Rapid communication

MRC

Received: 4 June 2014 Revised: 12 August 2014 Accepted: 9 September 2014 Published online in Wiley Online Library: 8 October 2014

(wileyonlinelibrary.com) DOI 10.1002/mrc.4157

Quantification of multiple compounds containing heterogeneous elements in the mixture by one-dimensional nuclear magnetic resonance spectroscopy of different nuclei using a single universal concentration reference

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One-dimensional (1D) quantitative NMR (qNMR) is a useful tool for concentration determination due to its experimental simplicity and the direct proportionality of the integrated signal area to the number of nuclei spin. For complex mixtures, however, signal overlapping often in one-dimensional quantitative ^{1}H NMR (1D ^{1}H qNMR) spectrum limits the accurate quantification of individual compound. Here, we introduced employing joint 1D qNMR methods of different nuclei, such as ^{1}H and ^{31}P (or/and ^{19}F), to quantify multiple compounds in a complex mixture using a single universal concentration reference. When the concentration ratio of several compounds containing different elements in a complex mixture is of interest, the result calculated from measured intensities from 1D qNMR of different nuclei is independent of the gravimetric error from the reference. In this case, the common reference also serves as a 'quantitative bridge' among these 1D qNMR of different nuclei. Quantitative analysis of choline, phosphocholine, and glycerophosphocholine mixture is given as an example using trimethylphosphine oxide ($(CH_3)_3P(O)$) as concentration reference. Compounds containing multiple elements, such as tetramethylammonium hexafluorophosphate ($N^+(CH_3)_4PF_6^-$), are proposed as the common concentration reference for ^{1}H, ^{13}C, ^{15}N, ^{31}P, and ^{19}F qNMR for the quantitative analysis of complex mixture containing these different elements. We anticipate that the proposed joint 1D qNMR approach using a universal concentration reference will be a valuable alternative for simultaneous quantification of multiple compounds in a complex mixture due to its accuracy and single and simple sample preparation. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: joint 1D qNMR; universal concentration reference; trimethylphosphine oxide

Introduction

Besides providing structural information in chemical research, NMR has also been widely applied as a quantitative spectroscopic analytical tool because of its non-destructive character.[1] Usually, one-dimensional (1D) quantitative NMR (qNMR) is often used for concentration determination because of its experimental simplicity and the direct proportionality of the signal intensity to the number of nuclei spin (concentration of targeted compounds). Among these, 1D proton qNMR (^{1}H qNMR) is the most frequently used method for quantitative analysis due to the universal existence of ^{1}H in natural products, metabolites, and chemical compounds synthesized. Although ^{1}H qNMR is regarded as a routine quantitative analytical tool because of its universality and sensitivity, accurate concentration of individual compounds can only be obtained from the integrated intensities of the resolved proton signal.[2]

Previously, ^{1}H, ^{13}C, and ^{31}P qNMR spectra have been, respectively, reported as a quantitative tool.[3–5] Their application in pharmaceutical analysis has been reviewed by Holzgrabe.[6] Examples of ^{1}H, ^{13}C, ^{31}P, and ^{19}F qNMR are, respectively, given for quantitative analysis of drug impurities and the composition of drug products, such as, codergocrine, heparins, orphenadrine, the mixture of ingredients of a dosage form, phospholipids, and for monitoring the catabolic pathways of fluorinated drugs.[6,7] However, many substances were difficult to be accurately quantified by qNMR of only one nucleus due to signal overlapping. For example, quantification of choline and the related compounds phosphocholine (PC) and glycerophosphocholine (GPC) in a biological sample only by ^{1}H qNMR is hindered by signal overlapping.[8]

To overcome the signal overlapping issue in ^{1}H NMR spectrum, ^{31}P edited ^{1}H NMR spectroscopy was previously applied for quantification of PC and GPC mixtures.[8] In addition, 1D ^{1}H qNMR was extended to 2D NMR for quantitative purpose, such as ^{13}C-^{1}H HSQC experiments.[9–11] However, besides more time consuming for quantitative analysis, 2D qNMR concomitantly brought about the problem of loss of the direct proportionality of the signal intensity to the concentration, which has to be calibrated by referring to standard curve, theoretical calculation, or back extrapolation to obtain the signal-specific attenuation factor. Previously, some compounds such

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as sodium 3-trimethylsilyl[2,2,3,3-D_4]propionate (*TSP-d_4*) and maleic acid are proposed as concentration reference for ^{1}H qNMR,[3] and compounds such as trifluoroacetic acid and methylphosphonic acid are proposed as concentration reference for ^{19}F and ^{31}P qNMR.[12]

