeatability validation solutions.

	Sucrose standard solution(0.5 mg/mL)	Trehalose standard solution(0.5 mg/mL)	S1 (trehalose)	S2 (sucrose)	S3 (trehalose)
Average area	627.30	579.55	1734.67	2154.04	6779.34
SD (%)	9.32	9.67	22.52	32.40	63.50
RSD (%)	1.49	1.67	1.30	1.50	0.94

Table 3
Recoveries of accuracy and precision validation solutions.

%Level of validation solution	S1(trehalose) %Recovery (n = 6)	S2 (sucrose) %Recovery (n = 6)
Level-50	103.83 ± 2.11	101.88 ± 3.01
Level-100	103.11 ± 2.92	104.69 ± 1.83
Level-150	97.19 ± 1.73	98.64 ± 2.20

Table 4
Precision results of accuracy and precision validation solutions.

%Level of validation solution	S1(trehalose) %RSD (n = 6)	S2(sucrose) %RSD (n = 6)
Level-50	2.03	2.95
Level-100	2.83	1.75
Level-150	1.77	2.23

concentrations. The SDs, RSDs, and recovery rates were then calculated. The results are shown in Table 5, and the chromatograms are shown in Fig. 4. The RSDs of each of the three samples were 0.83 %, 1.82 %, and 1.18 %, which were less than 5 %. The recovery rates were 94.95 %, 106.35 %, and 98.60 %.

Table 5
Concentrations, SDs, and RSDs of sucrose or trehalose in S1,S2, and S3.

	S1 (trehalose)	S2 (sucrose)	S3 (trehalose)
1	18.74	20.72	53.50
2	18.96	21.01	53.58
3	19.20	21.41	54.78
4	19.10	21.12	53.90
5	18.96	21.71	54.90
6	19.01	21.65	54.74
Average concentration	18.99	21.27	54.23
Concentration SD (%)	0.16	0.39	0.64
Concentration RSD (%)	0.83	1.82	1.18
Recovery rate (%)	94.95	106.35	98.60

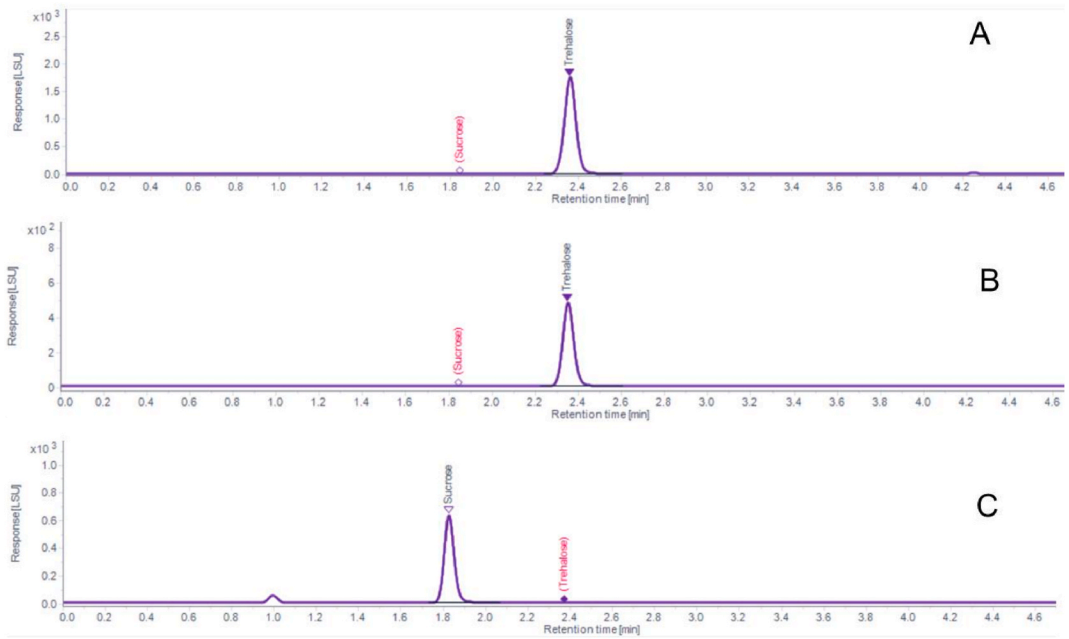

Fig. 4. Chromatograms of sample S1, S2 and S3(A: sample S1; B: sample S2; C: sample S3).

