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Signal Enhancement in HPLC/Micro-Coil NMR Using Automated Column Trapping

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Abstract

A new HPLC-NMR system is described that performs analytical separation, pre-concentration, and NMR spectroscopy in rapid succession. The central component of our method is the online pre-concentration sequence that improves the match between post-column analyte peak volume and the micro-coil NMR detection volume. Separated samples are collected on to a C18 guard column with a mobile phase composed of 90% D₂O/10% acetonitrile-D₃, and back-flashed to the NMR micro-coil probe with 90% acetonitrile-D₃/10% D₂O. In order to assess the performance of our unit, we separated a standard mixture of 1 mM ibuprofen, naproxen, and phenylbutazone using a commercially available C18 analytical column. The S/N measurements from the NMR acquisitions indicated that we achieved signal enhancement factors up to 10.4 (±1.2)-fold. Furthermore, we observed that pre-concentration factors increased as the injected amount of analyte decreased. The highest concentration enrichment of 14.7 (±2.2)-fold was attained injecting 100 µL solution of 0.2 mM (~4 µg) ibuprofen.

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is demonstrably one of the most powerful analytical tools for molecular structural elucidation, while liquid chromatography (LC) is the most widely employed technique for quantitative and qualitative separation of different compounds from a mixture. Thus, a logical choice for synchronized separation and structural elucidation of compounds from a mixture is the union of LC and NMR, and the first examples of coupled LC-NMR methods were reported in 1978 by Watanabe and Niki¹, and Bayer and co-workers.² However these methods suffered from insufficient NMR sensitivity, which made them impractical for general analytical applications at the time. The last decade of advances in NMR technology, such as development of stronger magnets, cryogenic probes³, micro-coil probes⁴ and solvent suppression techniques⁵ has dramatically enhanced NMR sensitivity, allowing LC-NMR coupling to develop. Consequently, LC-NMR has been employed in variety of applications such as stereo-chemical studies^{6,7}, combinatorial^{8,9} and environmental¹⁰ chemistry, analyses of natural products¹¹⁻¹⁴ and phytochemistry^{15,16}, metabolites studies¹⁷⁻¹⁹, drug discovery²⁰, and proteomics.²¹

In its earliest days, LC-NMR methods required mg amounts of analyte in order to obtain sufficient S/N, whereas the newly developed micro-coil NMR probes currently offer detection limits below 10 ng.²² In addition, compared to saddle coil geometries used in most commercial probes, solenoidal micro-coils offer an increase in S/N of two to three fold.⁴ Solenoidal micro-coils are positioned horizontally, perpendicular to the external magnetic field. This

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arrangement makes micro-coil probes amenable to flow-through design that can be interfaced with a variety of standard and capillary separation techniques.²³ In addition to the improvement in the sensitivity, the use of capillary micro-coil probes with effective volume in μL range has significantly reduced the amount of deuterated solvents needed to carry out LC-NMR analysis, thus making them economically feasible.

A remaining challenge is how to match the LC elution volume to effectively match the smaller NMR active volume. Essentially, one needs to compress the analyte into a smaller volume for more sensitive detection made available by the smaller micro-coil receiver volume that is minimized in order to increase the mass sensitivity of NMR measurements.²⁴ In order to achieve the highest feasible analyte concentration in a minimum solvent volume, two different approaches have been employed. The first relies on concentrating samples after separation, and involves either solid phase extraction (SPE),^{13,16,17,25-29} or the use of a guard column.^{30,31} Employing SPE, Xu and co-workers achieved sensitivity enhancements of 8 to 30-fold using high injection volumes,²⁹ while Griffiths *et al.*³¹ reported signal increase of 2.1-fold using a guard column for the pre-concentration. A second approach focuses on minimizing the elution peak volume directly in the chromatographic separation prior to NMR acquisition. Methods associated with this approach include capillary liquid chromatography (capLC),^{14,32-34} capillary electrophoresis (CE),³⁵ and capillary isotachopheresis (cITP).^{36,37} cITP has been shown to be useful in concentrating analytes for NMR detection by up to 2-3 orders in magnitude.³⁶ However, cITP and CE are limited to charged analytes.

