[技术帖] 药品中有关物质HPLC方法验证

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药品中有关物质HPLC方法验证

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METHOD VALIDATION FOR HPLC ANALYSIS OF RELATED SUBSTANCES IN PHARMACEUTICAL DRUG PRODUCTS
药品中有关物质HPLC方法验证

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3.1 INTRODUCTION 介绍

In this chapter we outline the general requirements for analytical method validation for HPLC analysis of related substances in pharmaceutical products. Most of the discussion is based on method validation for pharmaceutical products of synthetic origin. Even though most of the requirements are similar for other types of pharmaceutical drug products (e.g., biopharmaceutical drug products), detailed discussion of method validation for other types of pharmaceutical drug products is outside the scope of this chapter. The discussion focuses on current regulatory requirements in the pharmaceutical industry. Since the expectations for method validation are different at different stages of the product development process, the information given in this chapter is most suitable for final method validation according to the ICH requirements to prepare for regulatory submissions (e.g., NDA). Even though the method validation is related to HPLC analysis, most of the principles are also applicable to other analytical techniques (e.g., TLC, UV).
在本章中，我们主要介绍药品中有关物质HPLC方法验证的通则要求。其中大部分讨论是基于合成药品的方法验证。尽管其它类型药品（例如生物制品）的方法验证要求基本类似，但本章不进行讨论。我们的讨论集中在当前制药行业的法规要求。由于在产品研发过程中，不同阶段对方法验证的期望不同，本章中的信息比较适合于根据ICH要求准备法规注册资料（例如NDA申报）时所进行的最终方法验证。尽管本章讨论的是HPLC方法的验证，但其实大部分原则也适用于其它分析技术（例如TLC和UV）。

3.2 BACKGROUND INFORMATION 背景介绍

3.2.1 Definitions 定义

Definitions for some of the commonly used terms in this chapter are given below.
本章中常用术语定义如下：

- Drug substance (active pharmaceutical ingredient): a pharmaceutical active ingredient. 药用物质（活性药物成分、原料药）：一种药用活性成分

- Related substances: impurities derived from the drug substance and therefore not including impurities from excipients. Related substances include degradation products, synthetic impurities of drug substance, and manufacturing process impurities from the drug product. 有关物质：从原料药衍生出的杂质，不包括辅料中的杂质。有关物质包括降解产物、原料药合成杂质，在制剂生产过程中产生的杂质

- Authentic sample: a purified and characterized sample of a related substance. 认可样品：经过精制和表征确认的某个有关物质的样品。

Unlike reference standards, authentic samples may not be of high purity.
与对照品不同，经认可的样品可能不具有那么高的纯度

However, the purity of an authentic sample has to be determined before use. Authentic samples are used in method development to identify related substances in the analysis. In addition, they are used extensively to prepare the spiked samples in method validation.
但是，经认可的样品的纯度必须在使用前进行检测。认可样品可以用于方法开发阶段，在分析过程中用以识别有关物质，在方法验证被广泛用于制备加样样品。

Spiked sample: a sample added with a known amount of related substances, prepared from authentic samples during method development or validation.
加样样品：加入了已知数量有关物质的一份样品，在方法开发或验证过程中采用认可样品制备

Control sample: a representative batch of drug substance (or drug product).
控制样品：原料药（或制剂）具有代表性的批次

Typically, control samples are tested in all analyses to ensure consistency in method performance across different runs. Sometimes, they are used as part of the system suitability test to establish the run-to-run precision (e.g., intermediate precision, reproducibility).
最典型的做法是在每次检测中均对控制样品进行测试，以保证所使用的在不同次的检测中具有一致的表现。有时，控制样品也作为系统适用性测试的一部分用以建立不同次检测中的精密度（例如，中间精密度，重复性）

Response factor: the response of drug substance or related substances per unit weight. Typically, the response factor of drug substance (or related substance) can be calculated by the following equation:
响应因子：原料药或有关物质每单位重量所产生的响应。一般来说，原料药（或有关物质）的响应因子可以采用以下公式进行计算

