Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 328–334
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Ibuprofen metabolite profiling using a combination of SPE/column-trapping and HPLC–micro-coil NMR

Danijel Djukovic [a], Emmanuel Appiah-Amponsah [a], Narasimhamurthy Shanaiah [a], G.A. Nagana Gowda [a], Ian Henry [a], Mike Everly [a], Brian Tobias [b], Daniel Raftery [a,*]

[a] *Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907, United States*
[b] *Pfizer, Inc., 10646 Science Center Drive, San Diego, CA 92121, United States*

Received 19 October 2007; received in revised form 13 December 2007; accepted 18 December 2007
Available online 28 December 2007

Abstract

Solid-phase extraction and column-trapping preconcentration are combined to enhance HPLC–nuclear magnetic resonance (HPLC–NMR) and applied to metabolite profiling in biological samples. Combining the two signal enhancement techniques improved the NMR signal substantially such that we were able to identify 2-hydroxyibuprofen, carboxyibuprofen, and unmetabolized ibuprofen molecules from a small urine sample after a therapeutic dose of ibuprofen. The hyphenated SPE/column-trapping method resulted in an excellent overall signal enhancement of up to 90-fold.
© 2008 Published by Elsevier B.V.

Keywords: Metabolite profiling; ^{1}H NMR; Micro-coil; Solid-phase extraction; Ibuprofen

1. Introduction

Nuclear magnetic resonance (NMR) in combination with liquid chromatography (LC) is one of the most promising techniques for isolation, quantification, and structural elucidation of metabolites from biological fluids such as urine, blood, or plasma. The first coupled LC–NMR methods were reported in 1978 by Watanabe and Niki [1] and by Bayer et al. [2]. However, because of the poor sensitivity of NMR at that time, the method was somewhat impractical for broader analytical applications. Later improvements in LC–NMR technology expanded its use to a variety of analytical applications such as stereo-chemical studies [3], combinatorial [4] and environmental [5] chemistry, analyses of natural products [6] and phytochemistry [7], drug discovery [8], proteomics [9] and metabolite studies [10–13]. The inherently low sensitivity associated with NMR measurements is a major challenge for the development of LC–NMR methods suitable for isolating and detecting low concentration analytes such as those observed in metabolite profiling. Many efforts have been made to minimize this limitation. Advances in NMR technology, such as the development of stronger field magnets, cryogenic probes [14], solvent suppression techniques [15], and improvements in probe design [16] have dramatically improved NMR sensitivity, thus making hyphenated LC–NMR a very capable analytical tool for metabolite studies.

The introduction of on-flow solenoidal micro-coil probes [16] has significantly facilitated LC–NMR hyphenation. In their earliest days, NMR measurements required microgram amounts of analyte to obtain sufficient signal-to-noise (S/N), whereas the newly developed micro-coil NMR probes currently offer detection limits below 10 ng [17]. Solenoidal micro-coils offer an increase in S/N for mass limited samples due to the close proximity of the detection coil to the sample, as well as the fact that the solenoid geometry offers an enhancement of two to threefold compared to saddle coils [16]. Solenoidal micro-coils are positioned horizontally, perpendicular to the external magnetic field. This arrangement makes micro-coil probes ideal for flow-through design, which in turn facilitates NMR coupling to different separation techniques [18]. In addition to the improvement in sensitivity, the use of capillary micro-coil probes with effective volumes in the microliter range has significantly reduced the amount of deuterated solvents needed to carry out NMR analysis. However, the detection of low concentration analytes, especially from complex matrices, is still problematic.

The development of on-flow micro-coil NMR probes with active volumes of 10 μL and below has allowed a better match

* Corresponding author. Tel.: +1 765 494 6070; fax: +1 765 494 0239.
E-mail address: raftery@purdue.edu (D. Raftery).

0731-7085/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.12.035

between the eluted chromatographic peaks and NMR detection volumes. For example, capillary liquid chromatography (capLC) [19] and capillary electrophoresis (CE) [20] produce peaks with volumes in the microliter range, and are thus ideally suited for micro-coil NMR detection. Alternatively, capillary isotachophoresis (cITP) [21] has been shown to be useful in concentrating analytes for NMR detection by up to three orders in magnitude [22]. The major disadvantages of cITP and CE are that these two separation methods are time consuming and limited to charged samples. After the chromatographic separation, eluted analytes can also be preconcentrated to provide a better match between the LC elution volume and the smaller micro-coil NMR active volume. In essence, prior to the NMR acquisition one needs to constrict the sample to a smaller volume in order to obtain an enhanced NMR signal. Two strategies have been used. The first is based on post-chromatography sample concentration, and includes either solid-phase extraction (SPE) [10], or the use of a trapping (or guard) column [23–25]. Employing SPE, Xu and Alexander achieved sensitivity enhancements of 8–30-fold using high injection volumes [26], while our research group reported analyte enrichments of up to 14-fold using a C18 trapping column [23]. Another alternative is to construct micro-coil NMR probes in an intermediate volume range that provide a better match to eluted volumes and thus simplify hyphenation [27].

