Use of hybrid capillary tube apparatus on 400MHz NMR for quantitation of crucial low quantity metabolites using aSICCO signal

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Quantitative nuclear magnetic resonance (qNMR), Broad Band Fluorine Observe (BBFO), Centre for Drug evaluation and Research (CDER), multiple reaction monitoring (MRM), Electronic Reference To access In vivo Concentrations (ERETIC), artificial Signal Insertion for Calculation of Concentration Observed (aSICCO), recombinant CYPs (rCYPs), Hydrophilic-Lipophilic-Balanced (HLB), 3, 5-Dinitrobenzene (DNB), Liquid Chromatography-Mass Spectrometry (LC-MS). Alkaline phosphatase (ALP)

Abstract

Metabolites of new chemical entities can influence safety and efficacy of a molecule and often times need to be quantified in preclinical studies. However, synthetic standards of metabolites are very rarely available in early discovery. Alternate approaches such as bio-synthesis needs to be explored to generate these metabolites. Assessing the quantity and purity of these small amounts of metabolites with a non-destructive analytical procedure becomes crucial. Quantitative nuclear magnetic resonance (qNMR) becomes the method of choice for these samples. Recent advances in high field NMR (>500 MHz) with the use of cryoprobe technology has helped to improve sensitivity for analysis of small microgram quantity of such samples. However, this type of NMR instrumentation is not routinely available in all laboratories. In order to be able to analyze microgram quantities of metabolites on a routine basis with lower-resolution 400MHz NMR instrument fitted with a BBFO room temperature probe a novel hybrid capillary tube set up was developed. To quantitate the metabolite in the sample an aSICCO method that introduces an internally calibrated mathematical signal was used after acquiring the NMR spectrum. The linearity of aSICCO signal was established using Ibuprofen as a model analyte. The limit of quantification of this procedure was 0.8 mM with 10K scans which could be improved further with the increase in the number of scans. This procedure was used to quantify three metabolites-phenytoin from fosphenytoin, dextrophan from dextromethorphan and 4-OH-diclofenac from diclofenac and is suitable for mini-biosynthesis of metabolites from in vitro systems.

Introduction

The importance of identification and characterization of metabolites in early stage drug development has been highlighted in the guideline on "Safety Testing of Drug Metabolites" issued by the U.S. Food and Drug Administration Centre for Drug evaluation and Research (CDER) (Timmerman et al., 2010; Briggs et al., 2014; Timmerman et al., 2016). This guidance suggests that metabolites that circulate at 10% or more of total drug related material in humans at steady state needs to be further characterized in safety studies, which may require re-analysis of IND-enabling toxicity studies with quantitative determination of metabolite levels, or conducting fresh safety studies to match the exposure levels of the metabolites observed in humans. These studies are often conducted in the absence of synthetic metabolite standards. Additionally, proactive assessment of metabolite levels in IND toxicity studies anticipated to be major in humans is a common mitigation strategy.

Advances in mass spectrometry have contributed to the improvements in identification and characterization of metabolites. However, getting the exact structure from a mass spectrum is still challenging. For any LCMS-based quantification assay, for example multiple reaction monitoring (MRM), a qualified metabolite reference standard is needed (PicÃ and BarcelÃ, 2008; Zhou and Yin, 2016). Thus, many unknown metabolites detected in LCMS profiling experiments at discovery stage cannot be quantified due to lack of suitable reference standards (Scalbert et al., 2009). Synthesis of many metabolite standards is chemically challenging so alternate strategies such as the biological synthesis of metabolites followed by their isolation and quantification has become routine in drug discovery (Tao et al., 2009; Huttanus et al., 2016). Certification of these biologically generated metabolites using the classical methods of analysis is challenging, since there is very limited quantity available. So there is a need for a method of quantification of these

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metabolites that should be sensitive and nondestructive so that recovered sample solutions could be used as reference standard for subsequent analyses.

Nuclear magnetic resonance (NMR) spectroscopy is becoming an important tool for the quantitation of small and large molecules (Bharti and Roy, 2012; Pauli et al., 2014). NMR has excellent reproducibility and quantitative accuracy (Wishart, 2008; Nagana Gowda and Raftery, 2015). The response of the signal from NMR is proportional to molar concentrations of the analyte and is independent of the type of other atoms present which makes it a more accurate quantitative technique compared to other traditional analytical methods of quantitation. At the same time, it is a non-destructive technique so analytes can be recovered after analysis for further use (Simmler et al., 2014).

Quantitation with qNMR has been done by utilizing both an internal as well as an external standard (Bharti and Roy, 2012; Pauli et al., 2014; Barding et al., 2012; Siddiqui et al., 2013; Rundlẵf et al., 2014; Simmler et al., 2014). There are several criteria for a compound to be used as NMR internal standard: a) it should be available in pure form, b) it should have well resolved and distinguished signal(s) from the analyte peak of interest, c) it should be inert when mixed with analyte, and d) it should be nonvolatile (Saito et al., 2009; Weber et al., 2014). Use of external reference standards for qNMR was helpful to overcome a few of these limitations like compatibility and signal interference issues because in this method, standard and analyte sample with the same volume is taken separately in identical precise NMR tubes and a spectrum is acquired separately (Huang et al., 2014).

Another reported method for quantitation with NMR uses ERETIC (Electronic Reference To access In vivo Concentrations) signal as a reference. Applications of ERETIC signal on low

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resolution instruments is limited due to variation in response factor caused by variation of several experimental factors (Barantin et al., 1997; Farrant et al., 2010; Liang et al., 2010; Esteve et al., 2012; Jung et al., 2016). Another challenge in the ERETIC signal method is the insertion of the reference signal into the acquisition data which requires specialized hardware and pulse sequences. Walker et al. reported the use of a mathematically generated aSICCO signal (artificial signal insertion for calculation of concentration observed) inserted post-acquisition as a reference for the quantitation of metabolites. Any region in the analyte spectra can be selected for the insertion of aSICCO reference signal. The magnitude of the inserted signal can be modified to appropriately match the intensity of the sample resonances. The major limitation of Walker's aSICCO signal technique is that it uses high field magnets (500MHz and above with respect to ^{1}H frequency) and requires high sensitivity probe such as microprobe or cryoprobe (Walker et al., 2011; Walker et al., 2014).

Another major challenge in the metabolite identification and quantification is the choice of appropriate NMR solvents to get better resolution of signals. Use of incorrect solvent may lead to improper signal with poor resolution or merging peaks. Generally, metabolite samples are very limited in quantity and difficult to recover and reuse so there is no opportunity to try different solvents to get the best possible resolution.

For improving the sensitivity in NMR experiments use of Shigemi NMR tubes have been reported (Krunic et al; 2015) on 900 MHz instrument with cryoprobe. These tubes are matched in terms of magnetic susceptibility to each of the solvents they are identified with. But there are certain limitations with these tubes. These can only be used for the specified solvent, reported for the high sensitivity NMR instruments, available only till 3 mm diameter, have no option to add another solvent for screening, are very costly and do suffer with appearance of air bubbles between the

plunger and the solvent at high temperature which tends to destroy the field homogeneity (Jean-Michael et al 2008). In order to extend the application of the aSSICO signal method for metabolite quantitation we have developed a novel hybrid capillary tube set up which could be used on 400MHz NMR instrument with a room temperature BBFO probe. This configuration could also be used to screen multiple NMR solvents for structure elucidation of crucial small quantity metabolites using the same sample without recovery or contamination with uncommon solvents.

Materials and Methods

Chemicals and Reagents

Analytical grade Fosphenytoin, dextromethorphan, and diclofenac drug standards were purchased from Sigma-Aldrich (St. Louis, USA). HPLC grade solvents and chemicals such as acetonitrile (ACN), methanol, formic acid, malic acid, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and tris (hydroxymethyl) amino methane (Tris buffer) were purchased from Merck Specialties Private Limited (Mumbai, India). NADPH was purchased from SISCO research laboratory Ltd. (Mumbai, India). Recombinant cytochrome P450s (rCYP 2D6 and 2C9) were purchased from Corning Life Sciences (Gurgaon, India). The rALP microsome was prepared by in house methods (Subramanian M et al., 2017). Oasis HLB cartridges were purchased from Waters India. All deuterated solvents such as DMSO-d6, Benzene-d6, and $CDCl_3$ were purchased from Eurisotop Inc (Saclay, France). All other solvents and reagents were highest grade commercially available materials and were used without further purification.

Equipment

The following equipment were used in these preparations unless otherwise noted: Eppendorf 5810 R centrifuge (Unit 4 112 Talavera Road, North Ryde NSW 2113Australia); Turbovap LV nitrogen evaporator: (Caliper life sciences, 23 Mill Street Arcade, NY 14009United States); HLB cartridges -Oasis HLB 1cc extraction cartridges (Milford, Massachusetts, United States); 100 μL X-type syringe (CTC analytics AG, Zwingen, Switzerland); Cyclo-Mixer (Remi laboratory instruments, Remi house, Mumbai, India). HPLC column SunFire C18 (Waters, Milford, MA 01757, USA), HPLC column Inetrsil-ODS (GL Sciences Inc. Tokyo, Japan).

Preparation of calibration standards and internal standard solution

In this study, Ibuprofen was used as model analyte for checking the calibration and linearity of aSSICO and traditional qNMR methods. 3, 5-Dinitrobenzene DNB was used as an internal standard for quantification using the traditional method, whereas maleic acid was used for external calibration using the aSICCO method. DNB is a nonvolatile compound, soluble in a variety of solvents common to drug metabolism studies and has a sharp resonance at δ 8.9 ppm. A stock solution of 3,5-dinitrobenzene (DNB) and ibuprofen were prepared by dissolving 6.12 mg and 10.6 mg solid in 5.00 mL (5.77mM) and 2.00 mL (25.7 mM) DMSO-d6, respectively. A series of five standard solutions ranging from 0.80 mM (8.24 µg) to 12.85 mM (132.68 µg) concentration of the Ibuprofen were prepared by serial dilution of the stock solution with DMSO-d6. Each sample contained a 2.35 mM 3, 5 DNB acid as an internal standard. All solutions were vortexed and stored in the refrigerator when not in use.

Generation and calibration of aSICCO Signal

NMR-SIM (Bruker TopSpin, Version 3.2) was used to create an aSSICO signal. A single pulse sequence (zg) with uncoupled resonance was used to generate an artificial signal with 1s as relaxation time. The chemical shift of the artificial signal was decided based on the spectral data of the metabolite of interest. A line broadening of 1 Hz was used to reproduce the processing of the isolated sample. Before each experiment, the aSSICO signal was calibrated with a known concentration of a standard solution with constant parameters set for both known standard solution and unknown sample data collection. Phase and baseline were manually corrected and the aSSICO signal and signal from standard were integrated equally. An aSSICO signal relative concentration was assigned using the equation below (Walker et al., 2011)

$$\text{Conc of aSSICO signal} = \text{Conc.of Std} \; \times \; \frac{IC}{IS} \; \times \; \frac{NS}{NC}$$

Where IC = Integral of aSSICO signal, IS = Integral of standard, NS = Number of protons in standard, NC = Number of protons in sSSICO signal

The acquisition and processing parameters such as pulse length, relaxation delay, and sweep width were kept identical for both analyte and the calibration data. After generating an artificial signal and calibration data set, both the data points were merged with the help of macro in TopSpin. A scaling factor which is variable within the macro is used to set aSSICO signal similar to that of resonance signal. The chemical shift variable was also available in macro which allowed setting of aSSICO signal chemical shift value as per the profile of the analyte.