3.2.2 Different Types of Related Substance Analysis 有关物质分析的不同类型

Area Percent. In this approach, the level of an individual related substance is calculated by the following equation:
面积百分比（面积归一法）：采用本方法时，单个有关物质的水平使用以下公式计算

$$\%\text{related substance} = \frac{\text{area}_{\text{related substance}}}{\text{total area}} \times 100\%$$

where the area related substance is the peak area of the individual related substance and the total area is the peak area (i.e., response) of the drug substance plus the peak areas of all related substances. This is one of the simplest approaches for related substance analysis because there is no need for a reference standard.
其中，有关物质面积是该有关物质的峰面积，总面积是原料药加上所有有关物质的峰面积（即响应值）。这是有关物质分析中最简单的方法之一，因为它不需要对照品。

This is particularly important during the early phase of the project when a highly purified reference standard is not available. It is the preferred approach as long as the method performance meets the criteria described below.
在项目早期，无法获得高纯度的对照品时，该方法尤为重要。只要方法表现符合以下标准，这种方法其实是可以优先选择的。

Linearity over a Wide Range of Concentration. Since the areas of the related substances (typically, less than 1%) and drug substance (typically, more than 95%) are summed, it is important that the method is linear from the concentration of related substances (e.g., 1%) to that of the drug substance (e.g., 95%). However, in some cases, the peak shape of the drug substance may not be totally symmetrical at such a high concentration. Therefore, the response may not be linear in such a wide concentration range, and the use of area percentage may not be appropriate. If the response of the analyte is nonlinear at higher concentrations, the related substances would be overestimated. Although this is conservative from a safety perspective, it is inaccurate and therefore unacceptable.
在较宽的浓度范围内具有线性。由于有关物质的面积（典型的结果是低于1%）和原料药的面积（典型的结果是高于95%）要进行相加，因此方法在有关物质的浓度（例如1%）至原料药浓度（例如95%）范围内呈线性就很重要。但是，有些情况下，原料药的峰形在浓度较高时可能并不完全对称，这时，响应值可能在较宽的浓度范围内无法呈线性，可能就不适合采用面积百分比方法。如果被分析物质在高浓度时不呈线性，有关物质结果可能会虚高。尽管从安全角度来说这样更保守，但它毕竟是不准确的，因此是不能接受的。

Sample Concentration (Method Sensitivity). To maintain linearity at the concentration range of the drug substance, scientists may try to lower the sample concentration to improve peak shape for the drug substance. However, if the sample concentration is too low, it will affect the method sensitivity, and the ability to detect low levels of related substances may not be adequate.
样品浓度（方法灵敏度）。为了使原料药保持在线性浓度范围内，研发人员可以尝试降低样品浓度，以改善原料药的峰形。但是，如果样品浓度太低，会对方法的灵敏度产生影响，则对低水平的有关物质检测能力可能就不够了。

Response Factor. The response factors of the related substances should be similar to that of the drug substance (i.e., relative response factors close to unity).
响应因子。有关物质的响应因子应与原料药的相近（即相对响应因子接近统一）。

Otherwise, a response factor correction must be used in the calculation.
如果达不到，则在计算中必须代入响应校正因子。

High–Low. This approach can be used to overcome the limitation of linear range in the area percent method discussed above. In this approach, samples are prepared at a concentration (i.e., high concentration) similar to that of the area percent method (Figure 3.2). In addition, the high concentration sample solutions are diluted further, to low concentrations (Figure 3.3). Samples from both high and low-concentration solutions are injected for analysis. In the injections of the high concentration, the responses of all related substances are determined as these small peaks are detectable. The high sample concentration is used to allow all related substances to be detected and quantitated. In the injection of low-concentration sample, the response of the drug substance is determined. Low concentration is used to ensure that the response of the drug substance is within the linearity range.
高-低浓度法（自身稀释对照法）。高-低浓度法可以用来克服上述面积百分比方法中线性范围的缺点。采用高-低浓度法时，样品溶液配制浓度（即高浓度）与面积百分比方法相近（见图3.2），同时，将高浓度的样品溶液进一步稀释至低浓度（图3.3）。将高浓度和低浓度样品溶液分别进样，采用高浓度图谱中的有关物质响应值，因为这时所有这些小峰均可以被检测到。同时，采用低浓度样品图谱中的原料药响应值。高浓度样品溶液的目的是使用所有有关物质均可被检出及定量，而低浓度样品溶液的目的是保证原料药的响应在线性范围内。

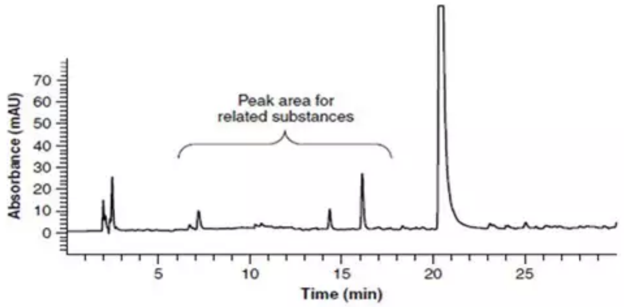

Figure 3.2. chromatogram from high concentration.

