2025 Volume 10, Issue 4
Journal of Medicines Development Sciences
ISSN: 2382-6371(Online) ISSN: 2382-6363(Print)

Validation Strategy for Monoclonal Antibody Purity Detection Methods Based on Risk Assessment - a Review of the Core Technical Requirements of the 2025 Version of ChP and USP

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Abstract: Verification of monoclonal antibody (mAb) purity detection methods is a key link to ensure drug quality. However, in the current validation practice of size exclusion high-performance liquid chromatography (SEC-HPLC) and capillary electrophoresis (CE), there are outstanding problems such as inconsistent technical implementation and "formalization" of projects, and there are many disputes over the selection of validation projects. Among them, the SEC-HPLC area normalization method is the most widely used semi-quantitative analysis method for polymers in enterprises. The selection and implementation of verification projects are particularly chaotic. At the same time, methods such as ion exchange chromatography (IEC) often fail to achieve baseline separation, and their verification logic has also become the focus of the industry. This article is based on the 2025 version of the Pharmacopoeia of the People's Republic of China (ChP 2025), the latest specifications of the United States Pharmacopoeia (USP) and the results of special literature research, systematically sorting out the chaos and core disputes in enterprise verification, focusing on interpreting the verification project design logic of the SEC-HPLC area normalization method, explaining the core reasons for choosing this method for purity testing of biological products, and focusing on IEC that does not reach baseline separation. The testing method proposes verification ideas, starting from the perspective of method characteristics, pharmacopoeia requirements, and risk control, and summarizes a unified verification plan design idea to provide enterprises with a technical reference that is both compliance and practical, and promote the standardized development of industry verification practices.
Keywords: monoclonal antibody; purity detection; method validation; SEC-HPLC; area normalization method; ion exchange chromatography

Online publication: December 26, 2025

1. The practical chaos of corporate SEC-HPLC and CE verification

Combining industry survey data and practical application feedback, the technical implementation chaos of monoclonal antibody purity detection method verification is particularly prominent in the two mainstream analysis technologies of size exclusion high-performance liquid chromatography (SEC-HPLC) and capillary electrophoresis (CE). The specific analysis can be carried out from the following dimensions:

1.1. Inconsistency in verification implementation

1.1.1. SEC-HPLC precision calculation is confusing

Some companies use "absolute RSD of aggregate peak area" as the precision judgment index, ignoring the defect that this index cannot reflect the relative fluctuation of purity results; standard operations should calculate "aggregate peak area percentage RSD" to truly reflect the repeatability of the detection method for purity analysis.

1.1.2. Lack of verification of CE reduction conditions

Only 30% of companies will verify the impact of reduction reagent concentration, incubation temperature and time on test results, resulting in fragment proportion deviations of more than 10% in different batches of tests; while CE technology is highly sensitive to the consistency of the reduction degree, the lack of this verification will directly affect the comparability of results.

1.1.3. There are significant differences in the design of verification projects

The linearity and accuracy designs of the area normalization method are not unified among companies. Since biological products do not have high-purity reference substances and cannot be spiked and recovered, resulting in large differences in plan design, there is a mismatch between verification and method use; some companies omit capillary column batch difference verification for CE and ignore the impact of column performance fluctuations on fragment separation.

1.2. Typical manifestations of "formal verification"

1.2.1. The durability verification is superficial

SEC-HPLC durability verification only briefly examines "mobile phase ratio ±5%" and does not pay attention to the impact of column efficiency decay on polymer separation, which is the main reason for polymer misjudgment. CE durability verification does not cover key parameters such as buffer pH ± 0.1 and capillary column batch differences, which is inconsistent with the requirements of USP <1053> "the impact of column batches and buffers needs to be verified".

1.2.2. Unreasonable linearity verification design

For monoclonal antibody samples with polymer content less than 1%, some companies still force verification of the linear range of "0.1%~5%". Poor repeatability at low concentration points leads to distortion of linear data; and some companies only focus on instrument linearity (relationship between response value and concentration) and do not conduct analytical linearity verification based on method characteristics, which violates the requirement of ChP 2025 General Principle 9101 "Linearity needs to match the purpose of the method".

1.2.3. Data reliability is out of control

Some companies have falsified linear verification data. There have been cases where false SEC-HPLC linear data resulted in excessive polymer content in marketed products that was not discovered in time, eventually triggering product recalls, highlighting the serious harm of formal verification to drug quality.