General method for *In vitro* incubations to generate metabolites

In the general method, the parent compound (typically 30–100 µM) was incubated in an in vitro system containing a source of drug metabolizing enzymes and cofactors necessary to effect the biotransformation of interest (Table 1). The reaction volume was typically a multiple of 1 ml depending on the requirement and turnover of the compound in the given matrix. All the incubations were performed at 37°C in a shaking incubator at 60 rpm. At the end of the incubation, the multiple reaction samples were pooled and passed through Oasis HLB (Hydrophilic-Lipophilic-Balanced) cartridges to remove the matrix interference and concentrate the metabolite(s) samples. A vendor (Waters) recommended protocol was used to process the samples through HLB (Zhang X et al). Around 10 µL of the pooled sample was loaded on the Orbitrap for the confirmation of the metabolite formation. The eluted samples from HLB cartridge were subjected to nitrogen evaporation for reducing the sample volume. Resulting concentrated samples were purified on analytical HPLC column. This general method for *In vitro* incubations to generate metabolites was applied for fosphenytoin, dextromethorphan, and diclofenac drug standards. The details of type of microsome and buffer conditions are given in (Table1)

HPLC conditions

Concentrated fractions from SPE after incubation were purified on Agilent 1200 series chromatographic system (Santa Clara, CA, USA) equipped with a high-pressure quaternary pump with PDA detector, heated column thermostat, and a Rheodyne universal loop injector with 100 µL injection capacity. Sample injection and data processing were controlled by Chemstation software. The purifition of fosphenytoin, dextromethorphan metabolites were carried out on Inertsil-ODS (250 mm×4.6 mm, 5 µm) column. The mobile phase consisted of 10 mM ammonium acetate buffer in water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 1ml/min. The gradient started with 20% B and ramped linearly to 100% B over 25 minutes, held at 100% B for 5 minutes, returned to the initial condition over 1.0 minute, and allowed to equilibrate for 4.0 minutes. Diclofenac metabolites were purified on SunFire C18 (250 mm × 4.6 mm, 5 µm) column. The mobile phase consisted of 0.1% TFA buffer in water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 1ml/min. The gradient started with 10% B and ramped linearly to 100% B over 25 minutes, held at 100% B for 5 minutes, returned to the initial condition over 2.0 minute, and allowed to equilibrate for 4.0 minutes. All the metabolites were monitored at 220 nm and 254 nm throughout the run time. During chromatography, the column temperature was maintained at room temperature. Fractions containing the metabolite(s) of interest were combined, and the organic solvent removed by nitrogen evaporation and the remaining aqueous sample was passed through an HLB cartridge for a second time, following the same protocol. The eluted organic sample that passed through the HLB cartridge was analyzed on the Orbitrap before nitrogen evaporation to verify the sample integrity. Remaining organic samples were dried under nitrogen.

NMR Sample preparation and analysis

After SPE purification, the Eppendorf tubes containing the dried samples were left under vacuum for a minimum of one hour. All the samples were reconstituted in 50 µL of high purity deuterated DMSO (typically "100%" grade DMSO-d6 which is 99.96 % deuterium enriched) solvent. After addition of solvent, sample tubes were sonicated for 2 min and vortexed on cyclomixer for 10 min. The resulting clear solution was transferred to the melting point capillary tube with the help of 100 µL micro syringe. After filling capillary tube to two third of its size (50 µl), it was carefully placed inside the regular 5 mm NMR tube filled with 600 µL of desired locking NMR solvent.

NMR spectra were recorded on Bruker 400-MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA) controlled by TopSpin software (version 3.2) and equipped with a 5 mm BBFO probe. One-dimensional proton spectra were recorded with a sweep width of 12,000 Hz, 32K data point and a relaxation delay of 2 sec. For calibration sample and the metabolite samples, 10K and 15K scans were accumulated respectively. All spectra were referenced using residual DMSO-d6 (^{1}H, δ 2.5ppm). Phasing, baseline correction, and integration was all performed manually. qNMR was performed using the aSICCO method (Walker, et al., 2011). For aSICCO method, a standard of known concentration of maleic acid was used as a calibrant. The concentration of the analyte was calculated as per the formula given below:

$$\text{mM of Analyte/ Conc of aSSICO signal} = \text{mM of IS/ Conc of Std} \times \frac{IA}{IS} \times \frac{NS}{NA}$$

IA = Integration of analyte protons (aSICCO signal)/ Integral of aSSICO signal

IS = Integration of internal standard protons/ Integral of standard,

NA = Number of protons in analyte/ Number of protons in standard,

NS = Number of protons in internal standard/ Number of protons in sSSICO signal

Quantitation of 4-hydroxy diclofenac metabolite by LC/MS/MS

All prepared samples were analyzed using an Acquity UPLC system coupled to an API 5400 quadrupole ion trap (Applied Biosystems, Canada).The chromatographic separation of analytes was achieved on an Acquity BEH C18 column (2.1 X 50 mm, 1.7 µm) using a binary gradient mobile phase consisting of 0.1% formic acid water (Mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 800 µL/min. Initially the column was held at 95% of A for 0.8 min and then linearly ramped to 50% of B over 0.4 min, followed by linear ramping to 90% of B over 0.4 min held at these conditions for next 0.5 min. The system was then returned to in initial conditions and equilibrated for next 0.5 min. The total chromatographic run time was 3 min. Mass spectroscopic analysis was performed with an electrospary ionization (ESI) source operated in negative ion mode with multiple reaction monitoring (MRM). The ESI source temperature was set at 6000C and ion spray voltage, cell entrance (EP) and exit potentials (CXP) were set at -4500, -10 and -10 volts, respectively. The curtain gas, nebulizer gas (Gas1) and heater gas (Gas2) were set at 40, 30 and 40, respectively. The quantification of analyte was achieved by monitoring m/z 312.2→230.2 (DP -70 and CE -35) for 4-OH diclofenac. The Analyst software (version 4.2) by Applied Biosystems was used for data acquisition and data analysis.

Results

Ibuprofen was selected as a model analyte for comparing the linearity and dynamic range of classical internal standard qNMR method using 3, 5-dinitrobenzene (DNB) as an internal standard vs the aSICCO signal as internal standard. 3, 5-dinitrobenzene (DNB) was selected as an internal standard due to its easy commercial availability. ^{1}H NMR spectra of ibuprofen dissolved in DMSO-d6 were recorded at various concentrations from 0.8 mM (8.24 µg) to 12.85 mM (132.68 µg). For each spectrum, 10k scans were acquired. Each sample contained 2.35 mM of DNB as an internal standard. For classical qNMR method using DNB as an internal standard, the linearity calculations were performed using signals at δ7.10 and δ8.90 for the aromatic protons of ibuprofen and the internal standard DNB, respectively. For the aSICCO method, the signals at δ7.10 and δ15.0 for aromatic proton of ibuprofen and artificially inserted aSICCO signal were used for linearity calculations, respectively. In both cases, the response was linear with correlation coefficients greater than 0.999 and intercepts not significantly different from 0 (Figure 1 and 2).

To demonstrate the utility of the aSICCO approach, quantitative estimation of major metabolites of Fosphenytoin, Dextromethorphan, and Diclofenac drug were performed using aSICCO as reference standard with 15 K scans so as to improve the sensitivity for the metabolite signal. Maleic acid standards used to calibrate the aSICCO signal were also acquired with 15 K scans. Reported methods of incubations were used to generate major metabolite for each of these drugs and metabolites were then isolated by SPE and HPLC chromatography and were concentrated for NMR analysis.

Phenytoin Metabolite

Fosphenytoin is a phosphate ester derivative of phenytoin that is susceptible to hydrolysis and forms phenytoin as a major metabolite (Fischer et al., 2003) Fosphenytoin was incubated with rALP to generate phenytoin as a single metabolite (Figure 3 and Supplemental Figure S1). As per the UV chromatogram 90 % conversion of parent to phenytoin was observed. The isolated metabolite was confirmed by matching the retention time (RT) and mass with the known standard. The overall recovery of phenytoin metabolite was found to be 84% on w/w basis. The phenytoin metabolite has been previously characterized using ^{1}H NMR, MS and tandem mass spectrometry techniques (Annesley et al., 2001). Crude material obtained after drying was reconstituted in DMSO-d6 and ^{1}H-NMR was recorded, simultaneously 0.45 mM of the maleic acid standard in DMSO-d6 was also acquired to calibrate the aSICCO signal (Figure 4). The aromatic proton of phenytoin at δ 7.1 with integral of 4.3 and aSICCO signal at δ 14 with integral of 1 was used for qNMR calculations. The calculated concentration of the isolated metabolite was found to be 1.5 mM by the aSICCO qNMR method.

Dextrorphan metabolite

Dextromethorphan is rapidly converted into the active metabolite dextrorphan by the cytochrome P450 enzyme CYP2D6 (Schadel et al., 1995). After *in vitro* incubation using the general procedure described in experimental section, dextrorphan was isolated using analytical HPLC (Figure 5). As per the UV chromatogram, 65 % conversion was observed for dextrorphan metabolite from the parent drug during *in vitro* incubation (Supplemental Figure S2). The isolated metabolite was confirmed by matching the RT and mass with the known standard. The overall recovery of pure dextrorphan metabolite was found to be 69.7 % on w/w basis. ^{1}H NMR was recorded for the isolated metabolite in DMSO-d6. Simultaneously, ^{1}H NMR of 8.14 mM of maleic acid standard was also acquired to calibrate the aSICCO signal (Figure 6). The aromatic proton of dextrorphan

at δ 6.8 with integral of 0.58 and aSICCO signal at δ8.5 with integral of 1 was used for qNMR calculations. The calculated concentration of the isolated metabolite was found to be 3.33 mM by qNMR using the aSICCO method.

4-Hydroxy diclofenac

Diclofenac was metabolized to 4-hydroxy diclofenac by polymorphic CYP2C9 (Dorado et al., 2003). After *in vitro* incubation using general procedure described in experimental section, 4-hydroxy diclofenac was isolated using analytical HPLC (Figure 7). As per the UV chromatogram 80 % conversion was observed for 4-hydroxy diclofenac from the parent drug during *in vitro* incubation (Supplemental Figure S3). The overall recovery of pure 4-hydroxy diclofenac was found to be 76 % on w/w basis.

^{1}H NMR was recorded for the isolated metabolite in DMSO-d6. Simultaneously ^{1}H NMR was acquired for 7.28 mM of the maleic acid standard to calibrate the aSICCO signal (Figure 8) The aromatic proton of 4-hydroxy diclofenac at δ 6.1 with integral of 1 and aSICCO signal at δ14 with integral of 1.17 was used for qNMR calculations. The calculated concentration of isolated metabolite was found to be 4.13 mM by qNMR using the aSICCO method.

Discussion:

The utility of NMR for metabolite structural characterization has been well established (Forseth et al., 2011; Caceres-Cortes and Reily, 2010). In recent years, with the advent of high field instrument and availability of cryo probes the ability of NMR to quantify metabolite in low microgram to nanogram quantities is possible (Spraul et al., 2003; Molinski et al 2010). However, these types of high end instrumentation are not available in all the laboratories. The work presented here provides some practical solution to utilizing a 400 mHz NMR with a 5 mm probe to conduct metabolite characterization and quantitation. A major limitation for the quantitation of drug metabolites with a 400 MHz NMR instrument is sensitivity, as the amount of metabolite generated from in vitro incubations is typically in the range of microgram to nanogram quantities.

