Validation of Isolated Metabolites from Drug Metabolism Studies as Analytical Standards by Quantitative NMR

Gregory S. Walker, Tim F. Ryder, Raman Sharma, Evan B. Smith, and Amy Freund

Pharmacokinetics, Dynamics, and Metabolism Department, Pfizer Global Research and Development, Groton, Connecticut (G.S.W., T.F.R., R.S.); Pharmacokinetics, Dynamics, and Metabolism Department, Pfizer Global Research and Development, La Jolla, California (E.B.S.); and Bruker BioSpin Corporation, Billerica, Massachusetts (A.F.)

Received September 13, 2010; accepted November 22, 2010

ABSTRACT:

In discovery and development, having a qualified metabolite standard is advantageous. Chemical synthesis of metabolite standards is often difficult and expensive. As an alternative, biological generation and isolation of metabolites in the nanomole range are readily feasible. However, without an accurately defined concentration, these isolates have limited utility as standards. There is a significant history of NMR as both a qualitative and a quantitative technique, and these concepts have been merged recently to provide both structural and quantitative information on biologically generated isolates from drug metabolism studies. Previous methodologies relied on either specialized equipment or the use of an internal standard to the isolate. We have developed a technique in which a mathematically generated signal can be inserted into a spectrum postacquisition and used as a quantitative reference: artificial signal insertion for calculation of concentration observed (aSICCO). This technique has several advantages over previous methodologies. Any region in the analyte spectra, free from interference, can be chosen for the reference signal. In addition, the magnitude of the inserted signal can be modified to appropriately match the intensity of the sample resonances. Because this is postacquisition quantification, no special equipment or pulse sequence is needed. Compared with quantitation via the addition of an internal standard (10 mM maleic acid), the signal insertion method produced similar results. For each method, precision and accuracy were within ±5%, stability of signal response over 8 days was ±5%, and the dynamic range was more than 3 orders of magnitude: 10 to 0.01 mM.

Introduction

The quantitative and qualitative assessment of metabolites from pharmacologically active compounds has traditionally been a topic of great interest within the pharmaceutical industry. This interest includes the assessment of reactive metabolites, the establishment of the pharmacological activity of metabolites, and the monitoring of metabolites in a clinical setting [metabolites in safety testing (MIST) activities] (Prakash et al., 2007; Kalgutkar, 2008; Nedderman, 2009).

The issuance of the MIST guidelines has increased the need for the quantitative monitoring of metabolites in preclinical and clinical studies (U.S. Food and Drug Administration, 2008). This document was intended to provide guidance on how to increase safety in human trials through having adequate exposure of metabolites in preclinical studies. In the past, this was accomplished first by structural characterization of an unknown metabolite using various liquid chromatography (LC), mass spectrometry (MS), and NMR techniques. Once the metabolite structure was defined, several milligrams were chemically synthesized and qualified for purity. The qualification process could include assessment of water content, combustion analysis to estimate inorganic impurities, and HPLC analysis to estimate organic impurities (Williams, 2006; Görög, 2008). This standard was then treated as any other chemical entity and could be used to produce standard curves or quality control samples or used in pharmacological assays.

It may also be advantageous to have a metabolite standard during drug discovery. When a compound is selected to advance from discovery to development, there are multiple factors that can differentiate a candidate from a series of related compounds. Understanding the exposure of reactive/active metabolites and clearance pathways and resolving pharmacokinetic/pharmacodynamic disconnects are parameters that are made easier by having an authentic metabolite standard. It is clear that there is a need in both discovery and development for qualified standards of metabolites.

Many metabolites are difficult, and therefore expensive, to chemically synthesize, for example, glucuronides, glutathiones, or oxidations that introduce chirality. Thus, chemical synthesis of metabolites for standards is often delayed to later stages of drug development. An alternative approach to chemical synthesis is the biological generation and isolation of metabolites. Using source material generated from in vivo or in vitro studies, isolates in the nanomole range can be produced (Espina et al., 2009). These isolates are usually adequate for the structural identification of a compound using MS and NMR;
however, there is seldom sufficient material to qualify these isolates as true analytical standards using gravimetric methods. As discussed above, the purity of a synthetically generated metabolite would be determined using a variety of techniques based on gravimetric analysis. However, at the levels achievable through biological generation, gravimetric analysis is often not feasible. Consequently, other methods must be used to determine the concentration/purity of an isolate. In situations, in which no qualified standard is available, the purity of an isolate would be determined by HPLC. An ideal HPLC detection method would be sensitive and specific and have a uniform response independent of molecular structure. UV, chemiluminescent nitrogen detection, evaporative light scattering detection, refractive index, and/or MS have all been evaluated for such a purpose. All of these hyphenated detection schemes lack either sensitivity (refractive index and chemiluminescent nitrogen detection) or specificity (UV) and/or a uniform molar response (UV and MS) (Kuo et al., 2001; Yurek et al., 2002).