4. Conclusion

We established an HPLC-ELSD method and optimized the various parameters, including the chromatographic column, mobile phase system, salt concentration, ammonium acetate ratio, elution mode, column oven temperature, injection volume, atomization temperature, evaporation temperature, and protein removal method [8,18,19]. This resulted in an efficient HPLC method that is capable of simultaneously determining the sucrose and trehalose concentrations in monoclonal antibodies without mutual interference. The methodological validation conducted in accordance with the ICH Q2 [19] guidelines showed that this method offers excellent specificity, accuracy, precision, and linearity, with a detection limit of 0.001 mg/mL.

Hydrophilic interaction liquid chromatography (HILIC) is a commonly used method for the separation of polar analytes inadequately retained on typical reversed-phase columns. The columns we considered were based on Poroshell cores coupled with different chemically bonded phases. Poroshells utilize a superficially porous particle technology in which the particles have a solid core and a porous outer shell. This structure allows for high-efficiency separation while maintaining lower backpressure. This design not only enhances the analysis speed, but it also provides excellent resolution in the separation of complex mixtures. HILIC Plus columns are non-bonded silica columns, and HILIC-OH5 columns contain a polyhydroxy phase bonded onto superficially porous silica particles. HILIC-Z columns contain a zwitterionic phase bonded onto superficially porous silica particles. This provides a hydrophilic and charged surface, which is suitable for highly polar or ionized compounds that remain stable under high pH or high-temperature conditions. We screened the above three types of columns and fully utilized the advantages of the HILIC-Z column's Poroshell core and zwitterionic phase to perform the quantitative analysis of trehalose and sucrose in a single experiment.

We explored the determination of multiple excipient contents in therapeutic monoclonal antibodies using a single HPLC method compared with methods such as HPLC-RID and HPLC–electrospray ionization mass spectrometry (HPLC-ESI-MS). This method does not require complex pretreatment, making the operation simpler and more efficient than in traditional methods, and it provides an important reference for the formulation development and quality control of therapeutic monoclonal antibodies. However, when using this method, several points must be considered. First, many sugar compounds exhibit anomeric isomerism. Some types of Hilic columns (e.g., Hilic-OH5) might separate anomeric isomers at lower column temperatures; therefore, when using such columns, it is advisable to operate at higher temperatures to avoid the separation of anomeric isomers. Second, due to the varying linear range and sensitivity of different ELSD detector models, if the compound response exceeds the linear range, it is necessary to reduce the injection volume or decrease the intensity of the light source to ensure that the control substance fits well within this concentration range. Alternatively, the standard curve range should be adjusted to 0.2–1.0 mg/mL. Moreover, the samples exceeding the linear range should be diluted with 50 % acetonitrile to bring them into the linear range.

According to the concept of quality by design, the quality-control method of monoclonal antibodies is closely related to early drug development, and the development of prescription formulas takes a long time and has high costs. The method developed in this study can quantify both sucrose and trehalose at an early stage of prescription development, which can greatly reduce the time and cost required for quality control, and provide assistance for further rapid development of monoclonal antibodies.

CRediT authorship contribution statement

Xiaojuan Yu: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Zhanlei Xing:** Methodology. **Lan Wang:** Conceptualization. **Chuanfei Yu:** Conceptualization.

Data availability statement

Data will be made available on request.

Funding disclosure

This work was supported by the National Natural Science Foundation of China (grant No. 2170020522) and the National Key Research and Development Program (grant No. 2021YFF0600804).

Declaration of competing interest

The authors declare that they do not have any conflicts of interest.

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