Short Communication

In Vivo Magnetic Resonance Imaging To Detect Biliary Excretion of 19F-Labeled Drug In Mice

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Running Title Page

Running title: $^{19}$F-Drug Detected in Mouse Gallbladder by in vivo MRI

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Nonstandard abbreviations:

MR, magnetic resonance
PET, positron emission tomography
RF, radiofrequency
ABSTRACT:

Isoflurane is an inhaled halogenated hydrocarbon anesthetic commonly used for animal research. In our quest to develop a method for measuring bile acid transport in live animals using $^{19}$F magnetic resonance (MR) imaging, it occurred to us that isoflurane, which contain five fluorines per molecule and is likely widely distributed, would provide an excellent test drug to evaluate the merits of this approach. Experiments in 20-28 g male C57BL/6 mice were performed using a Bruker Biospec 7.0 Tesla 30-cm horizontal bore scanner with a 30-mm $^{19}$F/$^1$H dual-tuned surface coil (Bruker) used to transmit and receive radiofrequency signals at 300.28 MHz for $^1$H and 282.55 MHz for $^{19}$F nuclei. Proton MR imaging was used to identify the mouse gallbladder in vivo which was subsequently verified by anatomical dissection. Subsequent experiments in mice inhaling 1.5% isoflurane for 1 to 2 h revealed robust $^{19}$F signals from the gallbladder, verified by overlying $^1$H and $^{19}$F signals. No $^{19}$F signal was detected in mice anesthetized with non-halogenated anesthetics. The presence of isoflurane in gallbladder bile of isoflurane-treated mice was verified using liquid chromatography-mass spectrometry. Gallbladder bile isoflurane content ranged from 3.2 to 4.7 $\mu$g. The data presented here provide proof-of-concept that this novel approach can be used for in vivo measurement of biliary excretion of both existing and novel $^{19}$F-labeled drugs.
Introduction

In the course of studying bile acid transport using an in vitro assay system, we observed that many commonly-used drugs inhibit the function of the major intestinal bile acid transporter, the apical sodium-dependent bile acid transporter (ASBT; gene name SLC10A2) (Zheng et al., 2009). These ASBT inhibitors include 3-hydroxy-3-methylglutaryl-coenzyme A inhibitors (statins) and dihydropyridine calcium-channel blockers; statins and calcium-channel blockers are amongst the most commonly prescribed drugs in the United States. Inhibition of ASBT results in reduced intestinal uptake of bile acids and increased fecal bile acid concentrations, a risk factor for colon pathology, including cancer (Flynn et al., 2007). Hence, side-effects deriving from drug-induced inhibition of ASBT could pose potential, currently unappreciated risk.

Based on these observations we decided to develop a new in vivo assay to measure the ability of drugs to inhibit bile acid transport. Currently available methods to measure bile acid transport in vivo are limited to 75Se-25-homocholic acid-taurine (75SeHCAT) (Boyd et al., 1981; Fracchia et al., 1998) and luciferase-FXR imaging (Houten et al., 2007). 75SeHCAT, which was not approved by the U.S. Food and Drug Administration and available only in Europe, requires administration of a radioactive agent (Pattni et al., 2009) and its production was recently discontinued by the supplier (European Amersham catalogs). Luciferase-FXR imaging is applicable only in transgenic mice expressing the reporter gene and provides very limited anatomical detail (Houten et al., 2007). Hence, to measure in vivo bile acid transport we conceived of an innovative, non-radioactive approach; 19F-labeled bile acid tracers and in vivo magnetic resonance imaging (MRI) in mice.

19F is particularly well suited to monitor distribution of administered agents; 19F is second only to 1H in sensitivity for MRI and background 19F signal in animals and humans are negligible (Jiang et al., 2009). Positron emission tomography (PET) was considered but rejected for this purpose because PET probes are radioactive (e.g. 18F), more costly (cyclotron needed to create 18F for each use) and have short radiation half-lives (109 min for 18F). In mice, stable non-radioactive 19F-labeled tracers can be
imaged for days (Jiang et al., 2009).