We recently designed and evaluated an automated HPLC–micro-coil NMR system capable of performing on-line chromatographic separation, signal enhancement, and NMR acquisition [23]. The core component of our system was the preconcentration step based on a column-trapping technique. Anti-inflammatory drug samples were trapped on a guard column with a mobile phase composed of 90% D_2O/10% acetonitrile-d_3 (CD_3CN), and then *back-flushed* to the NMR micro-coil probe with 90% acetonitrile-d_3/10% D_2O. By employing this preconcentration step, we achieved signal enhancements of up to 14-fold.

The objective of our current effort has been the application of our previously developed signal enhanced HPLC–NMR on a real biological sample such as urine. As will be shown below, we successfully performed ibuprofen metabolite profiling of a urine sample collected from a healthy donor following a 200 mg dose of ibuprofen. In order to maximize the NMR signal, solid-phase extraction (SPE) was added prior to the HPLC–NMR procedure (see Fig. 1). The work describes the unique combination of SPE and column trapping in order to reduce the amount of biological sample needed to perform HPLC–NMR analyses. By combining the two techniques, we obtained a signal enhancement of up to 90-fold, which in turn enabled us to identify two known ibuprofen metabolites and unmetabolized drug from 1 mL of urine using a micro-coil NMR probe at 300 MHz. Confirmation of the structures was provided by a series of two-dimensional experiments at 500 MHz.

2. Experimental

2.1. Reagents

Ibuprofen tablets (200 mg, distributed by Topco Associates LLC, Skokie, IL, USA) were purchased over the counter. Acetonitrile-d_3 (99.8%) and deuterium oxide (D_2O,

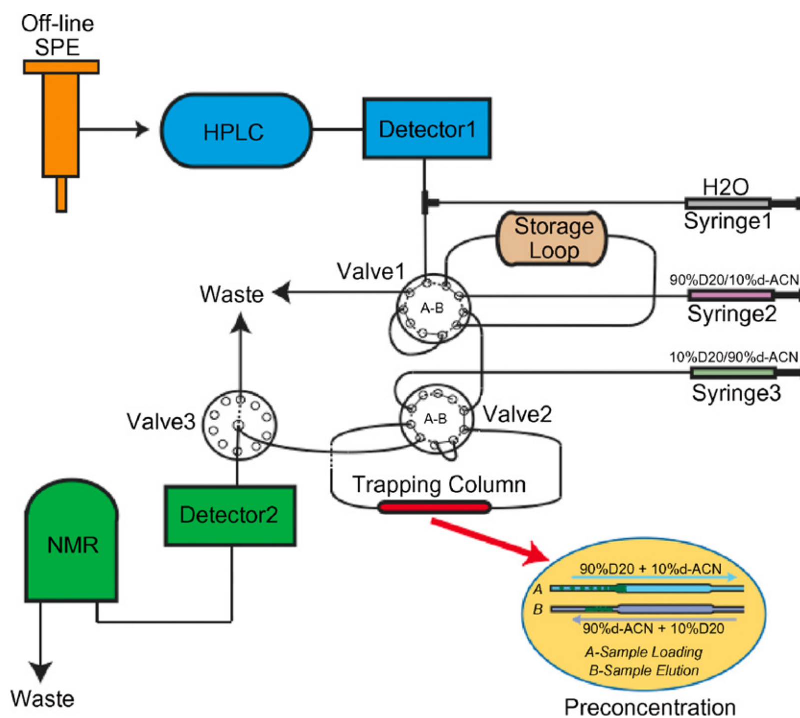

Fig. 1. Schematic diagram of the SPE/LC/column-trapping/micro-coil NMR unit showing the major components of the system. The inset represents the column-trapping preconcentration sequence during which (A) the sample is loaded on to the trapping column and (B) the analyte is eluted from the guard column to the NMR micro-coil probe.

99.9%) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC-grade acetonitrile (CH_3CN, 99.8%), methanol (MeOH, 99.9%), and potassium hydroxide (KOH, pellets, 98%) were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA), while phosphoric acid (H_3PO_4, 85%), HPLC-grade glacial acetic acid, *t*-butyl methyl ether (MTBE, 99.8%), sodium acetate (NaOAc, anhydrous), trifluoroacetic acid (TFA, 98%), and sodium azide were obtained from Fisher Scientific (Pittsburgh, PA, USA). Water was dispensed from an EASYpure II UV water purification system (Barnstead International, Dubuque, IA, USA).

