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Quantitative analysis of Cefpodoxime Proxetil raw material and dry suspension using proton nuclear magnetic resonance

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Abstract

This study established a simple, rapid, and accurate nuclear magnetic resonance (NMR) quantitative method to determine the cefpodoxime proxetil content in raw materials and dry suspensions, and evaluated the uncertainty. The relative content of cefpodoxime proxetil *iso*-A and *iso*-B was also analyzed based on ^{1}H NMR. In this study, an internal standard method was used, with DMSO-d_6 as the solvent and maleic acid and 1,3,5-trimethylbenzene as internal standards, to calculate the concentration of the samples. Quantitative analysis was performed using both the traditional quantitative NMR (traditional qNMR) and the quantitative Global Spectral Deconvolution (qGSD) method. The linearity range, quantitation limit, precision, robustness, and accuracy of these quantitative analysis methods were validated. The results indicated that both internal standards and integration methods met the requirements of the "9101 Guidelines for Analytical Method Validation" in the 2020 edition of the Chinese Pharmacopoeia. Compared to the traditional qNMR, qGSD has unique advantages in accurate quantitative analysis in complex systems. By using the combined HPLC-SPE-NMR technique, cefpodoxime proxetil *iso*-A and *iso*-B were enriched and analyzed, and their NMR data were accurately assigned. The quantitative analysis results were in line with the requirements of the Chinese Pharmacopoeia.

Keywords: qNMR; qGSD; cefpodoxime proxetil; uncertainty; HPLC-SPE-NMR

1 Introduction

Cephalosporin antibiotics comprise a series of semi-synthetic antibiotics derived from cephalosporin C, an antimicrobial component extracted from the culture filtrate of natural fungi. This series includes antibiotics such as cephalothin, ceftriaxone, cefazolin, cephalexin and cefpodoxime proxetil (CPDX-PR) [1]. In industrial production, cephalosporin drugs are semi-synthetic products. Cephalosporins are primarily produced by fermentation using Cephalosporium spp., followed by the amide formation to obtain 7-ACA,

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which is then modified to create semi-synthetic cephalosporins [2]. CPDX-PR is a third-generation cephalosporin and the prodrug of cefpodoxime (CPDX). The structure of CPDX-PR is shown in Fig. 1. The drug itself has no antibacterial activity. After oral administration, it is absorbed through the intestines and hydrolyzed by nonspecific esterase in the intestinal wall to CPDX, and then it has pharmacological activity. Its antibacterial action is based on inhibiting the synthesis of bacterial cell walls. CPDX-PR is highly stable to β-lactamase and exhibits potent antibacterial activity against traditional resistant pathogens as well as a broad spectrum of Gram-positive and Gram-negative bacteria, making it highly versatile [3-8]. CPDX-PR exists in two diastereomeric forms (CPDX-PR *iso*-A and CPDX-PR *iso*-B) due to the presence of an asymmetric carbon atom in the ester group of the drug [9]. It is documented in Chinese Pharmacopoeia 2020 Edition (ChP 2020 Edition) Part II. The quantification of CPDX by high-performance liquid chromatography (HPLC) in ChP 2020 Edition shows a relatively long detection time. The pharmacopoeia indicates that the ratio of the peak area of CPDX-PR *iso*-B to the combined peak areas of *iso*-A and *iso*-B should be between 0.50 and 0.60.

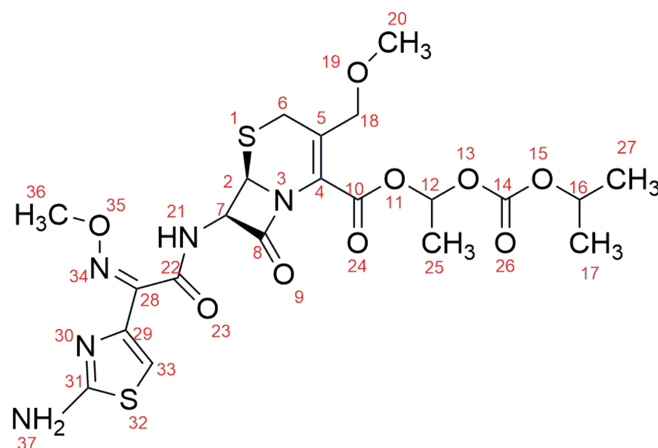

Fig. 1 Structure of CPDX-PR

In the field of medicine research, HPLC or UV spectrophotometry is commonly used for substance quantification [10]. In recent years, however, nuclear magnetic resonance spectroscopy (NMR) has been included in various national pharmacopeias, such as the ChP 2020 Edition, the United States Pharmacopeia 2023 Edition (USP 2023), the British Pharmacopoeia 2023 Edition (BP 2023), the European Pharmacopoeia 11.2 Edition (EP 11.2), the Korean Pharmacopoeia 10th Edition (KP 10) and the Japanese Pharmacopoeia 18th Edition (JP 18). NMR spectroscopy has unique advantages in quantitative analysis, such as simple sample preparation, rapid detection, no need for highly pure reference standards, non-destructive sample analysis, and the combination of qualitative identification and quantitative determination in a single method [11-17]. Quantitative nuclear magnetic resonance (qNMR) uses NMR signal peaks to quantify the target compounds within a sample, simultaneously providing both structural and quantitative information. The fundamental principle is that the integrated intensity of the NMR signal peaks is proportional to the molar concentration of protons in the compound. Usually, quantitative analysis can be performed by comparing the peak areas caused by protons on specific groups in the molecule, regardless of the chemical properties of the atomic nucleus. For example, Zhao et al. used 2,3,5-triiodobenzoic acid as the internal standard

and deuterated methanol as the solvent, employing ^{1}H qNMR to simultaneously determine the contents of saikosaponin a, saikosaponin b1, saikosaponin b2 and saikosaponin d in Bupleurum Chinenees DC [18]. The results of this method were accurate, making it a reliable analytical method for the quality control of Bupleurum medicinal materials. Additionally, qNMR, as a promising reference method for quantifying organic substances, is characterized by strong selectivity, accuracy and speed, rendering it highly suitable for the purity determination of reference materials. Guo et al. used the qNMR method to accurately determine the purity of the aflatoxin B1 reference material (GBW(E)100599) [19]. This is the first report on preparing a reliable aflatoxin B1 purity reference material, which improves the accuracy, traceability and comparability of aflatoxin B1 determinations in various foods, ultimately ensuring the quality and safety of food. Wang et al. established a ^{1}H NMR method suitable for the quantification of hyperoside reference material, and the results were consistent with the mass balance method [17]. Currently, qNMR has been widely applied in various fields. For example, in agriculture, Well et al. used the qNMR method to determine the purity of different herbicides [20]. In the food industry, Norma et al. used the qNMR method for the quantitative determination of caffeine in sugar-free energy drinks [21].

