

Mass Balance Method for Purity Assessment of Organic Reference Materials: for Thermolabile Materials with LC-UV Method

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A mass balance method for purity assessment of thermolabile organic reference materials was established by combining several techniques, including liquid chromatography with UV/VIS detector (LC-UV), Karl-Fischer (K-F) Coulometry, and thermal gravimetric analysis (TGA). This method was applied to three fluoroquinolones like enrofloxacin, norfloxacin and ciprofloxacin. LC-UV was used to analyze structurally related organic impurities based on UV/VIS absorbance spectra obtained in combination with LC separation. For all three organic reference materials, the UV/VIS spectra of the separated impurities were similar to that of the major component of the corresponding materials. This indicates that the impurities are structurally related to the respective reference material sharing common chromophores. Impurities could be quantified by comparing their absorbances at the wavelength of maximum absorbance (λ_{max}). The water contents of the reference materials were measured by K-F Coulometry by an oven-drying method. The total inorganic impurities contents were assayed from ash residues in TGA analysis with using air as a reagent gas. The final purities estimated from results of those analytical techniques were assigned as (99.91 ± 0.06) , (97.09 ± 0.17) and $(91.85 \pm 0.17)\%$ (kg/kg) for enrofloxacin, norfloxacin and ciprofloxacin, respectively. The assigned final purities would be applied to the reference materials which will be used as calibrators for the certification of those compounds in matrix CRMs as starting points for the traceability of their certified values to SI units.

Key Words : Purity assay, Thermolabile organic substance, LC-UV, K-F coulometry, TGA

Introduction

Chemical measurement society makes several endeavors to secure the reliability of measurement results by minimizing bias and increasing accuracy in measurement. In order to achieve the compatibility and reliability of measurement results, it has been emphasized that the application of a well-characterized calibrator with its traceability to the International System (SI) of Units^{1,2} plays critical roles. As an effort to link traceability, National Metrology Institutes (NMIs) establish and maintain the procedures for the purity assessment of organic reference material as their core competences.³⁻⁵ Differential scanning calorimetry (DSC) or quantitative nuclear magnetic resonance (qNMR) is possible techniques to directly determine the mass fraction of the principal components in organic substances.^{6,7} However, these techniques are still used as confirmatory methods due to their inherent limitation such as insolubility of impurity in the melt and requirement of well-characterized standards and further validation. The mass-balance approach is generally accepted to assay the purity of organic reference materials through measuring all detectable impurities obtained by several analytical techniques.^{8,9} Previous work performed by our group¹⁰ introduced a case study on the purity assessment of Endosulfan-II based on the mass-balance approach. Due to the volatility of Endosulfan-II, GC-FID had been applied to analyze the structurally related organic impurities. However, various organic reference materials are sensitive to

high temperature, making them in-compatible with GC analysis and requiring that LC techniques be applied to separate main component and organic impurities. Unlike GC-FID which can show a consistent linear response to all organic compounds, UV detector has a limitation as a universal detector. However, the utilization of a photodiode array (PDA) detector can detect and characterize the major compound and impurities based on the information from their UV absorption spectra. In most of the pure substances either chemically synthesized or purified from natural sources, organic impurities are inherently structurally related with the major components. As a result, those impurities in a reference material show similar UV/VIS absorption spectra with the main component, usually because they share common chromophores resulting in their absorbance at the wavelength of maximum absorbance (λ_{max}) having similar appearances. Therefore, the chromatographical purity of the reference material can be obtained by comparing the peak areas of the main component and all of the detected impurities at λ_{max} . If there are impurities which have different UV absorbance spectrum from the main compound, their contents in the material should be carefully evaluated based on the relative area ratio of impurities at each characteristic wavelength, otherwise external calibration may be required for significant impurities.

This article describes an impurity analysis approach using LC-UV for heat sensitive organic reference materials including enrofloxacin, norfloxacin and ciprofloxacin, and the

establishment of purity assessment based on a mass balance method including Karl-Fischer coulometry and TGA analysis. This type of purity assessment will be applied to primary reference materials for the certification of certified reference materials (CRM) of defined organic species with linking traceability to SI units.

Materials and Methods

Reagents and Chemicals. Pure substances, enrofloxacin (Lot No. 91111), norfloxacin (Lot No. 10214), and ciprofloxacin (Lot No. 00526), were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) with the purpose to use as primary reference materials to certify the related CRMs. Organic solvents for LC mobile phases were purchased from Burdick & Jackson (Muskegon, MI, USA). Other chemicals such as formic acid or ammonium acetate were purchased from Sigma-Aldrich (St. Louis, USA).