In this paper, we proposed the joint 1D qNMR methods of different nuclei using a single universal concentration reference as general protocol for the quantitative analysis of a mixture. The joint 1D qNMR methods are much more convenient to be implemented and more time-effective compared to $HSQC_0$.[11,13,14] The concentration can be more straightforward obtained from the simple relationship of direct proportionality, avoiding standard curve preparation in advance[9] or complicated calculation of the signal attenuation factor in the 2D pulse sequence.[10] Choline, PC, and GPC are considered to be important metabolites in human tumor.[15] Previous studies have demonstrated that the relative ratios and concentrations of these metabolites associated with the development and progression of breast cancer.[16] In this study, we demonstrate that employing the joint 1D qNMR methods of ^{1}H and ^{31}P can accurately and efficiently quantify individual compound in the mixture of choline, PC, and GPC, using trimethylphosphine oxide ($(CH_3)_3P(O)$, TMPO) as the single universal concentration reference for both 1D ^{1}H and ^{31}P qNMR.

Materials and methods

To verify the applicability of the joint 1D qNMR methods of ^{1}H and ^{31}P, series of model mixtures of $(CH_3)_3P(O)$ TMPO (Adamas-beta, 98%) and tert-butylphosphonic acid ($(CH_3)_3CP(O)(OH)_2$ TBPA) (Across, 98%) with varying concentration ratios were prepared. A 177.5 mg TMPO and a 279.5 mg TBPA were weighed on an analytical balance of ±0.1 mg precision and put directly into a 10 ml volumetric flask; D_2O (Cambridge Isotope Laboratories, 99.9%) was added to the marked line to give final stock concentrations of 188.91 mM and 198.34 mM, respectively. The final NMR samples were prepared by mixing TMPO and TBPA to give the final concentration ratio at 1 : 0.105, 1 : 0.525, 1 : 1.05, 1 : 2.10, 1 : 4.20, 1 : 6.30, 1 : 8.40, 1 : 10.50, 1 : 21.00, and 1 : 42.00, in which the final concentration of TMPO was fixed at 9.446 mM (1 : 20 dilution of its stock), except for the last two samples with the concentration ratios of 1 : 21.00 and 1 : 42.00, in which the final concentration of TMPO was 4.723 and 2.362 mM, respectively. Accordingly, the concentration of TBPA was varied at 0.9917 mM, 4.9585 mM, 9.917 mM, 19.834 mM, 39.668 mM, 59.502 mM, 79.336 mM, 99.17 mM, 99.17 mM, and 99.17 mM). These mixtures were put into 5 mm NMR tube for NMR data collection.

To further demonstrate the application of the joint 1D qNMR methods of ^{1}H and ^{31}P, a model mixture of choline (choline chloride, 99%, Adamas-beta), PC (choline glycerophosphate, 98%, Adamas-beta), and GPC (phosphocholine chloride calcium salt tetrahydrate, 98%, Adamas-beta) was prepared, and the concentration of each of them was accurately and efficiently measured using TMPO as the single concentration reference for both ^{1}H and ^{31}P qNMR spectra. Choline (74.9 mg), PC (48.1 mg), and GPC (54.8 mg) were dissolved in 2 ml D_2O, resulting stock concentrations of 265.546, 91.630, and 81.436 mM, respectively. The final sample was prepared by mixing TMPO (50 µl, 188.91 mM), choline (50 µl, 265.546 mM), PC (50 µl, 91.630 mM), and GPC (50 µl, 81.436 mM) into D_2O with the total final volume of 1000 µl. The sample of 600 µl was then transferred into an NMR tube for NMR data collection.

All NMR data were collected at 298 K on a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm Smartprobe. Operating at 400.1318 (^{1}H) and 161.9799 (^{31}P) MHz, respectively.

1D ^{1}H qNMR data with ^{31}P decoupled and 1D ^{31}P qNMR data with ^{1}H decoupled using Bruker standard pulse program 'zgig' were collected with carrier frequency of ^{1}H and ^{31}P set at 4.7 and 27.0 ppm, respectively. For quantitative purpose, it is crucial to allow all spins from different molecules to be fully relaxed (>99.9% recovery). In 1D ^{1}H qNMR, the recycle delay, d1, was set to 25 s, but in 1D ^{31}P qNMR, d1, it was set to 65 s, which are larger than five times of the longest ^{1}H/^{31}P T_1 of the spins in the sample (^{1}H/^{31}P T_1 values are obtained from typical inversion-recovery experiment, using Bruker standard pulse program 't1ir1d'). In 1D ^{1}H qNMR, 32 K data points were acquired with spectral width of 21 ppm and number of scans (NS) of 4, and acquisition time of 1.946 s, resulting total experimental time of about 2.5 min. The signal-to-noise ratios of all interested peaks were larger than 900. In 1D ^{31}P qNMR, 64 K data points were acquired with spectral width of 100 ppm and number of scans (NS) of 16, and acquisition time of 2.027 s, resulting total experimental time of about 22.5 min. The minimum signal-to-noise ratio obtained was about 78.

