Purity determination of cyclophosphamide hydrate by quantitative $^{31}$P-NMR and method validation

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Summary

Recently, quantitative NMR (qNMR), especially $^1H$-qNMR, has been widely used to determine the absolute quantitative value of organic molecules. We previously reported an optimal and reproducible sample preparation method for $^1H$-qNMR. In the present study, we focused on a $^{31}P$-qNMR absolute determination method. An organophosphorus compound, cyclophosphamide hydrate (CP), listed in the Japanese Pharmacopeia 17th edition was selected as the target compound, and the $^{31}P$-qNMR and $^1H$-qNMR results were compared under three conditions with potassium dihydrogen phosphate (KH$_2$PO$_4$) or O-phosphorylethanolamine (PEA) as the reference standard for $^{31}P$-qNMR and DSS-$d_6$ as the standard for $^1H$-qNMR. Condition 1: separate sample containing CP and KH$_2$PO$_4$ for $^{31}P$-qNMR or CP and DSS-$d_6$ for $^1H$-qNMR. Condition 2: mixed sample containing CP, DSS-$d_6$, and KH$_2$PO$_4$. Condition 3: mixed sample containing CP, DSS-$d_6$, and PEA. As conditions 1 and 3 provided good results, validation studies at multiple laboratories were further conducted. The purities of CP determined under condition 1 by $^1H$-qNMR at 11 laboratories and $^{31}P$-qNMR at 10 laboratories were 99.76±0.43% and 99.75±0.53%, respectively, and those determined under condition 3 at five laboratories were 99.66±0.08% and 99.61±0.53%, respectively. These data suggested that the CP purities determined by $^{31}P$-qNMR are in good agreement with those determined by the established $^1H$-qNMR method. Since the $^{31}P$-qNMR signals are less complicated than the $^1H$-qNMR signals, $^{31}P$-qNMR would be useful for the absolute quantification of compounds that do not have a simple and separate $^1H$-qNMR signal, such as a singlet or doublet, although further investigation with other compounds is needed.

Key words
quantitative $^{31}P$-NMR; cyclophosphamide hydrate; absolute purity; certified reference material
Introduction

Quantitative NMR (qNMR), especially $^1$H-qNMR, has emerged as an absolute quantitation method for small molecules. In the Japanese Pharmacopoeia (JP), including the 18th edition scheduled for publication in 2021, 19 compounds evaluated using $^1$H-qNMR are listed as HPLC analytical standards in the assay of crude drug section.\textsuperscript{1-8} To establish HPLC reference standards with purities determined by $^1$H-qNMR in the JP crude drug section, there are several problems to better understand. We previously resolved issues related to 1) establishing reference standards for $^1$H-qNMR, 2) signals from impurities in $^1$H-qNMR reference standards and targeted marker compounds, and 3) the peak unity of the signals of targeted marker compounds in HPLC.\textsuperscript{9-12} We also reported that humidity affects the purity of hygroscopic reagents. Humidity control before and/or during weighing is essential for reproducible analysis, and an indication of the absolute amount (not the purity value) that is unaffected by water content is vital for hygroscopic products determined by $^1$H-qNMR.\textsuperscript{13,14} We also developed an optimal and reproducible method for the sample preparation of hygroscopic marker compounds of crude drugs such as ginsenoside Rb\textsubscript{1} for $^1$H-qNMR.\textsuperscript{13,14}

Additionally, we have reported that the absolute quantitation of chemical drugs such as nabumetone and ipriflavone is feasible using $^1$H-qNMR.\textsuperscript{12} Recently, the absolute purity of indocyanine green, a hygroscopic chemical drug listed in the Japanese Pharmaceutical Codex 2002, was determined by $^1$H-qNMR toward adaption of the drug into the JP. A comparison of absolute indocyanine green purities under three different humidity control revealed that using a constant temperature and humidity box resulted in the lowest variability.\textsuperscript{15}

As described above, we developed an optimal and reproducible $^1$H-qNMR sample preparation method suitable for the respective properties of targeted compounds. However, depending on the purity and structure of the compound, selecting a quantitative signal can be difficult due to overlapping and complexity. To overcome this limitation, other nuclear measurements were considered potentially useful because of their signal simplicity, although their sensitivity is worse...
than that of $^1$H-NMR. Since the relative sensitivity of $^{31}$P-NMR is better than that of $^{13}$C-NMR, and $^{31}$P nuclei exist in relatively large quantities in chemical drugs, we investigated $^{31}$P-qNMR. There have been few reports on $^{31}$P-qNMR. Kato et al. reported measuring the absolute purity of synthetic phosphatidylecholine using $^{31}$P-qNMR, and the results were consistent with those determined by $^1$H-qNMR using an internal standard. However, the investigated compound was not a chemical drug, and they did not report validation data.