The clarity and completeness of quantitative signal peaks are crucial to the accuracy of qNMR. According to the qNMR Internal Standard Reference Data published by the Bureau International des Poids et Mesures (BIPM), there should be no interfering signals within 100 times the half-width of the quantitative signal peak. However, based on the above selection principles, in complex samples with numerous NMR signal peaks, it is difficult to obtain the required quantitative signal peaks by using traditional integration methods. The quantitative Global Spectral Deconvolution (qGSD) method can be used to solve the problem. qGSD is an NMR signal processing technique that employs mathematical models to decompose complex NMR spectra, facilitating the identification and quantification of individual components. This method utilizes Lorentzian or Gaussian line shape functions to fit signal peaks within the spectrum, enabling the deconvolution of overlapping complex signal peaks. This approach provides complete peak shapes and integral information that satisfy quantitative requirements [22].

The mass balance method is currently the most widely used content analysis method, which requires that the sum of the content of active ingredients, organic impurities, moisture, residual solvents, and inorganic impurities must be 100 [23]. Consequently, the results of the mass balance method tend to be influenced by multiple factors, including the lack of traceable external reference standards and the limitations of liquid chromatography methods in detecting certain impurities [24]. Therefore, it is necessary to find a quantification method that is not affected by these factors to corroborate the results of the mass balance method, and qNMR is usually a good choice. Since qNMR has the advantages of determining absolute content and qualitative analysis, it is particularly valuable when the results of qNMR differ from those of the mass balance method [24]. Zhang determined that the purity of propyl acetate was 99.36% using the mass balance method, and that of high-purity propyl acetate was 99.61% using qNMR [25]. Further investigation revealed that the raw n-propyl acetate contained a high level of moisture impurity. After dehydration through adsorption, the purity was increased to 99.63% by mass balance method, which was consistent with the qNMR result. Similarly, when using the mass balance method to determine the purity of the CPDX-PR reference material, the results were also affected by the aforementioned

factors. This study established a quantitative NMR method for the rapid determination of the content of CPDX-PR in raw materials and formulations, based on the ChP 2020 Edition "0441 NMR Spectroscopy Method", "9101 Guidelines for Analytical Method Validation" and BIPM "qNMR Internal Standard Reference Data (ISRD)", using both the traditional qNMR method and the qGSD method, and validated this content determination method. Through HPLC-SPE-NMR technology, the two isomers of CPDX-PR and their NMR data were accurately assigned, and the relative contents of the two isomers were analyzed. This study provides evidence and reference for the purity determination of CPDX-PR standard materials using the mass balance method, expands new methods for content determination of CPDX-PR raw materials and formulations, and provides an example of the application of qGSD in the analysis of substance content in complex systems.

2 Materials and methods

2.1 Instruments and chemicals

NMR spectra were acquired on a BRUKER AVANCE III HD 600 MHz NMR Spectrometer (Bruker, Switzerland), using a 5 mm Dual Resonance Probe BBO 600S3 BBF-H-D-05 Z SP and Xpress 60-position Automatic Sampler. HPLC spectra were recorded on a 1260 Infinity II HPLC (Agilent, USA). HPLC-SPE-NMR System (Bruker, Switzerland) was used to separate and enrich CPDX-PR *iso*-A and *iso*-B.

CPDX-PR (batch number: 130517-202004, RSM, 75.0%) was provided by the National Institute for Food and Drug Control (China). CPDX-PR dry suspension (batch number: 350323, 350123, 100 mg/5 mL) was purchased from Guangzhou Nanxin Pharmaceutical Co., Ltd. Dimethyl sulfoxide (DMSO-d_6, 99%) was purchased from Cambridge Isotope Laboratories (USA). 1,3,5-Trimethoxybenzene (batch number: BCCH9679, 99.91% ± 0.28%) was purchased from Sigma-Aldrich (USA). Maleic acid (batch number: 22001, 99.91%) was purchased from the National Institute for Food and Drug Control (China). Methanol (Chromatography Grade) was purchased from Thermo Fisher Scientific (USA), and Ultrapure Water was purchased from Hangzhou Wahaha Group Co., Ltd.

2.2 Method

2.2.1 Sample preparation

An internal standard substance stock solution of certified 1,3,5-Trimethoxybenzene and maleic acid reference standard (RS) was prepared with the concentration of 4 mg/mL in DMSO-d_6, respectively. The active pharmaceutical ingredient (API) working solution of CPDX-PR RS was prepared with the concentration of 20 mg/mL in DMSO-d_6. The working solution of CPDX-PR dry suspension was prepared with the concentration of 300 mg/mL in DMSO-d_6. The HPLC-SPE-NMR sample solution was prepared with the concentration of 10 mg/mL in methanol. All solutions were dissolved by ultrasound for 10 min, and the dry suspension were ultrasonicated and then the supernatant was taken for detection.

2.2.2 HPLC-SPE-NMR

Chromatographic analysis was performed using an Agilent 1260 Infinity II HPLC system with a CAPCELL PAK C_{18} MGIII column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of water-methanol (55:45) at a flow rate of 1 mL/min and detection was carried out at 240 nm. The column temperature was set to 40 °C, with an injection volume of 30 μL. SPE was performed using Resin GP (code: Y10, 10 μm, column: E).

2.2.3 NMR data processing method

NMR data were processed using MestReNova 15.0.1 and TopSpin 3.5. Quantification peaks for the sample were as follows: Peak 1: δ_H 5.17-5.21 (H-2, quantified using traditional qNMR), Peak 2: δ_H 4.12-4.17 (H-18, quantified using qGSD), Peak 3: δ_H 3.84 (H-36, quantified using qGSD method). Maleic acid quantification peak: δ_H 6.26; 1,3,5-Trimethoxybenzene quantification peak: δ_H 6.09. The resulting spectra were manually phased and baseline-corrected, with manual integration and peak integration selected using qGSD.

