Feasibility Evaluation of Detecting Hydroxymethylphosphine Oxide In Vivo by $^{31}$P-MRS

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ABSTRACT

Application of organophosphorus compounds in biomedicine is attractive because the $^{31}$P nucleus is very amenable to study by nuclear magnetic resonance (NMR) techniques, particularly, by in vivo $^{31}$P magnetic resonance spectroscopy ($^{31}$P-MRS). The water-soluble organophosphorus compounds that are non-toxic, exhibit metabolic stability, and show a unique resonance peak in $^{31}$P NMR spectroscopy, which could be ideal to be used as probes for $^{31}$P-MRS. Here we evaluated the in vivo feasibility of potentially using a hydroxymethylphosphine oxide as a novel probe for $^{31}$P-MRS studies using tris (hydroxymethyl) phosphine oxide (THPO) as an example. THPO was synthesized, injected in the normal C57 mice, and $^{31}$P spectra were acquired before and after injection with the coil located on the regions of interest. The NMR signal from the region of interest appeared within one minute of THPO injection. The compound was stable in vivo as no metabolites of THPO were observed. No toxicity was observed after THPO injection in mice. The peak concentrations of THPO in liver and kidney were reached within 15 min and 60 min respectively. THPO was excreted exclusively in urine without undergoing any metabolism indicating that it is very stable under in vivo conditions. These initial studies in normal C57 mice clearly demonstrate that THPO possess the essential characteristics required for a potential MRS probe. Based on the current preliminary results, we suggest that HMPs, when incorporated into targeted drugs (peptides, proteins, antibodies, etc.), may serve as novel $^{31}$P probes for monitoring the drug distribution in vivo by MRS. (Int J Biomed Sci 2010; 6 (3): 228-232)

Keywords: $^{31}$P-MRS; hydroxymethylphosphine oxide; MRI; NMR

BACKGROUND

Application of organophosphorus compounds in biomedicine is attractive because the $^{31}$P nucleus is very amenable to study by nuclear magnetic resonance (NMR) techniques, particularly, by in vivo $^{31}$P magnetic resonance spectroscopy ($^{31}$P-MRS). The advantages of using $^{31}$P-MRS are: a) $^{31}$P is a desirable NMR nucleus with 100% natural abundance, high NMR sensitivity (~6.6% of $^1$H), relatively large g value (10,830 rad/gauss), and a large chemical shift range (1); b) the technique is intrinsically noninvasive; c) there is no radioactivity involved and therefore the compounds are inherently stable with a long shelf life, allowing studies at later time points after injection when the blood background is diminished; and d) it is possible to obtain high-resolution anatomic $^1$H magnetic resonance imag-
Hydroxyethylphosphine oxide detection in vivo by $^{31}$P-MRS

By 31P-mrs

www.ijbs.org    Int  J  Biomed  Sci    V ol. 6  No. 3    September   2010

ing (MRI) in conjunction with the $^{31}$P NMR spectra, which provide context in terms of organs and tissue heterogeneity, with no need for imaging after processing and co-registration procedures.

The organophosphorus compounds that are non-toxic, soluble in biologically compatible solvents, exhibit metabolic stability, and show a unique resonance peak in $^{31}$P NMR spectroscopy, are ideal to be used as probes for $^{31}$P-MRS. Here we discuss the utility of a tris (hydroxymethyl) phosphine oxide (THPO) as a probe for $^{31}$P MRS. Little is known regarding the toxicity of THPO. THPO was not found to be mutagenic in Salmonella and did not inhibit acetyl cholinesterase in vitro (2, 3). THPO was nominated by the National Toxicology Program in 2005 to undergo toxicity tests, since it is a by-product and metabolite of tetrakis (hydroxymethyl) phosphonium chloride (THPC), a reactive flame retardant widely used with cellulosic and cellulosic blend fabrics, including FR-treated infant sleepwear and work clothes (4).

Katti et al. have reported the utility of hydroxymethylphosphines as chelating ligands for labeling biomolecules with $^{99m}$Tc/$^{188}$Re for developing targeted diagnostic/therapeutic radiopharmaceuticals (5-7). However, no organophosphorus compound has ever been utilized for any other diagnostic application. To our knowledge, this is the first report that describes the utility of an organophosphorus compound as an extrinsic $^{31}$P-MRS probe. $^{31}$P-MRS is currently used to determine intrinsic phosphorus metabolite concentrations in human tissues such as muscle and brain (8, 9).