Cyclophosphamide hydrate (CP), an anticancer drug, is an organophosphorus compound listed in the JP 17th edition (Fig. 1). In this study, CP was selected as the compound of interest for absolute quantification using $^{31}$P-qNMR, and the results were compared with those determined by $^1$H-qNMR. In addition, validation studies of $^{31}$P-qNMR at multiple laboratories were conducted utilizing the purity determination of CP.

Results and Discussion

1. Optimization of CP purity determination by $^1$H- and $^{31}$P-qNMR

1-1. Selection of appropriate solvent and reference standards for $^1$H- and $^{31}$P-qNMR

To select the appropriate solvent, acetone-$d_6$, CDCl$_3$, CD$_3$OD, and D$_2$O were preliminary used for the $^1$H- and $^{31}$P-qNMR analysis of CP. D$_2$O was selected (Fig. 2a, 2b) because CP existed as two conformers in acetone-$d_6$, CDCl$_3$, and CD$_3$OD (data not shown). From the viewpoint of solubility, sodium 4,4-dimethyl-4-silapentanesulfonate-$d_6$ (DSS-$d_6$; IUPAC name: sodium 3-(trimethylsilyl)propane-1-sulfonate-1,1,2,2,3,3-$d_6$), an SI-traceable certified reference material (CRM), was selected as the $^1$H-qNMR reference standard. Previous reports state that potassium dihydrogen phosphate (KH$_2$PO$_4$), triphenyl phosphate, and phosphonoacetic acid can be used as commercially available CRMs for $^{31}$P-qNMR. We chose KH$_2$PO$_4$ ($\delta$P: approximately 0.7 ppm) due to its solubility and the chemical shift of CP ($\delta$P: approximately 15.9 ppm) (Fig. 1, 2b). Separately, $O$-phosphorylethanolamine (PEA; IUPAC name: 2-aminoethyl dihydrogen phosphate) was also assessed in terms of purity by $^1$H-qNMR with SI-traceable DSS-$d_6$ and then used as a reference.
1-2. Relaxation delay time for $^{31}$P-qNMR measurements

For quantitative NMR experiments using the internal standard method (AQARI: accurate quantitative NMR with internal reference substance), a relaxation delay time exceeding seven times longer than $T_1$ is required for stabilization. The $T_1$ values of the $^{31}$P signals of CP, PEA, and KH$_2$PO$_4$ in D$_2$O were 2.5, 4.6, and 8.0 s, respectively. Therefore, the relaxation delay time for $^{31}$P-qNMR was set to 60 s or more.

1-3. Hygroscopicity of CP

The hygroscopicity of CP was investigated by moisture adsorption and desorption analysis prior to quantitative NMR measurements. Under the conditions of 15–80% relative humidity, the weight of CP did not change (0.1% or less in 1 h) (data not shown). Therefore, sample preparation was performed under 15–80% relative humidity.

1-4. Optimization of $^1$H- and $^{31}$P-qNMR measurements

Next, to determine the optimum $^1$H- and $^{31}$P-qNMR measurement conditions, the following three conditions were examined in Laboratory A (Table 1). Condition 1: DSS-$d_6$ and KH$_2$PO$_4$ were used as quantitative reference standards for $^1$H- and $^{31}$P-qNMR, respectively, with separate quantitative samples. Condition 2: a mixed solution of CP, DSS-$d_6$, and KH$_2$PO$_4$ was prepared. Condition 3: a mixed solution of CP, DSS-$d_6$, and PEA was prepared. PEA was assessed for purity by $^1$H-qNMR with DSS-$d_6$ as the standard and used as a reference standard for CP quantitation by $^{31}$P-qNMR. The devices and parameters for sample preparation and $^1$H- and $^{31}$P-qNMR measurements in Laboratory A are summarized in Tables 2–4. $^1$H- and $^{31}$P-qNMR spectra of CP prepared under the three conditions are shown in Fig. 2a and b, Fig. 2c and 2d, and Fig. 2e and f, respectively, and the quantitative data for each condition are shown in Table 1.

Under condition 1, the $^1$H-qNMR spectra of CP could be measured without any problems. Signals a and b of CP were selected as quantitative candidates considering their sufficient separation and height (Fig. 2a). For $^{31}$P-qNMR, a slight signal attributed to a CP-derived degradant was observed at...
approximately 9 ppm beginning from 6 h after preparation (data not shown). However, this signal was not problematic if the sample was measured quickly after preparation (Fig. 2b). The purities of CP determined by $^1$H-qNMR were 99.37±0.20% (signal a) and 99.99±0.16% (signal b), where the quantitative signal values for a were slightly lower (Table 1). The purity of CP determined by $^{31}$P-qNMR was 99.54±0.69%; thus, the quantitative CP purities determined by $^1$H-qNMR (signals a and b) and $^{31}$P-qNMR were almost the same (Table 1). Additionally, the time course of the purity of CP determined by $^{31}$P-qNMR up to 32 h after preparation was examined. Since the determined purity was relatively stable until 12.5 h after preparation, and an apparent reduction in purity occurred after 17 h, we concluded that the data obtained within 12.5 h after sample preparation was acceptable (Fig. 3).