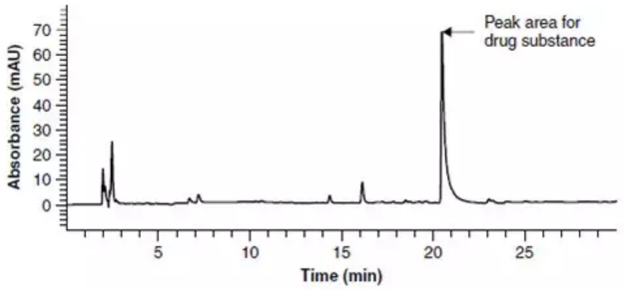

Figure 3.3. chromatogram from low concentration.

3.3.6 Range 范围

ICH definition: The *range* of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analytes in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity (Figure 3.13).

ICH定义：分析方法的范围是指样品中（包括这些浓度）被分析物质的最高和最低浓度（数量）的区间，要证明分析方法在此区间具有适当水平的精密度、准确性和线性（见图3.13）。

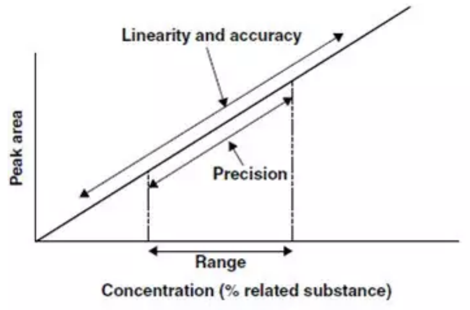

Figure 3.13. Range.

图3.13 范围

Typically, linearity and accuracy determination covers a wide concentration range (e.g., 50% of the ICH reporting limit to 150% of specification). However, the concentration range for precision will be limited by the availability of sample of different related substance levels. Therefore, to ensure an appropriate method validation range with respect to precision, it is critical to use samples of low and high levels of related substance in precision experiments (e.g., fresh and stressed samples).

典型情况是，线性和准确度测试覆盖一个较宽的浓度范围（例如ICH报告限的50%至质量标准的150%）。但是精密度的浓度范围会受到不同有关物质水平的样品是否可以获得的限制。因此，要保证精密度试验具有适当的方法验证范围，关键是在试验中使用含有较高和较低水平的有关物质的样品（例如，新鲜的和强降解的样品）。

3.3.7 Robustness 耐用性

ICH definition: The *robustness* of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal use.

ICH定义：分析方法的耐用性是其它保持不受方法参数的较小变化影响的能力，说明其中常规使用中的可靠程度。

General Considerations. This is to verify that the method performance is not affected by typical changes in normal experiments. Therefore, the variation in method conditions for robustness should be small and reflect typical day-today variation. Experimental design (e.g., Plackett–Burman screening, factorial design) is very useful to investigate multiple parameters simultaneously. Critical parameters are identified during the method development process. Only these critical method parameters should be investigated for robustness. Common critical method parameters can be divided into two categories:

一般考虑。该试验是为了确认方法表现不会受到常规检测中一些典型的变化的影响。因此，耐用性试验中的条件变动应较小，反映较为典型的常规变动。试验设计（例如，Plackett–Burman 筛选试验，因素设计）对于调查多因素模拟非常有用。在方法开发阶段，要对关键参数进行识别。耐用性试验只需要对这些关键参数进行调查。一般关键参数分为两类。

1. *HPLC conditions*

HPLC条件

a. HPLC column (lot, age, brand)

色谱柱（批号、使用时长、品牌）

b. Mobile-phase composition (pH ± 0.05 unit, percent organic ± 2%)

流动相比例（pH ± 0.05 unit, 有机相百分比 ± 2%）

c. HPLC instrument (dwell volume, detection wavelength ± 2 nm, column temperature ± 5?C, flow rate)