2.2.4 ^1H qNMR analysis

2.2.4.1 Selection of quantitative peak

The NMR spectra are shown in Fig. 2 (A) and Fig. 2 (B). In traditional qNMR, quantification peaks should exhibit good peak symmetry, separation, and high signal-to-noise ratio (S/N). Therefore, δ_H 5.17-5.21 was selected as quantitative peak 1. According to BIPM regulations, there should be no interfering signals within 100 times the half-peak width of the quantification peak. δ_H 4.12-4.17 and δ_H 3.84 don't meet this condition; there is mutual interference between the two peaks, and other impurities under the main peak affect integration. Consequently, qGSD was used to calculate and quantify the interfering signal peaks. Different from traditional integration methods, qGSD deconvolves the frequency domain spectrum and can provide reliable information even in cases of overlapping peaks. δ_H 4.12-4.17 was chosen as quantitative peak 2, and δ_H 3.84 as quantitative peak 3 for qGSD analysis, as shown in Fig. 2 (C). There is only one signal peak of maleic acid, which does not interfere with the signal peak of CPDX-PR. Therefore the quantitative peak of maleic acid is δ_H 6.26. 1,3,5-trimethoxybenzone has two signal peaks, δ_H 6.09 and δ_H 3.71. But the distance between the peak at δ_H 3.71 and the peak at δ_H 3.84 (H-36) of CPDX-PR affects the accuracy of integration. Therefore, the peak at δ_H 6.09 of 1,3,5-Trimethoxybenzone was chosen in this study.

2.2.4.2 Relaxation delay time

The setting of relaxation delay time (D_1) is closely related to the T_1 relaxation time. The accuracy of T_1 directly affects the integrated intensity of NMR peaks, which in turn influences the determination of the target substance content in the sample. The effect of different delay time on the peak area ratio of quantification peaks to internal standard peaks was investigated to select the optimal delay time for subsequent experiments. Relaxation time (T_1) of proton resonance lines was tested using inversion-recovery experiments. The results showed that, when using maleic acid as the internal standard, the T_1 value was 2.995 s for the maleic acid peak (δ_H 6.26), 2.142 s for sample quantification peak 1 (δ_H 5.17-5.21, -CH), 0.866 s for sample quantification peak 2 (δ_H 4.12-4.17, $-CH_2$) it was, and 2.142 s for sample quantification peak 3 (δ_H 3.84, $-CH_3$). When using 1,3,5-trimethoxybenzene as the internal standard, the T_1 value was 3.147 s for the 1,3,5-trimethoxybenzene peak δ_H 6.09), 2.164 s for sample quantification peak 1 (δ_H 5.17-5.21), 0.858 s for sample quantification peak 2 (δ_H 4.12-4.17), and 0.858 s for sample quantification peak 3 (δ_H 3.84). To ensure the accuracy of the integration results, D_1 should be at least ten times the T_1 of the quantification signal. Therefore, D_1 was set to 36 s when using maleic acid as the internal standard, and to 39 s when using 1,3,5-trimethoxybenzene as the internal standard. The results indicated that these settings met the measurement accuracy requirements.

Fig. 2 A: ^{1}H NMR spectrum of CPDX-PR and 1,3,5-trimethoxybenzene (^{1}H 600 MHz, DMSO-d_6); B: ^{1}H NMR spectrum of CPDX-PR and maleic acid (^{1}H 600 MHz, DMSO-d_6); C: Comparison of CPDX-PR spectrum between ordinary NMR spectrum and spectrum extracted by qGSD (^{1}H 600 MHz, DMSO-d_6)

2.2.4.3 Optimization of NS

The number of scans (NS) refers to the number of times that the NMR signal is repeated in the acquisition process. Too small NS will reduce the signal-to-noise ratio of the spectrum, thus affecting the accuracy of quantification. Conversely, too many scans will consume a significant amount of experimental time, so it is necessary to set an appropriate number of scans. Tests with NS values of 8, 16, 24, and 32 showed that when NS was set to 16, the S/N for all quantification peaks was greater than 1200. According to the Guide to NMR Method Development and Validation – Part I: Identification and Quantification published by the European Network of Testing and Calibration Laboratorie [26], the higher the S/N, the lower the uncertainty of NMR measurement. When S/N is 1200, the uncertainty is

0.1%. To balance uncertainty with instrument time, NS was determined to be 16.

2.2.4.4 Optimization of RG

Receiver gain (RG) is a crucial parameter used to adjust the strength of the received signal to optimize the S/N ratio, thereby improving the signal quality and data accuracy of the experiment. However, an excessively high RG can lead to signal loss, necessitating the selection of an appropriate RG. Testing with RG values of 32, 64, 80, 96, 112, and 128 revealed that ADC overflow occurred when RG value exceeded 80. Therefore, RG was set to 64.

2.2.5 qNMR method

Using DMSO-d_6 as the solvent and 1,3,5-trimethoxybenzene as the internal standard, the following parameters were used for ^{1}H qNMR test: zg90 pulse sequence, temperature of 298 K, spectral width (PW) of 20.0269 Hz, center frequency (O_1) of 4801.20 Hz, time domain data points (TD) of 65536, acquisition time (AT) of 2.73 s, D_1 of 39 s, dummy scans (DS) of 2, NS of 16, and RG of 64.

Using DMSO-d_6 as the solvent and maleic acid as the internal standard, ^{1}H qNMR tests were performed with the same parameters as above, except that D1 was set to 36 s.