Phase and baseline corrections were carried out automatically in Bruker Topspin. The signal intensities from the mixtures of TBPA and TMPO were all obtained by peak fitting using NMR software Mnova (Mestrelab Research,Spain). For the mixture of choline, PC, and GPC, because of the partial overlapping of the signals of the methyl groups in 1D ^{1}H qNMR spectrum, their overall intensities are summed together by integration over a region (2.67–3.36 ppm) covering all of them, while for other isolated peaks, the intensities are integrated individually over a range at least 40 times of the full-width at half-height, capturing about 97% of the peak intensity with the assumption of a Lorentzian lineshape. Random noise regions from 2.28 to 2.53 ppm in 1D ^{1}H qNMR and from 30.46 to 33.78 ppm in 1D ^{31}P qNMR are integrated, respectively, resulting integrations of −0.0021 and 0.0026, which manifest that the effect of the spectral noise on the obtained peak integration is ignorable.

Results

Theoretical basis of qNMR is that the intensity *I* of a certain peak in the spectrum is directly proportional to the number of the corresponding nuclei spins, *N*.[6,7] In case there is no signal overlapping, the concentration of the compound S (C_s) can be easily calculated from the measured peak intensities of certain characteristic peaks in 1D qNMR of the compound S,[6] that is

$$C_s = \frac{n_{Ref} \cdot I_S}{n_S \cdot I_{Ref}} C_{Ref} \quad (1)$$

in which I_S and I_{Ref} are the characteristic peak intensities of the compound S and reference in 1D qNMR, n_S and n_{Ref} are the number of the magnetic equivalent spin nuclei at the corresponding chemical shift of compound S and reference, respectively, (such as for methyl group, the number of protons, n_S is 3). C_s and C_{Ref} are the molar concentration of the compound S and reference, respectively.

We demonstrated the applicability of 1D ^{1}H qNMR and 1D ^{31}P qNMR for concentration measurement using the series of model mixtures of TMPO and TBPA with varying concentration ratios prepared as aforementioned. Peak intensities in 1D ^{1}H qNMR spectrum are measured at 1.07 ppm (group $(CH_3)_3C$- of TBPA with $n_{H/S} = 9$)

and 1.51 ppm ($3 \times CH_3$ groups of TMPO with $n_{H/Ref} = 9$). The concentration of TBPA is calculated using Eqn (1) using TMPO as the concentration reference. The obtained concentrations of TBPA show very good agreement with the gravimetric concentrations with a linear correlation coefficient of 0.991 and a linear equation of $C_{exp} = 1.006C_{grav}$. Triplicated measurement is made at the gravimetric concentration of TBPA at 99.17 mM, the obtained concentrations of TBPA from 1D ^{1}H qNMR are 104.49, 97.83, 91.56 mM, giving the estimated relative error of 1.22% and the relative precision of 6.52%. Similarly, in 1D ^{31}P qNMR spectrum, peak intensities are measured at 36.79 ppm (P(O) of TBPA with $n_{P/S} = 1$) and 53.08 ppm (P(O) of TMPO with $n_{P/Ref} = 1$). The concentration of TBPA is calculated with Eqn (1), again using TMPO as concentration reference. The obtained concentrations of TBPA as well showed good agreement with the gravimetric concentrations with a linear correlation coefficient of 0.986 and a linear equation of $C_{exp} = 1.001C_{grav}$. As aforementioned, the relative measurement error of 0.05% and relative precision of 10.27% were given by triplicated measurements from 1D ^{31}P qNMR, which are comparable to the counterparts from 1D ^{1}H qNMR spectrum.

Using the model mixture in the preceding text, the dynamic range of the absolute concentration that can be accurately measured is about 100 times (from 0.9917 to 99.17 mM), while the dynamic range of the molar concentration ratio between the sample (TBPA) and the reference (TMPO) is even larger, about 420 times (from 1 : 0.105 to 1 : 42.00), from both ^{1}H and ^{31}P qNMR spectra. The molar concentration ratios can be accurately obtained by varying the concentration of the reference TMPO. It is worth noting that the dynamic range reported here was specifically generated from the data in this study; the limits of the dynamic range of measurable concentration or concentration ratio were not attempted.