In the $^1$H-qNMR spectrum measured under condition 2, signals estimated to be from CP-derived degradants were observed at approximately 4.1, 3.5, and 2.0 ppm beginning from 2.5 h after preparation (Fig. 2c). In the $^{31}$P-qNMR spectrum, a small signal was observed in the shoulder of the KH$_2$PO$_4$ signal (Fig. 2d). In addition, similar to the $^1$H-qNMR spectrum, a slight signal estimated to be from a CP-derived degradant was recorded at approximately 9 ppm beginning from 2.5 h after preparation (data not shown). The purities of CP determined by $^1$H-qNMR were 99.42±0.04% (signal a) and 100.09±0.08% (signal b), while the purity of CP determined by $^{31}$P-qNMR was 97.66±1.80% with a high variation (Table 1). The relative sensitivity of $^{31}$P-qNMR is 7% that of $^1$H-qNMR, meaning that the $^{31}$P-qNMR measurement time required to obtain enough S/N for quantification is approximately 200 times longer than that required for $^1$H-qNMR. The difference in the obtained purity values with variations by $^1$H-qNMR and $^{31}$P-qNMR apparently reflect this. In other words, under condition 2, the samples decomposed gradually, suggesting that condition 2 is inappropriate for $^{31}$P-qNMR.

Under condition 3, impurity signals derived from PEA at approximately 3.8 ppm and 4.12 ppm were observed in the $^1$H-qNMR spectrum (Fig. 2e). In contrast, the $^{31}$P-qNMR spectrum did not contain any impurities (Fig. 2f). The quantitative purities of CP determined by $^1$H-qNMR were
99.28±0.11% (signal CP-a) and 99.93±0.19% (signal CP-b), where the average was slightly lower for signal CP-a (Table 1). For the determination of CP purity by $^{31}$P-qNMR, the purity of PEA, which was selected as another $^{31}$P-qNMR reference standard, was first calculated as 99.28±0.10% (signal PEA-a) using DSS-$d_6$ as the $^1$H-qNMR reference standard. It should be noted that this PEA purity was calculated by including the slight impurity signal at approximately 4.12 ppm. Next, the purity of CP was calculated by $^{31}$P-qNMR based on the calculated purity of PEA, and the result was 99.96±0.19%. As the purities determined by $^1$H-qNMR and $^{31}$P-qNMR were almost the same, we believe that condition 3 is also acceptable for $^{31}$P-qNMR measurements (Table 1).

From the above results, conditions 1 and 3 were considered appropriate for the subsequent validation studies of $^{31}$P-qNMR at multiple laboratories by comparison with established $^1$H-qNMR data. In addition, we selected signal b of CP as the quantitative signal for $^1$H-qNMR because it had the highest intensity and is a simple triplet signal without any interference by impurities. Next, validation studies were conducted.

2. Validation studies of $^{31}$P-qNMR at multiple laboratories under conditions 1 and 3

Validation studies of $^{31}$P-qNMR with conditions 1 and 3 for the purity determination of CP were conducted. Table 2 summarizes the devices and parameters used for sample preparation in each laboratory, and Tables 3 and 4 summarize the devices and parameters for $^1$H-qNMR and $^{31}$P-qNMR measurements in each laboratory, respectively.

2-1. Purity determination of CP under condition 1

Under condition 1, the CP purity determined by $^1$H-qNMR at 11 laboratories was 99.76±0.43% (Table 5), and that determined by $^{31}$P-qNMR at 10 laboratories was 99.75±0.53% (Table 6). The results were in good agreement.

2-2. Purity determination of CP under condition 3

Under condition 3, the CP purity determined by $^1$H-qNMR at five laboratories was 99.66±0.08% (Table 7). The purity of PEA, which was used as the quantitative reference standard in subsequent


$^{31}$P-qNMR measurements, determined by $^1$H-qNMR at five laboratories ranged from 98.85% to 99.77%, as shown in Table 8. The CP purity determined by $^{31}$P-qNMR at five laboratories was 99.61±0.53% (Table 9), which also agreed well with that determined by $^1$H-qNMR (Table 7).