An alternative to these hyphenated methods for qualifying a metabolite standard is quantitative NMR analysis (Espina et al., 2009; Vishwanathan et al., 2009). Determining the concentration of an isolate by NMR has several advantages. Because a given resonance within an NMR spectrum is associated only with the atoms of the molecule of interest, the technique is very specific, which eliminates the need for salt, water, or purity corrections. In addition, the signal response from an NMR experiment is proportional to the molar amount of hydrogen atoms present, independent of the type of bonding arrangement. Last, one of the great benefits of NMR analysis is the nondestructive nature of the technique, enabling the entire sample to be used for other purposes after qualification is complete.

Historically, quantitative NMR used a separate chemical entity added to the sample as an internal standard (Malz and Jancke, 2005; Pauli et al., 2007, 2008). Selecting an appropriate internal standard can be difficult. A good internal standard is nonvolatile, is soluble in a variety of solvents, and resonates at a frequency that does not interfere with any of the analyte resonances. Addition of additional compounds to a pure isolate may interfere with subsequent chromatographic or enzymatic analysis. As an alternative, an electronic reference signal can be added to the NMR signal during the acquisition process and used as a quantitative standard (Akoka et al., 1999; Molinier et al., 2006; Ziarelli et al., 2008). Although this approach circumvents the problems of adding a chemical internal standard, it requires specialized hardware and software. Proposed in this article is a method to quantitatively add a reference signal to a sample’s spectra postacquisition. Use of an artificially generated signal as a quantitative reference eliminates all of the above liabilities.

**Materials and Methods**

**Chemicals.** PNU-90152 (delavirdine; N-[2-[(4-[3-(propan-2-ylamino)pyridin-2-yl]piperazin-1-yl]carbonyl]-1H-indol-5-yl]methanesulfonamide) and carbaz eran were synthesized by Pfizer (Groton, CT). Meloxicam, tolbutamide, verapamil, buspirone, enalapril, omeprazole, caffeine, 4-nitrobenzyl glutathione (NBG), and maleic acid were purchased from Sigma-Aldrich (St. Louis, MO) (Fig. 1). Acetonitrile and water were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All other solvents and reagents were of the highest grade.

![Fig. 1. Structure of the nine compounds used in assessment of linearity and dynamic range of the internal standard method and the aSICCO method of qNMR.](image-url)
commercially available and were used without further purification. All deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

NMR spectra were recorded on one of two instruments, either a Bruker DRX 600-MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA) controlled by XWin-NMR (version 3.5) and equipped with a 2.5-mm BBI probe or a Bruker Avance 600 MHz system controlled by TopSpin (version 2.0), equipped with a 5-mm TCI CryoProbe. One-dimensional spectra were recorded using a sweep width of 12,000 Hz and a total recycle time of 7.2 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance the signal/noise ratio. Samples were dissolved in 0.2 ml of DMSO-d6 “100%” (Cambridge Isotope Laboratories, Inc.) and placed in 3-mm diameter tubes. For quantitative results to be consistent, the same tube size must be used throughout a study for both samples and standards. All spectra were referenced using residual DMSO-d6 (1H δ = 2.5 ppm and 13C δ = 39.5 relative to tetramethylsilane, δ = 0.00). Phasing, baseline correction, and integration were all performed manually. If needed, the BIAS and SLOPE functions for the integral calculation were adjusted manually. T1 inversion recovery experiments and calculations were performed using the automated program provided by Bruker.

COSY and multiplicity edited HSQC, and heteronuclear multiple quantum coherence spectroscopy data were recorded using the standard pulse sequence provided by Bruker. Two-dimensional experiments were typically acquired using 1000 × 128 data with 16 dummy scans. The data were zero-filled to a size of 1000 × 1000. Unless otherwise noted, for two-dimensional experiments, a relaxation delay of 1.5 s was used between transients.

