Validation of Standardized High-Performance Thin-Layer Chromatographic Methods for Quality Control and Stability Testing of Herbals

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In herbal medicinal products the entire herbal drug or an herbal drug preparation is regarded as the active pharmaceutical ingredient, regardless of whether constituents with defined therapeutic activity are known. In quality control and stability testing of herbal medicinal products, fingerprint chromatograms are used as powerful tools to evaluate and compare the composition of compounds in such products. To fulfill the International Conference on Harmonization and Good Manufacturing Practice-based regulatory requirements in pharmaceutical quality control, chromatographic fingerprint analysis needs to be validated. Based on a standardized methodology, this paper provides a comprehensive concept for evaluating validation parameters for planar chromatographic fingerprinting by considering the stationary phase, sample application, developing solvent, chromatogram development, plate labeling, derivatization, documentation, and chromatographic equipment. Validation parameters addressed include stability of the analyte, selectivity, robustness testing, and method reproducibility.

In the United States, plant-derived products with health claims are sold as herbal dietary supplements, so-called "botanicals," for which so far only few official standards have been established for quality control. In Europe, plant-derived products for medicinal use are under the legal control of the approval procedure for drugs and medicinal products. Thus, for production and testing of such products, Good Manufacturing Practice (GMP) applies, and quality testing must meet International Conference on Harmonization (ICH) standards with respect to specification, validation, and stability testing. Quality control of herbal medicinal products (HMPs) is a challenging analytical task, because the entire herbal drug or herbal drug preparation is regarded as the active substance, regardless of whether constituents with defined therapeutic activity are known. Visualization (so-called fingerprinting) of the entire pattern of compounds present in an herbal drug or preparation is therefore fundamental in the quality and stability testing of HMPs and the respective starting materials. Fingerprint chromatograms with a visible pattern of bands provide fundamental data. Ideally, to achieve maximum information, several suitable methods should be used for fingerprinting different groups of constituents, which possibly originate from different biosynthetic pathways. Semiquantitative analyses are typically used to demonstrate the consistency and stability of materials. With the help of quantitation marker compounds, the production and composition of HMPs are controlled. High-performance thin-layer chromatography (HPTLC, planar chromatography) is an ideal tool for the analysis of herbals and offers several advantages (1). In its traditional form, thin-layer chromatography (TLC) has a long record in almost all pharmacopeias for its use in the identification of botanical raw materials. However, HPTLC is not limited to identification. It can also be used for control of batch-to-batch consistency in the stability testing of herbals and for purposes of control throughout the entire manufacturing process of HMPs. Typical tasks include the following: identification and quality control of herbal drugs as raw materials; quality control of herbal drug preparations, e.g., comminuted or powdered herbal drugs, extracts, tinctures, fatty or essential oils, expressed juices, or processed resins or gums; and monitoring of intermediate stages of the manufacturing process, as well as quality control of the finished product.

In the European Union (EU), quality standards and specifications for herbal drugs, their preparations, and HMPs are regulated in the guidelines "Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Herbal Drugs, Herbal Drug Preparations, and Herbal Medicinal Products" (2) and "Note for Guidance on Quality of Herbal Medicinal Products" (3). The use of TLC methods for purposes of quality control is explicitly permitted in those guidelines.

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As primary sources for TLC fingerprint methods for identity control of HMPs and starting materials such as plant material, crude drugs, and extracts, the *European Pharmacopoeia*, the *U.S.Pharmacopeia/National Formulary*, the *British Herbal Pharmacopoeia*, and the *Chinese Pharmacopoeia* may be consulted. Although none of these methods represent the state-of-the-art in modern HPTLC, they still have the status of validated methods.

For any other TLC/HPTLC method used in quality testing, the EU guidelines "Note for Guidance on Validation of Analytical Methods: Definitions and Terminology" (4) and "Note for Guidance on Validation of Analytical Procedures Methodology" (5) require validation data. Validation is also required if a pharmacopeial method is optimized or adapted for specific tasks in quality control or if a method is taken from other reliable sources such as the American Herbal Pharmacopeia (AHP), the Indian Herbal Pharmacopoeia, or Plant Drug Analysis: A Thin Layer Chromatography Atlas (6). Of all the sources, only the AHP monographs feature modern HPTLC methods for identification. In the United States, for botanicals with curative claims, a draft guidance of the U.S. Food and Drug Administration (FDA) points in the same direction, placing such products under tight regulations for quality control (7); a similar approach for dietary supplements from the Food Advisory Committee Dietary Supplement Working Group of the FDA for ingredient identity records and retention was initiated recently (8). An excerpt from an internal White Paper on Standardization drafted by the American Herbal Products Association (9) points in the same direction.