2.2. Urine collection and purification

Urine samples were collected from a healthy donor before and 4 h after a single 200 mg therapeutic dose of ibuprofen. The sample collected before taking the drug was used as a control sample. Sodium azide (0.1%, w/v) was added to the freshly collected urine in order to prevent bacterial growth. The urine samples were purified by centrifugation using Centriprep filters (cat. no. 4321, Millipore, Bedford, MA, USA). The procedure was provided by the manufacturer. Urine samples were pipetted into two filters, 15 mL each, and the filters were spun at 3000 rpm for 30 min, and afterwards the supernatant was collected. Purified samples were stored at −80 °C. All urine samples were collected and processed according to an approved IRB protocol at Purdue University.

2.3. SPE procedure

In order to further purify the urine samples and to maximize the concentration of ibuprofen metabolites, solid-phase extraction was performed by employing Oasis Max SPE cartridges ($6\,cm^3$/150 mg, part #186000370, Waters Corporation, Milford, MA, USA), and following the procedure provided by the manufacturer. Briefly, 1 mL of 10-M KOH was added to 10 mL of urine, and the sample was heated at 60 °C for 15 min. After cooling to the room temperature, the pH was adjusted to 2 with phosphoric acid, and the sample was diluted 50/50 with reagent water. The cartridge was conditioned with MTBE, MeOH, and H_2O, 3 mL each. 10 mL of diluted sample was loaded onto the cartridge. Afterwards, the cartridge was washed first with 3 mL of 50-mM NaOAc, and then with 4 mL of MeOH. The purified sample was eluted with a 4-mL MTBE/MeOH/TFA (89:10:1) solution. Finally, the elution solvent was evaporated with nitrogen, and the sample was reconstituted in a 0.4 mL solution of 30% acetonitrile/70% water. 100 μL of the reconstituted sample (corresponding to approximately 1 mL of pure urine) was used for each HPLC/NMR analysis.

2.4. HPLC

The HPLC system was composed of an LC-10AS Pump and SCL-10A System Controller (Shimadzu Corporation, Kyoto, Japan), 6-port injection valve (Rheodyne, CA, USA), and SPD-10A UV–vis Detector (Shimadzu Corporation, Kyoto, Japan). Fused silica tubes, 125 μm ID, and stainless steel fittings were used as the transfer lines and connectors, respectively (Upchurch Scientific, WA, USA). The HPLC system was operated using Shimadzu EZStart 7.2 software. Analytical separation was performed on 150 mm × 2.1 mm Hypersil GOLD C18 column (Thermo Electron Corporation, MA, USA) using the following gradient elution protocol: 85% H_2O/15% CH_3CN (5 min) ⇒ linear ramp to 45% H_2O/55% CH_3CN (10 min) ⇒ 45% H_2O/55% CH_3CN (15 min). The solvents were adjusted to pH 3 with acetic acid. The flow rate was 150 μL/min.

2.5. Column-trapping preconcentration

Following analytical separation and prior to NMR acquisition, the ibuprofen metabolites from urine were preconcentrated on a 50 mm × 1.0 mm Aquasil C18 guard column (Thermo Electron Corporation, MA, USA). The detailed column-trapping preconcetration procedure was previously described [23] and is shown schematically in the inset of Fig. 1. Briefly, after performing solid-phase extraction, a 100 μL aliquot of urine containing the ibuprofen metabolites was injected into the HPLC. As peaks of interest eluted off the analytical column, valve 1 was switched to forward the sample to the 500 μL storage loop. Simultaneously, syringe 1 was activated in order to infuse pure H_2O at the rate of 200 μL/min so as to decrease the percentage of organic solvent. After the peak of interest was parked in the storage loop, syringe 2 was triggered at the flow rate of 100 μL to transport the previously stored metabolite on to the trapping column. After analyte loading, syringe 2 was stopped, valve 2 was switched, and syringe 3 was activated at the flow rate of 10 μL/min to elute the sample off the guard column and move it towards the NMR micro-coil probe. At the same time, an arrayed NMR acquisition was activated in order to detect the sample as the NMR signal reached its highest intensity. At that point, the sample was stopped in the NMR probe by switching valve 3. All three switching valves were VICI valves (Valco Instruments, Houston, TX, USA), and the stainless syringes were purchased from Harvard Apparatus (Holliston, MA, USA). The syringe pumps and VICI valve modules were controlled using LabView National Instruments software (Austin, TX, USA).