2. Application logic and verification project interpretation of area normalization method

2.1. The core reason for choosing the area normalization method for purity testing of biological products

Combining the characteristics of biological products, testing needs and industry practices, the SEC-HPLC area normalization method has become a mainstream method for semi-quantitative analysis of polymers. Biological products such as monoclonal antibodies have complex structures, making it difficult to obtain high-purity, high-stability polymer commercial reference standards. However, the area normalization method does not need to rely on reference substance calibration. The relative proportion of polymers can be calculated through the peak area percentage, avoiding the industry pain point of "unable to accurately quantify without qualified reference substances". In scenarios such as process screening in the drug development stage and intermediate control in the production process, it is necessary to quickly determine whether the polymer content is within a reasonable range. The area normalization method is easy to operate and has a short detection cycle (single sample detection time <30 minutes). It can achieve high-throughput analysis and meet the needs of enterprises for "efficient quality control." SEC-HPLC is the gold standard for the separation of molecular size variants. It can efficiently separate monomers, dimers and higher-order polymers. The area normalization method is highly adaptable to the separation principle of this technology - after separation based on molecular size differences, the relative content of each component is directly reflected by the peak area ratio, without the need to establish an additional complex quantitative calibration curve. Both ChP 2025 and USP recognize the area normalization method for semi-quantitative analysis of biological product aggregates. ChP 2025 General Chapter 0512 clarifies that this method is suitable for "relative purity assessment". USP <129> also uses it as a recommended method for screening molecular size variants, providing a compliance basis for corporate applications.

2.2. Area normalization method verification project design and implementation specifications

Combining the 2025 version of the "Pharmacopoeia of the People's Republic of China" (ChP 2025, General Chapters 0512, 9101), the United States Pharmacopoeia (USP <621>, <1225>) and industry special research results, based on "Essence of Method - Pharmacopoeia Requirements - Practical Feasibility" Three-dimensional logic clarifies the design key points and implementation process of the area normalization method (SEC-HPLC) verification project, ensuring that the verification not only meets compliance requirements but also fits the technical characteristics.

2.2.1. Specificity/selectivity verification

ChP 2025 General Chapter 0512 requires that the resolution of the target component must meet the species regulations, and the blank solvent must not have interference peaks; USP <621> emphasizes that the target component must be effectively separated to ensure system applicability. Column efficiency attenuation will directly affect the separation effect of polymers and monomers, which can easily lead to misjudgment of polymers. Special verification is required to avoid this risk.

Take a blank solvent for detection (such as phosphate buffer) and inject it according to the established chromatographic conditions. Confirm that there are no interfering peaks between the peaks of polymers and monomers. Inject and analyze monoclonal antibody samples containing known polymers (such as dimers and multimers) to ensure the separation of polymers and monomers. Simultaneously record the number of theoretical plates of the system suitability solution (number of theoretical plates for monomer peaks ≥ 5000) and the tailing factor (0.8~1.2) to ensure that the chromatographic system is in a stable state.

2.2.2. Precision verification

ChP 2025 General Chapter 9101 requires that the continuous injection peak area RSD must meet the variety requirements. General Chapter 0512 further clarifies that the SEC-HPLC polymer peak area percentage RSD is ≤2.0% (n=6), and can be relaxed to ≤10% when the content is <1%; USP <1225> stipulates that the precision of low-content components can be appropriately adjusted. The core of the area normalization method is relative proportion calculation, and the verification core needs to be proportional stability.

Take the same batch of monoclonal antibody samples, prepare 6 test solutions according to the method, continuously inject samples and analyze, calculate the polymer peak area percentage RSD, which must meet the limit requirements of the corresponding content level; at the same time, it can assist in monitoring the total peak area RSD to troubleshoot the injection volume or instrument response fluctuation. Intermediate precision verification (if required): Analyze the same batch of samples (n=6) by different operators on different days using the same model of instrument, and aggregate peak area percentage RSD to ensure the stability of the method within the range of routine operating variation.

2.2.3. Durability verification

ChP 2025 General Chapter 0512 requires the investigation of the impact of mobile phase pH ±0.2 and column temperature ±5°C on the results; USP <621> stipulates that parameter adjustment must meet the premise of system applicability, focusing on column temperature and flow rate fluctuations. If necessary, the impact of column efficiency decay on detection needs to be verified. The mobile phase ratio has minimal impact on molecular size separation, and verification needs to focus on key parameters.