According to the guidelines mentioned above, qualitative HPTLC must be validated only with regard to selectivity. However, especially in connection with stability testing, data concerning robustness and reproducibility of methods are necessary as well.

It is the aim of this paper to present a standardized approach to modern HPTLC methodology, which can be applied to most tasks in the analysis of herbals. The central focus is on qualitative methods for quality control and stability testing. For the first time a proposal for the consequent validation of standardized qualitative methods is outlined and discussed on the basis of practical examples.

Standardized Methodology for Qualitative and Semiquantitative HPTLC

General

Qualitative and semiquantitative HPTLC methods are among the most suitable methods available for fingerprint analysis in the quality control of herbals. The technique features several advantages over other chromatographic fingerprint techniques (e.g., liquid chromatography, gas chromatography): many samples can be analyzed in parallel, and the separation can be optimized for a certain group of compounds in a very complex mixture of natural origin, representing a wide polarity range. By applying normal-phase HPTLC, the compounds of interest can be analyzed in the central region of the chromatogram. The more hydrophilic compounds in the sample remain at the application position, while the more lipophilic compounds migrate with the solvent front. However, all components remain detectable; thus, HPTLC fingerprints always represent the entire mixture of analytes applied for testing. Various selective detection methods are available in HPTLC, providing additional information about the identity of the analytes through derivatization and visualization. The most striking feature of HPTLC is the possibility of presenting and communicating the chromatographic result as an electronic image. Modern instrumentation enables image documentation according to GMP/Good Laboratory Practice (GLP).

To enhance selectivity, many parameters can be used to affect the chromatographic results. Most of the parameters can be varied independently. This makes HPTLC a technique of very high flexibility. However, it must also be acknowledged that ignoring the importance of any of the parameters affecting chromatography will result in difficulties with the reproducibility of a given method. As an open system, HPTLC is also susceptible to environmental factors, e.g., temperature, humidity, oxygen, and light.

To achieve reliable, reproducible, and valid results, HPTLC methods should be set up according to a highly standardized methodology. The following parameters should be considered.

Stationary Phase

HPTLC can be regarded as the most advanced form of modern TLC. It uses HPTLC plates featuring small particles with a narrow size distribution. As a consequence, very homogenous layers with a smooth surface can be obtained. The developing distance (typically 6 cm) and developing time (7–20 min) are significantly decreased, whereas the separating power can even be increased. Because of the smaller size of the plates, the developing chambers in HPTLC are also smaller. Therefore, less elution solvent is needed, and the disposal problem is smaller.

In quality control and stability testing of herbals, HPTLC plates coated with Silica Gel 60 F254 are used predominantly. However, for special analytical problems, such as fingerprinting of very polar or nonpolar constituents or separation of similar components, other plates such as reversed-phase or chemically bonded phases could be appropriate. Chiral stationary phases are available for investigation of optically active compounds.

All plates need to be stored under appropriate conditions. Before use, they should be inspected under white and UV light to detect damage and impurities in the adsorbent. It is advisable to prewash the plates to improve the reproducibility and robustness of the results. Generally plates are predeveloped with methanol, but mixtures of methanol and ethyl acetate or even the mobile phase of the method may be used. The plates are developed to the upper edge over a defined period of time, normally dried at 120°C for 20 min (for methanol), reconditioned to ambient temperature, and equilibrated with relative humidity in a suitable dust-free container. The direction of solvent migration should be marked with a soft pencil on the upper edge of the plate.

Sample Application

The sample can be applied manually or automatically. To obtain optimum resolution and sensitivity, the spreading of the sample spot or band during application must be as small as possible. To handle the required small volumes, automated sample application is preferred. The 2 principal technical solutions for transfer of samples onto the layer are contact spotting and spray-on application. Because the sample solvent during spot application always produces circular chromatography, which may limit the achievable resolution during development of the plate, spray-on application is preferred for the best separation. Typically, samples are applied on HPTLC plates as 8 mm bands, 8 mm from the lower edge of the plate, with the first application position 15 mm from the left edge of the plate.