In our lab, we have developed an automated HPLC-NMR system (Figure 1), which is capable of performing analytical separation, pre-concentration, and NMR acquisition in rapid succession. The core component of our system is the pre-concentration sequence, which is based on the method pioneered by Kokkonen *et al.*³⁰, and extended to LC-NMR by Griffiths and Horton.³¹ Our system offers significantly higher enhancement factors and lower detection limits, and it is almost completely automated under software control. A sample is trapped on to a guard column with a mobile phase composed of 90% D_2O /10% acetonitrile- D_3 , and then *back-flushed* to the NMR micro-coil probe with 90% acetonitrile- D_3 /10% D_2O as shown in the inset of Figure 1.

To evaluate this system, we performed a series of experiments involving separation, pre-concentration, and NMR acquisition of non-steroidal anti-inflammatory drugs (ibuprofen, naproxen, and phenylbutazone) in a mixture. As we will show below, the advantages of our system are: (1) it yields an excellent signal enhancement factor of up to 14.7 (± 2.2)-fold, (2) deuterated solvents are not required for chromatography, (3) has almost 100% sample recovery, (4) is very fast compared to SPE-NMR²⁹ or other methods involving sample drying, and (5) it has low sensitivity detection limit of $\sim 4 \mu\text{g}$ of sample per injection. Moreover, our LC-NMR unit is universal as it can be basically used for any separable mixture by simply making column and solvent changes.

EXPERIMENTAL SECTION

Reagents

Ibuprofen (99%), Naproxen (98%), and Phenylbutazone (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile- D_3 (d-ACN, 99.8%) and Deuterium Oxide (D_2O , 99.9%) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). HPLC-grade Acetonitrile (ACN, 99.8%) was from Mallinckrodt Baker Inc. (Phillipsburg, NJ), and Phosphoric Acid (H_3PO_4 , 85%) was from Fisher Scientific (Pittsburgh, PA). Water was dispensed from EASYpure II UV water purification system (Barnstead International, Dubuque, IA). Mixtures for HPLC-NMR runs were prepared by dissolving ibuprofen, naproxen, and

phenylbutazone in 50% H₂O/50% ACN. Standard samples were prepared by dissolving each analyte in 10% D₂O/90% d-ACN.

HPLC

The HPLC unit was composed of a LC-10AS Pump and SCL-10A System Controller (Shimadzu, Japan), 6-port injection valve (Rheodyne, CA), 150×2.1 mm Aquasil C18 analytical column (Thermo Electron Corporation, MA) and SPD-10A UV/Vis Detector (Shimadzu). Fused silica tubes, 125 μm ID, and stainless steel fittings were used as the transfer lines and connectors, respectively (Upchurch Scientific, WA). HPLC was operated using Shimadzu EZStart 7.2 software. All separations were performed applying reverse phase isocratic mobile-phase conditions (40% H₂O + 60% ACN + 0.1% H₃PO₄).

Pre-concentration (Figure 2c and 2d)

The inlet of the Aquasil C18 trapping column, 50×1.0mm (Thermo Electron Corporation) was connected to Syringe 2 (high-pressure stainless steel syringe, Harvard Apparatus, MA) via Valves 1 and 2 (two-position valves, Valco, TX), and Storage Loop (multi-position valve containing 500 μL storage-loops, Valco). Syringe 2 contained 90% D₂O/10% d-ACN. The outlet of the guard column was attached to Syringe 3 (high-pressure syringe pump, Harvard, MA) via Valve 2. Syringe 3 was filled with 10% D₂O/90% d-ACN. Pre-concentration was achieved by loading samples on to the trapping column with 90% D₂O/10% d-ACN (inset of Figure 2c), and *back-flashing* them to the NMR with 10% D₂O/90% d-ACN as shown in the inset of Figure 2d. Fused silica tubes, 125 μm ID, and stainless steel fittings, both from Upchurch, were used as the transfer lines and connectors, respectively. All switching valves as well as the storage-loop valve were interfaced to Valco Control Modules. The syringes were mounted on high-pressure programmable pumps (PHD 2000, Harvard Apparatus). Both, the pumps and Valco Control Modules were controlled employing LabView National Instruments software.

