Homo- and Heteronuclear Multiple-Quantum Filters for Measurement of NMR Isotope Shifts

E. WREN WOOTEN,* †+ RAJESH K. DUA,* GARRY D. DOTSON,* AND RONALD W. WOODARD*

*College of Pharmacy and †Biophysics Research Division, The University of Michigan, Ann Arbor, Michigan 48109

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The measurement of NMR isotope shifts as mechanistic probes can be complicated by mixtures of isotopomers. Homo- and heteronuclear NMR techniques based on multiple-quantum filtration are presented and shown to be a useful aid in measuring such shifts. The effects of $^1$H/$^2$H substitution and $^{16}$O/$^{18}$O substitution on the nuclear shielding of $^1$H, $^2$C, and $^3$P in a multiply labeled phosphoenolpyruvate are measured and interpreted qualitatively in terms of their rovibrational origins. © 1994 Academic Press, Inc.

INTRODUCTION

The effect of isotopic substitution on nuclear shielding has long been used as a probe in mechanistic chemistry and enzymology (1-3). For example, the difference in the shift of $^{13}$C when directly bonded to $^{16}$O or $^{18}$O is often used to determine whether a C–O bond is broken during a chemical reaction (4). Observation and measurement of isotope shifts are typically carried out using a single NMR tube containing the labeled and unlabeled species. As previously pointed out, however, one major obstacle in such studies is the presence of mixtures of isotopically labeled species (3). Mixtures often arise from incomplete stereo- or regiospecificity of the labeling method. The spectral complexity resulting from the combined effects of scalar couplings and multiple isotope shifts can often make the observation of the desired shift extremely difficult. This can be especially problematic when $^{18}$O-labeled water is used to incorporate $^{18}$O by acid-catalyzed exchange. The use of "unnormalized" water provides a much cheaper and more readily available source of $^{18}$O than its "normalized" counterpart; however, the unnormalized reagent typically contains >90% deuterium, which usually becomes incorporated to some degree during the exchange. Several NMR spectral-editing techniques have been suggested to circumvent the problem of mixtures and have largely involved using spin echoes (4, 5), DEPT-based methods (6, 7), or extensions of the two (7). Here we present editing techniques based on multiple-quantum filtration (MQF).

† To whom correspondence should be addressed.

Homonuclear multiple-quantum filtration (HoMQF) techniques (8, 9) are well-established for spectral simplification and are often used as a means to simplify the spectra of biomolecules. HoMQF typically involves the creation of multiple-quantum coherences between protons. The coherences are then separated and detected (usually indirectly) on the basis of their responses to the phase of RF pulses on the protons. Heteronuclear multiple-quantum filtration (HeMQF) is a similar technique closely related to INEPT (10), DEPT (11, 12), and methods like them (13), except MQF uses phase shifts instead of flip angles for spin system discrimination. HeMQF involves creating multiple-quantum coherences between, for example, protons and one or more X nuclei. In contrast to HoMQF, the coherences are then separated and detected (usually indirectly) according to the RF phase of the proton pulses, the X pulses, or both. Multiple-quantum filtration methods are therefore useful as "molecular selectors" to select out subspectra corresponding to individual populations of molecules, because the possible multiple-quantum coherences that can be created are determined by the coupling topology of the molecule and the values of the scalar coupling constants. We show that by using homo- and heteronuclear multiple-quantum filtration, we can obtain subspectra for the various isotopomers in a multiply isotopically labeled phosphoenolpyruvate. In so doing, we can measure precisely the effects of $^1$H/$^2$H substitution and $^{16}$O/$^{18}$O substitution on the nuclear shielding of $^1$H, $^2$C, and $^3$P.

MATERIALS AND METHODS

Synthesis of $[2-^{13}$C,2-18O,3,3-H(D)] Phosphoenolpyruvate

$[2-^{13}$C] Sodium pyruvate (99%) purchased from Cambridge Isotope Laboratory, was suspended in CCl$_4$, first converted into pyruvic acid by the addition of 1.1 equivalent of concentrated HCl and then into bromopyruvic acid by treatment with bromine via the method reported by Stubbe and Kenyon (14). The 3-bromo-[2-13C] pyruvic acid was converted into 3-[2-13C,2-18O,3,3-H(D)] bromopyruvate by the exchange method of Sprinser et al. (15) except that
unnormalized water, purchased from Cambridge Isotope Laboratory (18O, 98%; D, 90%), was utilized. The NaCl present from the initial sodium salt was removed by filtration once the labeled bromopyruvic acid was dissolved in anhydrous diethyl ether. The ethereal solution of labeled bromopyruvic acid was converted into enolpyruvyl phosphate by treatment with P(OCH3)3 under Perkov reaction conditions and then into its monocarbamylamine salt by the method reported by Clark and Kirby (16). The melting point range was 143–144°C, in agreement with the previously published range of 143–146°C (17). The compound was seen to be pure by NMR. The structure of phosphoenolpyruvate (PEP) and the isotopic labeling positions are shown in Fig. 1. The resulting compound is a mixture of labeled molecules with varying degrees of incorporation of deuterium and 18O, giving rise to as many as eight possible structures.

