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# Detection Technique for Determination of Residual Solvents in the API Tacrolimus

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## Abstract

**To establish the detection technique for determination of the residual solvents ethanol, ethyl acetate, butyl acetate, N-hexane or cyclohexane, toluene, acetonitrile and acetone popularly used as the solvents for preparation of the active pharmaceutical ingredient (API) tacrolimus as reported, in order to control the quality of the API, to be in line with the requirement as stated in ICH Q3C.**

## Keywords

**Tacrolimus, Ethanol, Ethyl acetate, Butyl acetate, N-hexane, Cyclohexane, Toluene, Acetonitrile, Acetone, Residue, Organic solvent.**

## 1. INTRODUCTION

Tacrolimus is a well-known active substance. It is a calcineurin-inhibitor immunosuppressant. It is indicated for the prophylaxis or therapy of organ rejection in adult and pediatric patients receiving allogeneic liver, kidney, heart, lung, pancreas, small intestine, or bone marrow transplants, in combination with other immunosuppressants. It also has curative effect on Autoimmune diseases (AID), indicated for Vernal keratoconjunctivitis (VKC), Atopic dermatitis, Rheumatoid arthritis, Lupus nephritis, Ulcerative colitis, Interstitial pneumonia associated with polymyositis/dermatomyositis, and Myasthenia gravis.

Tacrolimus belongs to twenty-three ring macrolide antibiotics, it is usually prepared by traditional fermentation method, then isolated and purified to obtain high purity active substance via purification technology. The typical isolation and purification technology as reported[1] [2] [3] [4] [5] include extraction after sheet frame filtration, extraction via ceramic membrane, exaction on fluidized bed, decoloration and adsorption on macroporous resin, solvents extraction, normal/reversion phase silica gel resin chromatography, preparation column isolation, crystallization and so on. All these process techniques need to use plenty of solvents, and the most popular solvents include ethanol, ethyl acetate, butyl acetate, N-hexane or cyclohexane, toluene, acetonitrile and acetone.

Tacrolimus was initially born on April 2, 1993 in Japan, which has been listed on Ph. Eur.[6], JP[7] and USP[8]. The tacrolimus monograph on all the three pharmacopeia do not describe the analytical procedure for determination of residual solvents, and the general chapter requires that the category and limit of the residual solvents should comply, i.e. in line with the requirement of ICH Q3C[9]. In order to effectively control the residual solvents in the API, ensuring the security of pharmaceutical, the text provides method and idea on how to establish the method for determination of residual solvents in the API obtained from aforementioned typical processes as reported, so as to provide method accordance for the quality control of the API tacrolimus, based on the actual analysis experience, also by reference to related methods as reported[10] [11] [12] [13] [14].

## 2. METHOD DESIGN AND DEVELOPMENT

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Tacrolimus API is usually in the form of Tacrolimus monohydrate, there is neither acidic nor alkalic base on the molecule. As reported on IF usage instruction for the Innovator drug Prograf® injection, its partition coefficient is more than 1000 (1-octanol/water), the US FDA review report of Prograf describes that it is a lipophilic molecule, practically insoluble in water and n-hexane. Aforementioned three pharmacopeia describe that the substance is very soluble in methanol, anhydrous ethanol and dichloromethane, freely soluble in DMF, acetone and 95% ethanol, soluble in 96% ethanol, practically insoluble in water and n-heptane.

## 2.1. Solvents property

Solvents property[15] is shown in Table 1:

**Table 1.** Solvents property

| Solvent | Boiling point | Polarity |
|---|---|---|
| Acetone | 57°C | Strong |
| Methanol | 65°C | Strong |
| N-hexane | 69°C | Non or weak |
| Ethyl acetate | 77°C | Medium |
| Ethanol | 79°C | Strong |
| Cyclohexane | 81°C | Non or weak |
| Acetonitrile | 82°C | Medium |
| Toluene | 111°C | Medium |
| Butyl acetate | 126°C | Medium |

## 2.2. Selection of detector type

The solvents to be determined belong to hydrocarbons, without any strong electronegativity substance, eg. halogen, so flame ionization detector (FID) can be selected. Detection temperature is usually more than column temperature and not less than 150°C, and it is proposed the detection temperature is 250~300°C, based on the actual analysis experience and with reference to the literatures.

## 2.3. Selection of solvent for dissolution of sample

The selected solvent should have high purity (Chromatographic pure is recommended), good solubility for sample and a little higher boiling point than that for the solvents to be determined. N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), dimethyl sulfoxide (DMSO), N,N-dimethylacetamide (DMA), etc. are commonly used solvents. Based on the API solubility and the property of the solvents to be determined, it is considered DMF, NMP and DMA are suitable to dissolve the sample, after screening test, NMP is proposed as the solvents for dissolution of sample.