Sample concentration could be enriched by carrying out multiple incubation reactions but isolating the required metabolite from large incubation volumes is a challenging task. Another way of increasing the sensitivity would be the use of microprobe designed for small volume samples, such as a 1 mm probe, where sensitivity is increased by improved filling factors. Another advantage of such a probe is that the smaller sample volume ($\sim$ 12 µL) reduces the interference noise from the NMR solvent and residual water, which in turn enhances the analyte signal-to-noise (Schlotterbeck et al., 2002). However, this option requires dedicated high cost probes which may be a limiting factor in many laboratories. Earlier reports proved that the use of 1.7 mm id NMR tube for metabolite analysis was helpful to reduce the interference/noise from NMR solvent and residual water, yet the sensitivity gain from using a smaller diameter NMR tube in a 5 mm probe was insignificant since the rf coil was further away from the sample and interference from the air was compensating all the gain (Johnson et al. 2010; Schlotterbeck et al., 2002). Based on this available information, a unique hybrid capillary tube set up was developed which uses a 1 mm capillary tube

within a 5 mm outer NMR tube, in a 5 mm room temperature probe. With this strategy, capillary tubes were used as sample reservoir needing only 20-30 µl of the sample solution compared to 600-700 µl sample in 5 mm tubes with data acquired on a 5 mm probe. Practically this should increase the actual sample concentration and sensitivity by multiple folds but increased noise was observed in the spectra collected probably created by the air gap between the rf coil and the sample solution (Figure 9). To reduce the noise due to the air gap, the capillary tube containing 20-30 µL of the sample solution in dueterated solvent was placed inside a 5 mm tube containing 600 µL of same NMR solvent (Figure 9). This theoretically should reduce the interference due to air gap between the rf coil and the sample while maintaining the sensitivity gain due to increased sample concentration. To confirm this, NMR spectra of Ibuprofen was acquired in DMSO-d6 in capillary tube inserted in two separate 5 mm NMR tubes- one containing DMSO in the outer tube and other without solvent in the outer tube. Signal to noise ratio (S/N) was found to be 301 when the outer tube was filled with DMSO and 126 (Supplemental Figures S4A, S4B and S5) when outer tube was empty which supported the proposed hypothesis.

Linearity samples were acquired using 2 sec relaxation delay with 10000 scans and it was observed that the integration values for the all the characteristic signals were linear. For a fully relaxed proton AQ+D1 should be > 5 x T1 for the resonances being measured (Edwin D, 2000). T1 for the test analyte Ibuprofen was measured and was found to be 1.3 sec. Acquisition time (AQ) for this experiment was 1.99 sec and delay time (D1) was 2 sec under the experimental conditions used which suggested a total of relaxation delay as 3 times of T1. Usually signal amplitude drops as the recycle time (relaxation + acquisition time) gets shorter but there are two choices to compensate the loss: (1) either to cut down recycle time and accumulate more scans with a lower signal recovery for each scan , or (2) use a longer recycle time and fewer scans with each scan recovering

nearly 100% of the signal. Increasing the relaxation delay would have increased the total run time significantly so loss of signal amplitude with lower relaxation delay was compensated by increasing the number of scans to 15000 for metabolite samples and the aSICCO standards with 2 sec relaxation delay and the total acquisition time was 21 hrs. In order to confirm if 2 sec of delay time would have major impact on the quality of the data we have acquired two data at 2 and 10 sec relaxation delay and the s/n in both cases were found to be 1912 and 1989 respectively (Supplemental Figure S6 and S7). Even relative integration of all protons were also similar under both conditions as shown in figures S6 and S7 in supplementary data. Similar parameters were used for both analytes and the standard used for calibrating the aSICCO signal so effective loss in signal amplitude due to shorter relaxation delay should be compensated.

To avoid the breaking of capillary tube, the NMR acquisitions were performed without rotation of the sample tube. With this new hybrid capillary tube appratus, we were able to achieve the quantitation limit up to low µg level on 400 MHz instrument with room temperature probe. To test the utility and accuracy of this hybrid capillary tube apparatus quantitation of Ibuprofen was performed with a standard 400 MHz NMR using the aSICCO approach. ^{1}H NMR spectra of Ibuprofen at different concentrations were aquired using identical instrumental parameters. In all cases, the response of the analyte was linear.

Traditionally the incubated samples are being purified using chromatographic techniques prior to NMR acquisition (Walker et al., 2014). However, prior to introducing the samples on to the column it need to be concentrated. Initial attempts to concentrate the sample by rotary evaporation, Genevac or lyophilization led to degradation of metabolites (Supplemental Figure S8). Apparently the presence of water in the sample took much longer time to concentrate which caused the degradation. It was confirmed by LC/MS analysis of the samples during multiple stages of

purification. To avoid this degradation, after incubation sample was passed through SPE cartridges to remove the biological matrix and the water from the sample. A similar SPE concentration step was introduced after the collection of HPLC fraction to remove the water and buffer. Eluent from SPE was dried down under nitrogen and final residue was reconstituted in 50 µl of NMR solvent for acquisition (Figure 10). Overall sample recovery in this process was found to be between 76-86%. SPE steps were not completely optimized for this study as we used the commercial samples and it is well known that recovery from SPE is compound dependent and can vary from sample to sample. So there is ample opportunity to further improve the sensitivity for actual study samples by optimizing the SPE step and thereby improve the overall recovery.

Three drugs fosphenytoin, dextromethorphan, and diclofenac were incubated to generate the specified metabolites for quantification as per the protocol specified in experimental section. The corresponding metabolites phenytoin, dextrorphan and 4-Hydroxy diclofenac, were isolated with optimized protocol described above. qNMR was performed with the aSSICO method for metabolite quantitation. The calculated concentration of 4-hydroxydiclofenac obtained by aSICCO approach (4.59 mM) was comparable with quantification by LCMS (4.61 mM) technique which supports the accuracy of this method.

It was observed that due to a larger volume of solvent (DMSO-d_6) in the outer tube (~600µL) compared to the quantity of the sample in the capillary tube , DMSO-d_6 and residual water peaks were suppressing the peak of interest from analyte in the aliphatic region. This could be a limitation of this method if a proton in aliphatic region is needed for quantification of desired metabolite. To address this concern other dueterated solvents like benzene, pyridine or chloroform, which lack aliphatic protons, could be used for sample preparation instead of DMSO but these solvents may not be ideal if the recovered sample has to be further used as reference standard. With the hybrid

capillary tube set up, however, these deuterated solvents could be used in the outer 5 mm tube without contaminating the actual sample in capillary tube. This could help to isolate the aliphatic peaks without contaminating the actual sample with undesired solvents in the outer tube. To confirm this ibuprofen was selected as model analyte and the NMR sample was prepared in DMSO-d_6. Different solvents like benzene-d_6, pyridine-d5 and $CDCl_3$ were used in the outer 5 mm tube and the NMR spectra of all these samples were acquired using the same parameters (Figure 11). Spectra acquired with benzene-d6 and pyridine-d5 in outer NMR tube showed better separation of protons 7 and 9 of Ibuprofen which were merging with water peak in DMSO-d6 data. Spectra acquired in deuterated chloroform showed clearly separated signals for H7 and H9 which could be utilized for the quantitation if needed. This clearly indicated the effect of NMR solvent on the separation of signals from solvent peaks and suggested that this strategy could be successfully applied when there is a need for the separation of signals in the aliphatic region without contaminating the actual sample with these uncommon solvents. This could also be used if data is needed for the structural confirmation of the small quantity of crucial isolated metabolite.

In conclusion, we have developed a novel hybrid capillary tube apparatus which can be used to quantitate metabolites in the μg level concentration with a 5 mm room temperature NMR probe on a 400 MHz instrument. With the use of an aSICCO signal as the internal standard the recovered NMR sample could be further utilized as reference standard for biological quantitation. At the same time screening of different NMR solvents can be done in the outer tube to avoid contaminating the actual sample in the capillary tube or compromising the data quality. This novel set up gives the opportunity to perform metabolite characterization and quantifications with regular room temperature probes in 400 MHz frequency spectrometers without adding any extra resources for increasing the sensitivity of the instruments.

Acknowledgements:

Authorship Contributions

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Subramanian, Ramaswamy Iyer, Ramakanth Sarabu and Lakshmikant Bajpai

Wrote or contributed to the writing of the manuscript: Siddheshwar Kisan Chauthe, Ranjeet

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Legends for Figures

Figure 1: Comparative analysis of linearity and dynamic range of the classic internal standard qNMR method and the aSICCO method.

Figure 2: One-dimensional ^{1}H NMR spectra at LOQ of 0.8 mM (8.24 μg) ibuprofen and 2.35 mM of 3, 5-Dinitrobenzene (DNB) with aSSICO signal.

Figure 3: HPLC UV chromatogram of fosphenytoin metabolites depicting phenytoin as single major metabolite.

Figure 4: ^{1}H NMR of phenytoin depicting analyte signals in the aromatic region and aSICCO signal at δ14 ppm.

Figure 5: HPLC UV chromatogram of dextromethorphan depicting dextrorphan as a single major metabolite.

Figure 6: ^{1}H NMR of dextrorphan depicting analyte signals in the aromatic region and aSICCO signal at δ 8.5 ppm.

Figure 7: HPLC UV chromatogram of diclofenac depicting 4-Hydroxy diclofenac as a single major metabolite.

Figure 8: ^{1}H NMR spectrum of 4-hydroxydiclofenac depicting analyte signals in the aromatic region and aSICCO signal at δ 14 ppm.

Figure 9: Pictorial representation for evolution of hybrid capillary tube apparatus in 5mm NMR BBFO probe.

Figure 10: Schematic diagram showing steps followed in final optimized conditions in isolation and purification of metabolites.

Figure 11: ^{1}H NMR spectra of ibuprofen in different NMR solvents depicting solvent effect to minimize the interference of aliphatic region impurities.

Table 1: In vitro incubations conditions for generation of metabolites of interest
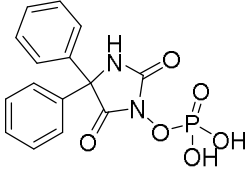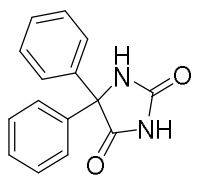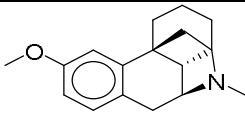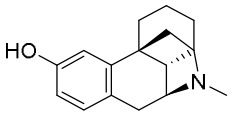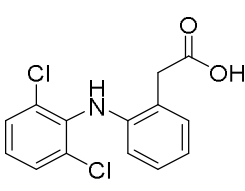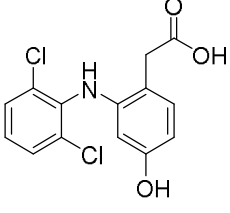
Name of the parent drug	Name of the metabolite	microsomes used	Concentration of drug	Buffer
Fosphenytoin	Phenytoin	rALP (0.1mg/ml)	30µM	Tris buffer (100mM pH 7.4)
Dextromethorphen	Dextrorphan	rCYP2D6 (0.5mg/ml)	50 µM	KH2PO4 (100 mM, pH 7.4)
Diclofenac	4-OH diclofenac	rCYP2C9 (0.5mg/ml)	50 µM	KH2PO4 (100 mM, pH 7.4)