LC/UV Analysis. The purity of the reference materials were analyzed by Waters 2695 HPLC (Milford, USA) with Waters 2998 PDA (Milford, USA) as detector. Impurity analysis was conducted with XDB-Phenyl (I.D.: 3.0 mm, length: 150 mm, particle size: 3.5 μm) column and mobile phase consisted of acetonitrile and water containing 10 μM EDTA.¹¹ A gradient mobile phase program was developed to separate impurity peaks from main components for each reference material.

Mass fractions of the main component and detected impurities in the reference material were determined as following:

$$P_{\text{LC-UV}} = \frac{A_{\text{main}}}{A_{\text{main}} + \sum_i A_{\text{impurity},i}} \quad (1)$$

$$P_{\text{impurity},i} = \frac{A_{\text{impurity},i}}{A_{\text{main}} + \sum_i A_{\text{impurity},i}} \quad (2)$$

where, $P_{\text{LC-UV}}$ and $P_{\text{impurity},i}$ are the mass fractions of the main component and impurity i , respectively, A_{main} is the peak area of the main component, and $A_{\text{impurity},i}$, the peak area of impurity i on the LC chromatogram (at λ_{max}) of the reference material. Note that equations (1) and (2) do not include the contents of impurities undetected by the LC-UV analysis such as inorganic impurities and water.

Karl-Fischer Coulometry. The water contents of the organic reference materials were measured by a C30 Karl-Fischer (K-F) coulometric titrator connected with an oven-drying sample changer (Mettler-Toledo, Switzerland). To minimize the influence from ambient atmosphere, the K-F coulometry system and balance were placed and operated inside of a glove-box filled with nitrogen. Details of preparation and operation for measurement were described in our previous paper.¹⁰ The drying oven temperature was set up for each organic compound according to its melting point, for instance, 150 $^{\circ}\text{C}$ for all three reference materials. Six blank pre-dried empty vials and a single sample vial

were measured in the present study. The six blank runs were done for the measurement of the system blank (sum of water introduced from carrier gas and residual water in the vial). Water content in the sample was calculated as followings

$$P_{\text{water}} = (\text{ICEQ}/10.712 - \text{Time} \times \text{Drift} - \text{Blank})/m \times C \quad (3)$$

where, P_{water} is the mass fraction (kg/kg) of water in the reference material, ICEQ is a total consumed electric charge, which is converted to total detected mass of water (in μg). Time is a total K-F coulometric measurement time in minute (10 minutes in this case), Drift ($\mu\text{g}/\text{minute}$) is a systematic water content measured by K-F coulometric titrator before the analysis, Blank is the system blank measured from the six empty vials, m is a sample weight (in g) used for a K-F coulometric analysis, and C is a constant, 1×10^6 , to convert $\mu\text{g}/\text{g}$ unit to kg/kg.

Thermo Gravimetric Analysis (TGA). Ash analysis with a thermo gravimetric analyzer (TGA/DSC1, Mettler Toledo, Switzerland) was applied to measure the total amount of non-volatile inorganic impurities. Details of experimental set-up were as described previously.¹⁰ In this study, air was introduced into the TGA oven chamber to burn out organic compounds. An external micro-balance (MX5, Mettler Toledo, Switzerland) was used for weighing the alumina pan before and after loading sample and after sample run by the TGA. In this way, the weight of sample (W_{sample}) was loaded for measurement and the weight of total residual inorganic impurities ($W_{\text{non-volatile residues}}$) that remained on the pan were precisely determined by weighing-by-difference with the standard uncertainty of 0.7 μg . The content of total non-volatile impurities ($P_{\text{non-volatile residues}}$) can be estimated by following equation.

$$P_{\text{non-volatile inorganic residue}} = \frac{W_{\text{non-volatile inorganic residues}}}{W_{\text{sample}}} \quad (4)$$

Results and Discussion

Analysis of Structurally Related Organic Impurities by LC-UV. LC-UV analysis was carried out to determine the structurally related organic impurities in reference materials of three fluoroquinolones including enrofloxacin, norfloxacin and ciprofloxacin. Analyses of these fluoroquinolones were performed with XDB-Phenyl column and mobile phases which consist of acetonitrile and water with 10 μM EDTA. Prior to impurity analysis, various concentrations of the reference material solutions were prepared in methanol, and used to check the dynamic range (range of linear response) of the detector. Considering intensities of the main compound and detectable impurity peaks, LC/UV analysis was conducted with an appropriate concentration of sample solution within the dynamic range (in the case of enrofloxacin, 100 mg/kg in methanol). A typical chromatogram with UV absorption spectra (obtained by the PDA) for enrofloxacin and the separated peaks is shown in Figure 1. The chromatogram was drawn based on the absorbance at 278 nm. In