Before proceeding with the complex and time-consuming development of $^{19}$F-labeled bile acid tracers, several key issues had to be addressed. First, for reasons of cost and efficiency, an animal model is needed to measure bile acid up-take by in vivo MR imaging. After excretion from the liver, bile acids are stored and concentrated in the gallbladder. Hence, gallbladder imaging would be most likely to result in a $^{19}$F signal of sufficient strength for detection. Because rats lack a gallbladder, mice would serve this purpose, and were previously used as test animals for studies of bile acid transport (Dawson et al., 2003). Second, a fluoridated test agent was required to validate the approach. Fluoridated hydrocarbons, particularly 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane) (Fig. 1), are commonly used to anesthetize mice, and wide tissue distribution as well as limited hepatic metabolism of this agent, is reported (Kharasch et al., 1993; Wissing et al., 2000; Kharasch, 2008) [Forane (Isoflurane) package insert]. Hence, isoflurane which contains five fluorines per molecule (Fig. 1) appeared to be an ideal test agent. Moreover, among the volatile halogenated anesthetics, isoflurane appears to undergo the least amount of defluorination (Kharasch et al., 1993). Almost 30 years ago, it was shown that high-resolution $^{19}$F magnetic resonance spectra from isoflurane could be obtained from a surface coil centered over the brain of a living rabbit (Wyrwicz et al., 1983).

Therefore, in the present study, we investigated whether isoflurane could be measured in mouse gallbladder bile using in vivo MRI. To verify the presence of isoflurane in gallbladder bile and estimate the limits of detection, we used liquid chromatography-mass spectrometry (LC-MS). The data presented here provide proof-of-concept that this novel approach can be used for in vivo measurement of biliary excretion of both existing and novel $^{19}$F-labeled drugs.
Methods

Animals. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). Mouse studies were approved by both the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and the Research and Development Committee at the VA Maryland Health Care System. C57BL/6 mice were obtained from Jackson Labs, Bar Harbor, ME and housed under identical conditions in a pathogen-free environment with a 12:12-hour light/dark cycle and free access to standard mouse chow and water for one week prior to treatment. Mice were fasted overnight before imaging studies.

Live Animal MRI. In vivo experiments were performed on a Bruker Biospec 7.0 Tesla 30-cm horizontal bore scanner using Paravision 5.0 software (Bruker Biospin MRI GmbH, Germany). A Bruker 30-mm $^{19}$F/$^1$H dual-tuned surface coil was used to transmit and receive radiofrequency (RF) signals at 300.28 MHz for $^1$H and 282.55 MHz for $^{19}$F nuclei. Mice (male C57BL/6; 20 to 28 g body weight) were anesthetized in an animal chamber with a gas mixture of O$_2$ (1 L/min) and isoflurane (3%; IsoFlo, Abbott Laboratories, North Chicago, IL). Animals were then placed supine in a Bruker animal bed and the RF coil was positioned and fixed with surgical tape in the region of interest on the animal body. The animal bed was moved to the center of the magnet and the isoflurane level was changed to 1.5% and maintained at this level for the remainder of the experiment. An MR-compatible small-animal monitoring and gating system (SA Instruments, Inc., New York, NY) was used to monitor animal respiration rate and body temperature. Mouse body temperature was maintained at 36-37°C using a warm water circulator.

Localization of the gallbladder was attained by first obtaining images using $^1$H-MRI. Three-slice (axial, mid-sagittal, and coronal) scout rapid acquisition with fast low angle shot MR imaging (FLASH) was used to first localize the volume of interest. High resolution proton density-weighted anatomic images were acquired using rapid acquisition with relaxation enhancement (RARE) sequence in the axial view with repetition time = 1847 or 2631 ms, echo time = 11 ms, RARE factor = 8, field of view = 6
x 6 mm², slice thickness = 1 mm, matrix size = 400 x 400, in-plane resolution = 0.15 x 0.15 mm², number of slices = 12 or 24, and number of averages = 8. Total acquisition time was not more than 18 min.

Low resolution ¹⁹F images were acquired using FLASH sequence in the same region of the anatomic ¹H MRI with repetition time = 123 or 245 ms, echo time = 6 ms, excitation pulse angle = 30 degree, field of view = 6 x 6 mm², matrix side = 32 x 32, in-plane resolution = 1.875 x 1.875 mm², slice thickness = 4 mm, number of slices = 3 or 6, and number of averages from 600 to 1200. Total acquisition time was less than 2 h and 36 min. Mice inhaled isoflurane from 50 to 94 min prior to ¹⁹F MRI experiments.

**Liquid chromatography- mass spectrometry (LC-MS).** In parallel studies, to measure isoflurane content in gallbladder bile, mice were dosed with isoflurane using the same protocol described for live animal MRI. Mice were euthanized immediately after completing isoflurane inhalation and the gallbladders removed and weighed. Tissue samples were transported in tightly sealed containers with minimal head-space and stored on ice prior to sample preparation. The gallbladder was ground using a tissue homogenizer in the presence of 75:25::acetonitrile:water solvent. Homogenized tissue was centrifuged (9600 g for 1 min; 4°C) and isoflurane content of the supernatant was measured using LC-MS. LC-MS was conducted using a TSQ Vantage (Thermo Scientific) mass spectrometer, with Accela 1250 pump and PAL HTC-Accela1-TM autosampler. The column was a Phenomenex Gemini C18 (50 X 4.6 mm, 3 u, 110 A). The mobile phase (1.2 mL/min, water and acetonitrile) was a gradient of: 0 - 0.5 min, 40% ACN; 0.5 – 1.5 min, 40% to 95% ACN; 1.5 – 2.5 min, 95% ACN; 2.5 – 2.6 min, 95% to 40% ACN; and 2.6 – 3.6 min, 40% ACN. MS was performed using an APCI probe, in negative mode; ion transition m/z 183 → 163 was used to quantify isoflurane. Data collection and processing used Xcalibur 2.3.0 with LC Quan 2.6.0 software.
Results and Discussion