2.2.6 Computing formula

The content of CPDX (P_x) was determined using an absolute quantification method, where the relative peak area ratio of the sample to the internal standard was measured and used to calculate the content by the internal standard method. The calculation formula is as follows:

$$P_x(\%) = \frac{A_r \times N_s}{A_s \times N_r} \times \frac{M_s \times m_r}{M_r \times m_s} \times W_r$$

A_r: the peak area of the quantification peak of the internal standard; N_s: the number of protons in the quantification peak of the sample; N_r: the number of protons in the quantification peak of the internal standard; M_s: the molecular weight of the sample, with the molecular weight of CPDX being 427.455; M_r: the molecular weight of the internal standard, where the molecular weight of 1,3,5-trimethoxybenzene is 168.1898 and the molecular weight of maleic acid is 116.0722; m_s: the mass of the sample used for measurement; m_r: the mass of the internal standard used; W_r: the purity of the internal standard, with the purity of 1,3,5-trimethoxybenzene being 99.91% and the purity of maleic acid being 99.91%.

3 Results and discussion

3.1 Validation of the methodology

3.1.1 Linearity

The linearity was investigated by preparing 8 mg, 9 mg, 10 mg, 11 mg, and 12 mg CPDX-PR in 0.5 mL internal standard substance stock solution, respectively. Using the ratio of the sample peak area to the internal standard peak area as the y-axis and the ratio of the sample concentration to the internal standard concentration as the x-axis, the regression equation and correlation coefficient were obtained. Great linearity across the range from 12 mg/mL to 18 mg/mL of CPDX was proven by high results of R shown in Fig. 3.

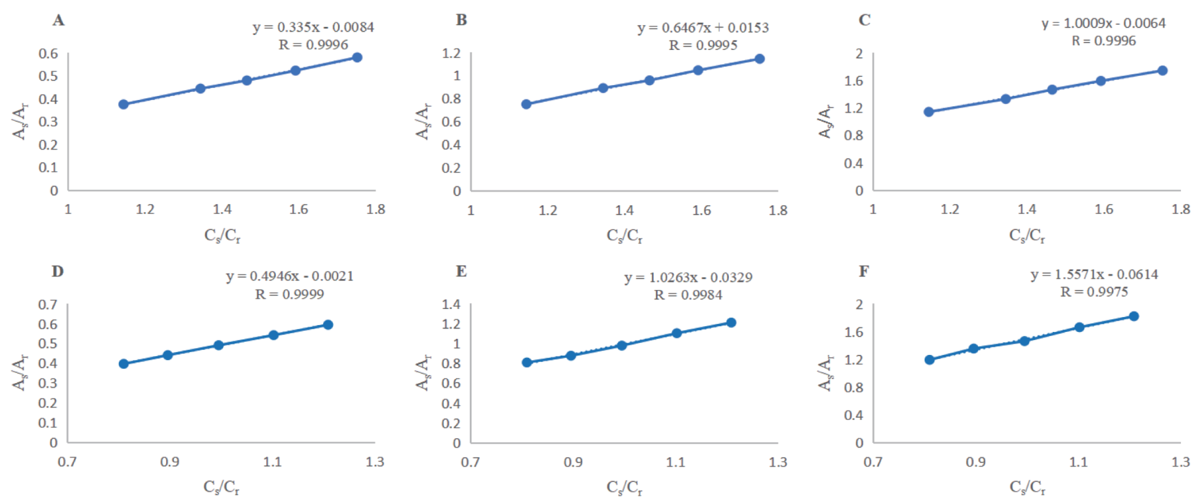

Fig. 3 A is a standard curve graph using the peak area ratio of peak 1 (δ_H 5.17-5.21) and the peak of 1,3,5-trimethoxybenzene (δ_H 6.09) as the x-axis; B is a standard curve graph using the peak area ratio of peak 2 (δ_H 4.12-4.17) and the peak of 1,3,5-trimethoxybenzene (δ_H 6.09) as the x-axis; C is a standard curve graph using the peak area ratio of peak 3 (δ_H 3.84) and the peak of 1,3,5-trimethoxybenzene (δ_H 6.09) as the x-axis; D is a standard curve graph using the peak area ratio of peak 1 (δ_H 5.17-5.21) and the peak of maleic acid (δ_H 6.26) as the x-axis; E is a standard curve graph using the peak area ratio of peak 2 (δ_H 4.12-4.17) and the peak of maleic acid (δ_H 6.26) as the x-axis; F is a standard curve graph using the peak area ratio of peak 3 (δ_H 3.84) and the peak of maleic acid (δ_H 6.26) as the x-axis

3.1.2 Repeatability

6 parallel samples of the API working solution of CPDX-PR RS were prepared using maleic acid and 1,3,5-Trimethoxybenzene as internal standards and measured according to the experiment parameters described in section 2.2.4. The spectra were processed using traditional qNMR and qGSD. The calculated purity of CPDX ranged from 73.85% to 74.67%, with RSD ranging from 0.60% to 0.88%. The detailed results were shown in Table 1. These results indicated that the established ^{1}H qNMR method exhibited good reproducibility.

Table 1 Repeatability, robustness and recovery of CPDX by qNMR (traditional qNMR vs qGSD)

		Maleic acid			1,3,5-Trimethoxybenzene		
		δ_H 5.17-5.21 traditional qNMR	δ_H 4.12-4.17 qGSD	δ_H 3.84 qGSD	δ_H 5.17-5.21 traditional qNMR	δ_H 4.12-4.17 qGSD	δ_H 3.84 qGSD
Repeatability	*Px*/%	73.85	74.67	74.18	74.05	74.38	74.37
	RSD/%	0.60	0.88	0.88	0.60	0.70	0.88
Robustness	standard	73.93	75.14	74.92	74.66	74.45	73.92
	RSD/%	0.26	0.93	0.87	0.29	0.57	0.93
Recovery	Average recovery	98.50	100.17	99.16	98.69	98.88	99.26
	RSD/%	0.67	0.80	0.90	0.68	0.88	0.80

3.1.3 Robustness

Under standard conditions, with fixed magnetic field strength, pulse sequence, TD, NS, O1, AT, RG, D1 and sample temperature, the PW (16 ppm and 24 ppm) and the NS (24 and 32) were varied. The measurement for each condition was repeated. The RSD of the CPDX purity results obtained under standard conditions versus those obtained with varied spectrum widths and scan numbers ranged from 0.26% to 0.93%, as detailed in Table 1. The results indicated that the established ^{1}H qNMR method exhibited good robustness.