Figure 1:
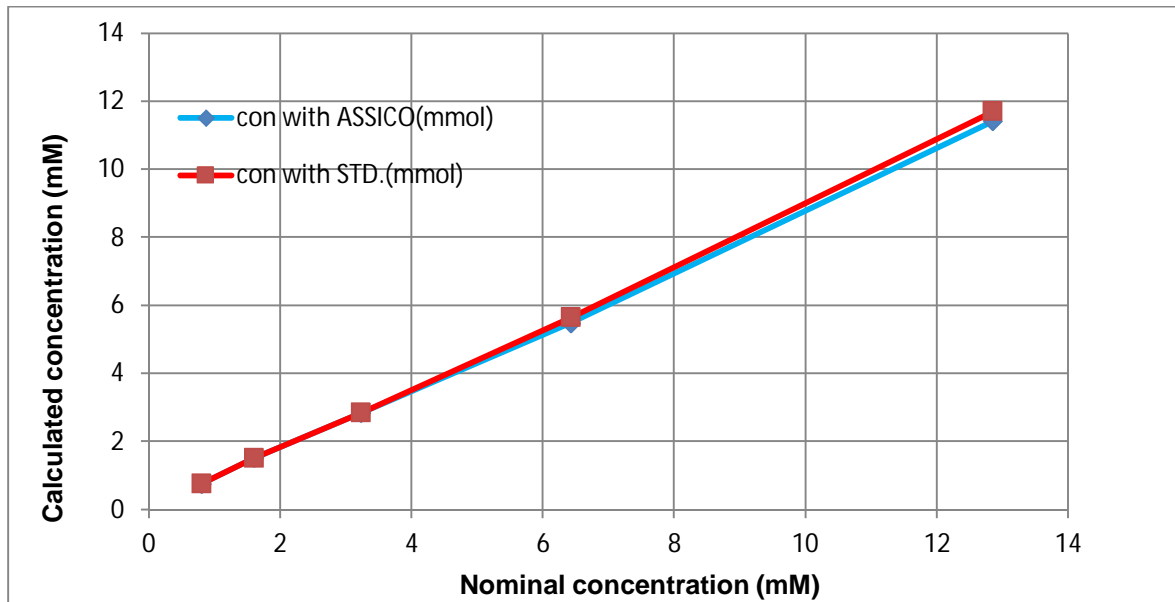
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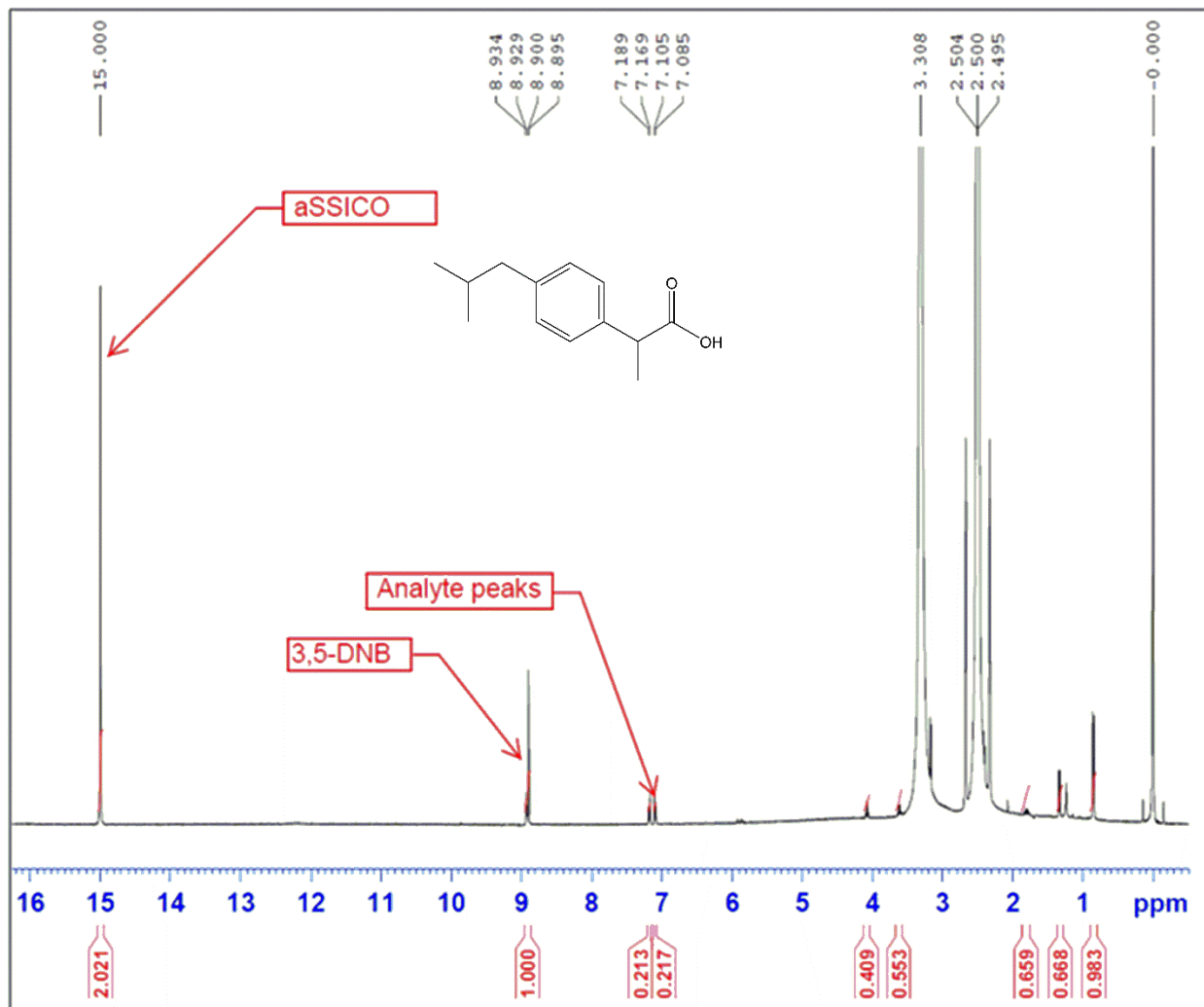
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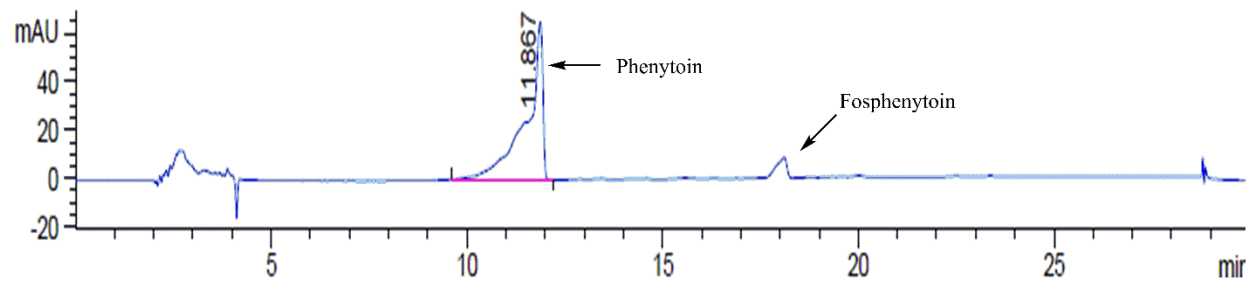
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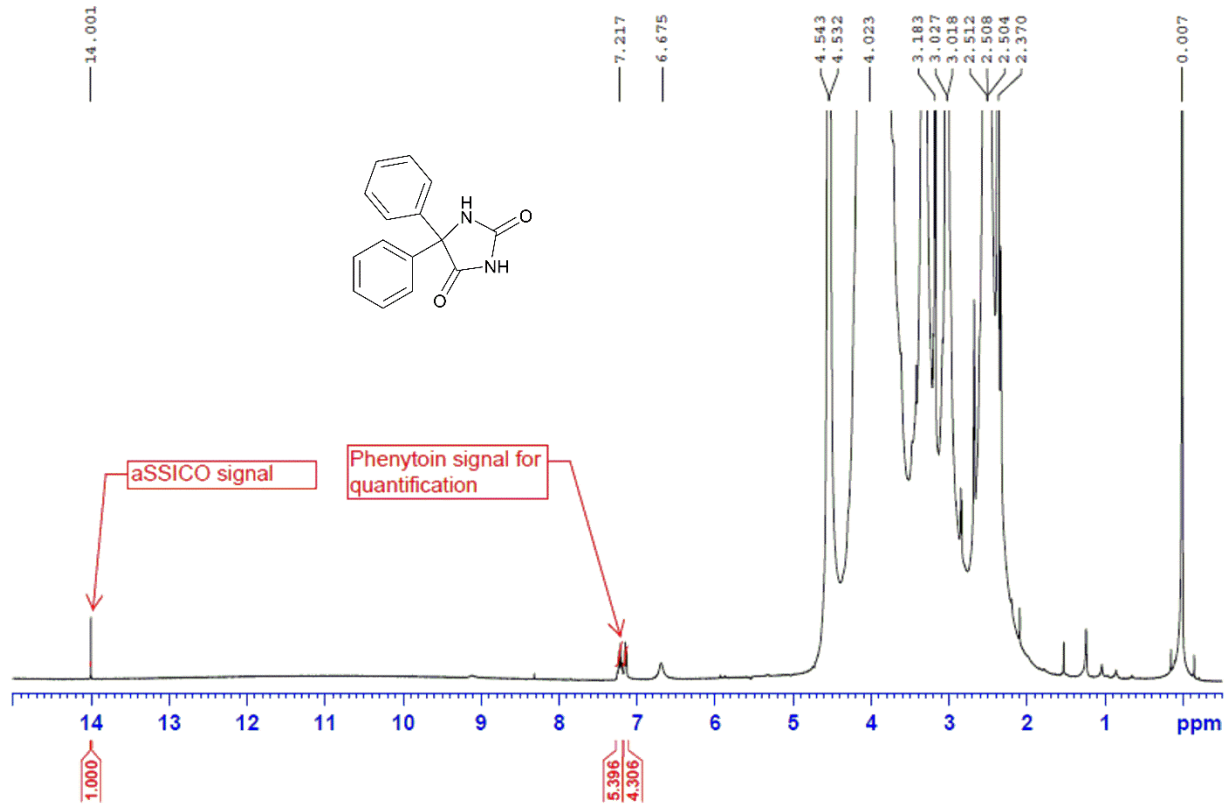
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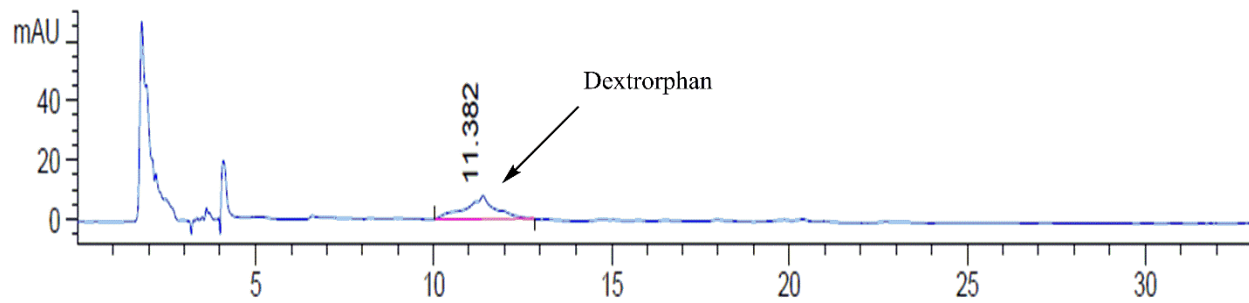
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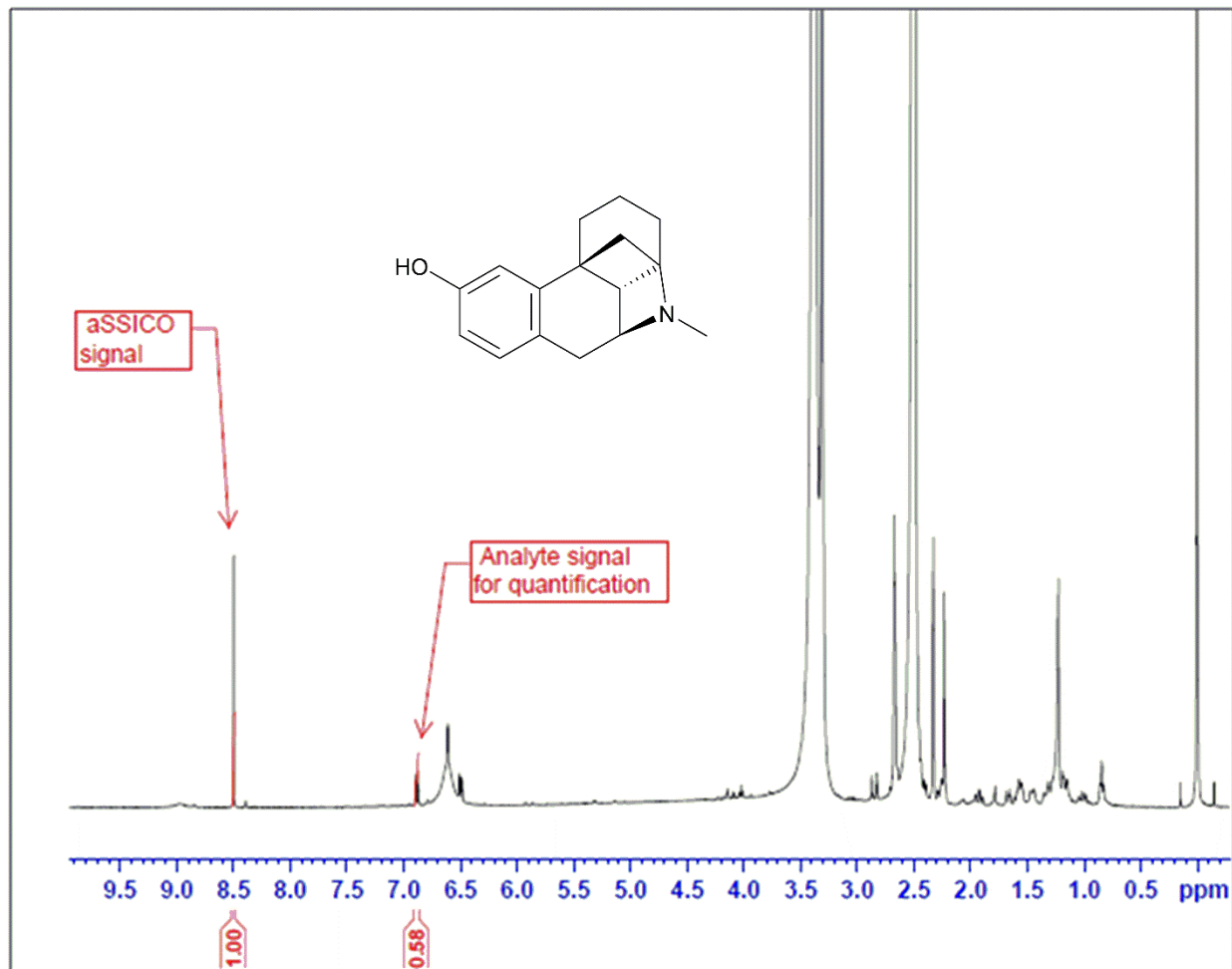