Exhalation is the major route of elimination for halogenated hydrocarbon anesthetics like isoflurane (Fig. 1). Based on both in vitro and in vivo data, it is estimated that liver metabolism, primarily by CYP2E1, contributes only 0.1 – 2% of isoflurane elimination (Kharasch, 2008; Kharasch et al., 1993). The major metabolite resulting from CYP2E1 metabolism of isoflurane is trifluoroacetyl chloride (Kharasch, 2008; Kharasch et al., 1993; Njoku et al., 1997). Minimal metabolism by the liver is thought to be the major reason that isoflurane poses a lower risk of hepatotoxicity than other halogenated anesthetics, like halothane (Christ et al., 1988; Kharasch, 2008; Njoku et al., 1997).

Although isoflurane is widely distributed (Wissing et al., 2000), no report has investigated its distribution into bile. Nevertheless, we focused our studies on imaging isoflurane in the gallbladder; exobiotics can be excreted by the liver into the biliary tree and both stored and concentrated in the gallbladder. Consequently, we reasoned that we were most likely to detect the strongest $^{19}$F signal in gallbladder bile. The mouse gallbladder is well revealed by proton MRI in the anterior upper abdomen (Fig. 2A). The identity of the gallbladder on MR imaging was confirmed by immediate post-imaging animal dissection (Fig. 2B).

Our initial experiments used the same scan resolution for proton and fluorine imaging. However, due to the relative weaker sensitivity of fluorine, in subsequent studies we modified MR imaging to obtain 4-mm slice thickness for $^{19}$F images instead of the 1-mm slice thickness used for $^1$H images. These modifications resulted in a stronger $^{19}$F signals. As shown in Fig. 3, following isoflurane anesthesia, MRI revealed a robust $^{19}$F signal in the mouse gallbladder (two representative examples are shown). No $^{19}$F signal was detected in the gallbladder of control mice that were anesthetized with subcutaneous injection of ketamine and xylazine instead of inhaled isoflurane (data not shown). This localization of $^{19}$F signal in the gallbladder suggests isoflurane is distributed into bile.

To assess $^{19}$F MRI results, parallel studies using LC-MS to measure isoflurane were
performed in five mice. Four mice were treated with inhaled isoflurane as described in Methods and one control mouse received subcutaneous non-halogenated anesthetics (no isoflurane). As an unplanned control, the gallbladder of one of the four isoflurane-treated mice was emptied of bile prior to removal, such that no isoflurane was detected in that mouse.

Table 1 lists the amount of isoflurane in each mouse gallbladder. No isoflurane was found in gallbladders from the two control mice; one that did not receive isoflurane and one whose gallbladder emptied before assay. In the remaining three isoflurane-treated mice, 4.72, 3.25 and 3.52 μg isoflurane was detected in the gallbladder (mean ± SE, 3.83 ± 0.45 μg) (Table 1). Although the volume of gallbladder contents was not measured, we estimated isoflurane concentration in gallbladder bile based on published data relating gallbladder volume to mouse weight (Graewin et al., 2004). This analysis yielded gallbladder bile isoflurane concentrations of 2.2, 1.3 and 1.4 mM, respectively (Table 1). This suggests that the limits of detection for isoflurane using 19F MRI are at least 1 to 2 mM.

Regarding the use of this approach for measuring bile acid transport, an advantage of MR imaging of the gallbladder is that bile acids are stored in this organ. It is estimated that after an overnight fast, at least 80% of the bile acid pool is stored in the gallbladder (Nilsell, 1990). The amount of 19F-labeled bile acid tracer that accumulates in the mouse gallbladder during an overnight fast will depend on the test dose, stability, absorption, mouse body weight and other factors. Nonetheless, using conservative estimates, we calculate that gavaging mice with 19F-labled chenodeoxycholic acid (15-300 μg/g body weight) will result in ~2-15 mM 19F-labeled bile acid in the gallbladder (assuming ~50% 19F-labeled bile acid accumulates in the gallbladder, vol. ~20 μl). These values are above the limits of detection for isoflurane in the present communication (1-2 mM).