### 2.3. Comparison of absolute CP purities

Under both conditions 1 and 3, the CP purities determined by $^1$H- and $^{31}$P-qNMR were almost the same. These data clearly suggested that the absolute quantitation of CP using both $^{31}$P-qNMR and $^1$H-qNMR is possible. Under condition 1, KH$_2$PO$_4$, an SI-traceable CRM, was used as a reference standard for $^{31}$P-qNMR to determine the CP purity. On the other hand, under condition 3, PEA was used as an alternative reference standard for $^{31}$P-qNMR, and its purity was calculated based on the SI-traceable CRM DSS-$^d_6$. As a result, a good quantitation value was obtained despite the inclusion of two weighing errors. The lower S/N of $^{31}$P-qNMR may be one reason for the slightly larger variation in CP purity values determined by $^{31}$P-qNMR, compared with the $^1$H-qNMR (Tables 3 and 4).

Additionally, the quantitative value of CP determined by titration of chloride ion caused by alkali degradation was 99.1% (data not shown); the method used (European Pharmacopoeia (EP) method) was described in the EP10.0. The value was almost the same as, but slightly smaller than that by $^1$H-qNMR and $^{31}$P-qNMR (Table 5–7, 9). This might be because the EP method is more complex, and includes a degradation process compared with qNMR methods; both of which are simple direct absolute determinations. Since qNMR is a more convenient and clean analysis than titration, our proposed method is useful for the quantitative assay of CP as a substitute of titration.

### Conclusion

In this study, we focused on a $^{31}$P-qNMR absolute determination method and selected the organophosphorus compound CP listed in the JP 17th edition as the target compound. After the optimum measurement conditions were set, validation tests at multiple laboratories were conducted. The purities of CP determined by $^1$H-qNMR and $^{31}$P-qNMR under condition 1 were 99.76±0.43%
and 99.75±0.53%, respectively, and those determined under condition 3 were 99.66±0.08% and 99.61±0.53%, respectively. These data suggested that the CP purities determined by $^{31}$P-qNMR are in good agreement with the established $^1$H-qNMR values. Since the $^{31}$P-qNMR signals are less complicated than the $^1$H-qNMR signals, $^{31}$P-qNMR would be useful for the absolute quantification of compounds that do not have a simple and separate $^1$H-qNMR signal such as a singlet or doublet, although further investigation with other compounds is necessary.

**Experiments**

1. Facilities

A total of 11 investigators from 11 laboratories (A–K) performed separate experiments.

2. Reagents, reference standards for $^1$H-qNMR and $^{31}$P-qNMR, and solvents

CP was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). DSS-$d_6$ (MW = 224.36), a CRM traceable to the National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology, was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) and used as the reference standard for $^1$H-qNMR. KH$_2$PO$_4$ (MW = 136.09), a qNMR CRM traceable to the International System of Units (SI) (TraceCERT®), was purchased from Sigma-Aldrich (MO, USA) and used as a reference standard for $^{31}$P-qNMR. PEA as another reference standard for $^{31}$P-qNMR was purchased from TCI. D$_2$O (>99.8% atom% D) was used as a deuterated solvent for qNMR, as shown in Table 2.

3. Instruments and equipment

An ultra-micro balance and micro balance with readabilities of 0.0001 and 0.001 mg, respectively, were used (Table 2). One 700 MHz NMR spectrometer equipped with a cryogenic probe and three 600 MHz, two 500 MHz, and five 400 MHz NMR spectrometers equipped with normal (room temperature) probes were used for $^1$H-qNMR measurements (Table 3). For $^{31}$P-qNMR measurements, three 600 MHz, two 500 MHz, and six 400 MHz NMR spectrometers equipped with normal probes were used (Table 4).
4. Preparation of sample solutions

4.1. NMR validation test

Approximately 5–20 mg of each reagent (CP) and approximately 1–6 mg of the reference standard for $^1$H-qNMR (DSS-$d_6$) or $^{31}$P-qNMR (KH$_2$PO$_4$ or PEA) were precisely weighed, placed in the same vial for each tare, and dissolved in the NMR solvent (D$_2$O) (1–2 mL). From the sample solution, precisely 0.6 mL was sealed in an NMR sample tube (Table 2).

4.2. Humidity conditions

Condition 1: The observed humidity’s in the 11 laboratories were 26–72%. Condition 2: The observed humidity in one laboratory was 66%. Condition 3: The observed humidity’s in the five laboratories were 51–73% (data not shown). CP, DSS-$d_6$, KH$_2$PO$_4$, and PEA were equilibrated for 0.5–3 h under each condition before weighing (Table 2).

5. Conditions for qNMR

Table 3 shows the devices and parameters employed for $^1$H-qNMR measurements in each lab. A reference standard for $^1$H-qNMR (DSS-$d_6$) was used as the chemical shift reference signal (0 ppm). The δ values are expressed in ppm. The observed spectral width was 20–25 ppm, and a digital filter was used. The center of the spectrum was set at 5 ppm, and the pulse width was set to the time at which a 90° pulse was obtained. The acquisition time was 4 or 8 s (Lab. I), the digital resolution was 0.125 or 0.25 Hz, and the delay time was 60 s. An auto FG shim or a Topshim was used for shim adjustment. The determination temperature was set at 22–30°C. $^{13}$C decoupling was performed with MPF8 or MPF9. Scans were performed 8 or 32 (Lab. G) times, and a dummy scan was performed two or four times.