Selection of Internal Standard. An internal standard for a generalized assay must meet several criteria: it must be nonvolatile, be soluble in a variety of solvents, and resonate at a frequency that does not interfere with any of the analyte resonances. For this study, we chose 10 mM maleic acid, which is nonvolatile, is soluble in a variety of solvents common to drug metabolism studies (DMSO-d6, D2O, methanol, and acetonitrile), and has a sharp resonance at δ 6.26.

Generation and Calibration of aSICCO Signal. The aSICCO signal was generated using NMR-SIM (Bruker BioSpin) software. The artificial signal was generated using the zg pulse sequence as an uncoupled resonance with a relaxation time of 1 s. The chemical shift of the artificial signal was chosen on the basis of the data acquired for the sample of interest. A line broadening of 1 Hz was used to reproduce the processing of the isolated sample.

To quantitatively calibrate the aSICCO signal (assign a molar quantity), a separate data set was acquired for a standard of 10 mM maleic acid. This calibration data set was recorded with the same parameter set as the unknown. As with the unknown NMR spectra, the phase and baseline were manually corrected. Both the maleic acid singlet at 6.26 ppm and the aSICCO singlet were integrated, and a relative molar concentration was assigned to the computer-generated reference signal using the equation:

$$C_{comp} = 10 \text{mM} \times (nm/nc) \times (ric/rim)$$

where Ccomp is the relative molar concentration of the computer-generated signal, nm is the number of protons integrated for maleic acid = 2, nc is the number of protons (theoretical) integrated for the computer generated signal = 1, ric is the relative integration of the computer-generated signal, and rim is the relative integration of 10 mM maleic acid.

After calculation of the relative concentration of the computer-generated signal against a 10 mM maleic acid standard, an identical signal was merged into the metabolite’s FID spectrum to allow select interference-free resonance(s) to be assigned a molar value. Transformed NMR spectra were phase-corrected and manually baseline-corrected before integration of all resonances. Standard concentrations for metabolites were calculated using the equation

$$C_{met} = C_{comp} \times \frac{nc}{nmet} \times \frac{rimet}{ric}$$

where Cmet is the molar concentration of the metabolite, Ccomp is the relative molar concentration of the computer-generated signal, nc is the number of protons (theoretical) integrated for the computer generated signal = 1, nmet is the number of protons integrated for the metabolite, rimet is the sum of the number of protons integrated for the computer-generated signal, and ric is the relative integration of the computer-generated signal.

Critical acquisition and processing parameters such as sweep width, pulse length, and relaxation delays must be the same for both the analyte and the calibration data. Once the aSICCO and calibration data sets are generated, the sets are merged using a macro written specifically for TopSpin. A variable scaling factor within the macro is used to ensure that the aSICCO signal is similar in intensity to that of the analyte resonances. There was also a chemical shift variable contained in the macro that allowed the chemical shift of the aSICCO signal to be modified. All statistical analyses (mean, S.D., slope, and correlation coefficients) were calculated using Excel (Microsoft, Redmond, WA).

Preparation of Carbazeran Metabolite. Human cystolic aldehyde oxidase (AO) was prepared using ammonium sulfate fractionation. Carbazeran (10 mg) was incubated in 500 ml of potassium phosphate buffer (50 mM, pH 7.4, 37°C, 18 h) with human cystolic AO. Every 2 h, a fresh aliquot of the AO was added to the incubate. The reaction was terminated with acetonitrile (10% v/v). The suspension was centrifuged, and the supernatant was filtered through a 10,000-Da membrane. The filtrate was applied directly to a C18 preparative HPLC column (10 × 150 mm) at a 2.0 ml/min flow rate. The flow through was monitored for carbazeran and carbazeran lactam by MS. After complete addition, the preparative column was washed with 5 column volumes of 10% aqueous acetonitrile containing 0.1% formic acid (2.0-ml flow rate). A gradient of acetonitrile from 10 to 90% with 0.1% formic acid over 40 min was applied, and 0.5-min fractions were collected. Fractions showing m/z 377 and UV absorbance were pooled and evaporated to dryness under reduced pressure. Analytical HPLC analysis of this material showed a single UV peak with tandem mass spectrometry consistent with the expected carbazeran metabolite (93% purity based on UV integration). This material was used to make a stock solution of 1 mM in acetonitrile.