In summary, mice inhaling 1.5% isoflurane for 1 to 2 h revealed robust 19F signals in the gallbladder, verified by overlying 1H and 19F signals. No 19F signal was detected in mice anesthetized with non-halogenated agents. The presence of isoflurane in gallbladder bile of isoflurane-treated mice was confirmed using LC-MS; isoflurane
content ranged from 3.2 to 4.7 μg. This demonstrates the sensitivity of $^{19}$F MRI; the level of detection is at least 1-2 mM. The data presented here provide an important proof-of-concept that this novel approach can be used for in vivo measurement of biliary excretion of both existing and novel $^{19}$F-labeled drugs.
Acknowledgments

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Authorship Contributions

**Participated in research design:** Raufman, Xu, Cheng, Johnson, Gullapalli, and Polli

**Conducted experiments:** Raufman, Xu, Cheng, Khurana, Johnson, Shao, Kane, Shi, Gullapalli, and Polli

**Contributed new reagents or analytic tools:** Xu, Johnson, Shao, Kane, Shi, Gullapalli, and Polli

**Performed data analysis:** Raufman, Xu, Cheng, Johnson, Shao, Kane, Gullapalli, Polli

**Wrote or contributed to the writing of the manuscript:** Raufman, Xu, Cheng, Khurana, Johnson, Gullapalli, Polli
References


Footnotes

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Legends for figures

Figure 1. Chemical structure of 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane) highlighting the five fluorines, including the three equivalent fluorines bonded to the ethane portion.

Figure 2. Localization of the mouse gallbladder by live animal MR imaging and verification by anatomical dissection. A. High-resolution proton density-weighted anatomical image of the murine gallbladder (arrowhead) acquired using live animal MR imaging as described in Methods. B. Gallbladder location (arrow) and appearance was verified by dissection immediately after liver animal MR imaging. The mouse gallbladder, just to the left of midline, is attached superiorly by the falciform ligament. It is likely that the gallbladder appears less distended than in the MR image because of the effects of ketamine/xylazine anesthesia, given after the effects of isoflurane had abated, and as a consequence of surgical manipulation and stretching of the gallbladder to obtain the photograph.

Figure 3. Comparison of $^1$H anatomical MR imaging and $^{19}$F isoflurane signal in murine gallbladder. The results of two experiments in live mice are shown. The left panels show $^1$H anatomical imaging of the murine gallbladder (arrowheads indicate gallbladder). The middle panels show the $^{19}$F signal from the corresponding region. The right panels show a reconstruction overlay of the $^{19}$F and $^1$H images revealing that the $^{19}$F signal emanates from the gallbladder.
TABLE 1

Amount and calculated concentration of isoflurane in mouse gallbladder.

Isoflurane content of mouse gallbladder bile was measured using LC-MS; TSQ Vantage mass spectrometer, with Accela 1250 pump and PAL HTC-Accela1-TM autosampler. The column was a Phenomenex Gemini C18 (50 X 4.6 mm, 3 u, 110 A). The mobile phase (1.2 mL/min, water and acetonitrile) was a gradient of: 0 - 0.5 min, 40% ACN; 0.5 – 1.5 min, 40% to 95% ACN; 1.5 – 2.5 min, 95% ACN; 2.5 – 2.6 min, 95% to 40% ACN; and 2.6 – 3.6 min, 40% ACN. MS was performed using an APCI probe, in negative mode; ion transition m/z 183 → 163 was used to quantify isoflurane. Data collection and processing used Xcalibur 2.3.0 with LC Quan 2.6.0 software.

<table>
<thead>
<tr>
<th>Gallbladder weight (mg)</th>
<th>Gallbladder isoflurane (µg)</th>
<th>Calculated isoflurane concentration (mM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-isoflurane control</td>
<td>4.9</td>
<td>Not detected</td>
</tr>
<tr>
<td>Emptied-gallbladder control</td>
<td>3.3</td>
<td>Not detected</td>
</tr>
<tr>
<td>Isoflurane-treated mouse 1</td>
<td>26.0</td>
<td>4.72</td>
</tr>
<tr>
<td>Isoflurane-treated mouse 2</td>
<td>12.6</td>
<td>3.25</td>
</tr>
<tr>
<td>Isoflurane-treated mouse 3</td>
<td>17.3</td>
<td>3.52</td>
</tr>
</tbody>
</table>

*aCalculated isoflurane concentration assumes mouse gallbladder volume of 8 µL per 16 g mouse total body weight (Graewin et al., 2004).
Figure 3

$^{1}$H MRI  $^{19}$F MRI  $^{19}$F/$^{1}$H Overlay