The same batch of samples were tested under the conditions of column temperature (set value ±5°C), mobile phase pH (set value ±0.2), and flow rate (set value ±10%), and the changes in polymer proportion were recorded, and the system applicability still met the requirements. Select three SEC columns of the same model from different batches, analyze the same batch of samples, and record the RSD of the polymer peak area percentage to confirm that differences in column batches do not affect the detection results. If necessary, column efficiency attenuation verification needs to be performed: continuously inject 50 injections of the test solution, and examine the difference in polymer peak area percentage before and after, while ensuring that the system applicability still meets the requirements.

2.2.4. Solution stability verification

ChP 2025 General Chapter 9101 requires that the stability of the sample within the detection cycle be examined in conjunction with the purpose of the method; industry research shows that monoclonal antibody samples are prone to polymerization at room temperature, and a reasonable detection window period needs to be determined through verification.

Stability of sample solution: Take the prepared test solution and place it at room temperature for 0h, 4h, 8h, 12h, 24h, and at 4°C for 0h, 24h, 48h, and 72h. Inject samples for analysis according to the method, and calculate the difference between the polymer peak area percentage at different time points and 0h.

2.2.5. The limit of quantitation (LOQ) can be exempted, and the limit of detection (LOD) can be verified on demand

ChP 2025 General Principle 9101 clarifies that "validation is exempted when the LOQ is lower than 1/10 of the reporting limit"; there are differences in the response factors of different components (monomers, polymers) in the SEC-HPLC area normalization method, and the LOQ data cannot reflect the true lower limit of quantification, and has no practical application value. This method is used for semi-quantitative screening of polymers without the need to accurately quantify low-concentration impurities. If you need to confirm the detection capability of the method, you can verify the LOD by using the signal-to-noise ratio method (S/N=3), which requires LOD≤0.05% (in line with the derivatization requirements of ChP 2025 "QL is lower than the reporting limit 1/10"); by gradually diluting samples containing trace amounts of aggregates, determine the lowest concentration that can clearly identify the aggregate peak.

2.2.6. Accuracy (spiked recovery), exempt

ChP 2025 General Chapter 9101's spiked recovery requirements for accuracy (80% ~ 120%) are for accurate quantitative methods; USP <1225> defines accuracy as the closeness of the test results to the true value, which needs to rely on a reference substance of known purity. There is no commercial polymer reference substance for the SEC-HPLC area normalization method. It is difficult to accurately determine the purity of the homemade reference substance, and the response factors of polymers and monomers are greatly different (the response factor of dimers is about 1.8~2.2 times that of monomers), resulting in significant fluctuations in the spiked recovery rate (60%~140%), which cannot objectively reflect the accuracy of the method.

If regulatory requirements apply, use the forced degradation method. The monoclonal antibody sample undergoes thermal degradation (such as 60°C for 24 hours) or oxidative degradation (such as hydrogen peroxide treatment) to prepare a sample containing a high proportion of polymers. Mix it with normal samples in different proportions (such as 1:9, 3:7, 5:5) to prepare a series of test solutions. Test and analyze the consistency between the theoretical value and the measured value of the polymer ratio according to the method, indirectly proving the reliability of the method.

2.2.7. Linearity: Exemption

ChP 2025 General Chapter 9101 requires quantitative methods to verify linearity, and the range covers the actual detection concentration; USP <1225> stipulates that linear results must match precision and accuracy. The core of the area normalization method is the calculation of peak area percentage, which has nothing to do with the linear relationship with concentration, and the repeatability of low-concentration polymers (<0.1%) is poor, and forced verification will lead to data distortion.

If the registration application needs to provide linear data, samples containing different proportions of polymers (such as 0.5%, 1.0%, 2.0%, 5.0%, 8.0%) can be selected, tested according to the method, and linear regression is performed with the actual proportion of the polymer as the abscissa and the peak area percentage as the ordinate. The correlation coefficient $R \geq 0.995$ is required; at the same time, it is clearly stated in the report that this linearity is only a compliance form and has no actual technical significance, emphasizing the rationality of the exemption based on the characteristics of the method.

2.3. Validation ideas for IEC detection methods that fail to achieve baseline separation

Ion exchange chromatography (IEC) is a common method for detecting charge isomers of monoclonal antibodies, but its separation effect is easily affected by factors such as sample charge distribution, buffer pH, and chromatographic column performance. In practical applications, peak shapes often overlap and the resolution does not reach baseline separation (resolution <1.5). Combining the concept of ChP 2025 General Chapter 9101 "Dynamic Validation Based on Risk Assessment", USP <1225> statutory method validation requirements and the actual needs of charge variant detection of biological products, a targeted validation idea was developed.