NMR Spectroscopy

¹H NMR spectra were acquired on a 300 MHz Varian Inova spectrometer operating at 299.12 MHz. The spectrometer was equipped with a home-built capillary flow micro-coil probe with a ¹H channel and without a field-frequency lock. The probe contained an etched fused-quartz detection cell for enhanced fill-factor, and two standard fused-silica capillaries (360 μm OD, 150 μm ID) were attached with polyimide sealing resin (Supelco, Bellefonte, PA) to the ends of the detection cell for sample input and output flow. The coil and flow cell were kept immersed in FC-43, a magnetic susceptibility matching fluid designed to improve resolution by decreasing distortion due to discrepancies in magnetic susceptibilities of probe materials.³⁸ The active volume was ~ 3 μL.

HPLC-NMR Procedure

After a mixture is injected into LC injection valve, the system operates in four sequences as detailed in Figure 2:

Sequence 1: (Figure 2a)—Prior to the appearance of the sample in Detector 1, Valves 1 and 2 are kept in position A such that the HPLC mobile phase flows from Detector 1 to the waste while Syringe 2 is delivering 90% D₂O/10% d-ACN through Storage-Loop, Valves 1 and 2, Trapping Column, Valve 3, Detector 2, and NMR probe, sequentially.

Sequence 2: Sample Storing (Figure 2b)—When the first sample peak appears in Detector 1, Valve 1 is switched (using a previously determined time delay) to position B in order to forward the analyte to the storage loop. By switching Valve 1 to position B, Syringe

1 automatically infuses H₂O to decrease the organic component of the mobile phase, while Syringe 2 is disengaged in order to minimize the deuterated solvent consumption. Since all separations were performed using isocratic condition with 40% H₂O/60% ACN/0.1% H₃PO₄ and flow rate of 100 µL/min, Syringe 1 was set at 150 µL/min in order to limit the amount of organic solvent in the storage-loops to less than 25%.

Sequence 3: Sample Loading on to Trapping Column (Figure 2c)—After the first peak is collected in the storage-loop, Valve 1 is switched back to position A, which automatically disengages Syringe 1 and activates Syringe 2. At this point, solvent from Syringe 2 transports the previously stored sample on to the trapping column. If another peak appears in Detector 1 before pre-concentration of the previous sample was finished, Valve 1 is switched to position B in order to park the incoming sample in a new position in the storage loop. In our experiments, *Sequence 3* was five minutes long since the volume of the storage loops was 500 µL, and the loading flow rate was 100 µL/min.

Sequence 4: Sample Back-Flushing to NMR (Figure 2d)—After loading sample on the trapping column, Valve 2 is switched to position B. This operation triggers Syringe 3 and shuts down Syringe 2. Valve 1 is kept in position A until the next sample appears in Detector 1. Syringe 2 is now delivering 90% d-ACN/10% D₂O at the rate of 10 µL/min through the guard column in order to back-flash trapped sample from the guard column, through Valve 3 and Detector 2 (Uv/Vis detector SPD-6AV, Shimadzu), and on to the NMR probe. When the sample peak in Detector 2 is at its maximum value, arrayed NMR acquisition is activated. In case of stop-flow NMR analyses, the solvent flow inside the NMR capillary is stopped (by switching Valve 3) as the intensity of NMR signal arising from the sample reaches its maximum. Valve 3 has a dual function: It allows solvent to flow to waste in order to release the backpressure in the transfer line between the valve and NMR probe, and it also disconnects the NMR probe from the trapping column allowing simultaneous pre-concentration and NMR acquisition.

RESULTS AND DISCUSSION

HPLC/NMR Optimization

Preliminary experiments were conducted in order to optimize the HPLC-NMR system for simultaneous separation, pre-concentration, and NMR analyses of three anti-inflammatory drugs: ibuprofen, naproxen, and phenylbutazone. In order to minimize dead volumes, the transfer lines were kept as short as possible. It was also necessary to identify analytical and trapping columns, solvent compositions, and flow rates that concurrently offered good mixture separation, a minimum sample loss through the trapping column, maximum pre-concentration enhancements of each analyte, and acquisition of decent NMR spectra. The optimum conditions (see Table 1) allowed good separation of a mixture of ibuprofen, naproxen, phenylbutazone (1mM each, 100 µL injection volume) with almost no loss of the samples through the trapping column in the pre-concentration sequence. The retention times for naproxen, ibuprofen, and phenylbutazone were approximately 4, 17, 32 minutes, respectively (data not shown).