3.1.4 Recovery

The accuracy of this method was studied by preparing 8 mg, 10 mg, and 12 mg CPDX-PR in 0.5 mL internal standard stock solution and preparing three samples of each concentration. These samples were then measured according to the conditions outlined in section 2.4. The recovery rate of CPDX was calculated to be 98.50%-100.17%, with RSD of 0.67%-0.88% (maleic acid and 1,3,5-Trimethoxybenzene, traditional qNMR and qGSD), as detailed in Table 1. These results demonstrated that the established ^{1}H qNMR method had good accuracy.

3.1.5 Limits of quantitation (LOQ)

According to the Guide to NMR Method Development and Validation – Part I: Identification and Quantification published by the European Network of Testing and Calibration Laboratories [26], this study defined LOQ as the sample concentration at the S/N ratio of 1200. The sample of the API working solution of CPDX-PR RS was prepared, and the solution was diluted with solvent until the lowest quantitation peak reached an S/N ratio of 1200. The corresponding concentration was defined as the LOQ. The results showed that using maleic acid as the internal standard, LOQ was 5.68 mg/mL of CPDX, while using 1,3,5-trimethoxybenzene as the internal standard, LOQ was 5.78 mg/mL of CPDX.

3.2 Determination results of CPDX-PR

Three parallel samples of the API working solution of CPDX-PR were prepared and measured according to the conditions specified in section 2.2.5. Each sample was measured in triplicate. The CPDX content was analyzed by internal standard method, and the spectra were processed by traditional qNMR and qGSD method respectively. The results were shown in Table 2.

Table 2 Determination results of CPDX API content (traditional qNMR vs qGSD)

	Maleic acid	RSD/%	1,3,5-Trimethoxybenzene	RSD/%
δ_H 5.17-5.21 traditional qNMR	74.15%	0.85	74.26%	0.31
δ_H 4.12-4.17 qGSD	75.03%	0.74	74.01%	0.57
δ_H 3.84 qGSD	73.90%	0.11	74.96%	0.53

3.3 Determination results of CPDX-PR dry suspension

According to the conditions specified in section 2.2.5 (1,3,5-Trimethoxybenzene, qGSD), three parallel samples from different batchs of formulation solutions were prepared and measured respectively. Each sample was measured in triplicate. The CPDX content was calculated using the internal standard method. For batch 350323, the average content was 3.09% CPDX with the RSD of 1.71%. For batch 350123, the average content was 3.29% CPDX with RSD of 1.98%.

3.4 Isomer analysis of CPDX-PR

3.4.1 Purification and identification of CPDX-PR iso-A and CPDX-PR iso-B

The HPLC-SPE-NMR sample solution was prepared and subjected to chromatographic analysis according to the conditions specified in section 2.2.2. Fig. 4 showed that only two major peaks appeared in the chromatogram. According to the ChP 2020 Edition, CPDX-PR *iso*-A was eluted before CPDX-PR *iso*-B, allowing for the distinction between CPDX-PR *iso*-A and CPDX-PR *iso*-B. Different collection time intervals were set, and 13 injections were made continuously under the conditions described in section 2.2.2. CPDX-PR *iso*-A and CPDX-PR *iso*-B were enriched and purified on different SPE columns. The samples were dried with nitrogen for 40 min and then eluted with 250 μL of DMSO-d_6 into 3 mm NMR tubes. ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HSQC, HMBC, and *J*-HMBC experiments were conducted. The NMR data of CPDX-PR *iso*-A, CPDX-PR *iso*-B and CPDX-PR were accurately assigned based on the above experimental data, as detailed below:

CPDX-PR *iso*-A: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.62 (d, *J* = 8.1 Hz, 1H, H-21), 7.33 (s, 2H, H-37), 6.85 (q, *J* = 5.4 Hz, 1H, H-12), 6.75 (s, 1H, H-33), 5.81 (d, *J* = 4.0 Hz, 1H, H-7), 5.17 (d, *J* = 4.8 Hz, 1H, H-2), 4.81 (p, *J* = 6.2 Hz, 1H, H-16), 4.13 (d, *J* = 2.6 Hz, 2H, H-18), 3.84 (s, 3H, H-36), 3.63-3.50 (m, 2H, H-6), 3.20 (s, 3H, H-20), 1.49 (d, *J* = 5.3 Hz, 3H, H-25), 1.24 (overlap, 6H, H-17,27).

^{13}C NMR (150 MHz, DMSO-d_6) δ 168.52 (C-31), 163.81(C-8, *E*/*Z*), 163.77 (C-8, *E*/*Z*), 162.83 (C-22, *E*/*Z*), 162.73 (C-22, *E*/*Z*), 159.59 (C-10), 152.02 (C-14), 148.58 (C-28), 141.62 (C-29), 128.99 (C-5), 123.59 (C-4), 109.12 (C-33), 91.70 (C-12), 72.65 (C-16), 69.74 (C-18), 62.02 (C-36), 58.81 (C-77, *E*/*Z*), 58.70 (C-7, *E*/*Z*), 57.84 (C-2), 57.52 (C-20), 25.74 (C-6), 21.38 (C-17 and C-27), 19.14 (C-25).

CPDX-PR *iso*-B: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.63 (d, *J* = 8.2 Hz, 1H, H-21), 7.29 (s, 2H, H-37) 6.79 (q, *J* = 5.4 Hz, 1H, H-12), 6.74 (s, 1H, H-33), 5.83 (d, *J* = 4.2 Hz, 1H, H-7), 5.20 (d, *J* = 4.9 Hz, 1H, H-2), 4.79 (p, *J* = 6.2 Hz, 1H, H-16), 4.14 (d, *J* = 3.6 Hz, 2H, H-18), 3.84 (s, 3H, H-36), 3.65-3.50 (m, 2H, H-6), 3.20 (s, 3H, H-20), 1.48 (d, *J* = 5.4 Hz, 3H, H-25), 1.23 (overlap, 6H, H-17,27).

^{13}C NMR (150 MHz, DMSO-d_6) δ 168.50 (C-31), 164.08 (C-8, *E*/*Z*), 164.04 (C-8, *E*/*Z*), 162.89 (C-22, *E*/*Z*), 162.79 (C-22, *E*/*Z*), 159.61 (C-10), 151.89 (C-14), 148.70 (C-28), 141.90 (C-29), 128.64 (C-5), 123.68 (C-4), 109.18 (C-33), 92.07 (C-12), 72.69 (C-16), 69.80 (C-18), 62.03 (C-36), 58.82 (C-7, *E*/*Z*), 58.72 (C-7, *E*/*Z*), 57.80 (C-2), 57.50 (C-20), 25.82 (C-6), 21.37 (C-17 and C-27), 18.99 (C-25).