**NMR Spectroscopy**

Nuclear magnetic resonance spectra were recorded on the monocarboxylamine salt on a Bruker AMX500 spectrometer (11.75 T) at a probe temperature of 298 K using 5 mm high-resolution NMR tubes. Sample volumes were 500 μl at a concentration of 10 mg/ml in deuterium oxide (100 at%). For 1H spectra, a spectral width of 3000 Hz was employed, while for 13C and 31P, a spectral width of 2000 Hz was used. Spectra were obtained with a 1.0 s relaxation delay and 16,384 complex points in the time domain using simultaneous detection of real and imaginary components. The GARP sequence (18) was used for heteronuclear decoupling.

The time-domain data were apodized with an exponential (0.5 Hz for X nuclei) prior to zero-filling followed by Fourier transformation. The pulse sequences and phase cycles used are shown in Fig. 2.

Figure 2a shows the pulse sequence, phase cycle, and coherence-transfer pathways (19) used to obtain 1H subspectra for the diptroto and monoproto (E and Z) PEP species via HoMQF. The first pulse creates transverse magnetization which evolves under the proton chemical shifts and the homo- and heteronuclear scalar couplings. The second pulse refocuses all except the homonuclear coupling. The antiphase term due to this coupling reaches a maximum at the end of the second τ period for τ = 1/4JHH, when the third pulse converts the antiphase coherence into a linear combination of homonuclear zero-, single-, and double-quantum coherences. The fourth pulse then converts nonobservable coherence into single-quantum coherence and, together with the appropriate addition of signals in the receiver (19), serves to select or reject one or more of the coherence orders. The final τ − π − τ sequence allows the antiphase single-quantum coherence created by the fourth pulse to evolve into in-phase coherence prior to detection.

Figure 2b shows the pulse sequence, phase cycle, and coherence-transfer pathways used to obtain 13C and 31P sub-

![Figure 1](image_url)

**RESULTS**

Figure 3a shows the unfiltered 1H spectrum of PEP (i), the 1H spectrum of the diptroto species obtained by homonuclear double-quantum filtration (ii), and the monoproto species obtained by homonuclear single-quantum filtration (iii). Figure 3b shows the unfiltered 1H-decoupled 13C spectrum of PEP (i), the 13C spectrum of the diptroto species obtained by heteronuclear double-quantum filtration (ii), and the monoproto species obtained by heteronuclear double-quantum filtration (iii). Figure 3c gives the analogous spectra for 31P. No evidence for significant amounts of the dideterro compound was seen either in the unfiltered spectrum or by heteronuclear single-quantum filtration. Addition of the subspectra gives a sum comparable to the unfiltered spectrum in all three cases. The sets of proton subspectra allow the effects of deuterium on the proton chemical shifts to be ob-
tained. More interestingly, the X-nucleus subspectra allow
the isotope shift from substitution with an isotope at one
site to be measured in the presence and absence of sub-
titution at another site with a different isotope. The various
isotope shifts are tabulated in Table 1. The convention for
the sign of the isotope shift is that used by Hansen (3),
\[ \Delta X(Y) = \delta X(L) - \delta X(H) \]. \( \Delta X \) is the isotope shift on X
when substituted by an isotope Y, while \( \delta X(L) \) and \( \delta X(H) \)
are the X chemical shifts when substituted by the light and
heavy isotopes, respectively. Scalar coupling constants are
given in Table 2 and do not, within experimental error, reveal
any isotope effects.

**DISCUSSION**

The unfiltered spectra in Fig. 3(i) demonstrate the diffi-
c ulty of precise measurement of isotope effects in multiply
isotopically labeled mixtures. This is especially pronounced
in the case of phosphorus [Fig. 3(c)(i)]. Nonetheless, the
spectra in Fig. 3(ii and iii) show that MQF can give good
subspectra for the various components.