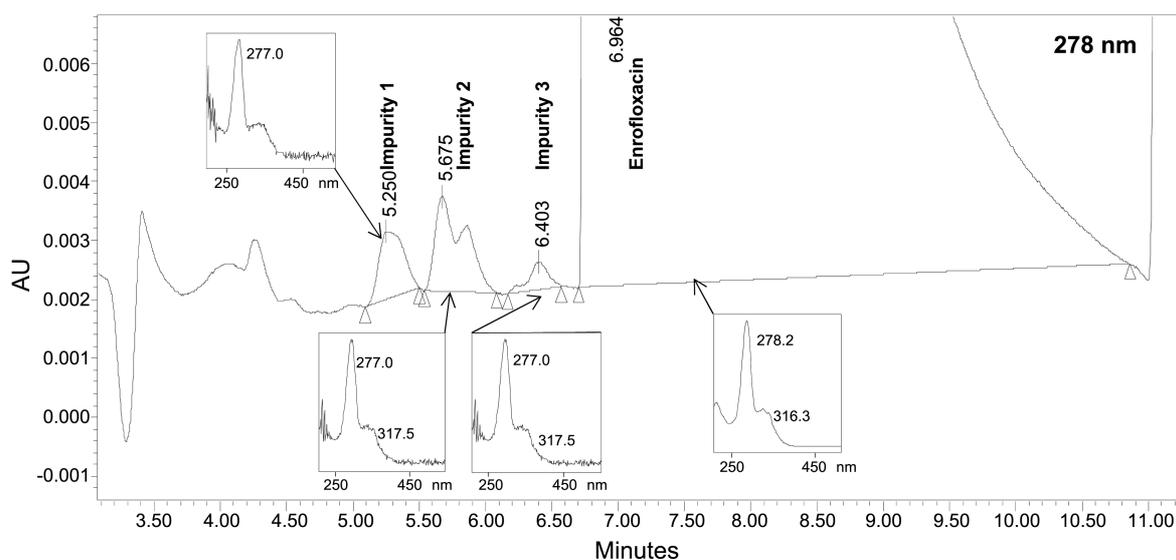


Figure 1. LC/UV chromatogram (278 nm) of the enrofloxacin reference material solution in methanol (100 mg/kg). Inserted UV spectra for the main component (enrofloxacin) and detectable organic impurities were obtained from the same LC run with photodiode array detector.