The most challenging aspect of the optimization was to avoid sample loss in the pre-concentration sequence. The best separation of the three analytes was obtained using gradient elution condition (50% to 90% acetonitrile) with the flow rate of 300 µL/min. However, we could not implement these conditions in our system because a considerable amount of phenylbutazone and naproxen passed through the trapping column during the sample loading. This was due to the excessive percentage of organic solvent entering the storage-loop. Thus, we had to switch to the isocratic condition shown in Table 1, thereby sacrificing slightly the quality of the chromatographic spectra (longer RT, broader peaks). The separation flow rate

was limited by the fact that Syringe 1 could, at best, infuse water at the rate of 150 $\mu\text{L}/\text{min}$. Any higher flow rate resulted in sufficient backpressure to stall the syringe pump. We also attempted to cut the percentage of the organic solvent in Syringe 2 below 10%, but as a result samples were precipitating in the transfer lines causing clogs due to the hydrophobic nature of the three compounds.

In liquid chromatography, the relative concentration of eluted sample is typically inversely proportional to the square root of column diameter (ID).¹⁴ Thus, we had expected to obtain the highest pre-concentration factors using trapping columns with a smaller radius. Nevertheless, after comparing the performance of several guard columns with IDs ranging from 0.3 to 2.0 mm, the best concentration enhancement results were surprisingly achieved with a column of 1.0 mm ID. This unexpected result was likely due the fact that the smallest ID columns were easily overloaded causing disruption in the solvent flow during the pre-concentration sequence.

On-Flow Analyses

After optimizing the instrument, we injected 100 μL of a mixture containing ibuprofen, naproxen, and phenylbutazone, each at a concentration of 1mM. The separation time was 32 min, and elution times between the peaks were sufficient such that no sample appeared in Detector 1 before the previous analyte was pre-concentrated and sent to the NMR probe. The time needed to elute samples from the trapping column to the NMR was approximately 4 min for phenylbutazone, 4.5 min for ibuprofen, and 5.5 min for naproxen due to their polarity differences, with naproxen being least, and phenylbutazone most hydrophobic of the three compounds. The total time for the separation, pre-concentration, and on-flow NMR-acquisition was 43 minutes. Arrayed NMR acquisitions were manually activated as the samples reached their peak maxima in Detector 2. A sample of ^1H stacked NMR spectra of ibuprofen is presented in Figure 3.

The major issue observed with on-flow NMR acquisition is significant peak broadening which results in a poor signal to noise ratio (S/N) and resolution. These problems were caused by the use of different solvent compositions in the pre-concentration sequence. Since the sample loading and back-flushing were performed with 90% $\text{D}_2\text{O}/10\% \text{d-ACN}$ and 10% $\text{D}_2\text{O}/90\% \text{d-ACN}$, respectively, interfacial mixing occurred as the sample entered the NMR observe volume. This mixing resulted in a shift of the water peak from 4.6 to 3.2 ppm as the percentage of deuterated water shifted from 90% to 10% (Figure 3). As a result of solvent mixing in the micro-probe, the obtained NMR spectra deteriorated due to the differences in magnetic susceptibility of the two solvents.^{39,40} Another possible reason for the NMR spectral deterioration was gas cavitation formation caused by the exchange of solvents under pressure in the pre-concentration step. However, the poor quality of the NMR spectra could be eliminated by stopping the sample in the active NMR volume, and waiting until the solvents equilibrated (or the small gas bubble passed through the NMR capillary), which took approximately five min. Interestingly, we observed that as the sample was equilibrating in the NMR probe, the water peak shifted back to ~ 4.6 ppm.