From the spectra in Fig. 3a, the deuterium isotope effect
on the proton shielding is easily observed. In the diproto-
species [Fig. 3a(ii)], the lines are complicated multiplets
due to the scalar coupling of each proton to the other proton,
FIG. 3. Unfiltered (i), diprotio (ii), and monoprotio (iii) species subspectra observing $^1$H (a), $^{13}$C (b), and $^{31}$P (c).
### Table 1
Isotope Shifts for Observed Nuclei in PEP for Various Combinations of Isotopic Substitutions A, B, C, D, and E

<table>
<thead>
<tr>
<th>Observed nucleus</th>
<th>A ((^{16}\text{O}^{18}\text{O},\text{H})</th>
<th>B ((^{16}\text{O}^{18}\text{O},\text{D})</th>
<th>C (\text{H/D},^{16}\text{O})</th>
<th>D (\text{H/D},^{18}\text{O})</th>
<th>E (^{16}\text{O}/^{18}\text{O},\text{D})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H}) (E proton)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>(^1\text{H}) (Z proton)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>(^13\text{C})</td>
<td>0.019</td>
<td>0.020</td>
<td>0.031</td>
<td>0.032</td>
<td>0.051</td>
</tr>
<tr>
<td>(^31\text{P})</td>
<td>0.018</td>
<td>0.019</td>
<td>-0.006</td>
<td>-0.005</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Note: As described under Results, the convention for the sign of the isotope shift is \(\Delta X(Y) = \Delta X(L) - \Delta X(H)\). The nomenclature found in parentheses is defined as follows: (\(^{16}\text{O}^{18}\text{O},\text{H}) and (\(^{16}\text{O}^{18}\text{O},\text{D}) represent the isotope shifts upon substitution of \(^{16}\text{O}\) with \(^{18}\text{O}\) in the diproto and monoproto forms, respectively. (\text{H/D},^{16}\text{O}) and (\text{H/D},^{18}\text{O}) represent substitution of H with D in the \(^{16}\text{O}\) and \(^{18}\text{O}\) forms, respectively. (^{16}\text{O}/^{18}\text{O},\text{D}) represents substitution of H and \(^{18}\text{O}\) with D and \(^{16}\text{O}\).

### Table 2
Homo- and Heteronuclear Scalar Coupling Constants in PEP for Various Combinations of Isotopic Substitutions A, B, C, and D

<table>
<thead>
<tr>
<th>Coupling pair</th>
<th>A ((^{16}\text{O}^{18}\text{O},\text{H})</th>
<th>B ((^{16}\text{O}^{18}\text{O},\text{D})</th>
<th>C (^{16}\text{O},^{18}\text{O})</th>
<th>D (^{16}\text{O},^{18}\text{O})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{13}\text{C}^{31}\text{P})</td>
<td>8.09</td>
<td>8.05</td>
<td>8.15</td>
<td>8.06</td>
</tr>
<tr>
<td>(^{13}\text{C}^{1}\text{H}) (E proton)</td>
<td>nd</td>
<td>3.91</td>
<td>nd</td>
<td>3.91</td>
</tr>
<tr>
<td>(^{13}\text{C}^{1}\text{H}) (Z proton)</td>
<td>nd</td>
<td>5.37</td>
<td>nd</td>
<td>5.37</td>
</tr>
<tr>
<td>(^{31}\text{P}^{1}\text{H}) (E proton)</td>
<td>nd</td>
<td>2.08</td>
<td>nd</td>
<td>2.08</td>
</tr>
<tr>
<td>(^{31}\text{P}^{1}\text{H}) (Z proton)</td>
<td>nd</td>
<td>1.71</td>
<td>nd</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Note: The nomenclature found in parentheses is defined as follows: (\(^{16}\text{O}^{18}\text{O},\text{H}) and (\(^{16}\text{O}^{18}\text{O},\text{D}) represent the coupling constants in the diproto and monoproto forms with \(^{16}\text{O}\), while (^{16}\text{O},^{18}\text{O}) and (^{16}\text{O},^{18}\text{O}) represent the corresponding coupling constants for forms with \(^{18}\text{O}\). nd, not determined.
topes is additive. This is demonstrated by the observation, for example, that in Table 1, columns A and C or columns B and D add up to give column E within experimental uncertainty. These last two observations can be attributed to small secondary dynamic factors; that is, the average $^{13}$C-O and $^{31}$P-O bond lengths do not change due to $^1$H/$^2$H substitution, and likewise for the $^1$H-$^12$C bond length upon $^{16}$O/$^{18}$O substitution. Such changes have been shown to be typically at least two orders of magnitude smaller than primary dynamic effects, the change in average bond length at the site of substitution (26). Finally, we note that while we have applied HeMQF to the measurement of NMR isotope effects, it is equally applicable to other situations where it is desirable to observe molecular subspectra within a complex mixture.

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REFERENCES