The concentrations of TBPA obtained from 1D ^{1}H qNMR are compared with their counterpart from 1D ^{31}P qNMR. Linear regression yielded a regression line of $C_{P,TBPA} = 0.9947C_{H,TBPA}$ with correlation coefficient of 0.996, demonstrating the consistency between ^{1}H and ^{31}P qNMR quantification using a single universal concentration reference, which contains the elements of both proton and phosphorus. Concomitantly, these results prop up the applicability of the joint 1D qNMR methods of ^{1}H and ^{31}P because of their consistency.

We demonstrate the application of the joint 1D qNMR methods of ^{1}H and ^{31}P using a model mixture of choline, PC, and GPC prepared as aforementioned. The concentration of each component can be accurately and efficiently measured using TMPO as the single concentration reference, although the signals of the methyl groups of choline, PC, and GPC are overlapped in 1D ^{1}H qNMR spectrum. The signals from PC (3.18 ppm) and GPC (−0.12 ppm) are well separated and also isolated from the signal of the reference, TMPO (53.06 ppm) in the ^{31}P spectrum, which makes it feasible to calculate the concentrations of PC and GPC from their individual integrated intensities. Based on the measured peak intensity of TMPO in 1D ^{31}P qNMR spectrum and its concentration known, the concentrations of PC and GPC are

$$C_{PC} = \frac{n_{P/TMPO} \cdot I_{P.PC}}{n_{P/PC} \cdot I_{P.TMPO}} C_{TMPO} \quad (2)$$

$$C_{GPC} = \frac{n_{P/TMPO} \cdot I_{P.GPC}}{n_{P/GPC} \cdot I_{P.TMPO}} C_{TMPO} \quad (3)$$

in which $I_{P.PC}$, $I_{P.GPC}$, and $I_{P.TMPO}$ are the peak intensities of PC (at 3.18 ppm), GPC (at −0.12 ppm), and TMPO (at 53.06 ppm), respectively; $n_{P/PC}$, $n_{P/GPC}$, and $n_{P/TMPO}$ are all 1 because there is only one phosphorus atom in each molecule of PC, GPC, and TMPO; C_{TMPO} is the known concentration of the reference, TMPO.

Using the identical sample with the same internal reference (no need for repetitive sample preparation), the signals of the methyl groups of choline (symbolized as C), PC, and GPC are overlapped in 1D ^{1}H qNMR spectrum. The overall intensity summed up over a region (2.67–3.36 ppm) covering all of them is directly proportional to the total amount of the nuclei spins. Because $n_{H/C}$, $n_{H/PC}$, $n_{H/GPC}$, and $n_{H/TMPO}$ are all 9, the concentration of choline can be calculated from Eqns ((2) and (3)) as

$$\begin{aligned} C_C &= \frac{I_{H.C+PC+GPC}}{I_{H.TMPO}} \cdot C_{TMPO} - C_{PC} - C_{GPC} \\ &= \left(\frac{I_{H.C+PC+GPC}}{I_{H.TMPO}} - \frac{n_{P/TMPO} \cdot I_{P.PC}}{n_{P/PC} \cdot I_{P.TMPO}} - \frac{n_{P/TMPO} \cdot I_{P.GPC}}{n_{P/GPC} \cdot I_{P.TMPO}} \right) \cdot C_{TMPO} \end{aligned} \quad (4)$$

In which $I_{H.\,C\,+\,PC\,+\,GPC}$ is the overall intensity of the overlapped methyl groups of choline, PC, and GPC; $I_{H.\,TMPO}$ is the intensity of a specific peak (at 1.51 ppm) of TMPO, and C_C, C_{PC}, and C_{GPC} are the molar concentration of choline, PC, and GPC, respectively. If only the conventional ^{1}H qNMR is used, it is difficult to accurately quantify the concentrations of choline, PC, and GPC because of the signal overlapping in the methyl region (2.67–3.36 ppm). However, if the joint 1D qNMR methods of ^{1}H and ^{31}P are applied, the absolute concentration of each component can be accurately obtained from the Eqns ((2), (3), and (4)), requiring only a single concentration reference TMPO. For the model mixture, using Eqns ((2), (3), and (4)), the concentrations of choline, PC, and GPC calculated from the integrated intensities over the regions are 14.079 mM, 4.214 mM, and 4.450 mM, respectively. The relative errors are 6.03%, 3.48%, and 2.88% for choline, PC, and GPC, respectively.