Results

With 10 mM maleic acid as the internal standard, the linearity and dynamic range of the classic internal standard qNMR method (ISTd) and the aSICCO approaches were compared using NBG as a model analyte. Spectra of NBG dissolved in DMSO-d6 were recorded at various concentrations from 0.01 to 10 mM. For each spectrum 120 scans were acquired. Each sample contained a 10 mM maleic acid

NBG 10 mM
Maleic Acid 10 mM
120 scans
* Indicates resonances used in linearity calculation

![Fig. 2. One-dimensional 1H NMR spectra of 10 mM NBG and 10 mM maleic acid. * resonances used in linearity and dynamic range calculations.](image_url)
internal standard. NBG was chosen as an analyte because of its commercial availability, diverse chemical shifts, and relevance to drug metabolism. Linearity calculations were performed using both the IStd (maleic acid) and the aSICCO signal at the following resonances: 2.01, 3.78, 4.58, 7.61, 8.18, 8.37, and 8.49 ppm (Fig. 2). For all resonances evaluated in both methods, the response was linear over 3 orders of magnitude with correlation coefficients greater than 0.999 and intercepts not significantly different from 0 (Table 1).

To further assess the robustness of these methods, a blinded study was performed in which 13 samples from nine compounds (Fig. 1) in concentrations from 0.10 to 37 mM were analyzed using both methods. These analyses were performed with the NMR analyst having no prior knowledge of analyte concentration or chemical structure. Compared with the known concentrations, the IStd and aSICCO methods had average errors of 3.4 and 5.1%, respectively. The correlations between nominal and measured concentrations were greater than \( r^2 = 0.999 \) (Fig. 3).

The day-to-day stability of these methods was also evaluated. For 8 consecutive days, a 0.10 mM tolbutamide sample was analyzed via both methods. The concentrations were calculated separately using each of the aromatic resonances. In all cases the deviation from the known concentration was less than 2%, and there was no observable trend in deviation over time (Fig. 4). These data were acquired on the NMR system equipped with the 2.5-mm room temperature probe.

The reproducibility of the incorporation of the aSICCO signal into an acquired data set was evaluated by systematically varying the chemical shift factor within the macro from 6.9 to 7.3 ppm and repeatedly incorporating a new artificial signal into the spectrum (Fig. 5). Integration of each of these signals had a relative S.D. of 0.2%. In a similar fashion, the linearity of the aSICCO signal was evaluated. In this case, a separate acquisition was performed, and both the chemical shift and magnitude variables of the macro were successively modified. The aSICCO signal had a correlation coefficient of 1.000 over 3 orders of magnitude.

---

**TABLE 1**

qNMR internal standard and aSICCO linearity and dynamic range data of NBG (0.01–10 mM NBG in DMSO-d6)

<table>
<thead>
<tr>
<th>NBG Chemical Shift</th>
<th>IStd method</th>
<th>aSICCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.01 ppm (7')</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>3.78 ppm (4')</td>
<td>0.999</td>
<td>1.000</td>
</tr>
<tr>
<td>4.58 ppm (2')</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>7.61 ppm (3/5)</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>8.18 ppm (2/6)</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>8.37 ppm (3')</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>8.49 ppm (3')</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>( r^2 )</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>IStd</td>
<td>1.000</td>
<td>0.098</td>
<td>0.00</td>
</tr>
<tr>
<td>aSICCO</td>
<td>1.000</td>
<td>0.59</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Fig. 3. Correlation between nominal and calculated concentrations of samples from blinded analysis study.
The effect of merging acquired data with artificial data was evaluated by assessing both the signal/noise ratio and the peak width of the maleic acid resonance before and after the addition of the aSICCO signal. This assessment was performed on the acquisitions with 448 transients. Five separate acquisitions were performed on the 10 mM maleic acid standard. The aSICCO macro was executed on each data set. Before the execution of the macro, the integration of the maleic acid resonance was $2.588 \times 10^9 \pm 0.040 \times 10^9$, the signal/noise ratio was $32,488 \pm 3440$ (signal was measured from 6.35 to 6.15 ppm; noise was measured from 6.0 to 5.8 ppm), and the peak width at half-height was $1.84 \pm 0.49$ Hz. After execution of the macro, the integration of the maleic acid resonance was $2.588 \times 10^9 \pm 0.044 \times 10^9$, the signal/noise ratio was $32,486 \pm 3464$ (signal was measured from 6.35 to 6.15 ppm; noise was measured from 6.0 to 5.8 ppm) and the peak width at half-height was $1.84 \pm 0.49$ Hz. All data indicate no analytically significant change to the native signal after the merging with the aSICCO signal.