METHODS

Materials

Tetrakis (hydroxymethyl) phosphonium chloride, triethylamine, and 30 wt% hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO). Tris (hydroxymethyl) phosphine (THP) was synthesized by slight modifications to the previously reported procedure (10). $^{31}$P NMR spectroscopy was performed on a Varian Mercury VX-300 NMR Spectrometer at the NMR Facility (University of Oklahoma, Norman, OK). CF-1 (19-21 g) male mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). All animal studies were conducted in accordance with the protocols approved by both the OUHSC and OMRF institutional animal care and use committees. All MR experiments were conducted using a 2.0 cm surface coil in a 7 Tesla 30 cm horizontal bore small animal MRI system (Bruker BioSpin MRI, Germany) at the Advanced Magnetic Resonance Center at the Oklahoma Medical Research Foundation, Oklahoma City, OK.

Tetrakis (hydroxymethyl) phosphine oxide (THPO)

To a solution of THP (3.4 g, 27.4 mmol) in water (6 ml) was added 30 wt% hydrogen peroxide in water (8.8 ml, 77.6 mmol). The reaction mixture was heated to 35 °C for 30 min. The progress of the reaction was monitored by $^{31}$P NMR. After completion of the reaction, the water was evaporated on rotary evaporator to obtain the THPO as a white solid (yield - 3.6 g, 93.8%). The THPO was used without any further purification. $^{31}$P NMR (D$_2$O, 121.47 MHz) δ (ppm) 48.9 (s, 1P); $^{13}$C NMR (D$_2$O, 75.45 MHz) δ (ppm) 52.2 (d, 3C).

$^{31}$P MRS

THPO frequency

To determine the frequency of the THPO, two 1.5 ml centrifuge tubes (one containing 1M phosphoric acid as a reference and the other containing 1M THPO solution) were placed at the isocenter of the magnet and a $^{31}$P single pulse spectrum was acquired with a repetition time (TR) of 3,000 ms, 2,048 points, an acquisition time of 146.23 ms and a spectral width of 14 kHz. The THPO frequency was determined to be 121.589 MHz.

T1 relaxation time of THPO

To identify the T1 relaxation time of the THPO, a series of $^{31}$P RARE images with varying TR were acquired on a 1.5 ml centrifuge tube containing 1M THPO solution (scan parameters: echo time (TE)=14.4 ms; TR=250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000 and 5,000 ms; matrix size=32 × 32 pixels; field of view=8 × 8 cm$^2$; 1 axial slice of 5 mm thickness). Regions of interest were drawn on the tube to get signal intensity measurements that were fitted to an equation linking signal intensity to TR:

$$S = C_1 - C_2e^{-\frac{TR}{T1}}$$

The T1 of THPO was determined to be 2,990 ms. As a consequence, a TR of 3,000 ms was chosen for subsequent experiments.

Animal experiments

Animal experiments were conducted at a frequency of 121.589 MHz using a $^{31}$P single pulse sequence over a spectral width of 8,000 Hz, 32 averages, a TR of 3,000 ms, 2,048 points, and an acquisition time of 1 min 36 s. A 72
mm-diameter \(^1\)H/\(^31\)P resonator was used for signal trans-
mission and the signal was received through a tunable
\(^1\)H/\(^31\)P flat surface coil positioned on the region of inter-
est of the sample (phantom or mouse). Normal CF-1 mice
were anesthetized initially using 2% isoflurane in oxygen
at 2 L/min, in a polypropylene induction chamber. When
fully anesthetized, a catheter was placed in the tail vein
and the animals were placed on the scanner bed. Anesthe-
sia was maintained through a nose cone with 1% isoflurane
in oxygen at 2 L/min. Body temperature was maintained
at 37 ± 1°C by using a water-circulated pad under the an-
imal. Animals were placed in a supine position for the liver
experiments, or on its side for the kidney experiments, and
the \(^1\)H/\(^31\)P surface coil was located on the organs of inter-
est. A quick \(^1\)H-T1-weighted morphological image was ac-
quired to guarantee the proper positioning of the surface
coil, and a \(^31\)P “baseline” spectrum was acquired. 100 µl of
4M THPO solution was then administered through the tail
vein, and \(^31\)P spectrum acquisition was resumed for 2 hours
(liver experiment) or 4 hours (kidney experiment). Follow-
ing MR experiments, the mice were euthanized, urine and
blood of the animal were collected, and \(^31\)P spectra were
acquired on these \textit{ex vivo} samples. \(^31\)P spectra were pre-
processed using the Bruker TopSpin software (apodiza-
tion, Fourier transformation, and phasing of spectra), and
then calibrated and integrated to provide peak areas using
Mathematica (Wolfram Research).