2.6. 1D NMR spectroscopy

Stopped-flow proton NMR spectra were acquired on a 300-MHz Varian Inova spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 299.12 MHz. The spectrometer was equipped with a home-built capillary flow ^{1}H NMR micro-coil probe without a field-frequency lock. The active volume of the NMR probe was ∼3 μL. Each ^{1}H spectrum was acquired with the following parameters: 512 transients, recycling delay (d1) = 4 s, acquisition time (at) = 0.5 s, transmitter power (tpwr) = 40 dB, and 90° pulse width (pw) = 4.1 μs.

2.7. 2D NMR spectroscopy

Since our micro-coil probe at 300 MHz was not equipped with a field-frequency lock, we performed 2D experiments on another NMR instrument. Unambiguous confirmation of the

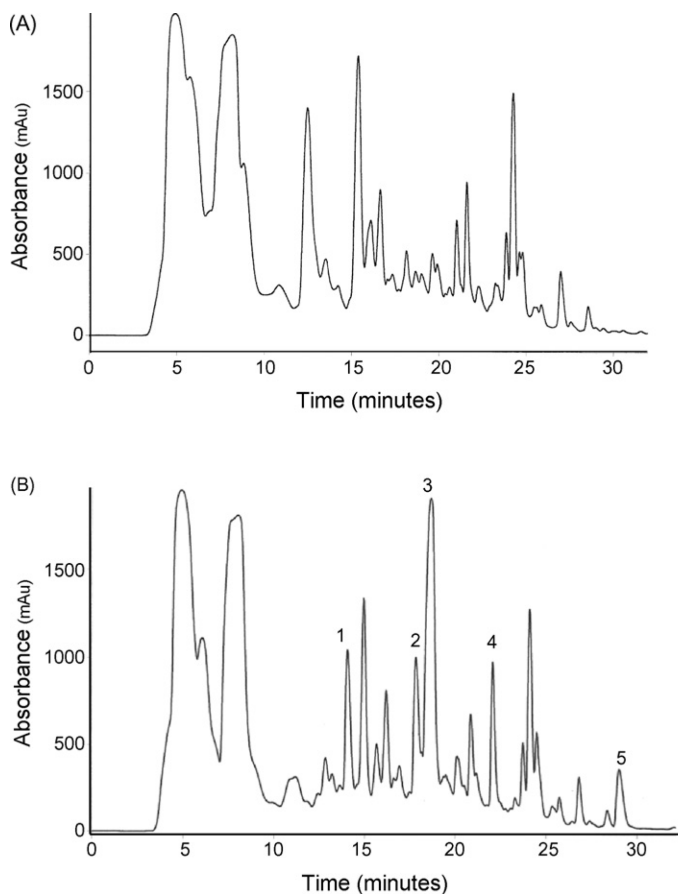

Fig. 2. HPLC–UV chromatograms of urine collected (A) before (control sample) and (B) after the dose of ibuprofen (sample containing potential drug metabolites). Both urine samples were from the same donor. The five potential metabolite peaks were recognized by visually comparing the two chromatograms.

metabolite structures previously detected by our preconcentration and stop-flow 1D NMR method at 300 MHz was made using two-dimensional experiments performed on a Bruker Avance 500 MHz instrument (Bruker BioSpin Corporation, Billerica, MA, USA) equipped with an inverse cryoprobe head and proton preamplifier cooling. Each LC peak (including all unlabeled peaks) from chromatogram B in Fig. 2 was fraction-collected directly from the analytical column. Due to the larger sample volume of the conventional 5 mm NMR tubes, pooled fractions were collected from ten sample injections. The solvents were removed by drying the samples over nitrogen gas, and the samples were then dissolved in CD_3CN for NMR measurements. Homonuclear and heteronuclear two-dimensional NMR experiments such as ^1H–^1H double quantum filtered correlation spectroscopy (DQF-COSY), sensitivity enhanced and multiplicity edited ^1H–^{13}C heteronuclear single quantum correlation (edited HSQC) and ^1H–^{13}C gradient enhanced heteronuclear multiple bond correlation (HMBC) experiments were performed. For the DQF-COSY experiments, a sweep width of 6500 Hz was used in both dimensions, and 400 t_1 increments were acquired, each of 2048 complex data points. The number of transients per t_1 increment was 16, and the relaxation delay was 2.5 s. Phase-sensitive data were obtained using the TPPI method. For the ^1H–^{13}C multiplicity edited HSQC and HMBC experiments, spectral widths of 6500 Hz and 25,000 Hz were used in the ^1H and ^{13}C dimensions, respectively. The number of t_1 increments was 256, and 32 transients, each of 2048 data points were acquired per t_1 increment. The recycle delay was set to 1.5 s. Phase-sensitive data for the edited HSQC experiment was obtained using echo-antiecho data. For the HMBC experiment, NMR data were obtained in magnitude mode without proton decoupling in the t_2 dimension. The resulting NMR data were zero filled to 1024 points in the t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by $\pi/2$ along both the dimensions.