HPLC仪器（死体积、检测波长± 2 nm，柱温± 5?C，流速）

2. *Sample preparation样品制备*

a. Sample solvent (pH ± 0.05 unit, percent organic ± 2%)

溶剂（pH ± 0.05单位,有机相比例 ± 2%）

b. Sample preparation procedure (shaking time, different membrane filters)

样品配制程序（振摇时长，不同孔径过滤膜）

c. HPLC solution stability

HPLC溶液稳定性

Other Considerations. Typically, the variations in robustness results are compared to the intermediate precision results to demonstrate that robustness is not affected significantly within normal day-to-day variation. When the related substance results are affected by some critical experimental parameters, a precautionary statement needs to be included in the procedure to ensure that this parameter is tightly controlled between experiments. For example, if percent organic of mobile phase affects the results significantly, the procedure should indicate the acceptable range for percent organic (e.g., 50% organic ± 2%)

其它考虑。一般来说，耐用性试验中的结果要与中间精密度结果进行比较，以证明在常规的不同天检测中，耐用性并不会受到显著影响。如果有关物质结果受到一些关键试验参数的明显影响，则在检验方法中要写明注意事项，以保证这些参数严格控制在范围内。例如，如果流动相中有机相比例会对结果形成显著影响，则检验方法中要注明有机相的可接受范围（例如，50%有机相± 2%）。

Built-in Robustness in Method Procedure. The following are some suggestions to improve method robustness:

分析方法自身耐用性。以下是如何改善耐用性的一些建议。

? *Weighing error*. Weighing error is usually the main source of error. Analytical procedure should ask for a weighing sample or standard of more than 10 mg to minimize weighing error. In addition, use two to three independent weighings in the standard curve and verify the nominal responses of these standard preparations to ensure that there is no significant weighing error. Alternatively, use area percent calculation to eliminate the need for weighing a small quantity of reference standard.

称重误差。称量误差一般是误差的主要来源。分析方法所要求的称样量，或对照品称样量应大于10 mg以减少称量误差。另外，在标准曲线中使用2-3个单独的称量，确认这些标准制备的名义响应值，以保证不会发生较大的称量误差。也可以采用面积百分比计算来消除对照品微量称量的需求。

? *Dilution error*. Pipette a volume of more than 5 mL, and avoid using volumetric flasks of less than 25 mL.

稀释误差。移液体积应大于5ml，避免使用少于25ml的容量瓶。

? *Sonication*. The efficiency of sonication is highly variable and depends on various factors (e.g., condition of the sonication bath, level of water, and position of flask in the sonication bath). Mechanical shaking is recommended, instead, and is much more reproducible.

超声。超声的效果具有高度不确定性，依赖于不同因素（例如超声浴池的条件、水位、容量瓶在超声浴池中放置的位置）。推荐采用机械振摇，因为其更具有重现性。

? *Mobile phase as sample solvent*. If possible, always use mobile phase as the sample solvent. This ensures the composition (e.g., percent organic, pH) of sample solution matches that of mobile phase and reduces the chance of any problem due to incompatibility of sample solvent and mobile phase.

流动相作为样品溶剂。应尽可能采用流动相作为样品溶剂，这样保证样品溶液的比例（例如有机相百分比，pH值）与流动相比例相当，减少由于样品溶剂与流动相不相容而产生问题的可能性。

Alternatively, always use sample solvent weaker than that of the mobile phase to ensure that the chromatography is not deteriorated. For example, in reversed-phase HPLC, use less organic solvent in the sample solvent than in the mobile phase.

所以，常常采用比流动相更弱的溶液作为样品溶剂，以保证色谱不会变差。例如，在反相HPLC中，样品溶剂使用有机溶剂比流动相更少。

? *Buffer*. Ensure that the buffer (p*Ka*) is appropriate for the pH of the solution.

缓冲液。保证缓冲剂(p*Ka*)与溶液的pH值相当。

In general, to provide appropriate buffering capacity, the pH of the solution should be within ±1 pH unit of the p*Ka* value of the buffer.

一般为了提供适当的缓冲能力，缓冲液的pH值应在缓冲物质的pKa值的±1 pH单位内。

? *Isocratic method*. Whenever possible, use isocratic HPLC condition, as this is affected less by the variation in flow rate, temperature, and dwell volume.