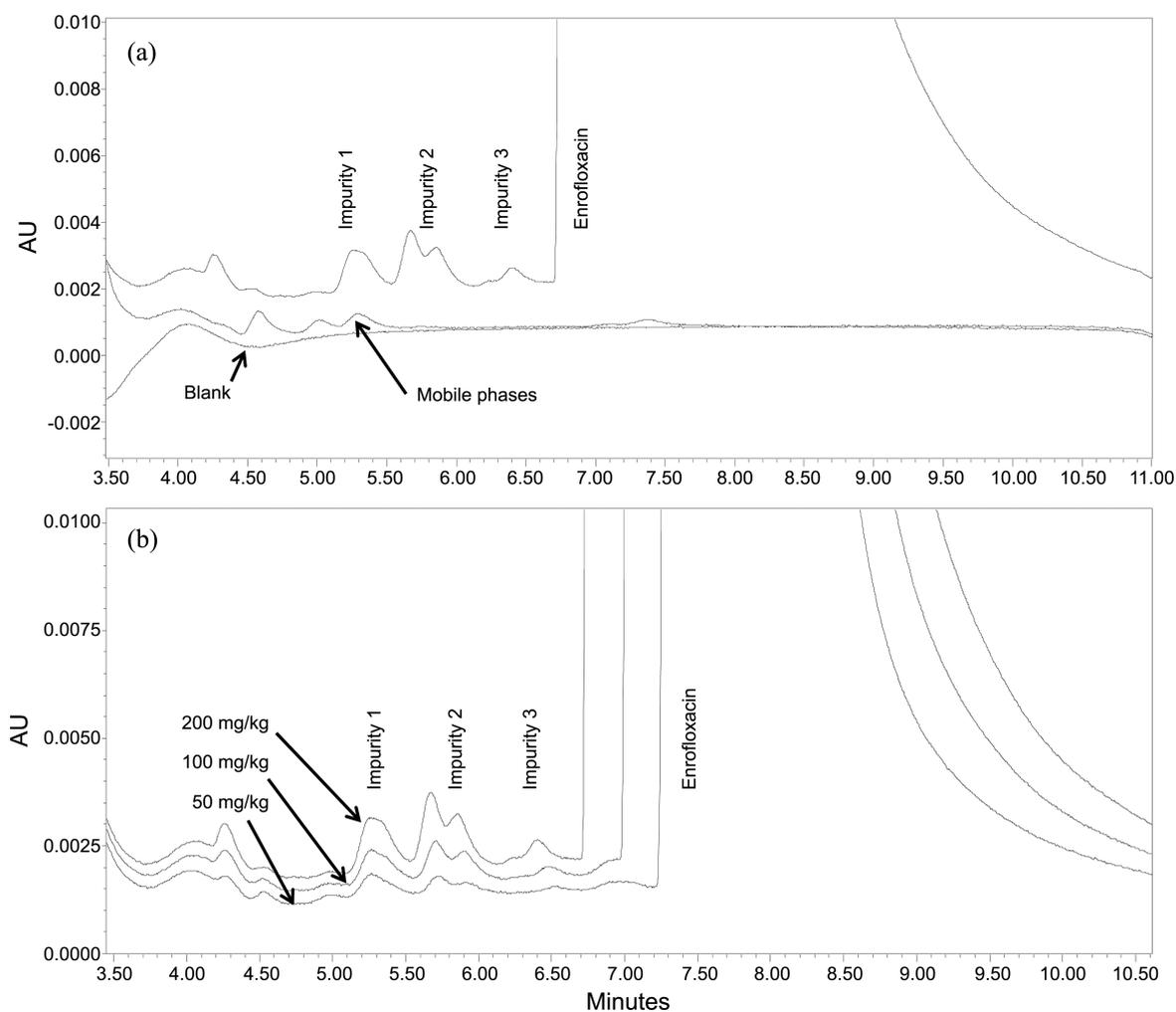


Figure 2. Overlaid chromatograms from LC run of the enrofloxacin reference material, mobile phases and methanol solvent blank (a), and of various concentrations (50–200 mg/kg) of enrofloxacin solutions (b).

addition to the main peak (peak 4) representing enrofloxacin, three additional peaks were obtained at 278 nm. These three

peaks were not found in either mobile phase or solvent blank (methanol) under identical LC conditions (Figure 2(a)), and

Table 1. Purities for enrofloxacin, norfloxacin and ciprofloxacin determined based on individual analytical methods for each reference material

Reference Materials	Lot No.	Purity assay by LC-UV,	Non-volatile Residues by TGA,	Water content by KFC,	Purity
		$P_{\text{LC-UV}}^a$ (%, kg/kg)	$P_{\text{non-volatile residue}}^a$ (%, kg/kg)	P_{water}^a (%, kg/kg)	$(P_{\text{material}})^b$ (%, kg/kg)
Enrofloxacin	91111	99.91 ± 0.004	0.00 ± 0.045	0.00 ± 0.040	99.91 ± 0.060
Norfloxacin	10214	99.91 ± 0.001	0.021 ± 0.015	2.803 ± 0.166	97.09 ± 0.167
Ciprofloxacin	00526	99.87 ± 0.001	0.000 ± 0.059	8.034 ± 0.103	91.85 ± 0.17

^aIn $P_{\text{LC-UV}}$, $P_{\text{non-volatile residue}}$, P_{water} , the number following “±” is the standard uncertainty. ^bIn P_{material} , the number following “±” is the expanded uncertainty with a level of confidence of 95% for the measurement value.

tend to increase the peak area with concentration of the main compounds (Figure 2(b)). These two criteria were used to confirm that peaks 1 to 3 were impurities from the reference material and not from the LC system. In comparing their UV spectra, we noticed that peaks 1 to 3 had very similar spectral patterns with the main compound. Though these peaks show weak absorption spectra due to low concentrations, they also have similar spectral patterns with enrofloxacin (Figure 1). Therefore, these three peaks were considered as organic impurities that were structurally related to the main compound, and 278 nm was suitable wave length to quantify those impurities in the selected enrofloxacin reference material. From the peak areas obtained from the chromatogram at 278 nm, the mass fraction of enrofloxacin ($P_{\text{LC-UV, enrofloxacin}}$) was estimated, according to Eq. (1), as 99.91% (kg/kg). Mass fraction for the three organic impurities in enrofloxacin was 0.09% (kg/kg). The standard uncertainty ($u_{P_{\text{LC-UV, enrofloxacin}}}$) during the purity assay by LC-UV was evaluated with the standard deviation ($s_{P_{\text{LC-UV, enrofloxacin}}}$) from multiple measurements ($n = 5$) (Table 1). All the impurities observed by LC-UV was less than 0.1%, and therefore small differences in their molar absorptivities from the corresponding main components are assumed to cause little influences in the observed purities.