**Carbazeran Phthalazone Metabolite.** To demonstrate the application of qNMR to a real system, we chose to examine the AO metabolism of carbazeran. Carbazeran is a phthalazone compound that is susceptible to AO and forms a phthalazone metabolite (Fig. 6) (Weishaar et al., 1983; Kaye et al., 1985). Whereas the phthalazone metabolite has been previously characterized using MS and tandem mass spectrometry techniques, a complete NMR characterization has not been performed. In the $^1$H spectrum of the isolated material, the O-methyl, the aminothyl, and the piperidine resonances are present and not significantly changed from those of the parent (Table 2). H5 and H8 of the phthalazine are also present and unmodified. The singlet attributed to H4 in carbazeran ($^1$H $\delta$ 9.11 and $^{13}$C $\delta$ 147.4) is absent, and a new singlet is present at $\delta$ 11.97. HSQC data indicate that this $^1$H resonance has no attached carbon. HMBC data from this resonance contain a cross peak to a carbon resonance at $\delta$ 159.1. All of these data are consistent with the oxidation of the phthalazine C4.

As described above, large-scale incubations were performed, and the metabolite was isolated via preparative chromatography. After exhaustive drying, a 1 mM solution was gravimetrically prepared in DMSO-d$_6$. HPLC analysis of the isolated material indicated a purity of 93%. Quantitative NMR (aSICCO method, using the aromatic resonances at $^1$H $\delta$ 7.22 and $^1$H $\delta$ 7.58) was performed on the above stock solution. qNMR results determined a concretion of 0.965 mM.

**Discussion**

Within the drug metabolism discipline, early discovery assessment of the importance of reactive metabolites (glutathiones or acyl glucuronides) or pharmacologically active metabolites is often hampered by the unavailability of quantitative information on these compounds. In development, preclinical and clinical studies are also often hindered by the lack of available synthetic standards.

Traditionally, quantitation of metabolites has been achieved in a two-step process. First metabolites are separated from endogenous matrix-related material and parent compound by various chromatographic techniques. Next, they are quantitated by comparison of the detector response of a metabolite in the matrix of interest to the response of a blank matrix similarly prepared that has been augmented with an authenticated synthetic standard. These steps are usually linked as hyphenated techniques, (LC-UV, LC-MS, and gas chromatography-flame ionization detector). Because even minor structural changes between a metabolite and a parent molecule may cause dramatic differences in the detector response, an authentic standard is required for accurate quantitation. Metabolites are often difficult to synthesize chemically, and, thus, studies requiring authentic standards are often delayed until later in development. This delay can be very costly if it is found that a drug candidate has either reactive metabolite or pharmacodynamic liabilities after development activities have been initiated.

An alternative to chemical synthesis of authentic standards is biological synthesis using in vitro or in vivo systems. These methods can produce metabolites in the range of hundreds of nanomoles (tens of micrograms), which is adequate for use as a standard in many assays. However, the qualification of these isolates via gravimetric analysis is not feasible. There are many aspects to qualification of a biological standard to consider. Drug-related impurities, impurities from endogenous materials, residual solvents, salts, and entrained water in the isolated material can all be factors in the assessment of an analytical standard generated from a biological source. The use of quantitative NMR as a method to qualify the biologically generated samples as analytical standards can circumvent all of the above-mentioned difficulties.

The signal response of an NMR system, unlike that of other spectroscopic techniques, is independent of the molecular motif of the
analyte. On a molar basis, the signal response from a nanomole of $^1$H from the aromatic resonances of a parent compound will give an equal response from a nanomole of $^1$H from a metabolite. This is not true with typical bioanalytical methods using MS or UV detection. In these methods, the structural changes between parent and metabolite frequently can cause significant, if not dramatic, changes in MS response.