Table 4 shows the devices and parameters employed for $^{31}$P-qNMR measurements in each lab. A reference standard for $^{31}$P-qNMR (KH$_2$PO$_4$ or PEA) was used as the chemical shift reference signal (0 ppm). The observed spectral width was 50 ppm, and a digital filter was used. The center of the spectrum was set at 8 ppm, and the pulse width was set to the time at which a 90° pulse was obtained. The acquisition time was 4 or 8 s (Lab. I); the digital resolution was 0.19, 0.23, or 0.25 Hz; and the
delay time was 60 or 70 s. Under condition 2, the delay times were 60 and 70 s for $^1$H- and $^{31}$P-qNMR, respectively, in Lab A. An auto FG shim or a Topshim was used for shim adjustment. The determination temperature was set at 22–30℃. $^1$H decoupling with inverse gated decoupling (No-NOE) was performed. Scans were performed 32 or 64 times, and a dummy scan was performed twice.

In principle, the measurements were performed three times for each sample following the internal standard method (AQARI) to ensure that the S/N of the quantitative signal was 100 or higher (Tables 3 and 4). Alice 2 for qNMR, Purity Pro, Delta (JEOL), and TopSpin (Bruker) were used for NMR data processing.

The trimethylsilyl peak of the reference standard for $^1$H-qNMR (DSS-d6) and the phosphorus peak of the reference standard for $^{31}$P-qNMR (KH$_2$PO$_4$ and PEA) were set at 0 ppm. Phase correction, baseline correction, and determination of peak integration ranges were performed manually or automatically. All integrated values in this study are expressed in terms of purity (%). The purity of the reagents was calculated using the following formula based on a previous study$^{21,22}$:

$$ P_{\text{sample}} = \left( \frac{I_{\text{sample}}}{I_{\text{std}}} \times \frac{H_{\text{std}}}{H_{\text{sample}}} \times \frac{W_{\text{std}}}{W_{\text{sample}}} \times \frac{M_{\text{sample}}}{M_{\text{std}}} \right) \times P_{\text{std}} $$

$P_{\text{sample}}$ = Purity of the sample (%)

$I_{\text{sample}}$ = Integral area of the sample signal

$I_{\text{std}}$ = Integral area of the reference standard signal

$H_{\text{std}}$ = Number of protons or phosphorus nuclei in the reference standard

$H_{\text{sample}}$ = Number of protons or phosphorus nuclei in the sample

$W_{\text{std}}$ = Weight of the reference standard

$W_{\text{sample}}$ = Weight of the sample

$M_{\text{sample}}$ = Molecular weight of the sample
\[ M_{\text{std}} = \text{Molecular weight of the reference standard} \]

\[ P_{\text{std}} = \text{Purity of the reference standard (\%)} \]

The following values were used for the calculations: number of methyl group protons in DSS-\textit{d}_6 (reference standard for \textsuperscript{1}H-qNMR), \( \text{CH}_3 \times 3 = 9 \); molecular weight of DSS-\textit{d}_6 = 224.36; molecular weight of CP = 279.10 (C\textsubscript{7}H\textsubscript{17}Cl\textsubscript{2}N\textsubscript{2}O\textsubscript{3}P); molecular weight of KH\textsubscript{2}PO\textsubscript{4} = 136.09; and molecular weight of PEA = 141.06 (C\textsubscript{2}H\textsubscript{8}NO\textsubscript{4}P).

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\textbf{Conflict of Interest}: The authors declare no conflict of interest.
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**Fig. 1** Structures of cyclophosphamide hydrate (CP), qNMR CRMs traceable to the SI reference standards of $^1$H- and $^{31}$P-NMR (DSS-$d_6$ and KH$_2$PO$_4$), and reference standard for $^{31}$P-NMR (O-phosphorylethanolamine (PEA))

*¹ IUPAC name: sodium 3-(trimethylsilyl)propane-1-sulfonate-1,1,2,2,3,3-$d_6$

*² IUPAC name: 2-aminoethyl dihydrogen phosphate
Fig. 2 ¹H-qNMR and ³¹P-qNMR spectra of CP under three different conditions. Condition 1: ¹H-qNMR spectrum of CP with DSS-d₆ in D₂O (a) and ³¹P-qNMR spectrum of CP with KH₂PO₄ in D₂O (b). Condition 2: ¹H-qNMR (c) and ³¹P-qNMR (d) spectra of CP with DSS-d₆ and KH₂PO₄ in D₂O, respectively. Condition 3: ¹H-qNMR (e) and ³¹P-qNMR (f) spectra of CP with DSS-d₆ and PEA in D₂O, respectively.
Fig. 3 Time course of CP purity (%) with KH$_2$PO$_4$ in D$_2$O determined by $^{31}$P-qNMR under condition
Table 1 Purity (%) of CP determined by $^1$H- and $^{31}$P-qNMR under conditions 1–3