CPDX-PR: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.61 (dd, *J* = 8.2, 7.0 Hz, 1H, H-21), 7.22 (s, 2H, H-37), 6.82 (dq, *J* = 36.4, 5.4 Hz, 1H, H-12), 6.73 (d, *J* = 2.1 Hz, 1H, H-33), 5.82 (ddd, *J* = 15.3, 8.2, 4.8 Hz, 1H, H-7), 5.19 (dd, *J* = 16.8, 4.9 Hz, 1H, H-2), 4.85 – 4.76 (m, 1H, H-16), 4.14 (dd, *J* = 4.7, 2.7 Hz, 2H, H-18), 3.84 (s, 3H, H-36), 3.65 – 3.49 (m, 2H, H-6), 3.20 (d, *J* = 2.7 Hz, 3H, H-20), 1.49 (dd, *J* = 5.5, 3.7 Hz, 3H, H-25), 1.27 – 1.21 (m, 6H, H-17,27).

^{13}C NMR (150 MHz, DMSO-d_6) δ 168.89 (C-31), 164.53 (C-8 of *iso*-B), 164.28 (C-8 of *iso*-A), 163.43 (C-22), 160.04 (C-10), 152.45 (C-14 of *iso*-A), 152.31 (C-14 of *iso*-B), 149.40 (C-28), 142.91 (C-29), 129.37 (C-5 of *iso*-A), 129.03 (C-5 of *iso*-B), 124.12 (C-4 of *iso*-B), 124.05 (C-4 of *iso*-A), 109.46 (C-33 of *iso*-B), 109.38 (C-33 of *iso*-A), 92.49 (C-12 of *iso*-B), 92.14 (C-12 of *iso*-A), 73.09 (C-16), 70.24 (C-18 of *iso*-B), 70.19 (C-18 of *iso*-A), 62.38 (C-36), 59.25 (C-7), 58.33 (C-2 of *iso*-A), 58.27 (C-2 of *iso*-B), 57.96 (C-20 of *iso*-A), 57.93 (C-20 of *iso*-B), 26.26 (C-6 of *iso*-B), 26.19 (C-6 of *iso*-A), 21.82 (C-17 and C-27), 19.59 (C-25 of *iso*-A), 19.43 (C-25 of *iso*-B).

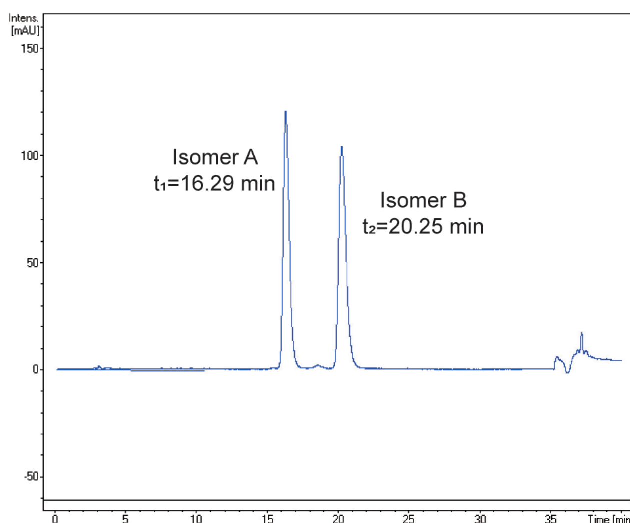

Fig. 4 HPLC spectrum of CPDX-PR

3.4.2 Analysis of relative content of CPDX-PR iso-A and CPDX-PR iso-B

The spectrum showed that the chemical shift deviation of protons at δ_H 6.78-6.87 (H-12, located at the chiral carbon) was the largest, with $\Delta\delta = 0.06$, which can be used as the characteristic signal peak for the relative quantitative analysis of isomers. According to the ChP 2020 Edition, the ratio of the peak area of CPDX-PR *iso*-B to the sum of the peak areas of CPDX-PR *iso*-A and CPDX-PR *iso*-B should be between 0.50 and 0.60. This study performed relative quantification of *iso*-A and *iso*-B by integration in the CPDX-PR spectrum. The results showed that the ratio of the peak area of CPDX-PR *iso*-B to the sum of the peak areas of CPDX-PR *iso*-A and CPDX-PR *iso*-B was 0.52, as shown in Fig. 5, as required by the ChP 2020 Edition.

3.5 Discussion

3.5.1 Selection of solvents

As the results showed, although CD_3OD also had good solubility, the solvent peak interfered with the sample. In contrast, both the sample and the internal standard dissolved well in DMSO-d_6 and the solvent peak did not interfere with the measurement. Therefore, DMSO-d_6 was selected as the solvent.

3.5.2 Selection of internal standards

Since the sample CPDX-PR is an antibiotic, considering that acidic or basic internal standards might interfere with it, we tested two internal standards: acidic maleic acid and 1,3,5-trimethoxybenzene. Neither interfered with the sample signal peaks. Methodological validation results indicated that both maleic acid and 1,3,5-trimethoxybenzene can be used as internal standards for CPDX quantification by qNMR. As for the determination of the CPDX-PR dry suspension, only one internal standard, 1,3,5-trimethoxybenzene, was selected in the study.