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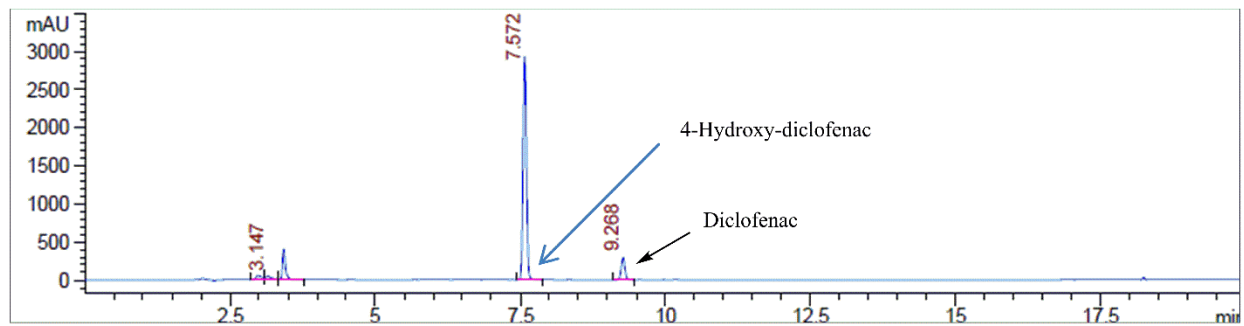
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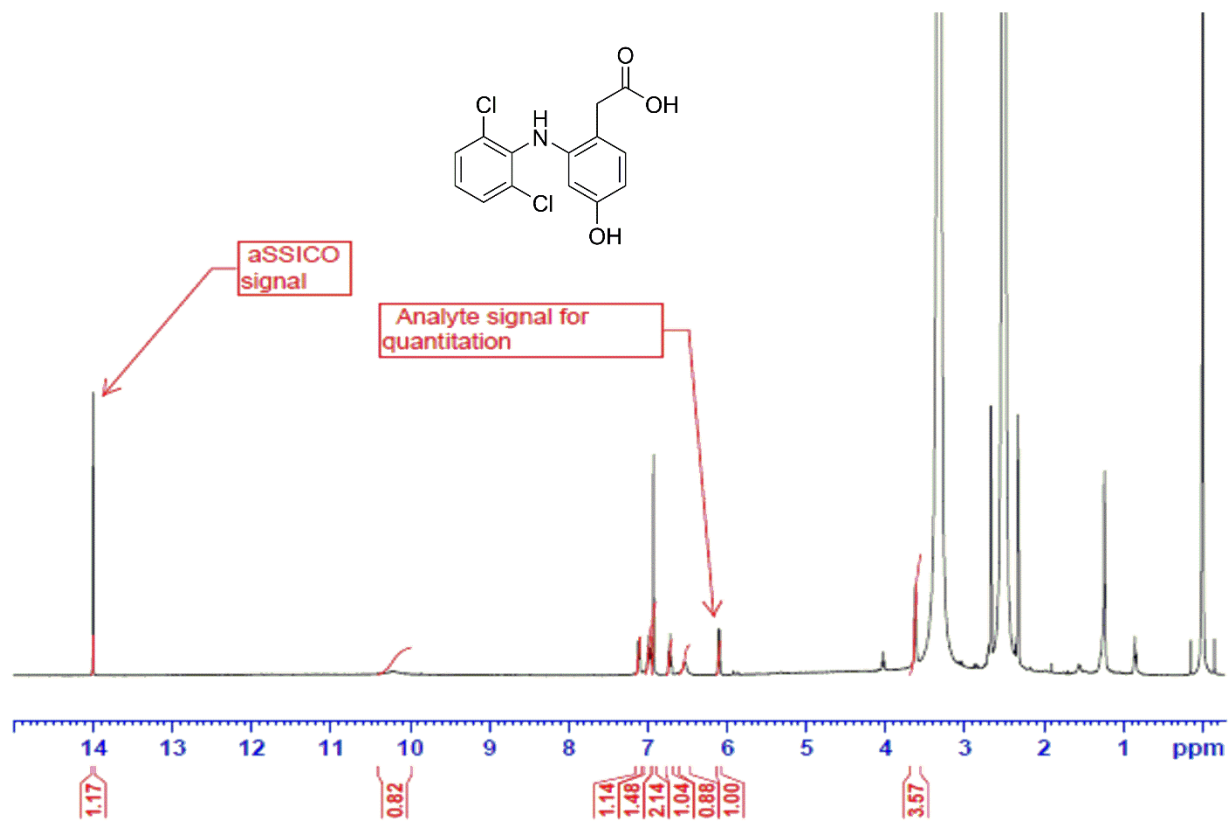
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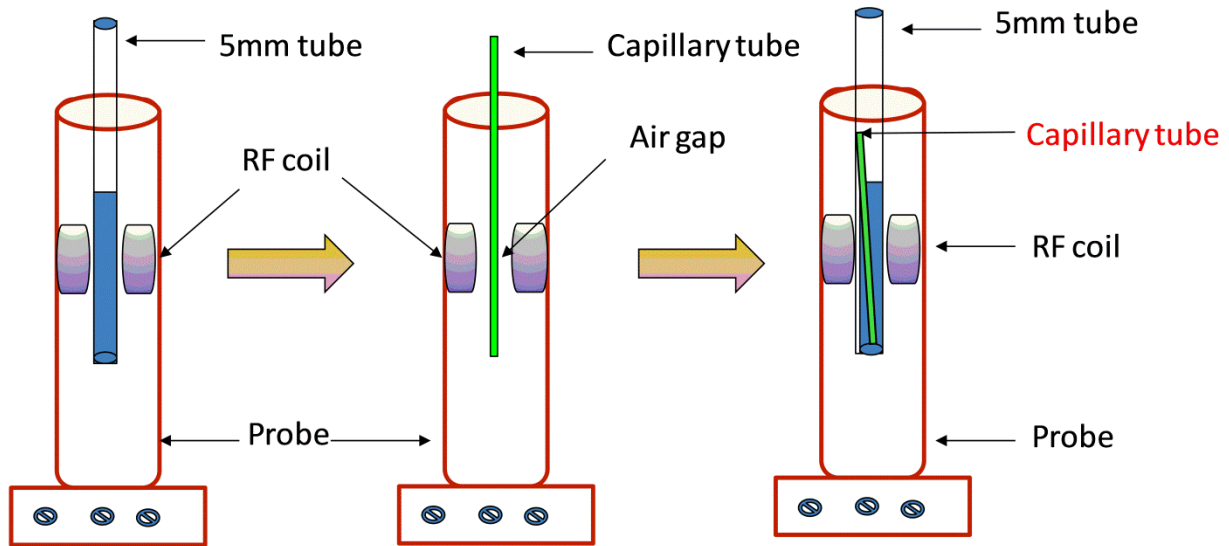
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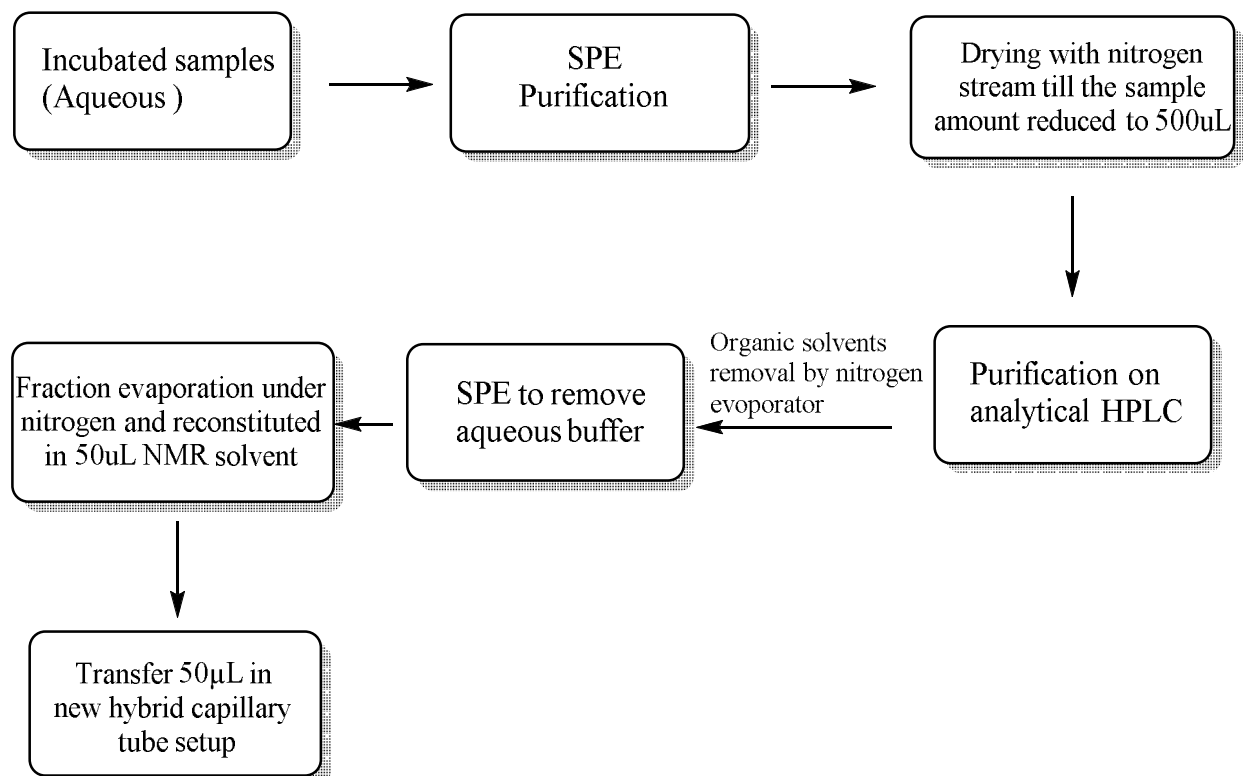
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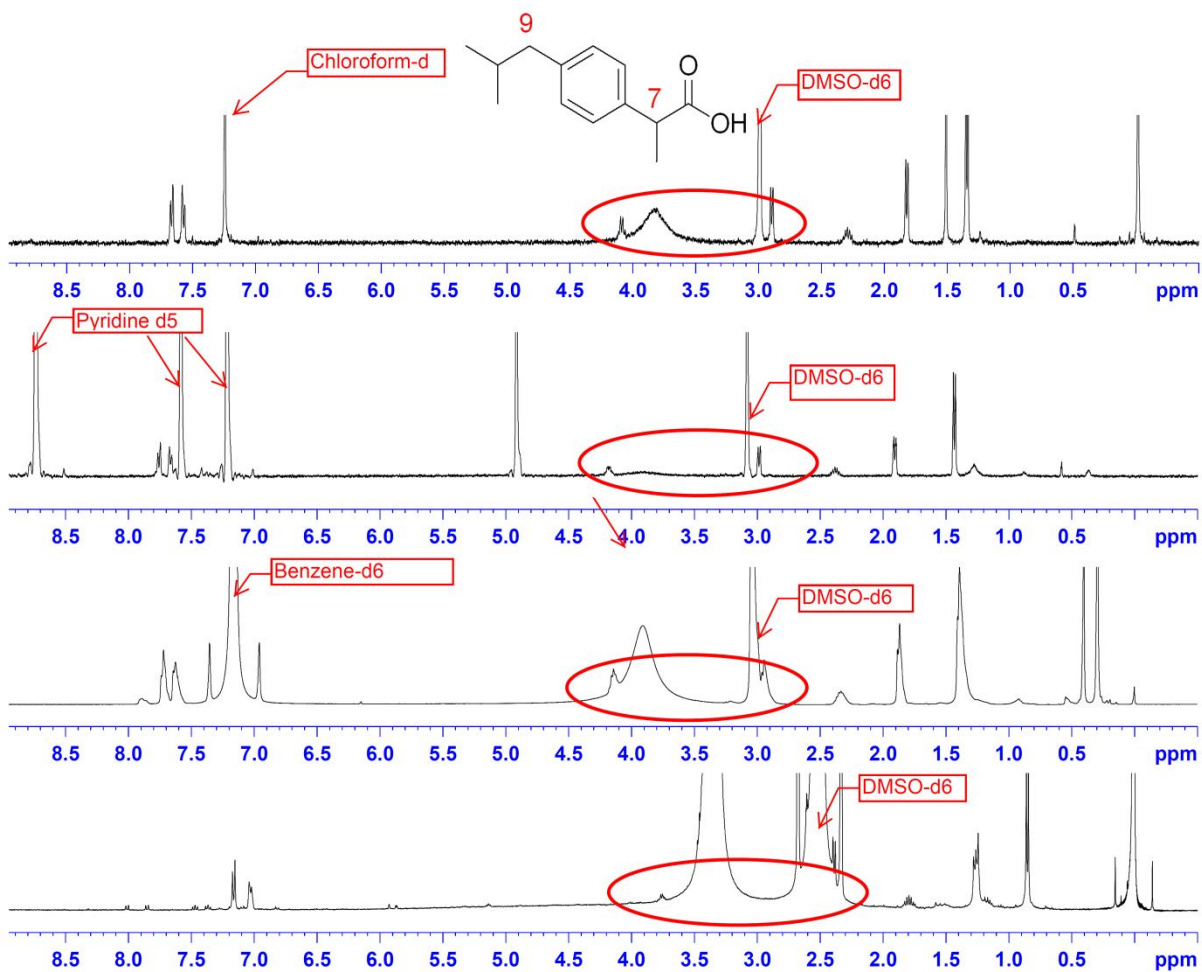
Supplementary information