等度方法。只要可能，应使用HPLC等度条件，因为等度条件受到流动速度、温度、死体积的影响会更小。

If gradient HPLC conditions have to be used, a simple linear gradient is preferred over multistep gradients. Complicated gradient conditions are more susceptible to differences between HPLC instruments (e.g., flow rate, dwell volume).

如果采用了HPLC梯度条件，则线性梯度优先于多步梯度。复杂的梯度条件在不同HPLC仪器上会产生更多差异（例如，流速、死体积）。

3.4 COMMON PROBLEMS AND SOLUTIONS 常见问题和解决方法

1. *Presentation of method validation data.* Table 3.2 provides a quick overview of the validation data.

方法验证数据列表。表3.2中列出验证数据。

2. *System suitability.* During the robustness testing of method validation, critical method parameters such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range for the system suitability results. As a result, system suitability results in these method validation experiments are very useful in determining the system suitability acceptance criteria. This is a very effective approach since the required system suitability results can be generated during method validation and no other special study is required. However, these results reflect the expected performance of the system, but not necessarily the minimum performance standard for acceptable results. For example, the minimum resolution of the critical pair from method validation may be 3.5; however, a resolution of 2.0 may still be acceptable as long as they are baseline resolved and all other chromatographic parameters remain acceptable.

系统适用性。在方法的耐用性测试中，关键方法参数，例如流动相比例和柱温进行了变动，以模拟不同天变化。这时，这些耐用性试验的系统适用性结果反映了系统适用性所期望的范围。该验证试验所得到的系统适用性结果对于决定系统适用性可接受标准非常有用。这种方法非常有效，因为

所要求的系统适用性结果可以在方法验证中产生，不需要进行其它特殊的研究。但是，这些结果反映的是对系统表现的期望，而不是可接受结果的最低表现标准。例如，方法验证中关键相邻峰最小分离度为3.5，但只要保证基线分离，所有其它色谱参数可以接受，分离度为2.0时仍可以接受。