Discussion

In case that signal overlapping in the conventional 1D ^{1}H qNMR spectrum hinders the accurate quantification of individual compound, here, we proposed employing joint 1D qNMR methods with single and simple sample preparation for quantification of multiple compounds in a complex mixture using a single compound consisting of multiple elements as universal concentration reference for qNMR of different nuclei. Using a model mixture of choline, PC, and GPC, we demonstrated that the quantification of individual compound can be obtained using TMPO as the universal reference for both 1D ^{1}H and ^{31}P qNMR. The accuracy is satisfactory. The deviation of the measured absolute concentrations of choline, PC, and GPC from their gravimetric concentrations can be possibly partially attributed to the gravimetric error in their stock solutions, which are prepared by weighing, and to the impurities in the chemical reagents used. Note that the certified purities of choline, PC, GPC, and TMPO (reference) are 99%, 98%, 98%, and 98%, respectively. Ignoring or including the satellite peak intensities in both ^{1}H and ^{31}P qNMR due to the coupling to the natural abundance of ^{13}C could also contribute to the deviation.[6] The NMR data, ^{1}H qNMR data with ^{31}P decoupled and ^{31}P qNMR data with ^{1}H decoupled, were collected with a double-resonance Smartprobe (^{1}H and ^{19}F, ^{31}P–^{15}N), with which ^{13}C decoupling cannot be simultaneously applied.

However, ^{13}C decoupling issue can be easily tackled if an H/C/BB or H/P/BB triple-resonance probe is available.

Furthermore, if only the relative concentration ratios of several compounds in a complex mixture are of interest, for example, the relative ratios of choline, PC, and GPC are associated with the development and progression of cancers as stated in the preceding text, the ratios can be calculated from the measured intensities from 1D qNMR spectra of both ^{1}H and ^{31}P, using Eqns ((2), (3), and (4)) as

$$\begin{aligned} C_C : C_{PC} : C_{GPC} &= \left(\frac{I_{H.C+PC+GPC}}{I_{H.TMPO}} - \frac{n_{P/TMPO}\cdot I_{P.PC}}{n_{P/PC}\cdot I_{P.TMPO}} - \frac{n_{P/TMPO}\cdot I_{P.GPC}}{n_{P/GPC}\cdot I_{P.TMPO}}\right) C_{TMPO} \\ &: \frac{n_{P/TMPO}\cdot I_{P.PC}}{n_{P/PC}\cdot I_{P.TMPO}} C_{TMPO} : \frac{n_{P/TMPO}\cdot I_{P.GPC}}{n_{P/GPC}\cdot I_{P.TMPO}} C_{TMPO} \\ &= \left(\frac{I_{H.C+PC+GPC}}{I_{H.TMPO}} - \frac{n_{P/TMPO}\cdot I_{P.PC}}{n_{P/PC}\cdot I_{P.TMPO}} - \frac{n_{P/TMPO}\cdot I_{P.GPC}}{n_{P/GPC}\cdot I_{P.TMPO}}\right) \\ &: \frac{n_{P/TMPO}\cdot I_{P.PC}}{n_{P/PC}\cdot I_{P.TMPO}} : \frac{n_{P/TMPO}\cdot I_{P.GPC}}{n_{P/GPC}\cdot I_{P.TMPO}} \end{aligned} \tag{5}$$

which are in fact independent of the gravimetric error from the reference. As shown in Eqn (5), the concentration term of the reference (C_{TMPO}) does not show up, implying that the gravimetric error of the reference TMPO is irrelevant to the measured concentration ratios. In this case, the single common concentration reference serve as a 'quantitative bridge' among these 1D qNMR spectra of different nuclei.

The joint 1D qNMR methods of ^{1}H and ^{31}P, which combines ^{1}H with ^{31}P NMR spectra for quantitative purpose, can be further explored. Compounds containing multiple elements, such as tetramethylammonium hexafluorophosphate ($N^{+}(CH_3)_4PF_6^{-}$), can be used as the common concentration reference for ^{1}H, ^{13}C, ^{15}N, ^{31}P, and ^{19}F qNMR for the quantitative analysis of compounds in a complex mixture containing these different elements. We anticipate that the proposed joint 1D qNMR approach using a single universal concentration reference will be a valuable alternative for simultaneous quantification of multiple compounds containing different elements in a complex mixture due to its accuracy and single and simple sample preparation.

Acknowledgements

K. H. acknowledges funding support from The Recruitment Program of Global Youth Experts; Kunming Institute of Botany, Chinese Academy of Sciences, and Yunnan Provincial Science and Technology Department (2012HA015).This study made use of the NMR Facility at Public Technology Service Center of Kunming Institute of Botany, Chinese Academy of Sciences.

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