3. Results and discussion

Solid-phase extraction of ibuprofen and its metabolites in urine resulted in a concentration enhancement of ∼10-fold. This value was determined as a ratio of the actual volume of urine loaded on the SPE cartridges and reconstituting volume (taking in consideration the manufacturer claim of >95% sample recovery). We tried to increase the concentration of ibuprofen-related material further by decreasing the volume of the reconstituting solvent, but this caused a significant peak broadening and resulted in peak overlap. We tried unsuccessfully to change the solvent conditions in order to eliminate this problem. The likely reason for the peak broadening was column overloading. In order to differentiate ibuprofen metabolites from the rest of the urine components, we performed a chromatographic separation of the urine extract containing ibuprofen-related compounds as well as a separation of the control urine sample under identical chromatographic conditions. By comparing the obtained chromatograms, we distinguished five peaks that were potential metabolites of interest. The HPLC–UV chromatogram of the extract containing potential metabolites as well as the chromatogram of a control urine sample are shown in Fig. 2.

After separating potential ibuprofen metabolites from the urine extract, each peak was trapped on the guard column with 90% D_2O/10% CD_3CN at 100 μL/min, and eluted to the NMR probe with 10% D_2O/90% CD_3CN at 10 μL/min. Three of the five preconcentrated potential metabolite peaks (peaks 2, 3, and 5 in Fig. 2) produced an NMR signal sufficiently strong for structural elucidation. We repeated each experiment five times in order to produce statistically reliable column-trapping enhancement measurements. The preconcentration factors were computed from the NMR S/N measurements of preconcentrated versus non-preconcentrated (directly from analytical column) peaks. The obtained column-trapping enhancement factors were 4.3 ± 0.7, 5.7 ± 0.7, and 8.8 ± 0.9 for peaks 2, 3, and 5, respectively.

After the analytical separation and column-trapping preconcentration, we acquired stop-flow 300 MHz 1D proton NMR spectra of peaks 2, 3 and 5 (Fig. 3). For comparison, we also obtained NMR spectra of the same peaks eluting directly off the analytical column without signal enhancement prior to the NMR acquisition. Peaks 1 and 4 did not produce a sufficient