FIG. 6. Aromatic portion of the $^1$H spectrum of the carbazeran phthalazone metabolite (bottom) and the same spectrum with the aSICCO signal (top).

TABLE 2

$^1$H and $^{13}$C chemical shift assignments of carbazeran and the AO metabolite

<table>
<thead>
<tr>
<th></th>
<th>$^1$H (Multiplicity, Integration, Coupling Constant)</th>
<th>$^{13}$C</th>
<th></th>
<th>$^1$H (Multiplicity, Integration, Coupling Constant)</th>
<th>$^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbazeran</td>
<td></td>
<td></td>
<td>Carbazeran M1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.11 (s, 1)</td>
<td>159.1</td>
<td></td>
<td>11.97 (s, 1)</td>
<td>149.2</td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.50 (s, 1)</td>
<td>147.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>7.58 (s, 1)</td>
<td>159.1</td>
</tr>
<tr>
<td>H$_2$CO-6</td>
<td>3.98 (s, 3)$^b$</td>
<td>124.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$CO-7</td>
<td>3.98 (s, 3)$^a$</td>
<td>153.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7.22 (s, 1)</td>
<td>106.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td></td>
<td>153.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'/6'</td>
<td>3.21/3.63 (cm, 2/2)</td>
<td>116.5</td>
<td></td>
<td>7.17 (s, 1)</td>
<td>105.3</td>
</tr>
<tr>
<td>3'/5'</td>
<td>1.82/2.08 (cm, 2/2)</td>
<td>48.5</td>
<td></td>
<td>1.76/2.02 (cm, 2/2)</td>
<td>48.6</td>
</tr>
<tr>
<td>4</td>
<td>4.78 (cm, 1)</td>
<td>31.3</td>
<td></td>
<td>4.71 (cm, 1)</td>
<td>31.3</td>
</tr>
<tr>
<td>Ethyl carbamate CO</td>
<td></td>
<td>69.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl carbamate NH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl carbamate CH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl carbamate CH3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td>15.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The methoxy $^1$H and $^{13}$C assignments may be reversed.
$^b$ Assignment cannot be made; no HMBIC cross peaks were observed.
$^c$ $^{13}$C assignments for C6 and C7 may be reversed.
response. Historically, what has hampered the use of NMR as a quantitative technique of metabolites has been its sensitivity. In disciplines in which the available sample mass is greater, pharmaceutical sciences, medicinal chemistry, and even natural products, qNMR has been long used to qualify and quantitate samples. Advances in cryoprobe technology and the reduction of the volume of these probes have together greatly enhanced the sensitivity of NMR systems (Dalsay and Molinski, 2009; Duarte et al., 2009; Xiao et al., 2009).

There are two established ways of incorporating a quantitative reference signal into an NMR spectrum: the use of a separate chemical entity and the electronic insertion of an artificial signal. The addition of a known quantity of a separate chemical entity to an NMR sample to act as a quantitative reference is established and works well under prescribed circumstances (Akoka et al., 1999; Pauli et al., 2005). However, there are limitations to this approach. The internal standard must have at least one isolated resonance free from interference from either the sample or the solvent resonances. The internal standard must be chemically stable, nonvolatile, and soluble in the NMR solvent of choice. When an analytical method is developed for a specific compound these requirements can be challenging to meet. When a generalized quantitative NMR assay designed for a variety of compounds is developed, the selection of the internal standard can be very challenging.

Furthermore, the addition of an internal standard to the sample may interfere with its subsequent use after quantitation. To thwart this difficulty, the residual solvent resonance has been used as an internal standard (Dalsay and Molinski, 2009; Wang et al., 2009). This concept also has limitations. Because the residual solvent line is also in any blank material that can be generated, it is not possible to establish that the quantitative reference signal is free from interference. In addition, the T1 relaxation times of deuterated solvents, relative to those of most protonated small molecules, are excessively long, resulting in a long interscan delay. In addition, because the manufacturers of NMR solvents guarantee only a maximum amount of protio contamination, the lots used as internal standards must be consistent.