| Condition | Reference standard for qNMR | Analyte | Solvent | $^1$H-qNMR Purity±S.D. (%) (n=3) | $^{31}$P-qNMR Purity±S.D. (%) (n=3) |
|-----------|-----------------------------|---------|---------|---------------------------------|-----------------------------------|
| 1         | DSS-$d_6$                   | CP      | D$_2$O  | Signal a: 99.37±0.20            | 99.54±0.69                        |
|           | DSS-$d_6$                   | KH$_2$PO$_4$ | CP   | Signal b: 99.99±0.16           |                                    |
| 2         | DSS-$d_6$                   | KH$_2$PO$_4$ | CP   | Signal a: 99.42±0.04            | 97.66±1.80                        |
|           |                             |         | D$_2$O  | Signal b: 100.09±0.08           |                                    |
| 3         | DSS-$d_6$                   | $\alpha$-Phosphoryl-ethanolamine (PEA) (Purity: determined by DSS-$d_6$) | CP | Signal a: 99.28±0.11          | 99.96±0.19                        |
|           |                             |         | D$_2$O  | Signal b: 99.93±0.19           |                                    |
|           |                             |         |        | PEA: 99.28±0.10                |                                    |

* Purity of DSS-$d_6$: 92.4% (CRM); purity of KH$_2$PO$_4$: 99.68% (CRM)

S.D.: Standard deviation
Table 2 Devices and parameters employed for sample preparation at different laboratories

| Sample preparation | A | B | C | D | E | F | G | H | I | J | K |
|--------------------|---|---|---|---|---|---|---|---|---|---|---|
| Reference standard for $^1$H-qNMR | | | | | | | | | | | DSS-d$_6$ (FUJIFILM Wako) |
| Reference standard for $^{31}$P-qNMR (condition 1) | | | | | | | | | | | KH$_2$PO$_4$ (SIGMA-ALDRICH) |
| Reference standard for $^{31}$P-qNMR (condition 3) | | | | | | | | | | | Reference standard for $^{1}$H-qNMR DSS-d$_6$ (Isotec) |
| Analyte | C-Phosphorylethanolamine (PEA) (TCI) | | | | | | | | | | PEA (TCI) |
| Solvent | D$_2$O: 99.9 atom%D (Isotec) | D$_2$O: 99.9 atom%D (Isotec) | D$_2$O: 99.9 atom%D (CIL) | D$_2$O: 99.9 atom%D (Alrich) | D$_2$O: 99.9 atom%D (CIL) | D$_2$O: 99.8 atom%D (Kanto) | D$_2$O: 99.9 atom%D (CIL) | D$_2$O: 99.9 atom%D (MERCK) | D$_2$O: 99.9 atom%D (Alrich) | D$_2$O: 99.9 atom%D (CIL) | D$_2$O: 99.9 atom%D (MERCK) |
| Solvent | Ultramicro balance | Ultramicro balance | Micro balance | Micro balance | Micro balance | Micro balance | Micro balance | Ultramicro balance | Ultramicro balance | Ultramicro balance | Ultramicro balance |
| Minimum indicated value (mg) | 0.0001 | 0.0017 | 0.001 | 0.001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Minimum weight (mg) | 0.3777 | 0.2951 | 0.1531 | 0.1498 | 0.0718 | 0.1531 | 0.0718 | 0.1531 | 0.0718 | 0.1531 | 0.0718 |

**Condition 1**

Preparation for $^1$H-qNMR

| Laboratory | A | B | C | D | E | F | G | H | I | J | K |
|------------|---|---|---|---|---|---|---|---|---|---|---|
| Reference standard for $^1$H-qNMR: DSS-d$_6$ (mg) | 1.1093 | 1.0545 | 2.147 | 1.996 | 2.107 | 1.0156 | 1.0128 | 1.0073 | 1.0093 | 1.0075 | 0.9508 |
| Analyte volume: CP (mg) | 6.5047 | 5.1956 | 10.003 | 10.382 | 10.385 | 5.0452 | 5.0646 | 5.3129 | 4.7470 | 5.0745 | 5.1522 |
| Equilibration period before weighing (h) | DSS-d$_6$: 1 h | DSS-d$_6$: 1 h | CP: 1 h | CP: 1 h | DSS-d$_6$: 3 h | DSS-d$_6$: 1 h | CP: 1 h | DSS-d$_6$: 0.5 h | DSS-d$_6$: 0.5 h | CP: 0.5 h | CP: 0.5 h |
| Reference standard for $^3$P-qNMR: KH$_2$PO$_4$ (mg) | 3.5049 | 2.914 | 2.958 | 5.996 | 3.021 | 3.1539 | 2.9957 | 3.2085 | 3.0782 | 3.0602 | 2.9928 |
| Analyte volume: CP (mg) | 10.2745 | 9.7551 | 4.883 | 19.597 | 9.806 | 9.9722 | 10.0758 | 10.1997 | 10.4469 | 10.1546 | 10.2215 |
| Equilibration period before weighing (h) | KH$_2$PO$_4$: 1 h | KH$_2$PO$_4$: 1 h | CP: 1 h | CP: 1 h | KH$_2$PO$_4$: 0.5 h | KH$_2$PO$_4$: 0.5 h | CP: 0.5 h | CP: 0.5 h | KH$_2$PO$_4$: 0.5 h | KH$_2$PO$_4$: 0.5 h | CP: 0.5 h |