Preparation of the Developing Solvent

The developing solvent system is chosen according to the adsorbent material used as the stationary phase and the chemical structure of the analytes to be separated. Generally, solvents with low toxic potential are preferred to lessen problems of solvent disposal. Solvent systems should be as simple as possible in their composition. Only solvents of known quality should be used. The solvent mixture can be optimized according to the procedure described by Reich and Blatter (1). Accurate volumetric measurements of the components of the developing solvents must be performed separately and precisely in adequate volumetric glassware before mixing and re-equilibration to ambient temperature. Provided that the mixtures are stable, larger volumes of developing solvents should be prepared to decrease volumetric errors.

Development of the Chromatogram

HPTLC plates are developed in flat-bottom chambers, twin-trough chambers, or horizontal-development chambers. In general, saturated twin-trough chambers fitted with filter paper offer the best reproducibility. The use of unsaturated chambers should be avoided because of decreased reproducibility, and often bent solvent fronts. In any case, the chamber type and configuration must be specified in the method. Usually, the developing distance is 60 mm from the application position. The plate is positioned in the front trough with the stationary phase facing the inside of the chamber. Longer developing distances do not improve separation, but they do produce more diffused bands.

Plate Labeling

According to GMP/GLP, the plates should be labeled unambiguously for identification. In general, a soft pencil or fluorescent pen is used to write the number in the upper right corner. The label should include the project number, the date of analysis, and the consecutive plate number. If GLP plates are used, the laser-edged number of the plate serves as the number for identification.

Derivatization

In quality control and stability testing of herbals, derivatization is necessary in most cases to visualize the analytes of interest and to generate specific fingerprints.

Most substances can be visualized by using nonspecific derivatizing reagents, for example, 10% sulfuric acid in methanol. If a specific substance or group of substances is to be detected, specific derivatization can be performed by using selective reagents, e.g., dimethylaminocinnamcaldehyde reagent for detecting procyanidins, or ninhydrin reagent for detecting nitrogen compounds.

If possible, derivatization reagents with low toxic potential are preferred. Before derivatization, the plates need to be dried for a defined period of time under defined conditions. Derivatization can be performed either by immersing the plates or by spraying the plates with a suitable reagent. For better reproducibility, immersion is the preferred derivatization technique. To induce or optimize the derivatization reaction, it may be necessary to heat the plates. Also, the conditions and time must be specified for this step.

Documentation

Each developed plate is documented under UV light at 254 nm, UV light at 366 nm, and white light. If a type of light does not produce usable information, that fact must be documented. If a plate is derivatized, images are taken before and after derivatization. Image labels should include the plate number as well as the derivatization and the illumination mode.

Video and digital documentation systems are widely used to document high-performance thin-layer chromatograms. The advantages of modern electronic documentation systems are instant images of the chromatograms/fingerprints, which can be easily edited, archived, and evaluated. With suitable software the documents can be saved and stored in compliance with GMP/GLP. Furthermore, the electronic image is available for densitometric evaluation, yielding analog curves of the chromatogram as well as semiquantitative and quantitative results.

Specific Requirements for Stability Testing for HMPs

In the case of HMPs and the respective starting materials, stability testing is a field of increasing importance because, in the EU, the ICH guidelines on stability testing also apply to plant-derived medicinal products. However, HMPs have particular characteristics, which must be considered appropriately, as stated in the draft guideline "Stability Testing of Products" Herbal Medicinal proposed by Forschungsvereinigung der Arzneimittel-Hersteller (FAH) expert group on stability testing. Thus, "the purpose of stability testing is to provide evidence on how the quality of the herbal drug preparation or the herbal medicinal product varies with the time under the influence of environmental factors, especially such as temperature, humidity, and oxygen, and enables recommended storage conditions, retest periods, and shelf-lives to be established" (10).



Figure 1. Stability testing of St. John's wort extract over a period of 18 months (11).

With regard to stability testing, the fact that the herbal drug preparation in its entirety is regarded as the active principle is of great importance. Thus, a mere determination of the stability of individual substances (markers) is not sufficient. It must also be shown that the other components of the herbal drug preparations remain constant in their proportional content.