Use of hybrid capillary tube apparatus on 400MHz NMR for quantitation of crucial low quantity metabolites using aSICCO signal

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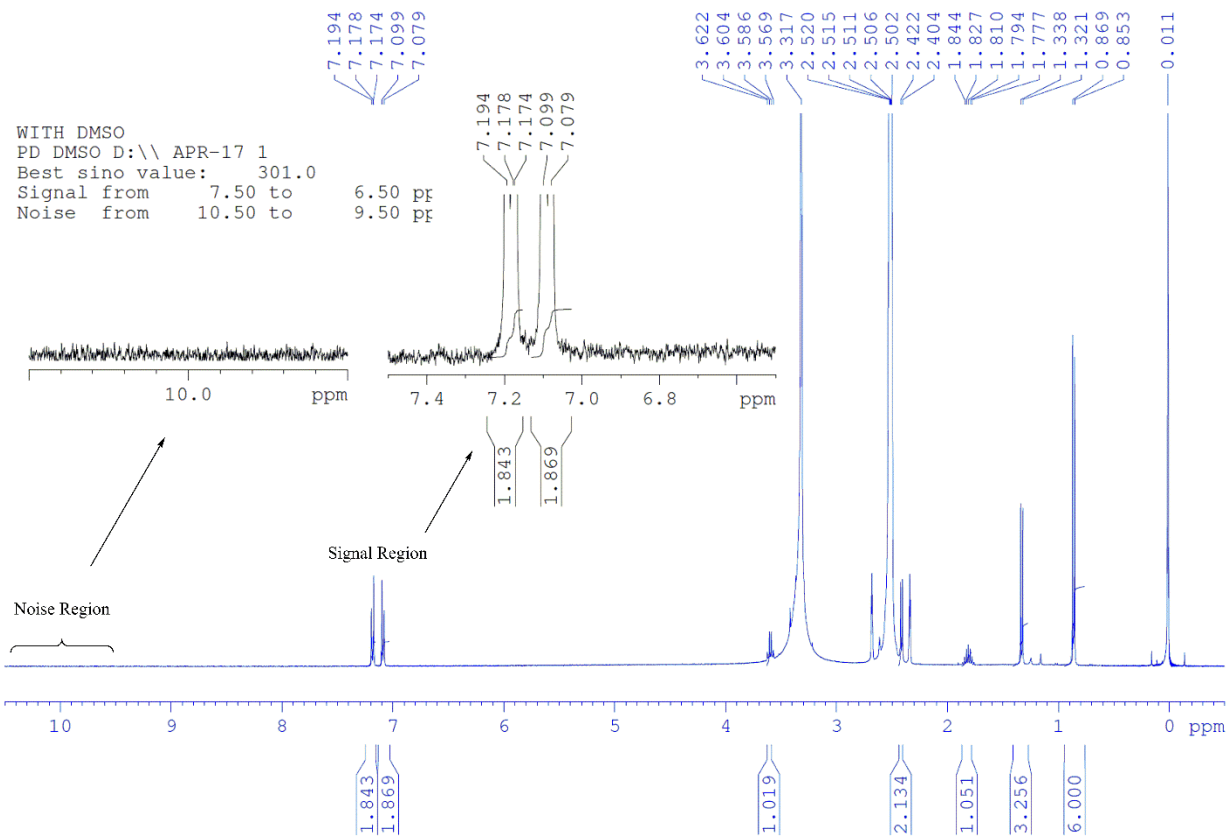
Figure S1A: ^{1}H NMR of Ibuprofen indicating signal to noise ratio (S/N) 301 when the outer tube was filled with DMSO.
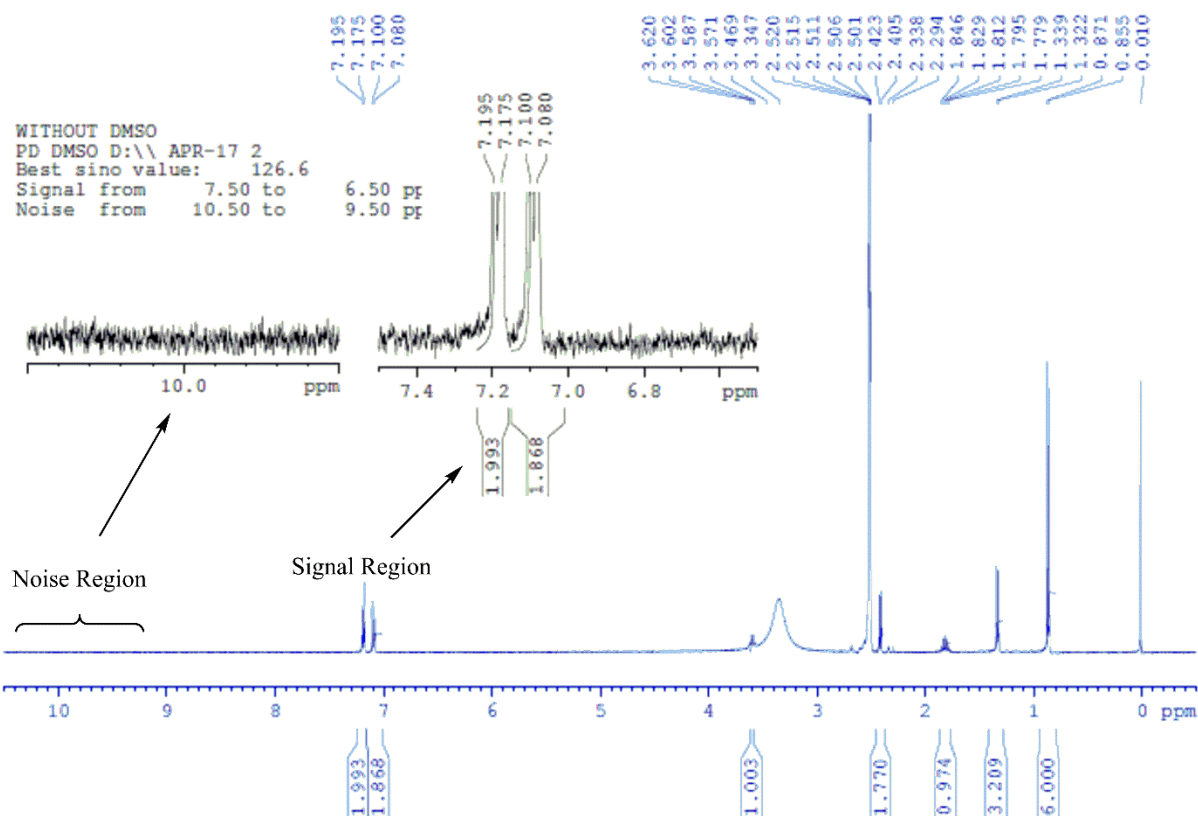
Figure S1B: ^{1}H NMR of Ibuprofen indicating signal to noise ratio (S/N) 126.6 when the outer tube was empty.
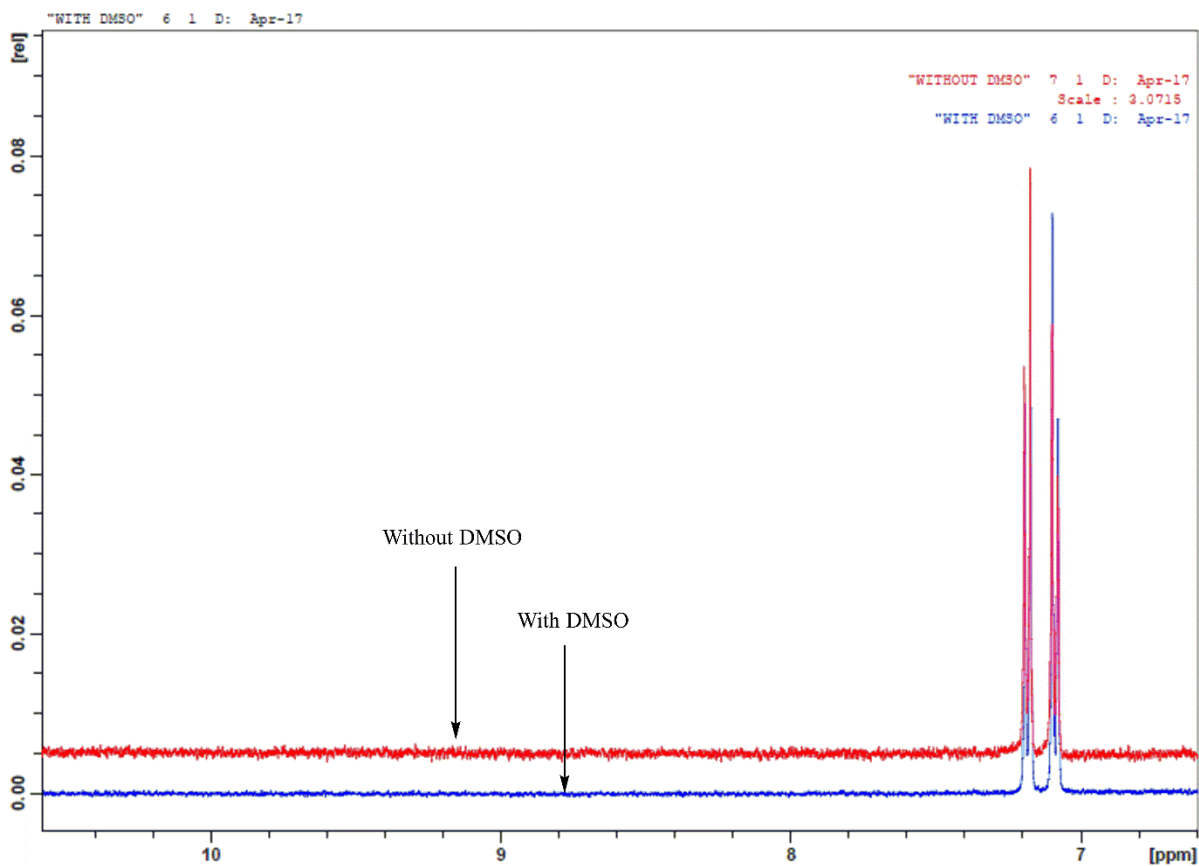
Figure S2: Overlay of ^{1}H NMR data of Ibuprofen showing comparative signal to noise ratio (S/N) with and without solvent in outer tube.
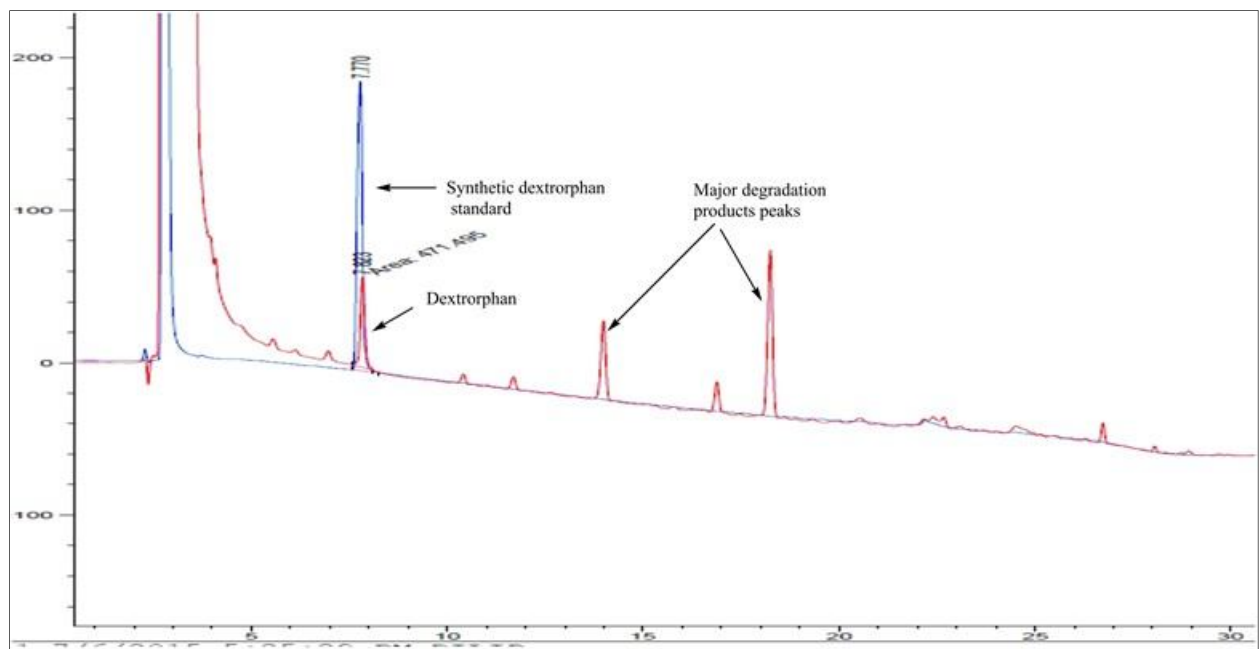
Figure S3: Overlay of Synthetic dextrorphan metabolite standard and NMR recovered dextromethorphen metabolite sample after degradation.
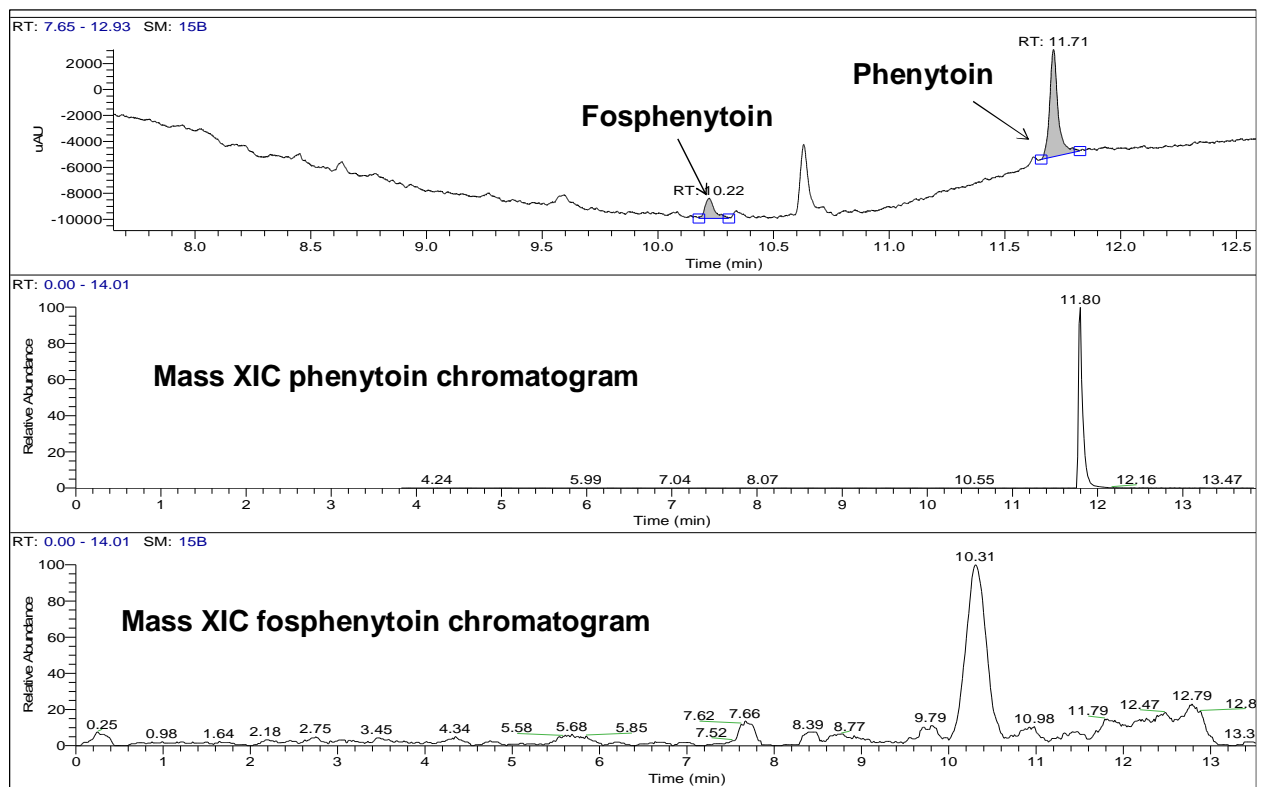
Figure S4: LCMS chromatogram of fosphenytoin metabolites depicting phenytoin as single major metabolite.
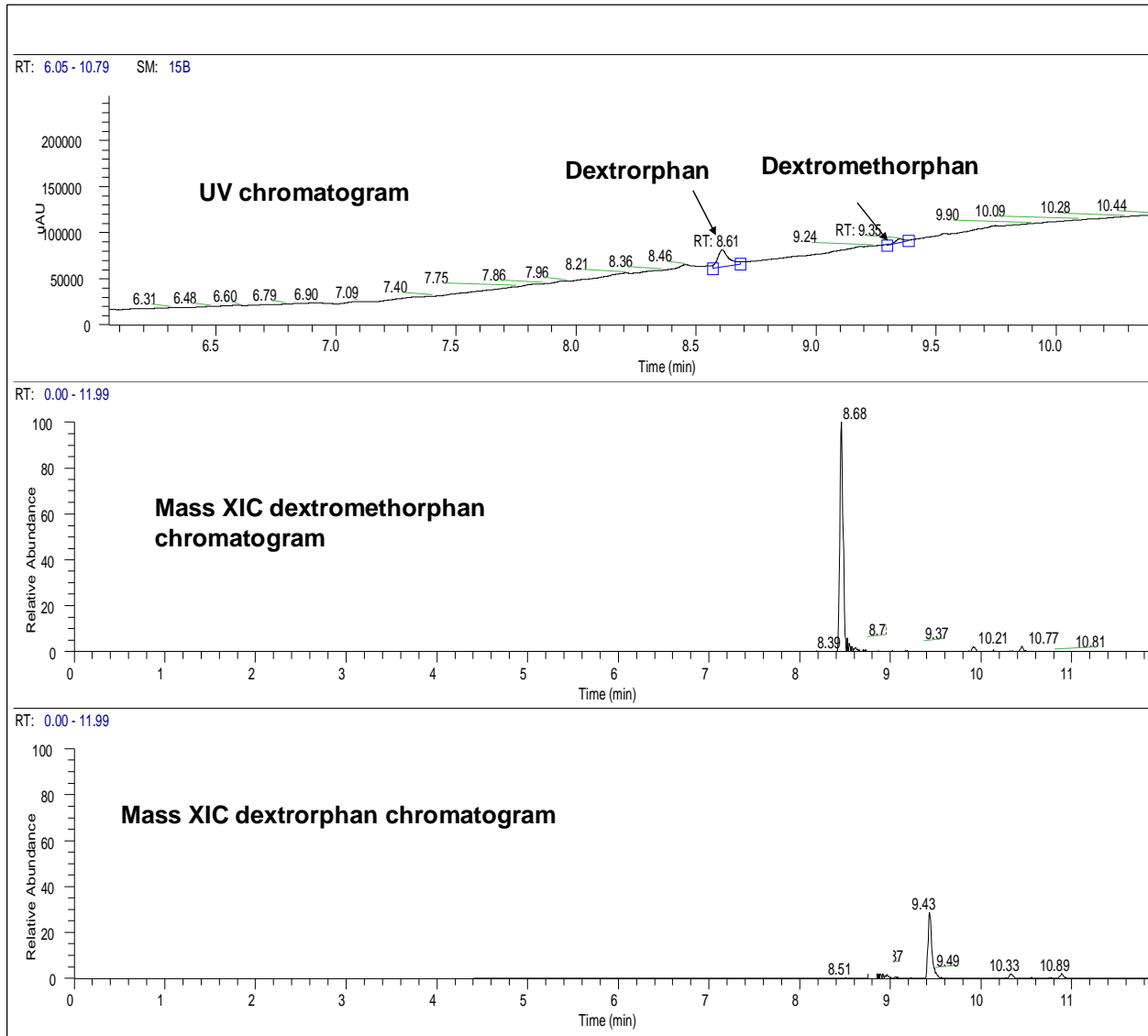
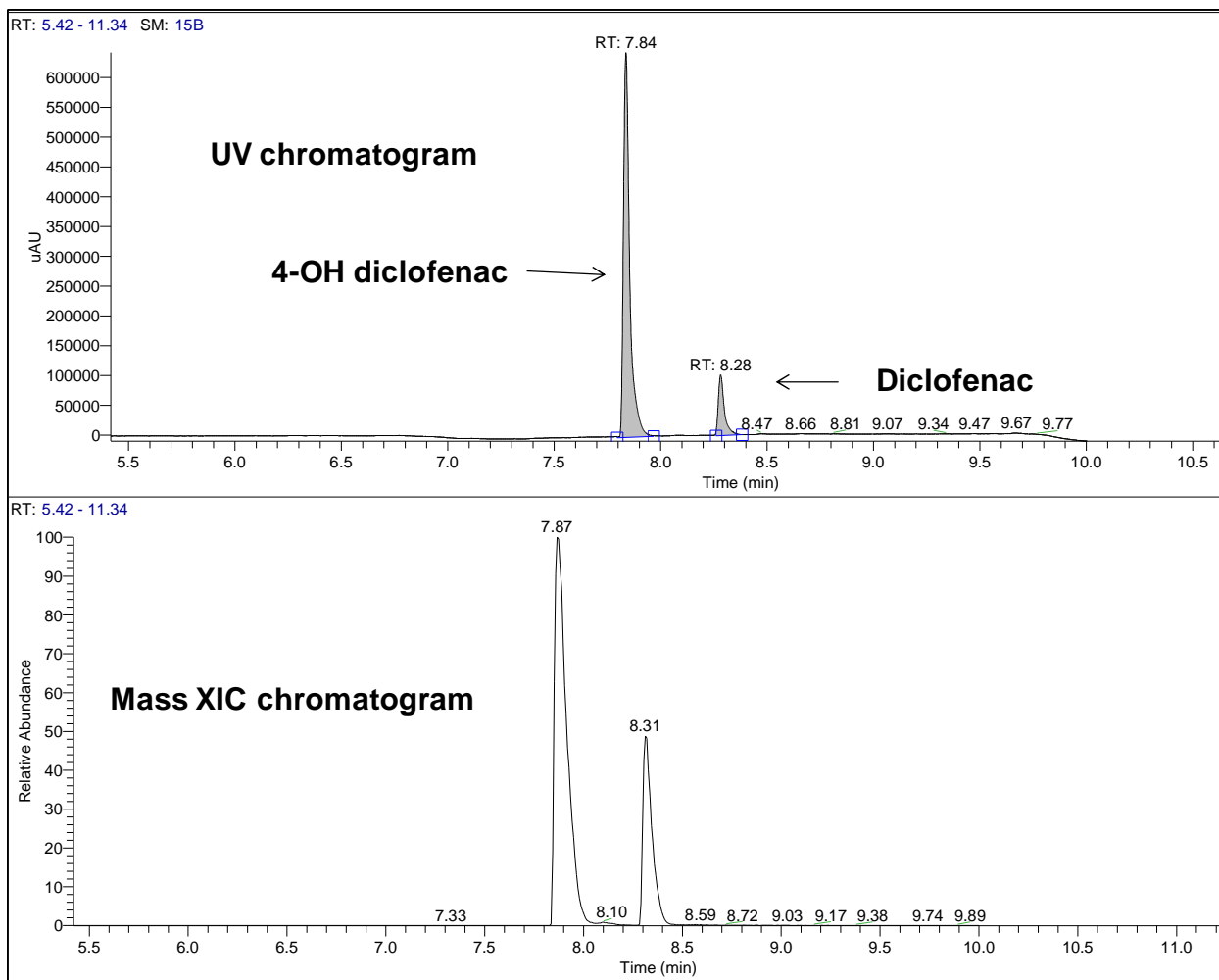**Figure S5:** LCMS chromatogram of of dextromethorphan depicting dextrorphan as a single major metabolite.