## 2.4. Selection of injection mode and procedure

Gas chromatography (GC) is usually used to determine the residual solvents, GC has two injection modes, headspace injection and direct injection, and headspace injection is more popular for it has less pollution to chromatographic column and detector. The headspace vial equilibration temperature is selected mainly based on the boiling point of the solvents to be determined. It is proposed the vial equilibration temperature is 80°C ~ 105°C, the equilibration time is 30 ~ 45min, and injection port temperature of the FID is 150°C ~ 220°C, based on the

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boiling point of the solvents to be determined, the actual analysis experience and with reference to the literatures.

## 2.5. Selection of chromatographic column

Chromatographic column has two types, packing column and capillary column, packing column is used for isolation of macromolecule, biomolecule, organic substances and so on for their quantitative analysis, while capillary column is used for isolation of small molecule compound, gas and volatile substance and so on. For this case is to determine residual solvents, capillary column is proposed.

The right column is selected mainly based on the polarity of the solvents to be determined, and the aspects listed below should be considered:

a. Stationary phase category: capillary stationary phase has four categories, polarity, medium polarity, weak polarity and non-polarity. For the method refers to many solvents, it is proposed to try with a medium polarity column with priority, then with polarity and weak polarity ones. And it is proposed to use a medium polarity column (6% cyanopropylphenyl-94% dimethylpolysiloxane or similar polarity stationary phase), which can obtain a good resolution.

b. Column length: generally if less solvents are involved and the matrix has no interference with the test, it is preferred to select a short column in order to shorten the running time, otherwise, if many solvents are involved, or the resolution is not good, or the matrix has interference with the test, a longer column may be adopted. It is proposed to use a 30m capillary column which can effectively isolate the solvents in a short time, with good resolution and without any matrix interference, based on the actual analysis experience and with reference to the literatures.

c. Inner diameter: the common size of inner diameter includes 0.25mm, 0.32mm or 0.53mm. Generally when the size is small, the column efficiency is higher, the peak shape is good, but the column pressure may increase. So a suitable size need to be selected based on the resolution between each solvent, when the resolution is not good, a little smaller size may be adopted. It is proposed to use a column with inner diameter at 0.53mm or close to this size which can effectively isolate the solvents, with good resolution and shape, and acceptable column pressure, based on the actual analysis experience and with reference to the literatures.

d. Stationary phase thickness: the common thickness is 0.1μm~5.0μm. The stationary phase thickness may affect the sample carrying capacity and the retention time of the substances to be determined. Suitable thickness may be selected to postpone the peak eluted too early, increasing the thickness may enlarge the column capacity. For the case, many solvents are involved, it is proposed to select a thicker stationary phase to improve the peak shape. So using a column with stationary phase thickness at 3.0μm or close to this size can obtain suitable retention time, resolution and running time, and good shape, based on the actual analysis experience and with reference to the literatures.

## 2.6. Carrier gas flow rate

Nitrogen is often used as the carrier gas, and the flow rate is determined based on the chromatographic column inner diameter, the recommended flow rate is adopted at priority upon different inner diameter, for example, as to the inner diameter of 0.53μm, the recommended flow rate is 3.0ml/min~50ml/min. In case the flow rate needed to be modified, it is reminded that decreasing flow rate to improve resolution may result in the decreasing of the column efficiency, which may reduce the sensitivity of the method, a suitable balance point should be found out to determine the best flow rate.

## 2.7. Heating mode

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As to gas chromatography, heating mode has significant effect on the resolution. Heating mode includes isocratic heating mode and gradient heating mode. Isocratic heating mode is used for the sample with boiling point range not so wide, and gradient heating mode is used for the sample with complex components, containing high boiling point and low boiling point components. In this case, the components are complex, gradient heating mode is proposed. The initial parameters may be set as per that stated on the method II of General chapter 0861, Chinese Pharmacopeia edition 2020[16], volume IV, then adjust the parameter if necessary to make sure an expected retention time of each solvent is obtained, for example, adjust the retention time of the first solvent peak by increasing or decreasing the initial temperature and adjust the heating rate to make the resolution between each solvent meet the requirement. The initial temperature for this case is proposed as 40℃~72℃, based on the actual analysis experience and with reference to the literatures.

## 3. METHOD AND RESULT

The text selects a group of four solvents ethanol, acetone, ethyl acetate and n-hexane based on a process technique as reported to perform the test, via headspace injection gas chromatography by external standard method, with the method and validation information summarized below,

### 3.1. Chromatographic condition

Detector: A hydrogen flame-ionization detector.

Column: DB-624, 30m×0.53mm, 3.0μm, or similar polarity column.