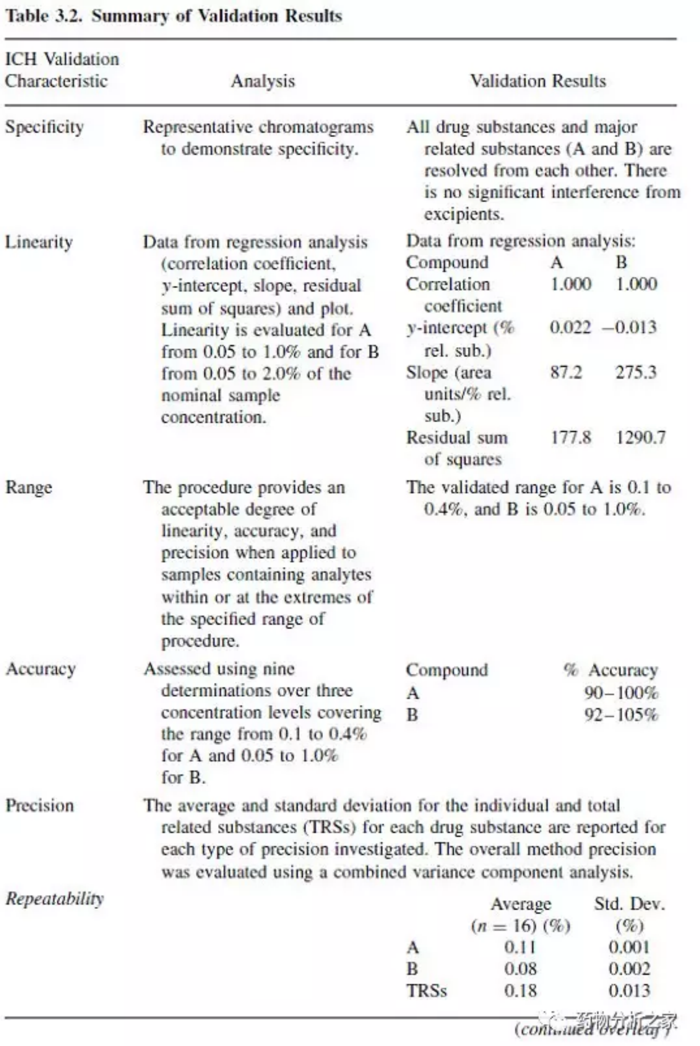

Table 3.2. Summary of Validation Results

ICH Validation Characteristic	Analysis	Validation Results
Specificity	Representative chromatograms to demonstrate specificity.	All drug substances and major related substances (A and B) are resolved from each other. There is no significant interference from excipients.
Linearity	Data from regression analysis (correlation coefficient, y-intercept, slope, residual sum of squares) and plot. Linearity is evaluated for A from 0.05 to 1.0% and for B from 0.05 to 2.0% of the nominal sample concentration.	Data from regression analysis: Compound — A — B Correlation coefficient — 1.000 — 1.000 y-intercept (% rel. sub.) — 0.022 — −0.013 Slope (area units/% rel. sub.) — 87.2 — 275.3 Residual sum of squares — 177.8 — 1290.7
Range	The procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing analytes within or at the extremes of the specified range of procedure.	The validated range for A is 0.1 to 0.4%, and B is 0.05 to 1.0%.
Accuracy	Assessed using nine determinations over three concentration levels covering the range from 0.1 to 0.4% for A and 0.05 to 1.0% for B.	Compound — % Accuracy A — 90–100% B — 92–105%
Precision	The average and standard deviation for the individual and total related substances (TRSs) for each drug substance are reported for each type of precision investigated. The overall method precision was evaluated using a combined variance component analysis.	
Repeatability		— Average ($n = 16$) (%) — Std. Dev. (%) A — 0.11 — 0.001 B — 0.08 — 0.002 TRSs — 0.18 — 0.013

(continued overleaf)

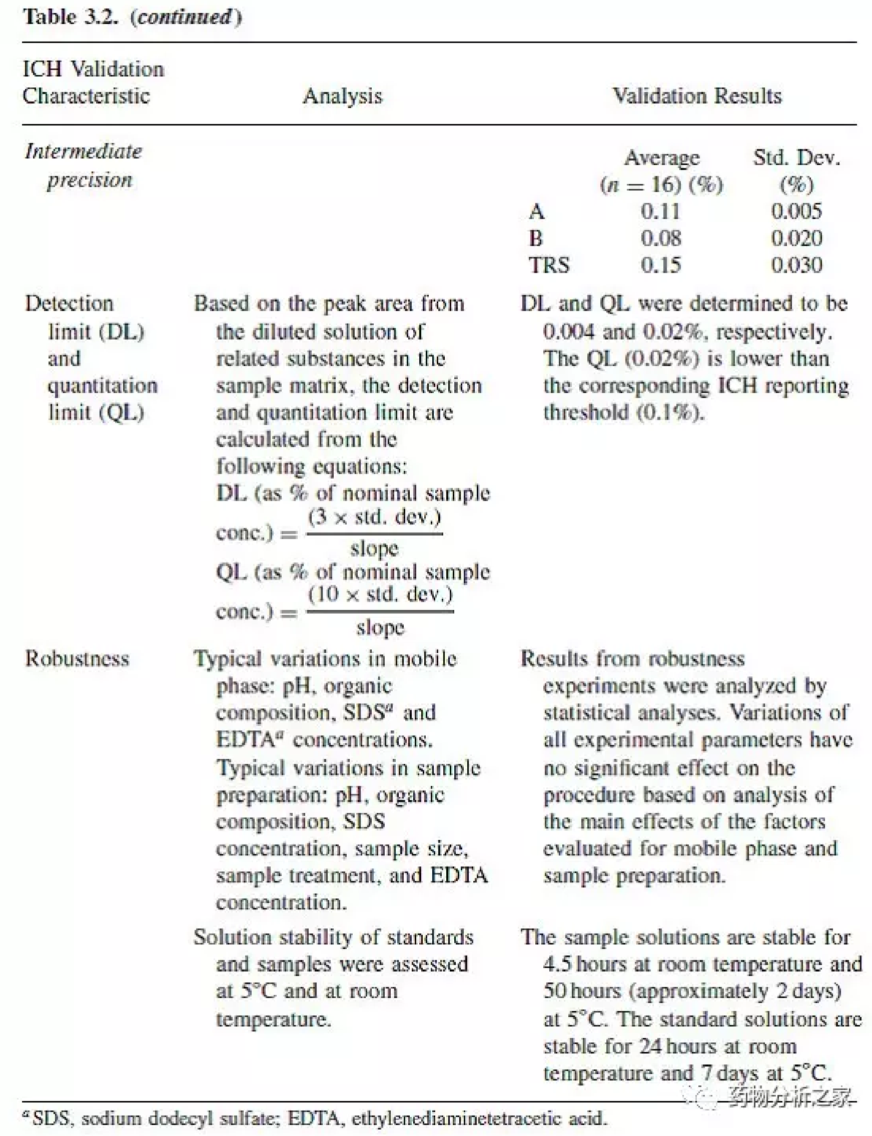

Table 3.2. (*continued*)