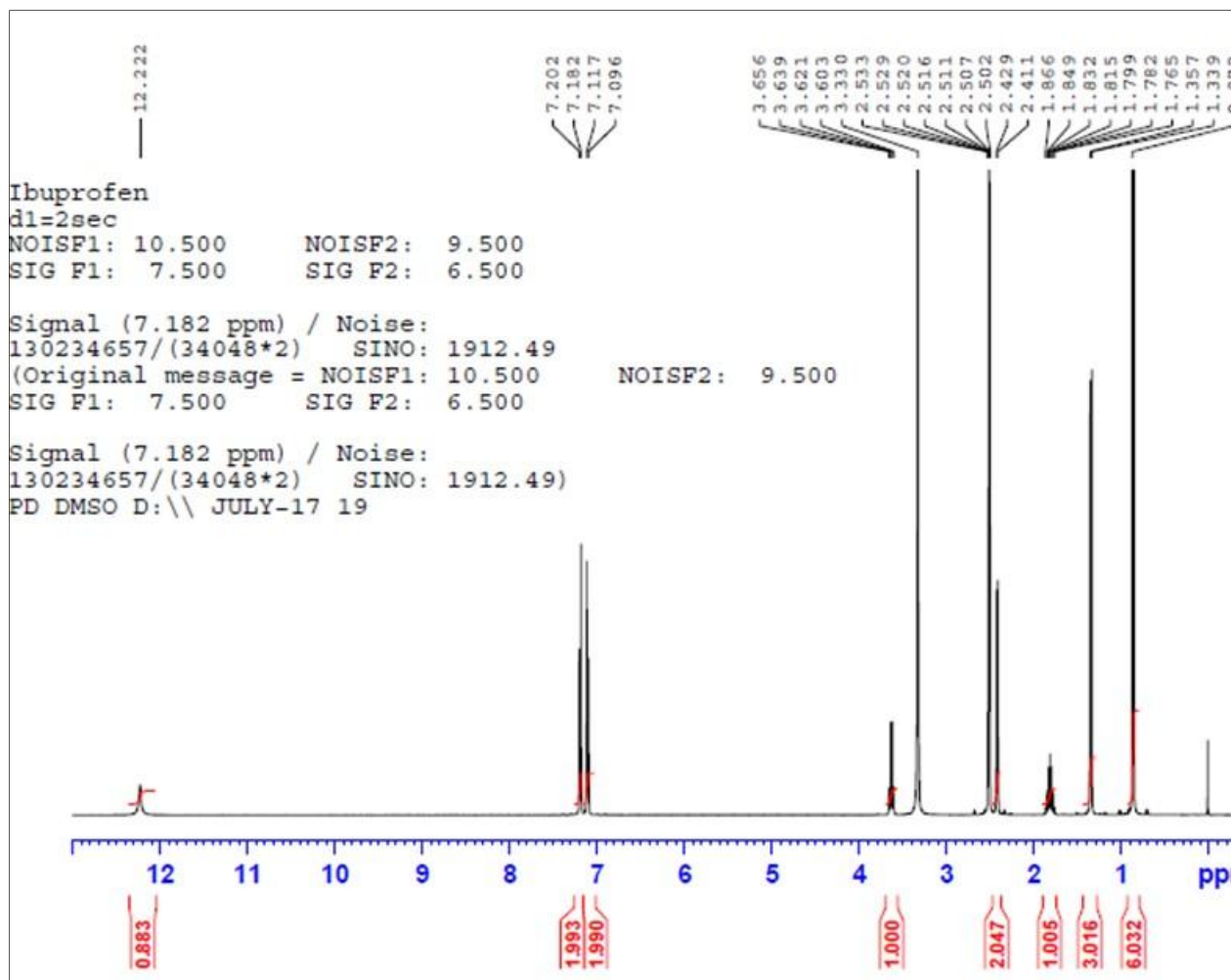**Figure S6:** LCMS chromatogram of diclofenac depicting 4-OH diclofenac as a single major metabolite.