Based on the relevant general requirements of the 2025 edition of the Chinese Pharmacopoeia, two core contents need to be clarified before the verification of this type of IEC method: First, the method positioning is limited to the screening of relative proportions of charge isomers and process consistency evaluation, and cannot be used for accurate quantification of individual charge isomers, and the scope of application and limitations need to be noted in the verification report; second, the acceptance criteria for resolution need to be set in conjunction with risk assessment, with separation ≥ 1.0 and peak-to-valley ratio ≥ 1.2 as thresholds to ensure the accuracy of overlapping peak integration. The core verification project needs to focus on six practical points: the specificity verification adopts the "reference substance positioning" scheme, which ensures that the overlapping peaks are all target charge isomers and have no interference from impurity peaks through reference drug positioning, matrix interference screening and column efficiency attenuation inspection; the precision verification takes the standardized integration parameters as the core and uses the peak area percentage RSD as the judgment index, while verifying the intermediate precision of different personnel, instruments and chromatographic columns; the durability verification expands the scope of operating parameter fluctuations and requires buffer solutions Adjustment of parameters such as pH and concentration; solution stability verification examines the change in the charge isomer ratio of the sample under different storage conditions, and stipulates the detection time limit after sample preparation to reduce the result deviation; accuracy verification abandons the traditional spike recovery method and adopts relative accuracy verification, which can be performed with capillary isoelectric focusing Comparison of electrophoresis (cIEF) results or comparison of different laboratory methods indirectly proves the reliability of the method; quantitative limit and linearity verification are exempted or simplified as needed, and limit inspection can be directly exempted. Relative proportion analysis only needs to verify the linear relationship covering the actual proportion interval, and clear requirements are put forward for the correlation coefficient. In addition, three supplementary risk control measures are required: incorporate standardized integration parameters into standard operating procedures (SOPs) and carry out personnel training and assessment to avoid human integration errors; establish quality control standards for chromatographic columns, verify separation effects before using new columns, and re-evaluate column performance after using more than 30 needles; retain the original integration data of all test spectra to ensure data traceability and comply with the data integrity requirements of the 2025 version of the Chinese Pharmacopoeia.

Combining the concept of “analytical method life cycle” of the 2025 edition of the “Chinese Pharmacopoeia” and the 2025 drug testing proficiency testing requirements of the State Food and Drug Administration, a life cycle-based hierarchical verification logic for monoclonal antibody analytical methods can be established: the R&D phase focuses on rapid screening of effective detection methods, SEC-HPLC only verifies specificity and repeatability, CE only verifies specificity and consistency with reducing conditions, IEC Only specificity and integral repeatability are verified; the application stage aims to meet registration compliance requirements, and key parameters are supplemented on the basis of verification in the R&D stage. SEC-HPLC adds durability verification of column efficiency attenuation and temperature fluctuations, CE adds intermediate precision verification of different personnel and column batches, and IEC adds intermediate precision and buffer stability verification; the post-marketing stage is oriented to ensure the reliability of large-scale production testing, focusing on production stability verification. SEC-HPLC verifies batch differences in column packing materials, CE verifies batch differences in reagents, and IEC Then verify the difference between the column batch and the buffer batch.

3. Conclusion

The chaos and controversy in the verification of monoclonal antibody purity detection methods are essentially manifestations of the imbalance between “compliance formalization” and “technical practicality”. The SEC-HPLC area normalization method has become a mainstream method for polymer analysis due to its core advantages of adapting to the complexity of biological products and meeting the needs of rapid screening. The core of its verification lies in “returning to the essence of the method” - reasonable selection of items based on semi-quantitative purposes, strict verification of key parameters such as specificity and precision, and exemption of quantitation limit and accuracy verification that are irrelevant to the purpose of the method.

For IEC methods that do not reach baseline separation, the core of verification is “risk controllability”. By clearly defining the method, setting reasonable separation thresholds, and strengthening integration repeatability and durability verification, reliable screening of the relative proportions of charge isomers can be achieved. The design of a unified verification scheme needs to be based on pharmacopoeia specifications as the bottom line, based on method characteristics, with risk control as the core, abandoning the “full coverage verification” thinking, reducing technical differences among enterprises through standardized operations and clear project selection principles, promoting the transformation of industry verification practices from “compliance and compliance” to “precision quality control”, and providing reliable technical support for monoclonal antibody quality assurance.

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Disclosure statement

The author declares no conflict of interest.

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