3.5.3 Selection of quantitative peaks

Quantitative peak signals should exhibit good peak symmetry, excellent resolution, and a high S/N. As shown in Fig. 2 (A) and Fig. 2 (B), the peak at δ_H 9.61 (H-21) and the peak at δ_H 7.22 (H-37) areas are unstable because they are the signal peaks

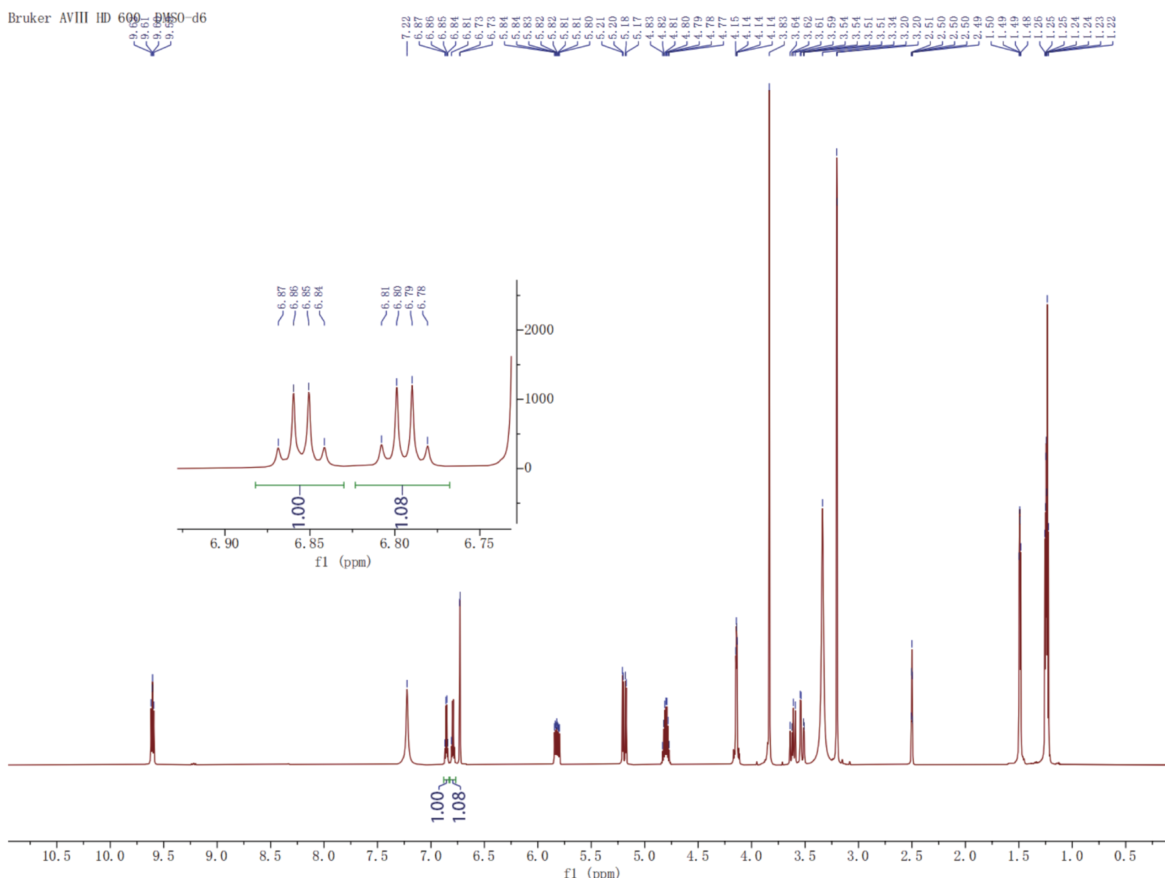

Fig. 5 ^{1}H NMR spectrum of CPDX-PR (^{1}H 600 MHz, DMSO-d_6)

of active protons. Two signal peaks, the peak at δ_H 6.82 (H-12) and the peak at δ_H 6.73 (H-33), are too close, and the separation is low, which affects the integration accuracy. The peak at δ_H 5.82 (H-7), the peak at δ_H 4.85-4.76 (H-16), and the peak at δ_H 3.65-3.49 (H-6) have complex peak shapes and low S/N, which is easy to increase the integration error. There is impurity signal interference at the baseline near the peak at δ_H 4.14 (H-18). The shapes of the peak at δ_H 3.84 (H-36) and the peak at δ_H 3.20 (H-20) are relatively simple, but there are water peaks near them, which interferes with them. The peak at δ_H 1.49 (H-25) and the peak at δ_H 1.27-1.21 (H-17,27) is complex in shape and far away from the internal standard quantitative peak, so the baseline correction may affect the accuracy of the integration. The peak at δ_H 5.17-5.21 (H-2) in the sample shows good resolution, a high S/N, and excellent peak symmetry. Therefore, this peak is selected as the sample quantitation peak for analysis using the traditional manual integration method. In this experiment, maleic acid and 1,3,5-trimethoxybenzene were selected as internal standards. The signal of maleic acid (δ_H 6.26) does not interfere with the sample peaks, nor does the signal of 1,3,5-trimethoxybenzene (δ_H 6.09). Therefore, maleic acid (δ_H 6.26), 1,3,5-trimethoxybenzene (δ_H 6.09), and the sample peak at δ_H 5.17-5.21 (Peak 1) are selected as the internal standard and sample quantitation peaks for analysis using the traditional qNMR. According to BIPM regulations, there should be no interfering signals in the range of 100 times the half-peak width of the quantitation signal peak. In addition, the

CPDX-PR dry suspension contains many excipients, and the signal peak of excipients interfere with that of CPDX-PR. Therefore, in cases with interference, qGSD can be used to process and exclude interfering signals before quantitative analysis. As long as the signal peaks are not completely overlapped, qGSD can be used for analysis.The signal peaks at δ_H 4.12-4.17 (Peak 2) and δ_H 3.84 (Peak 3) exhibit mutual interference and additional impurity signals. The traditional manual integration method fails the methodological validation in such cases, whereas qGSD meets the quantitative requirements. Thus, for the processing of complex spectra with interference, qGSD followed by integration is superior to traditional manual integration methods.

3.5.4 Comparison of determination results of CPDX-PR dry suspension by qGSD method and HPLC external standard method

In order to further validate the reliability of the qGSD method for quantifying formulations, the study used the HPLC external standard method as specified in the ChP 2020 Edition. For the batch number 350323, the content of CPDX-PR dry suspension was determined to be 3.24% (*n* = 3 RSD 1.15%) by HPLC, and 3.09% (*n* = 3 RSD 1.71%) by qNMR calculated on CPDX ($C_{15}H_{17}N_5O_6S_2$). For the batch number 350123, the content of CPDX-PR dry suspension was determined to be 3.44% (*n* = 3 RSD 1.50%) by HPLC, and 3.29% (*n* = 3 RSD 1.98%) by qNMR calculated on CPDX ($C_{15}H_{17}N_5O_6S_2$). The results of the two quantitative methods were basically consistent, indicating that the established qGSD method can be used to determine the content of CPDX-PR dry suspension.