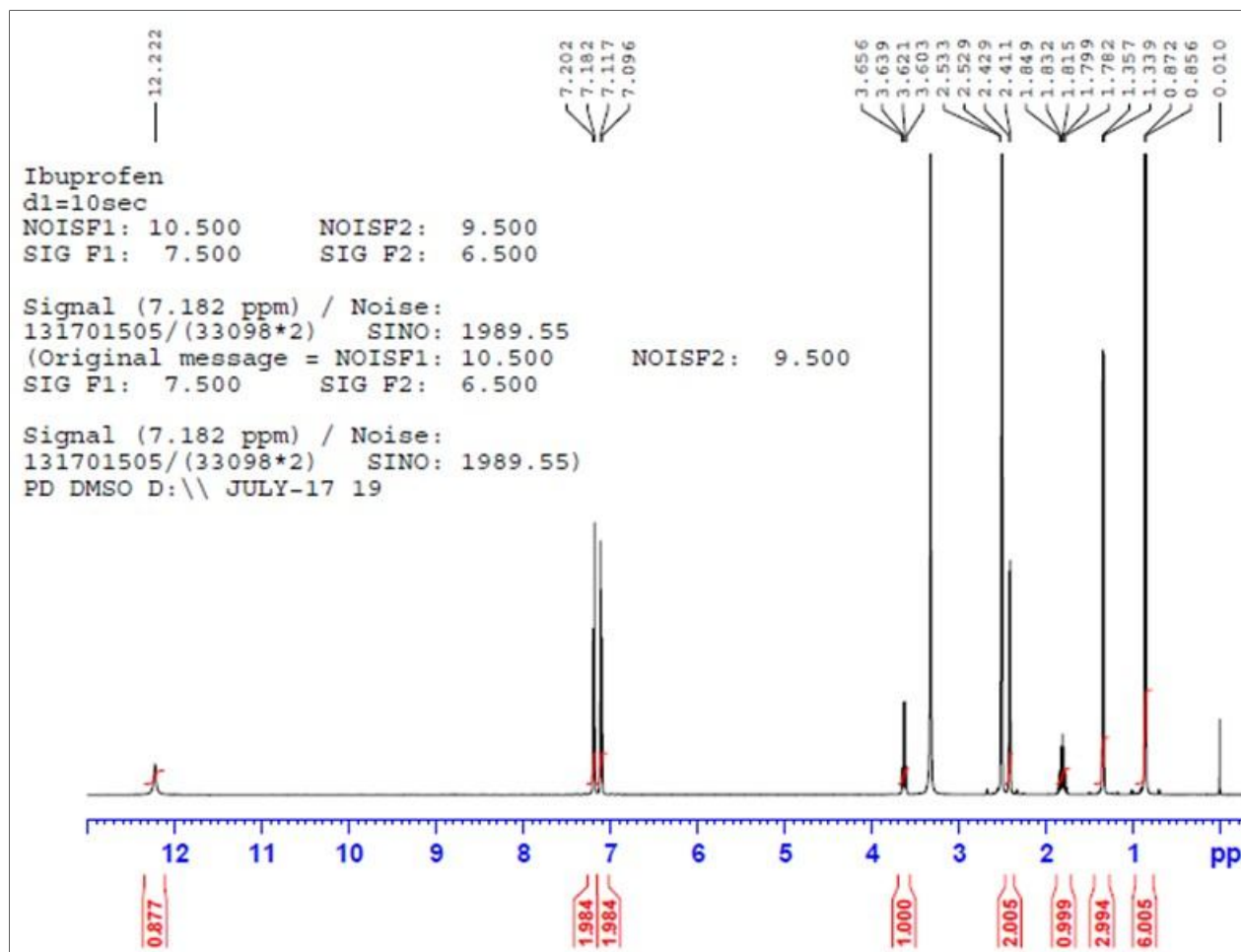**Figure S7:** ^{1}H NMR of Ibuprofen when data was acquired with 2S relaxation delay

Figure S8: ^{1}H NMR of Ibuprofen when data was acquired with 10S relaxation delay