ICH Validation Characteristic	Analysis	Validation Results
Intermediate precision		Average (n = 16) (%) Std. Dev. (%) A 0.11 0.005 B 0.08 0.020 TRS 0.15 0.030
Detection limit (DL) and quantitation limit (QL)	Based on the peak area from the diluted solution of related substances in the sample matrix, the detection and quantitation limit are calculated from the following equations: DL (as % of nominal sample conc.) = $\frac{(3 \times \text{std. dev.})}{\text{slope}}$ QL (as % of nominal sample conc.) = $\frac{(10 \times \text{std. dev.})}{\text{slope}}$	DL and QL were determined to be 0.004 and 0.02%, respectively. The QL (0.02%) is lower than the corresponding ICH reporting threshold (0.1%).
Robustness	Typical variations in mobile phase: pH, organic composition, SDS[a] and EDTA[a] concentrations. Typical variations in sample preparation: pH, organic composition, SDS concentration, sample size, sample treatment, and EDTA concentration.	Results from robustness experiments were analyzed by statistical analyses. Variations of all experimental parameters have no significant effect on the procedure based on analysis of the main effects of the factors evaluated for mobile phase and sample preparation.
	Solution stability of standards and samples were assessed at 5°C and at room temperature.	The sample solutions are stable for 4.5 hours at room temperature and 50 hours (approximately 2 days) at 5°C. The standard solutions are stable for 24 hours at room temperature and 7 days at 5°C.

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[a] SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetracetic acid.

表3.2 验证结果总结

ICH验证项目	分析	验证结果
专属性	典型图谱证明专属性	所有原料药和主要有关物质（A和B）能彼此分离。辅料对检测无明显干扰。
线性	回归分析数据（线性相关系数、Y轴截距、斜率、残差平方和）和绘图。 线性对A在名义样品浓度0.05-1.0%范围评估，B在名义样品浓度0.05%-2.0%范围评估	回归分析数据： 物质 A B 相关系数 1.000 1.000 Y轴截距 0.022 -0.013 （有关物质%） 斜率（面积 87.2 275.3 单位/有关物质%） 残差平均和 177.8 1290.7
范围	具备线性、准确度、精密度可接受度时分析物质的浓度范围	验证范围 A 0.1-0.4% B 0.05%-1.0%
准确度	采用A的0.1-0.4%之间，B的0.05%-1.0%之间的3个浓度9次检测来评估	物质 准确度% A 90-100% B 92-105%
精密度	各原料药中单个和总有关物质（TRSs）的平均值和标准偏差报告为各自精密度。方法总体精密度采用合并变量因素分析方法进行评估。	
重复性		平均值 标准偏差 （n=16）（%） （%） A 0.11 0.001、

		B 0.08 0.002 TRSs 0.18 0.013
中间精密度		平均值 标准偏差 （n=16）（%） （%） A 0.11 0.005 B 0.08 0.020 TRSs 0.15 0.030
检测限（DL） 定量限（QL）	基于有关物质在样品中的稀释溶液，按以下公式计算检测限和定量限 DL（以名义样品浓度的%计）=（3×标准偏差）/斜率 DL（以名义样品浓度的%计）=（10×标准偏差）/斜率	检测限和定量限测得分别为0.004和0.02% 定量限（0.02%）低于对应的ICH报告限阈（0.1%）。
耐用性	流动相典型变量：pH值、有机相比例、SDS和EDTA浓度 样品配制典型变量：pH值、有机相比例、SDS浓度、样品量、样品处理、EDTA浓度	耐用性试验结果采用统计学进行分析。 基于流动相和样品配制评估因素主要影响性分析，所有试验参数的变化对检验方法无显著影响。
	5?C和室温下标准溶液稳定性和样品溶液稳定性	样品溶液在4-5小时内室温下稳定，5?C 下50小时（约2天）内稳定。标准溶液室温下24小时内稳定，5?C下7天内稳定。

SDS：十二烷基硫酸钠

EDTA：乙二胺四乙酸

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