Column temperature: Maintain the temperature at 50°C for 5 minutes after injection, raise to 100°C at a rate of 10°C per minute, and maintain at 100°C for 2 minutes, then raise to 220°C at a rate of 30°C per minute, and maintain at 220°C for 9 minutes.

Injection port temperature: 220°C.

Detector temperature: 270°C.

Carrier gas: Nitrogen.

Flow rate: 3.0mL/minute.

Split ratio: 10:1. (Note: The split ratio can be modified in order to optimize sensitivity.)

Headspace operating parameters:

Oven temperature: 105°C.

Vial equilibration time: 30 minutes.

### 3.2. Solution preparation

Accurately transfer 0.2g of sample to a headspace vial, add 2.0mL of blank (N-methylpyrrolidone (NMP)), seal immediately, and use the solution as the sample solution. Prepare a solution with about 0.5mg/mL ethanol, 0.5mg/mL acetone, and 0.5mg/mL ethyl acetate in the blank (equivalent to 5000ppm ethanol, 5000ppm acetone, and 5000ppm ethyl acetate in sample), transfer 2.0mL of the solution to a headspace vial, seal immediately, and use this solution as the reference solution.

### 3.3. Specificity and System suitability

Take the diluent (blank), sample solution and reference solution in item 2 of Method and result, perform injection under the chromatographic condition in item 1 of Method and result, and the record the chromatograms. The result demonstrated that the baseline separated well for each solvent, all the resolutions were more than 1.5, the plate number for each solvent was

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more than 5000, the matrix had no interference with the test, and the relative standard deviation (RSD) of each solvent content were all less than 10.0%.

### 3.4. Linearity

Prepare mixed solutions respectively with concentrations of LOQ, 50%, 70%, 100%, 120%, and 150% that of reference solution, perform two injections for each concentration under the chromatographic condition in item 1 of Method and result, and the record the chromatograms. Calculate regression equation with peak area response as Y coordinate, and concentration as X coordinate, and correlation coefficient γ. The result demonstrated that the correlation coefficient γ for all the solvents were more than 0.99, which showed that the linearity of the method was good.

### 3.5. Limit of quantitation (LOQ) and limit of detection (LOD)

Take the concentration at S/N of approximately 10 as LOQ, and take the concentration at S/N of approximately 3 as LOD, respectively prepare LOQ solution and LOD solution, perform two injections for each concentration under the chromatographic condition in item 1 of Method and result, and record the chromatograms. The result demonstrated that the LOQ of ethanol, acetone, ethyl acetate and n-hexane were 7ppm, 2ppm, 4ppm and 0.8ppm, respectively, with the RSD of each solvent peak area response less than 15.0%, and the LOD of ethanol, acetone, ethyl acetate and n-hexane were 2ppm, 1ppm, 1ppm and 0.3ppm, respectively, which showed that the sensitivity of the method was high.

### 3.6. Precision

Take the reference solution in item 2 of Method and result and prepare six sample solutions spiked by 10% of the reference solution, perform injection by different analyst on different date with different equipment under the chromatographic condition in item 1 of Method and result, and record the chromatograms. The results demonstrated that the intra-day RSD and inter-day RSD of residual solvent content were all less than 10.0% and 15.0% respectively, which showed that the precision of the method was good.

### 3.7. Accuracy

Take the reference solution in item 2 of Method and result. Accurately transfer 0.2g of sample to a headspace vial, respectively with 2.0mL of blank, 50%, 100%, and 150% concentration of reference solution, seal immediately and dissolve, three portions for each concentration. Perform injection for each concentration of sample solution spiked and reference solution under the chromatographic condition in item 1 of Method and result, and record the chromatograms. The results demonstrated that all the recovery were within the range from 70% to 130%, which showed that the accuracy of the method was acceptable.

## 4. DISCUSSION

For the limitation of test condition, the text did not take actual test on all of the mentioned solvents, just with a group of the solvents tested, and the detection technique of the rest ones can be reference to the exiting analytical procedures as reported. Something should be considered during parameter adjustment, a. if the resolution is not good, gradient heating mode is firstly considered to be optimized, with initial temperature or heating rate modified; b. if the sensitivity is too low, suitably increase the sample solution concentration, adjust split ratio, or increase headspace vial equilibration temperature and such measures; c. if the recovery is somewhat low or high, it is guessed that sample solution could have absorption to the solvents, the matrix could have enhancement to the test, or the solvents were volatile, so some measures

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can be tried to optimize the method, such as decreasing the sample solution concentration, or taking measure to reduce the solvent volatile etc.

From all above, the text discussed the determination technique for the residual amount of the nine organic solvents adopted by the manufacturing process of the API tacrolimus as reported, the proposed analytical procedure and operations provide details on how to establish the analytical procedure for simultaneous determination of the residual solvents in the API, and the actual procedure for each case should be further developed and validated based on its purpose in future.

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