In this context, HPTLC is an interesting and powerful analytical technique. The stability of herbal drugs, herbal drug preparations, and HMPs is investigated by comparing the pattern of constituents of the entire extract, not only a part of it, as a fingerprint. In particular for stability testing, it is necessary to optimize, rigorously standardize, and validate both the chromatographic procedure and the appropriate documentation to obtain comparable fingerprint chromatograms. For an example, *see* Figure 1.

Validation of Qualitative Methods

Method validation cannot be separated from method development, because the performance parameters are finally acceptable only if the performance achieved passes validation. Validation, development, and optimization may be considered as an iterative process (12).

Validation starts with the preselected method. The first step, also called prevalidation, consists of examining the stability of the analyte; the second step is proofing the selectivity of the method and demonstrating appropriate robustness and reproducibility. The validation procedure should be based on a validation master plan, which specifies the acceptance criteria for each validation point. The results are documented in a validation protocol.

The validation of qualitative HPTLC fingerprint methods intended for use in quality control and stability tests of herbals should include the following validation parameters: stability of the analyte, selectivity, robustness, and reproducibility.

Stability of the Analyte

The stability of the analyte should be examined before chromatography (in solution and on the plate) and during chromatography. For that purpose the solution of analyte is prepared twice and applied 4 times on the plate.

A solution of the analyte is prepared and immediately applied on the plate. This solution and the plate are stored under ambient conditions for a defined period of time. After that defined period of time, a second solution of the analyte is prepared and immediately applied on the plate next to the previously applied sample. The set is used to investigate the stability of the analyte on the plate. Next, on the same plate, both solutions of the analyte (the solution stored over a defined period of time and the freshly prepared solution) are applied. This set evaluates the stability of the analyte in solution. After development of the HPTLC plate, the 4 fingerprints are compared. If the fingerprints are similar in all details, the analyte is stable in solution as well as on the plate during the tested time interval.

With a 2-dimensional HPTLC experiment, the stability of the analyte during chromatography is examined. For that purpose the solution of the analyte is applied as a spot in the lower left corner of the HPTLC plate. After development according to the method specifications (first dimension), the plate is thoroughly and appropriately dried. After turning the plate 90°, development in the second dimension is performed under similar conditions with fresh mobile phase. Before development in the second dimension, appropriate re-equilibration time is important if drying has not been performed under ambient conditions. The analyte is stable during chromatography if all components line up on the diagonal that connects the application position with the intersection of the 2 mobile phase fronts (Figure 2). Any spot appearing above or below the diagonal indicates instability of the corresponding analyte during chromatography. If the stability of the analyte is not satisfactory, the method must be optimized/changed, e.g., by development in the dark, with antioxidants, or with a different solvent system.

Selectivity

The selectivity of identity tests for herbal drugs and batch release can be demonstrated by comparing the fingerprint of the sample(s) with the fingerprints of authentic herbal drug material (e.g., voucher specimen), or reference substances of active pharmaceutical ingredients (in the case of herbal drugs with known therapeutically active compounds), or marker substances (in the case of herbal drugs with unknown therapeutically active compounds) and herbal materials that are known as adulterants.

All samples are chromatographed in parallel on the same HPTLC plate. The method is selective if the fingerprints enable a proper identification of the herbal drug. Adulterants must be distinguishable. In this regard not only the pattern of the bands but also their intensity may provide important information. Relevant bands of the sample(s) and the authentic herbal drug should be similar with respect to number, color,



Figure 2. Stability of the analyte examined in a 2-dimensional chromatogram of stinging nettle leaves. Stationary phase: HPTLC plates coated with Silica Gel 60 F254. Developing solvent: ethyl acetate-methanol-water-formic acid (50 + 4 + 4 + 2.5).

intensity, and relative position. Relevant bands are defined as bands representing accepted marker substances, compounds contributing to efficacy, or efficacious compounds. The fingerprints of the adulterants should be distinctly different from the fingerprints of the sample and the authenticated drug. The bands of the reference or marker compounds must be present in the fingerprint of the sample(s) and the authenticated drug. They should correspond in their relative positions and colors. However, fingerprints of herbal drugs and herbal drug preparations could be different, at least in the very lower and upper R_f range. As an example, *see* Figure 3. The plant material applied on track 2 does not comply with the specification although it was labeled as Black Cohosh Rhizome. The fingerprint is not similar to the fingerprint of the authenticated herbal drug on track 7.