**Condition 3**

Reference standard for $^1$H-qNMR: DSS-d$_6$ (mg) | 1.1726 | 1.0125 | | | | | | | | | 1.0602 | 1.0068 | 1.0523 |
| Reference standard for $^3$P-qNMR: PEA (mg) | 3.2419 | 3.0892 | | | | | | | | | 3.0628 | 3.035 | 3.0061 |
| Analyte volume: CP (mg) | 10.0026 | 10.3019 | | | | | | | | | 10.0818 | 10.0901 | 9.702 |
| Equilibration period before weighing (h) | DSS-d$_6$, PEA, CP: 1 h | DSS-d$_6$, PEA, CP: 1 h | | | | | | | | | DSS-d$_6$, PEA, CP: 2 h | | | |
| Laboratory | A   | B   | C   | D   | E   | F   | G   | H   | I   | J   | K   |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Observation nuclear | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H | ¹H |
| Spectrometer frequency | 600 MHz | 600 MHz | 500 MHz | 400 MHz | 500 MHz | 700 MHz | 400 MHz | 600 MHz | 400 MHz | 400 MHz | 400 MHz |
| Probe type | Normal | Normal | Normal | Normal | Normal | Cryogenic | Normal | Normal | Normal | Normal | Normal |
| Spectral width | 20 ppm | 20 ppm | 20 ppm | 20 ppm | 20 ppm | 25 ppm | 20 ppm | 20 ppm | 20 ppm | 20 ppm | 20 ppm |
| Pulse offset | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm | 5 ppm |
| Spinning | No | No | No | No | No | No | No | No | No | No | No |
| Digital filter | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Pulse angle | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° |
| Digital resolution | 0.25 Hz | 0.25 Hz | 0.25 Hz | 0.25 Hz | 0.25 Hz | 0.25 Hz | 0.25 Hz | 0.125 Hz | 0.25 Hz | 0.25 Hz | 0.25 Hz |
| Relaxation delay time | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s | 60 s |
| Acquisition time | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s |
| Measurement temperature | 22°C | 23°C | 25°C | 25°C | 23°C | 30°C | 25°C | 25°C | 25°C | 30°C | 25°C |
| ¹³C decoupling | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Decoupling sequence | MPF8 | MPF8 | MPF9 | MPF9 | MPF8 | MPF8 | MPF8 | MPF8 | MPF9 | MPF8 | MPF8 |
| Scan times | 8 | 8 | 8 | 8 | 8 | 8 | 32 | 8 | 8 | 8 | 8 |
| Dummy scan times | 2 | 2 | 2 | 2 | 2 | 4 | 2 | 2 | 2 | 2 | 2 |
| S/N (4.4 ppm: CP-a) (Condition 1) | 624 | 530 | 480 | 223 | 386 | 1527 | 610 | 252 | 230 | 350 | 204 |
| S/N (4.1 ppm: PEA) (Condition 3) | 931 | 830 | 970 | 970 | 970 | 970 | 555 | 297 |
Table 4  Devices and parameters employed for $^{31}$P-qNMR measurements at different laboratories