Following similar procedures, the chromatographic purities of reference materials of norfloxacin and ciprofloxacin were analyzed and results were summarized in Table 1. These findings indicate that noticeable organic impurities in the material are also structurally related with the main components and that their relative contents in the material can be estimated by comparing their peak areas on LC chromatogram at its λ_{max} , 278 nm. Similar patterns were obtained for both norfloxacin and ciprofloxacin reference materials. Two and five impurity peaks were detected in norfloxacin and ciprofloxacin reference materials, respectively.

Measurements of Water by Karl-Fischer (K-F) Coulometry. Measurement of the mass fraction of water (P_{water}) in the reference material was performed by a Karl-Fischer (K-F) coulometric titration method.¹⁰ The magnitude of the system blank and its standard deviation ($s_{\text{KFC system blank}}$) from multiple measurements have significant influence upon the accurate determination of water contents in organic materials when only limited amount of sample can be taken for analysis. This is especially true for pure organic substances that are usually very precious and supplied in small

amounts per unit. In this case, only a few mg of the sample is available for water analysis, therefore reducing the magnitude of the system blank is critical to improve measurement quality. In an effort to minimize these main factors, a Karl-Fischer (K-F) coulometric titrator was placed inside a glove-box filled with nitrogen gas passed through a moisture trap. The closed glove-box system helped to protect the moisture from environment and improved the reliability of results from K-F coulometry. Under the dried nitrogen atmosphere, the value of the system blank was down to around 160 μg compared to 390 μg in ambient air, and the standard deviation of multiple measurements for system blank ($s_{\text{KFC system blank}}$) was maintained at around 10 μg . Mass fractions of water (P_{water}) for enrofloxacin, norfloxacin and ciprofloxacin were measured as (0.00 ± 0.04)%, (2.80 ± 0.17)%, and (8.03 ± 0.10)%, respectively.

Mass Fraction of Non-volatile Inorganic Residues by TGA. Inorganic impurities can be introduced during synthesis or/and purification of the material. TGA measurement under inert gas can measure residues of inorganic impurities and non-volatile high-molecular weight organic impurities. However, most of the compounds that have low volatility or/and a thermo-labile property produce residues of black carbon soot in TGA analysis under inert gas. To overcome this phenomenon, it is necessary to conduct the analyses using infused air as the reagent gas to burn out all organic compounds in the sample. Therefore, the amount of residues from the TGA analysis in this study represents total non-volatile inorganic impurities. Due to limited sample size, a single TGA analysis was conducted for each reference material as discussed previously.¹⁰ The mass fractions of non-volatile impurities ($P_{\text{non-volatile residue}}$) were assigned as (0.02 ± 0.02)% (kg/kg) for norfloxacin, (0.00 ± 0.05)% (kg/kg) for enrofloxacin, and (0.00 ± 0.06)% (kg/kg) for ciprofloxacin. Here the uncertainty represents the standard uncertainty ($u_{\text{non-volatile residue}}$) which was estimated from the weighing uncertainty ($u_{\text{weighing-by-difference}}$) of 0.7 μg in 2 mg of sample (W_{sample}).

Determination of the Final Purity and Evaluation of its Uncertainty. All of the measurement results from individual analytical techniques are summarized in Table 1. The final purities of the enrofloxacin, norfloxacin and ciprofloxacin were calculated to be (99.91 ± 0.06), (97.09 ± 0.17) and (91.85 ± 0.17)% (kg/kg), respectively, which were determined using the following Eq. (6).

$$P = (1 - P_{\text{water}} - P_{\text{non-volatile residue}}) \times P_{\text{LC-UV}} \quad (5)$$

Here, the expanded uncertainties (level of confidence of 95%) were obtained by combining standard uncertainties of individual measurement results. The assigned values were produced by comprehensive examination and evaluation of the purities by a mass-balance method by applying several available analytical techniques to detect and quantify most of the possible major impurities. Therefore, the assigned purity by this best-practice approach is more reliable and scientifically reasonable under the current technical limits and economic burdens.

Conclusion

This study established the mass-balance method for purity assessment on thermolabile reference materials. The purities of three fluoroquinolones including enrofloxacin, norfloxacin, and ciprofloxacin were determined based on the results of LC-UV, Karl-Fischer coulometry and TGA analysis. The assigned results will be utilized to the reference materials which are subjected as calibrators for certification of matrix CRMs, intended to maintain the traceability to SI units.

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