The selectivity of identity tests in process control and batch release of herbal drug preparations (extracts) can be established by comparing the fingerprint of the sample(s) with the fingerprint of authentic herbal drug material (e.g., voucher specimen or plant material released and used for extract preparation), or authentic herbal drug preparation (reference extract), or reference substances of active pharmaceutical ingredients (in the case of herbal drugs with known therapeutically active compounds), or marker substances (in the case of herbal drugs with unknown therapeutically active compounds), and excipients used for extract preparation.

The method is selective if the fingerprints of the sample(s) and the authentic herbal drug preparation are similar with respect to number, color, intensity, and relative position of the bands. The bands of the reference or marker substances should be present in the fingerprints of the sample and the authentic herbal drug preparation and/or authentic herbal drug material. They should correspond in color and relative position. The

fingerprints of the authentic herbal drug and the authentic herbal drug preparation should be similar to the fingerprint of the sample except for the bands of the excipients. The bands of the excipients should not interfere with detection of the fingerprint of the sample and the authenticated extract. For an example, *see* Figure 4. The herbal drug, the intermediate product of extract preparation, and the final extract show fingerprints that are similar in number, position, and intensity of the zones. The excipients in lanes 5 and 6 do not interfere with the detection of the fingerprints of the herbal drug and the extract.

The selectivity of identity tests, in process control, and batch release of HMPs can be proven by comparing the fingerprint of the sample(s) with the fingerprint of authentic herbal drug preparation (type extract), or extract released for the HMP preparation, and reference substances of active pharmaceutical ingredients (in the case of herbal drugs with known therapeutically active compounds), or marker substances (in the case of herbal drugs with unknown therapeutically active compounds), and excipients used for the preparation of the HMP.

The method is selective if the bands of the reference or marker substances are similar in the fingerprints of the authentic herbal drug preparation and the HMP with respect to color and relative position. Their detection should not be disturbed by the excipients.

The fingerprints of the authentic extract material, or the extract material released for production, and the HMP should be similar with respect to number, color, intensity, and relative position of the bands, except for the bands of the excipients. For example, *see* Figure 5. The fingerprints of the extract and the HMP are similar in number, color, and relative position of the bands. The excipients do not interfere with the detection of the fingerprints in the chromatograms.



Figure 3. Selectivity of the identity test for Black Cohosh Rhizome drug. Method: Identification of Black Cohosh Rhizome (13). Track assignment: 1: adulterant of Black Cohosh Rhizome, 2: Black Cohosh Rhizome, 3 and 4: adulterants of Black Cohosh Rhizome, 5: reference actein, 6: reference 23-epi-26-deoxyactein, 7: Black Cohosh Rhizome authenticated drug.



Figure 4. Selectivity of identity test for Black Cohosh Rhizome extract. Method: Identification test of Black Cohosh Rhizome (13). Track assignment: 1: Black Cohosh Rhizome authenticated drug, 2: intermediate product of extract preparation, 3: extract, 4: reference, caffeic acid, 5 and 6: excipients.

Robustness and Reproducibility

The overall testing for robustness and reproducibility of a method may be different for individual HMPs and the respective starting materials and/or laboratory environments. It must address any specific problem typically encountered for a given herbal in the context of the intended use of the method, e.g., identification, in-process control, batch release, or stability testing.

Reproducibility

Because HPTLC methods still include several manual operations, which can lead to differences and even mistakes in handling, the determination of reproducibility in a collaborative test is very important. A reproducible chromatographic method requires an explicit, precise, and detailed written description of the entire procedure. All persons involved in the analysis must be well trained and familiar with all technical terms and regulations.

The method is reproducible if the results obtained by all analysts are similar within the acceptance criteria.

Robustness

The capacity of HPTLC methods to remain unaffected by small deliberate variations in several parameters, which may occur during long-term use in one laboratory, is evaluated during robustness testing. If methods are affected by variations in analytical conditions, they might not be appropriate for the intended use, e.g., stability testing. If robustness problems are detected and method optimization fails to resolve them, at least the critical parameter(s) has been identified and can be appropriately controlled in the future. In this respect, a system suitability test (e.g., a resolution test), that can be performed on each chromatogram, will be a useful tool. The following critical parameters in HPTLC should be evaluated in a robustness test.