| Laboratory | A          | B          | C          | D          | E          | F          | G          | H          | I          | J          | K          |
|-----------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Observation nuclear $^{31}$P | 242.8 Hz (1'H: 600 MHz) | 242.8 Hz (1'H: 600 MHz) | 200 Hz (1'H: 500 MHz) | 162 Hz (1'H: 400 MHz) | 202.5 MHz (1'H: 500 MHz) | 162 Hz (1'H: 400 MHz) | 243 Hz (1'H: 600 MHz) | 161.9 Hz (1'H: 400 MHz) | 162 Hz (1'H: 400 MHz) | 162 Hz (1'H: 400 MHz) |
| Spectrometer frequency $^{1}$H | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° |
| Probe type | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Spectral width | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm | 50 ppm |
| Pulse offset | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm | 8 ppm |
| Spinning | No | No | No | No | No | No | No | No | No | No |
| Digital filter | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Pulse angle | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° | 90° |
| Digital resolution | 0.23 Hz | 0.23 Hz | 0.25 Hz | 0.25 Hz | 0.19 Hz | 0.25 Hz | 0.25 Hz | 0.23 Hz | 0.25 Hz | 0.25 Hz |
| Relaxation delay time (Condition 1) | 70 s | 70 s | 70 s | 60 s | 70 s | 70 s | 70 s | 70 s | 70 s | 70 s |
| Relaxation delay time (Condition 3) | 60 s | 60 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s | 8 s | 4 s |
| Acquisition time | 4 s | 4.3 s | 4 s | 4 s | 5.2 s | 4 s | 4 s | 4 s | 8 s | 4 s |
| Measurement temperature | 22°C | 23°C | 25°C | 25°C | 23°C | 30°C | 25°C | 25°C | 25°C | 25°C |
| $^{1}$H decoupling | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) | Inverse gated decoupling (No-NOE) |
| Decoupling sequence | Waltz | Waltz | Waltz | Waltz | Waltz | Waltz | Waltz | Waltz | Waltz | Waltz |
| Scan times | 32 | 32 | 32 | 32 | 64 | 64 | 32 | 32 | 32 | 32 |
| Dummy scan times | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| S/N (Condition 1: 15 ppm) | 303 | 280 | 137 | 280 | 154 | 345 | 210 | 230 | 433 | 160 |
| S/N (Condition 3: 0 ppm) | 210 | 260 | 280 | 280 | 154 | 345 | 210 | 230 | 433 | 160 |
Table 5 Purities (%) of CP determined by $^1$H-qNMR under condition 1 at 11 laboratories

| Position | Laboratory | Average (%) of 11 labs | S.D. (%) of 11 labs |
|----------|------------|------------------------|---------------------|
|          | A          | B                      | C                   | D    | E    | F    | G    | H    | I    | J    | K    |       |
| Signal b | 3.7 ppm    | 99.99                  | 99.82               | 100.33 | 100.13 | 99.07 | 99.61 | 99.56 | 99.42 | 100.49 | 99.44 | 99.52 | 99.76 | 0.43  |
|          | S.D. (%)   | 0.15                   | 0.29                | 0.06   | 0.16   | 0.02  | 0.03  | 0.03  | 0.01  | 0.10  | 0.14  | 0.10  |       |

S.D.: Standard deviation

Table 6 Purities (%) of CP determined by $^{31}$P-qNMR under condition 1 at 10 laboratories

| Position | Laboratory | Average (%) of 10 labs | S.D. (%) of 10 labs |
|----------|------------|------------------------|---------------------|
|          | A          | C                      | D                   | E    | F    | G    | H    | I    | J    | K    |       |
| 15.3 ppm | 99.54      | 100.48                 | 99.82               | 98.87 | 99.88 | 100.37 | 99.31 | 100.32 | 99.53 | 99.36 | 99.75 | 0.53  |
|          | S.D. (%)   | 0.69                   | 0.10                | 0.25   | 0.85   | 0.10  | 1.03  | 0.10  | 0.04  | 0.19  | 0.13  |       |

S.D.: Standard deviation
Table 7 Purities (%) of CP determined by $^1$H-qNMR under condition 3 at five laboratories

| Position | Laboratory | Average (%) of 5 Labs | S.D. (%) of 5 Labs |
|----------|------------|-----------------------|--------------------|
| Signal b (3.7ppm) | A | B | G | J | K | |
| Average (%) | 99.62 | 99.79 | 99.67 | 99.59 | 99.65 | 99.66 |
| S.D. (%) | 0.33 | 0.18 | 0.06 | 0.14 | 0.05 | 0.08 |

S.D.: Standard deviation

Table 8 Purities (%) of PEA as a reference standard for $^{31}$P-qNMR determined by $^1$H-qNMR under condition 3 at five laboratories

| Position | Laboratory | Average (%) | S.D. (%) |
|----------|------------|-------------|----------|
| 4.1 ppm | A | B | G | J | K | |
| Average (%) | 98.85 | 99.00 | 99.77 | 99.14 | 99.27 | 0.28 |
| S.D. (%) | 0.28 | 0.08 | 0.26 | 0.11 | 0.27 | |

S.D.: Standard deviation

Table 9 Purities (%) of CP determined by $^{31}$P-qNMR calculated from PEA purity under condition 3 at five laboratories

| Position | Laboratory | Average (%) of 5 Labs | S.D. (%) of 5 Labs |
|----------|------------|-----------------------|--------------------|
| 15.1 ppm | A | B | G | J | K | |
| Average (%) | 100.33 | 99.58 | 99.87 | 99.36 | 98.91 | 99.61 |
| S.D. (%) | 0.30 | 0.91 | 0.45 | 0.20 | 0.35 | 0.53 |

S.D.: Standard deviation