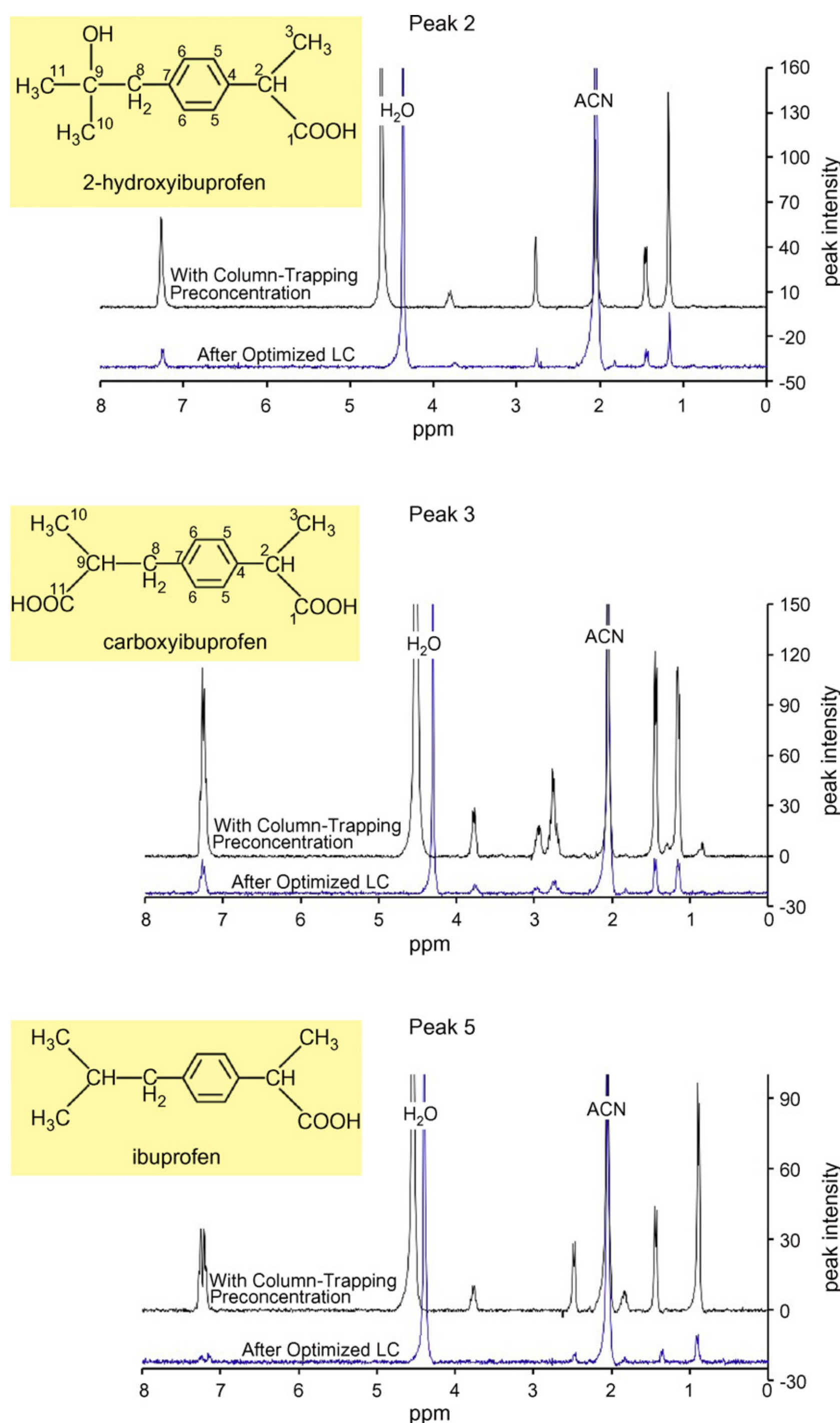

Fig. 3. 300 MHz ^{1}H NMR spectra of two ibuprofen metabolites and the unmetabolized drug after column trapping along with ^{1}H NMR spectra of the same compounds after optimized separation only. The slight variation in the chemical shift of the residual H_2O peaks in the NMR spectra obtained with and without preconcentration was due to the different CD_3CN/D_2O solvent ratios. Spectrometer parameters were nt = 512, d1 = 4 s, at = 0.5 s, tpwr = 40 dB, and pw at 90° = 4.1 μs. The acetonitrile peak at 2.06 ppm was used as the chemical shift reference. The column-trapping preconcentration factors were ∼4–9-fold. The overall signal enhancement was as high as 90-fold.

NMR signal. Based on previous reports [12,28–30], these two peaks were likely 1- and 3-hydroxyibuprofen, minor metabolites of ibuprofen. As these two metabolites are found in urine at significantly lower concentrations than 2-hydroxyibuprofen and carboxyibuprofen [29,30], the combined SPE/column-trapping approach was probably insufficient for NMR detection at 300 MHz. The column trapping enhancement factors were determined from the NMR spectra by measuring the S/N at the strongest (methyl) peak. Peak 5 was straightforward to assign to unmetabolized ibuprofen based on its 1D proton NMR spectrum in Fig. 3. From the spectra in the same figure, peaks 2 and 3 were structurally determined to be 2-hydroxyibuprofen

and carboxyibuprofen, respectively. The 2D NMR experiments described below positively confirmed that metabolites 2 and 3 were in fact 2-hydroxyibuprofen and carboxyibuprofen.

3.1. 2D NMR acquisition

Since the two chromatograms in Fig. 2 did not perfectly overlap, we fraction-collected each peak from chromatogram B in order to not only confirm the structures previously determined from 300 MHz 1D NMR data, but also to determine if any unlabeled peak was an ibuprofen metabolite. However, the 2D NMR data verified only that peaks 2, and 3 were in fact the previously determined metabolites and peak 5 was unmetabolized ibuprofen. The other peaks did not produce spectra related to ibuprofen or its metabolites.

To confirm the structural identity of the two ibuprofen metabolites (peaks 2 and 3; Figs. 2 and 3) detected at 300 MHz, two-dimensional DQF-COSY, edited HSQC and HMBC spectra were obtained at 500 MHz using a commercial 5 mm NMR cryoprobe. These experiments, specifically the heteronuclear 2D experiments, could not be obtained on the home built 300 MHz micro-coil flow probe since this probe was designed for only ^{1}H detection. DQF-COSY spectra of both metabolites showed cross-peaks arising from spin–spin coupling due to vicinal (three bond) as well as geminal protons (two bond; in case of non-equivalent methylene protons). Aromatic ring protons and the $CH–CH_3$ fragments (protons labeled 2 and 3 in Fig. 3) for both the metabolites and the $CH_2–CH–CH_3$ fragment in metabolite 3 (protons labeled 8, 9 and 10 in Fig. 3) were first tentatively identified by tracing the cross-peak positions along both frequency dimensions in the DQF-COSY spectra and taking into account the intensity and multiplicity of the signals in the 1D ^{1}H NMR spectra. Multiplicity edited HSQC spectra were useful in identifying carbon chemical shifts for the directly attached protons as well as the carbon multiplicity based on the position and sign of the cross-peaks (negative cross-peaks for CH and CH_3 groups and positive cross-peaks for CH_2 groups). From these assignments, the tentative proton assignments of the DQF-COSY spectra were confirmed. Subsequently, quaternary and carboxylic acid carbons were identified from the analysis of the HMBC spectra. Each proton signal in the HMBC spectra showed cross-peaks to carbons, which are up to three bonds away in the structure. Finally, using all proton and carbon assignments thus made from the combination of DQF-COSY, edited HSQC and HMBC spectra, the structures of the metabolites 2 and 3 were identified to be 2-hydroxyibuprofen and carboxyibuprofen, respectively. Complete ^{1}H and ^{13}C chemical shifts assigned to both metabolites are shown in Table 1.