To surmount all of these difficulties, an approach was developed in which an additional radiofrequency signal was introduced to the FID during the acquisition (ERETIC). This approach alleviated the aforementioned problems and allowed both the magnitude and the frequency of the reference signal to be modified based on the resonances in the sample. Although in concept this was an attractive alternative to addition of a chemical internal standard to a sample, it creates a different set of problems. To insert the reference signal into the acquisition data specialized hardware and pulse sequences are required. Although many laboratories have been successful using this technique, damage to the NMR system could result from attempting to run this experiment if the hardware is not appropriately configured. The postacquisition aSICCO method reported herein bypasses the shortcomings of both the internal standard and the ERETIC methods. Whereas the advantages of aSICCO may appear incremental over previous work, this approach results in significantly improved data quality and shortened time of analysis.

The benchmarks of any robust analytical assay are reproducibility, selectivity, linear response, and dynamic range. For this work, on the basis of the previous qNMR results and the expected use of the data, reproducibility and precision of 5% and linearity over 3 orders of magnitude were set as goals. In all cases, both methods, the addition of a chemical internal standard and the addition of a quantitative reference signal, met or exceeded these statistical parameters.

Mathematical manipulation of raw NMR data has been performed almost since the inception of Fourier transform NMR. However, the manipulation of an FID for quantitative purposes is a new concept. The merging of the data set presents obvious concerns over the effect on the original data. Is the artificial signal reproducible and linear once merged? In addition, is there an effect on the native signal? To assess the effect of merging the data, a 10 mM maleic acid sample was used. Two parameters, peak width at half-height and signal/noise ratio, were evaluated before and after the merger of the data. For the maleic acid resonance, the addition of the aSICCO signal had no analytically relevant effect. In addition, the aSICCO signal was linear over 2 orders of magnitude and reproducible.

The most appropriate assessment of the quantitation of a metabolite via qNMR is the comparison of the concentration of an isolated metabolite solution that has been prepared gravimetrically and qualified for purity by LC-MS with the concentration determined by qNMR. The aldehyde oxidase conversion of carbazeran to its phthalazine metabolite was used to compare these two processes. A 1 mM solution of the isolated material was prepared gravimetrically. The aSICCO analysis of this solution (0.965 mM) was nearly identical to the gravimetric concentration.

As discussed earlier in this article and elsewhere, the utility of having a qualified standard for quantification of metabolites in development is obvious (Dear et al., 2008). The benefit of having such a standard in discovery is also great. One example of the most frequent uses is as a standard in enzymatic assays. Enzymatic rates are often determined by measuring the disappearance of parent. However, when the compound turnover is low, the statistical ability to evaluate rates of depletion is limited. On the other hand, when a qualified standard is available, the appearance of a metabolite is much easier to quantify.

Another common use for biologically generated standards is the determination of pharmacological activity. There are often cases in discovery work in which there are questions about the pharmacological activity of metabolites. Activity of metabolites can be screened by fractionating an HPLC analysis and looking for activity in individual fractions. These fractions can then be correlated to the elution time of metabolites. When a biologically generated metabolite is structurally characterized and quantified by NMR, chemical synthesis of that metabolite may be eliminated. Our laboratories have made this a routine part of our discovery activities.

The use of NMR as a quantitative tool in drug metabolism studies significantly expands the influence of this class of instrumentation. In the past, after an NMR study the drug metabolism scientist would be returned a sample of known structure only. With the use of qNMR, the drug metabolism scientist is given a sample of known structure and quantity/purity. This sample is now available as a standard or substrate for a variety of metabolic studies that were previously not possible.

Acknowledgments
We acknowledge Doug Spracklin, R. Scott Obach, and Alfin Vaz for helpful discussions and manuscript review.

Authorship Contributions
Participated in research design: Walker, Ryder, and Sharma.
Conducted experiments: Walker, Ryder, and Sharma.
Contributed new reagents or analytic tools: Smith.
Performed data analysis: Walker and Ryder.
Wrote or contributed to the writing of the manuscript: Walker, Ryder, and Smith.
Other: Freund.

References


Wang Y, Huang S, and Reily M (2009) Development of the use of carbon-13 satellites of NMR solvents as internal standards for quantitative NMR of small molecule analytes at the microgram scale and applications in drug discovery, in *Abstracts of the 9th Northeast Regional Meeting of the American Chemical Society*, 2009 October 7–10; Hartford, CT.


Address correspondence to: Dr. Gregory S. Walker, Pfizer, Inc., Eastern Point Road, Groton, CT 06340. E-mail: gregory.s.walker@pfizer.com