Humidity and temperature.—The relative humidity and temperature of the environment can significantly affect HPTLC results. Typically, R_f values decrease with increasing temperature and decreasing humidity (Figure 6). Relative humidity and temperature are interdependent. If possible (avail-

able climate control unit), the influence of humidity at constant temperature should be investigated. Generally, the effects of humidity changes can be evaluated by preconditioning the HPTLC plate before chromatography over solutions (sulfuric acid, saturated salts), which establish an atmosphere of defined humidity. If no climate control unit is available, the analysis should be performed in an air-conditioned laboratory.

Chromatogram development.---It may be useful to com-pare the chromatograms developed in different chambers (horizontal development chamber/twin-trough chamber) and with different chamber saturation (saturated or unsaturated). Generally, with a given method only one chamber type in a specified configuration will lead to a valid result. However, in some cases, development of HPTLC plates in the saturated twin-trough chamber and in the saturated horizontal development chamber lead to similar results, so that it is possible to use both chambers. From a practical point of view, it is often more important to evaluate the effects of changes in the saturation time of ± 10 min. Another important parameter to be investigated is the developing distance; ± 5 mm of the specified developing distance should be investigated during a robustness test. If the resulting fingerprints are not similar within the acceptance criteria, the relevant development parameter must be explicitly specified in the operating protocol and procedure.

Stability of the developing solvent.—The stability of the developing solvents, which are susceptible to degradation and/or chemical reactions, should be evaluated. Mixtures of esters, alcohols, and/or acids are known to be unstable. The fingerprints obtained with a solvent mixture stored over a defined period of time must be similar to those generated with a freshly prepared solvent mixture. Otherwise, a shelf-life must be defined, or the developing solvent must be freshly prepared.

Derivatization.—For the derivatization procedure, all times and parameters, such as drying of the developed plate, amount and concentration of reagent, immersion time, drying after derivatization, heating (time and temperature), waiting times before detection, etc., should be investigated in the robustness test and, if necessary, clearly specified as important to be maintained exactly.



Figure 5. Selectivity of identification testing of an HMP containing extract of Black Cohosh Rhizome. Method: Identification test of Black Cohosh Rhizome (13). Track assignment: 1: reference, actein, 2: reference, 23-epi-26-deoxyactein, 3: authenticated extract, 4: HMP containing extract of Black Cohosh Rhizome, 5: excipients.



Figure 6. Influence of humidity and temperature on the fingerprint of authenticated Black Cohosh Rhizome drug. Method: Identification test of Black Cohosh Rhizome (13). Track assignment: 1: 27°C, 52% humidity; 2: 28°C, 50% humidity; 3: 30°C, 48% humidity; 4: 31°C, 47% humidity; 5: 32°C, 46% humidity.

Some derivatizing reagents lack stability. Therefore, it is necessary to determine the shelf-life of the reagent under specified storage conditions. The fingerprints obtained during the shelf-life of the reagent mixture must be similar; otherwise the reagent must be freshly prepared.

Adsorbent.—Batch-to-batch consistency of the adsorbent specified in the method should be evaluated. This is particularly important for chemically bonded phases. If the finger-prints on plates of different batches are not similar, or if the backgrounds on different plates are not similar, a number of plates from one batch or consistent batches sufficient for completion of the study must be stored under defined conditions.

It may be of interest to compare the performance of plates specified in the method with that of plates with the same specifications from a different manufacturer.

The operating protocol and procedure should allow only the use of batches showing similar performance.

Conclusions

The use of suitable equipment is one of the requirements for unlocking the full power and using all the advantages of modern HPTLC for quality control and stability testing of HMPs. To maximize the reproducibility and reliability of the results, it is also necessary to use a standardized methodology based on a theoretically and practically sound foundation. All parameters that can influence the chromatographic result must be explicitly defined in the method description. Also qualitative HPTLC fingerprint methods should be consequently validated; this means that the evaluation of selectivity, reproducibility, and robustness have to be established particularly before use in stability testing.

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