Attempts were made to identify additional ibuprofen metabolites, however, at 300 MHz, we were limited to the analysis of these three species. The most likely explanation for why we detected fewer ibuprofen metabolites than previously reported [12], is that we probably lost a number of metabolites that were conjugated with glucuronic acid during the SPE procedure. Ibuprofen, a carboxylic acid-containing drug, is metabolized in humans in a phase-I process into 1-, 2-, and 3-hydroxyibuprofen as well as carboxyibuprofen [28]. In a phase-II process, these four metabolites consequently conjugate with glucuronic acid and are excreted in urine as acyl glucuronides. Unchanged ibuprofen, carboxyibuprofen, 2-hydroxyibuprofen, both conjugated with glucuronic acid and free, account for approximately 80% of the drug dose [29]. If a urine sample is exposed to alkaline conditions, the excreted acyl glucuronides will be hydrolyzed resulting in cleavage of the phase-I metabolites from glucuronic acid [29,30]. Since we added KOH during our SPE procedure, we likely cleaved acyl glucuronides leaving only free metabolites to be extracted and detected. Nevertheless, the few separated metabolites from urine were sufficient to investigate the applicability of our SPE/column-trapping preconcentration concept for HPLC–NMR metabolite studies. For the future, improved SPE procedures at more moderate pH, improved sensitivity available at higher magnetic fields, and potentially better matching between the column trapping volume and the NMR detection coil [27] may allow the study of additional low concentration species.

Table 1
^{1}H and ^{13}C chemical shift assignments for the two ibuprofen metabolites

Carbon/proton number/type[a]	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
Peak 2 (2-hydroxyibuprofen)		
1 (COOH)	–	176.28
2 (CH)	3.693	45.71
3 (CH_3)	1.404	19.20
4 (C)	–	139.00
5,5′(CH)	7.185	128.18
6,6′ (CH)	7.204	132.15
7 (C)	–	140.20
8 (CH_2)	2.689	50.32
9 (C)	–	71.55
10 (CH_3)	1.114	29.90
11 (CH_3)	1.114	29.90
Peak 3 (carboxyibuprofen)		
1 (COOH)	–	176.21
2 (CH)	3.686	45.57
3 (CH_3)	1.398	19.19
4 (C)	–	140.03
5,5′ (CH)	7.211	128.95
6,6′ (CH)	7.165	130.56
7 (C)	–	140.48
8 (CH_2)	2.660, 2.923	39.99
9 (CH)	2.688	50.30
10 (CH_3)	1.106	17.59
11 (COOH)	–	177.94

NMR spectra were referenced to the residual acetonitrile peaks at 1.94 ppm and 118.69 ppm for proton and carbon, respectively.

[a] Refer to Fig. 3 for proton/carbon numbering of the nuclei for the two metabolites.

4. Conclusion

We have described the successful implementation of signal enhanced HPLC–NMR for the analysis of real biological samples such as human urine. By combining SPE and column-trapping preconcentration, along with micro-coil NMR detection, we were able to identify 2-hydroxyibuprofen, carboxyibuprofen, and unmetabolized ibuprofen from a small

amount of urine after an oral dose of 200 mg of ibuprofen. The hyphenated SPE/column-trapping preconcentration method provided an excellent overall signal enhancement of up to 90-fold. The major limitations of the present work are first, the incapability of performing 2D NMR acquisition using our micro-coil probe due to the current probe design; and second, an inability to detect larger number of drug metabolites caused by the use of a low magnetic field strength of 300 MHz. These limitations could be overcome by using a stronger NMR field, and by employing a micro-coil probe equipped with a field-frequency lock and both proton and carbon channels. Better matching of the micro-coil volume to the eluted volume could also provide additional enhancement of the signal.

Acknowledgements

The authors gratefully acknowledge the NIH (5R01-RR-018294) and Pfizer, Inc. for financial support, and thank the Amy Facility staff at Purdue for their technical assistance. The authors also thank the reviewer who made insightful comments about our SPE procedure.