RESULTS AND DISCUSSION

To demonstrate the proof-of-concept, we synthesized
THPO and evaluated the feasibility of observing this com-
 pound using \(^31\)P-MRS in normal CF1 mice. THPO was
synthesized in a two-step reaction as shown in Figure 1.
First, commercially available THPC was treated with tri-
ethylamine to obtain THP and then it was oxidized with
hydrogen peroxide to obtain THPO. THPO is soluble in
water at all concentrations due to the presence of hydro-
philic -CH\(_2\)OH substituents, and presents a single peak
in \(^31\)P NMR spectra with a chemical shift of ~49 ppm as
shown in Figure 2. This chemical shift is farther down
field than that of all indigenous phosphorous containing
compounds (<20 ppm) found in the body.

For \textit{in vivo} evaluation, we injected THPO solution in
the first mouse and acquired \(^31\)P spectra before and after
injection with the coil located between the kidneys and
the bladder, as shown in Figure 3. The NMR signal from
the region covering the kidneys and the bladder appeared
within one minute of injection. The compound was stable
\textit{in vivo} as no metabolites of THPO were observed. Only
a single peak corresponding to THPO was observed
throughout the imaging period of 61 min (Figure 3b). Af-
fter THPO injection, the mouse behaved normally and no
adverse reactions were observed.

To follow the clearance of THPO from the blood pool,
we injected two mice with THPO and assessed the liver in
one mouse for 2 h, and the kidney in the other mouse for 4
h (Figure 4). The mice were killed, and the blood and urine
were collected at the end of the imaging study. As shown in
the Figure 4b, the peak concentrations in the liver and the
kidneys were reached within 15 min and 60 min, respec-
tively. Since THPO is very hydrophilic, as expected it was
cleared predominantly through the kidneys into urine (Fig-
ure 5). The clearance from the kidneys was relatively slower
because THPO is a small hydrophilic and neutral molecule
that may be just cleared by glomerular filtration but not se-
creted by the tubules. As shown in Figure 5a, no THPO was
observed in the blood samples collected from the mice in-
dicating that THPO was completely cleared from the blood
pool by 2 h. THPO was excreted exclusively in urine without
undergoing any metabolism indicating that it is very stable
under \textit{in vivo} conditions (Figure 5b). More \textit{in vivo} studies
are needed to determine the pharmacokinetic properties of
THPO, as well as toxicity. These initial studies in normal
CF1 mice clearly demonstrate that THPO possess the es-
tential characteristics required for a potential MRS probe.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Figure1.png}
\caption{Synthesis of THPO. a) Triethylamine (10 eq), 8 h, room temp; b) 33 wt% H\(_2\)O\(_2\) (2.8 eq), 0.5 h, 35 °C.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Figure2.png}
\caption{\(^31\)P NMR spectrum of THPO.}
\end{figure}
Figure 3. a) Representative $^1$H-MRI image of a mouse (circle shows the region of interest observed by the surface coil); b) $^{31}$P spectra obtained from the region of interest.

Figure 4. a) $^1$H-MRI image from a representative mouse (circle shows the surface coil region of interest); b) Peak areas of the THPO resonance in liver and kidneys are plotted as a function of time after injection in two different mice.
New HMPO derivatives containing functional groups such as –COOH that allow conjugation to biomolecules (e.g., peptides, proteins, antibodies, etc.), as shown in Figure 6, are needed to increase the concentration of the probe at the targeted disease site. The hydrophilic nature of HMPOs facilitates their efficient clearance from the blood pool primarily through the renal/urinary pathway if they are separated from biomolecules due to any in vivo degradation process. Thus, this minimizes the background signal due to presence of free HMPO in the blood pool. However, the biodistribution and pharmacokinetic properties of the HMPO-biomolecule conjugate may predominantly be dominated by those of the biomolecule itself.

Based on the current preliminary results, we suggest that HMPOs, when incorporated into targeted drugs (peptides, proteins, antibodies, etc.), may serve as novel 31P probes for monitoring the drug distribution in vivo by MRS. However, due to the inherent low sensitivity, it is a challenging endeavor to develop a molecular-targeting MRS probe. We will undertake further studies to address the critical issue of sensitivity with HMPO based molecular-targeting MRS probes in the future.

ACKNOWLEDGEMENTS

This work was funded by the University of Oklahoma College of Pharmacy Startup Grant. We gratefully acknowledge the expert technical assistance of Debra Saunders during animal studies.

ABBREVIATIONS

HMPO, Hydroxymethylphosphine oxide; THPO, Tris (hydroxymethyl) phosphine oxide; THPC, Tetrakis (hydroxymethyl)phosphonium chloride; THP, Tris (hydroxymethyl) phosphine; MRS, Magnetic resonance spectroscopy; MR, Magnetic resonance; NMR, Nuclear magnetic resonance; ppm, Parts per million; TR, Repetition time; TE, Echo time.

CONFLICT OF INTEREST

The authors declare that no conflicting interests exist.

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