3.5.5 Isomer

There are two groups of signals at C-7, C-8, and C-22 in the ^{13}C NMR spectrum of CPDX-PR *iso*-A and CPDX-PR *iso*-B, which are considered to be caused by cis-trans isomerization of N=O double bonds [27]. The reason for this phenomenon is that the *Z*- isomer will be transformed into an *E*- isomer under acidic conditions or exposure to light [28].

3.5.6 Uncertainty

The sources of uncertainty in the NMR quantification method include NMR measurement uncertainty, molecular weight uncertainty, weighing uncertainty and internal standard purity uncertainty.

3.5.6.1 Uncertainty of NMR measurement uN

According to the Guide to NMR Method Development and Validation – Part I: Identification and Quantification published by the European Network of Testing and Calibration Laboratories [26], the measurement uncertainty is 0.1% when S/N is 1200.

3.5.6.2 Uncertainty of molecular weight uM

Calculation of the standard uncertainty for the relative molecular weight of CPDX and the internal standard (calculated according to the method outlined in the EURACHEM/CITAC publication “Quantifying Uncertainty in Analytical Measurement” [29,30]).

$$u(M) = \sqrt{\sum_{j=1}^{n} \left[N_j u_j\right]^2}$$

N_j: the number of atoms of element *j*; u_j: the uncertainty in the relative atomic mass of element *j*;

The molecular formula of CPDX is $C_{15}H_{17}O_6S_2$, with the molecular weight of 427.455. The standard uncertainty is:

$$u(Mx)=\sqrt{\sum_{j=1}^{n}\left[N_j u_j\right]^2}$$

$$\sqrt{\begin{array}{c}\left[15\times\left(0.0008\div\sqrt{3}\right)\right]^2+\left[17\times\left(0.00007\div\sqrt{3}\right)\right]^2+\\+\left[5\times\left(0.0002\div\sqrt{3}\right)\right]^2+\left[6\times\left(0.0003\div\sqrt{3}\right)\right]^2\\+\left[2\times(0.005\div\sqrt{3})\right]^2\end{array}}$$

$=0.0091$

The relative uncertainty of the relative molecular weight of CPDX is:

$u(Mx)/(Mx) = 0.0091 \div 427.455 = 0.0021\%$

The molecular formula of maleic acid is $C_4H_4O_4$, with the molecular weight of 116.0722. The standard uncertainty is:

$$u(Mstd1)=\sqrt{\sum_{j=1}^{n}\left[N_j u_j\right]^2}=$$

$$\sqrt{\begin{array}{c}\left[4\times\left(0.0008\div\sqrt{3}\right)\right]^2+\left[4\times\left(0.00007\div\sqrt{3}\right)\right]^2\\+\left[6\times\left(0.0002\div\sqrt{3}\right)\right]^2\end{array}}$$

$= 0.0020$

The relative uncertainty of the relative molecular weight of maleic acid is:

$u(Mstd1)/(Mstd1) = 0.0020 \div 116.0722$

$= 0.0017\%$

The molecular formula of 1,3,5-trimethoxybenzene is $C_9H_{12}O_3$, with the molecular weight of 168.1898. The standard uncertainty is:

$$u(Mstd2)=\sqrt{\sum_{j=1}^{n}\left[N_j u_j\right]^2}=$$

$$\sqrt{\begin{array}{c}\left[9\times\left(0.0008\div\sqrt{3}\right)\right]^2+\left[12\times\left(0.00007\div\sqrt{3}\right)\right]^2\\+\left[3\times\left(0.0002\div\sqrt{3}\right)\right]^2\end{array}}$$

$= 0.0042$

The relative uncertainty of the relative molecular weight of 1,3,5-trimethoxybenzene is:

$u(Mstd1)/(Mstd1) = 0.0042 \div 168.1898$

$= 0.0025\%$

3.5.6.3 Uncertainty of weighing u(m)

The minimum sample weight is 0.01 mg, and the sample and internal nominal sample sizes are 10 mg, 150 mg, and 20 mg respectively. According to the rectangular distribution, the calculation formula is as follows [25]:

$$u(w)=\frac{\Delta m}{k\times m}$$

Δm the balance accuracy, Δm=0.01 mg; k: rectangular distribution inclusion factor, $k=\sqrt{3}$; m: sample weighing quality, mg.

The uncertainties caused by weighing quality are 0.06%, 0.004% and 0.03%, respectively.

3.5.6.4 Purity uncertainty of internal standard $u(P_{std})$

The purity uncertainty of internal standard maleic acid and 1,3,5-trimethoxybenzene was obtained from the certificate of a reference substance, and the expanded uncertainty of maleic acid purity value is 0.06% (k = 2), so its standard uncertainty is 0.03%. The expanded uncertainty of the purity value of 1,3,5-trimethoxybenzene is 0.28% (k = 2), so its standard uncertainty is 0.14%.

3.5.6.5 Uncertainty of method u_{NMR}

The synthetic uncertainty of qNMR is the square sum of the above items. When maleic acid is used as the internal standard, the synthetic uncertainty is 0.0737%, and when 1,3,5-trimethoxybenzene is used as the internal standard, the synthetic uncertainty is 0.1553%.

4 Conclusion

This study established a quantitative analysis method for CPDX-PR active pharmaceutical ingredients and their formulations using proton nuclear magnetic resonance. Relative quantitative analysis of CPDX-PR isomers A and B was conducted, and the accurate assignment of isomers NMR data was performed for the first time. This method is fast, simple and reliable. The experimental results show that both maleic acid and 1,3,5-trimethoxybenzene can be used as internal standards for the content determination of this method. Content determination is a crucial step in the process of standard substance calibration and drug quality analysis. The principle of qNMR differs from chromatography methods, and they can complement and confirm each other. Quantitative peak signals should exhibit good peak symmetry, excellent resolution, and a high S/N.When traditional qNMR methods cannot select appropriate quantification signals in complex systems, the qGSD method can solve such problems.

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