References

[1] N. Watanabe, E. Niki, Proc. Jpn. Acad. Ser. B 54 (1978) 194–199.
[2] E. Bayer, K. Albert, M. Nieder, E. Grom, T. Keller, J. Chromatogr. 186 (1979) 497–507.
[3] M. Dachtler, T. Glaser, K. Kohler, K. Albert, Anal. Chem. 73 (2001) 667–674.
[4] J. Chin, J.B. Fell, M. Jarosinski, M.J. Shapiro, J.R. Wareing, J. Org. Chem. 63 (1998) 386–390.
[5] M. Godejohann, A. Preiss, C. Mugge, G. Wunsch, Anal. Chem. 69 (1997) 3832–3837.
[6] V. Exarchou, M. Krucker, T.A. van Beek, J. Vervoort, I.P. Gerothanassis, K. Albert, Magn. Reson. Chem. 43 (2005) 681–687.
[7] C. Seger, M. Godejohann, L.H. Tseng, M. Spraul, A. Girtler, S. Sturm, H. Stuppner, Anal. Chem. 77 (2005) 878–885.
[8] S.X. Peng, Biomed. Chromatogr. 14 (2000) 430–441.
[9] C.A. Daykin, O. Corcoran, S.H. Hansen, I. Bjornsdottir, C. Cornett, S.C. Connor, J.C. Lindon, J.K. Nicholson, Anal. Chem. 73 (2001) 1084–1090.
[10] M. Godejohann, L.H. Tseng, U. Braumann, J. Fuchser, M. Spraul, J. Chromatogr. A 1058 (2004) 191–196.
[11] K. Sohda, T. Minematsu, T. Hashimoto, K. Suzumura, M. Funatsu, K. Suzuki, H. Imai, T. Usui, H. Kamimura, Chem. Pharm. Bull. 52 (2004) 1322–1325.
[12] E. Clayton, S. Taylor, B. Wright, I.D. Wilson, Chromatographia 47 (1998) 264–270.
[13] M. Spraul, M. Hofmann, P. Dvrortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem. 65 (1993) 327–330.
[14] M. Spraul, A.S. Freund, R.E. Nast, R.S. Withers, W.E. Mass, O. Corcoran, Anal. Chem. 75 (2003) 1546–1551.
[15] S.H. Smallcombe, S.L. Patt, P.A. Keifer, J. Magn. Reson. A 117 (1995) 295–303.
[16] M.E. Lacey, R. Subramanian, D.L. Olson, A.G. Webb, J.V. Sweedler, Chem. Rev. 99 (1999) 3133–3152.
[17] K. Albert, On-line LC–NMR and Related Techniques, John Willey & Sons Ltd., Chichester, England, 2002.
[18] A.G. Webb, J. Pharm. Biomed. 38 (2005) 892–903.
[19] M.E. Lacey, Z.J. Tan, A.G. Webb, J.V. Sweedler, J. Chromatogr. A 922 (2001) 139–149.
[20] J. Schewitz, K. Pusecker, P. Gforer, U. Gotz, L.H. Tseng, A.K. Bayer, Chromatographia 50 (1999) 333–337.
[21] A.M. Wolters, D.A. Jayawickrama, J.V. Sweedler, J. Nat. Prod. 68 (2005) 162–167.
[22] R.A. Kautz, M.E. Lacey, A.M. Wolters, F. Foret, A.G. Webb, B.L. Karger, J.V. Sweedler, J. Am. Chem. Soc. 123 (2001) 3159–3160.
[23] D. Djukovic, S. Liu, I. Henry, B. Tobias, D. Raftery, Anal. Chem. 78 (2006) 7154–7160.
[24] L. Griffiths, R. Horton, Magn. Reson. Chem. 36 (1998) 104–109.
[25] P.S. Kokkonem, W.M.A. Niessen, U.R. Tjaden, J. van der Greef, Rapid Commun. Mass Spectrom. 5 (1991) 19–24.
[26] F. Xu, A.J. Alexander, Magn. Reson. Chem. 43 (2005) 776–782.
[27] I.D. Henry, G.H.J. Park, KCF R., B. Tobias, D. Raftery, Concepts Magn. Reson. B 33B (2008) 1–8.
[28] C.H. Johnson, I.D. Wilson, J.R. Harding, A.V. Stachulski, L. Iddon, J.K. Nicholson, J.C. Lindon, Anal. Chem. 79 (2007) 8720–8727.
[29] S.C. Tan, S.H.D. Jackson, C.G. Swift, A.J. Hutt, J. Chromatogr. A 701 (1997) 53–63.
[30] A.R.M. deOliveira, F.J.M. deSantana, P.S. Bonato, Anal. Chim. Acta 538 (2005) 25–34.