Stop-Flow Analyses

We repeated the previous experiment using the same mixture of the anti-steroidal drugs, but this time using a stop-flow technique. Each NMR spectral acquisition consisted of 512 transients in order to obtain good S/N. Since NMR acquisition for each sample required almost 65 minutes (5 min to equilibrate the sample in the NMR volume after pre-concentration, and 60 min for the acquisition), ibuprofen and phenylbutazone were parked in separate storage loops as we obtained the NMR spectrum of naproxen. Afterward, ibuprofen and phenylbutazone were sequentially pre-concentrated and analyzed by NMR. In order to

determine the signal enhancement factors, we compared the S/N measurements of the NMR spectra obtained after pre-concentration, to those after direct injection to the NMR probe, and to those observed under optimized LC-NMR conditions without any pre-concentration. In the latter case, we used deuterated solvents with the composition and flow rate presented in Table 1. All NMR measurements were acquired under the same NMR conditions. The ^1H NMR spectra of the pre-concentrated analytes as well as of the directly injected samples and optimized LC-NMR are presented in Figure 4.

The major challenge we encountered in the stop-flow NMR acquisition was the linewidth deterioration that occurred during the signal averaging. This was due to the lack of the lock-channel in the NMR probe, and to the analyte diffusion in the observed volume over a longer time period as reported *et al.*⁴¹ by Webb. After the samples were positioned and equilibrated in the NMR observe volume, the linewidth of the water peak was ~ 1.8 Hz. However, after 512 transients, the linewidth extended to ~ 5.5 Hz. Nevertheless, this did not significantly affect our spectra because the chemical shifts of the sample peaks were far enough from the solvent peaks. Even though the sample and solvent peaks did not overlap, we did not use solvent presaturation since the intensity of the solvent peaks was not sufficiently high to warrant solvent peak suppression in these test experiments.

Pre-concentration Enhancement Measurements

Pre-concentration factors were computed by comparing the S/N measurements of pre-concentrated samples to the S/N values obtained from 1 mM directly injected samples and from optimized LC-NMR runs, respectively. The obtained signal enhancement results are presented in Table 2. In all cases, data from 512 averaged transients were used.

In order to investigate the correlation between signal enhancement and injected sample concentration, we ran solutions of different concentrations containing only ibuprofen as the analyte. The ibuprofen concentration ranged from 0.2 to 2.0 mM, and the injected volume was kept at 100 μL . The enhancement factors were determined by comparing S/N measurements of pre-concentrated to directly injected samples. Each time the pre-concentrated analyte was stopped and equilibrated in the NMR active volume, we performed shimming to maximize spectral resolution and accuracy of S/N measurements. The number of scans per each NMR acquisition was 512. The dependence of enhancement factor on the sample concentration is demonstrated in Figure 5.

It is observed in Figure 5 that the pre-concentration enrichment decreased as the concentration of ibuprofen increased. The highest enhancement of $10.4 (\pm 1.2)$ -fold was achieved at the analyte concentration of 0.2 mM. At this concentration, we also determined the signal improvement of $14.7 (\pm 2.2)$ -fold respect optimized LC without pre-concentration sequence. The comparison of the NMR spectra of 0.2 mM ibuprofen obtained in the three modes is shown in Figure 6.

The significant dependence of signal enhancement upon sample concentration is likely due to the fact that an increase in the sample amount resulted in the analyte permeating deeper inside the trapping column during the loading sequence due to the decrease of relative surface area of the column packing material per sample unit. As a result, the relative concentration of the back-flushed sample with respect to the concentration of the injected sample decreased, resulting in the reduced signal enhancement. Moreover, for the concentrations over 1.6 mM there is a significant sample loss through trapping column during the pre-concentration loading.

Unfortunately, we were unable to study ibuprofen concentrations below 0.2 mM as we could not clearly distinguish the strongest sample peak at its maxima in the NMR active volume on-flow. This was due to the spectral deterioration caused by the use of the solvent gradient, or

the temporary presence of air bubbles produced by cavitations. We originally tried to determine the time elapsed from the appearance of peak maxima in Detector 2 to the sample reaching its peak maxima in the NMR probe, and set a timer that would automatically switch Valve 3. However, the time the sample took from Detector 2 to the NMR micro-coil was not perfectly uniform even though the back-flushing flow rate was constantly kept at 10 $\mu\text{L}/\text{min}$. Thus, we decided to stop incoming samples in the NMR active volume manually. As a result, we could only process pre-concentrated samples that produced NMR signals strong enough to be visually distinguished from the background noise. In contrast, we did not encounter the same problem when performing NMR analyses of the samples coming directly from LC. As a result, we were able to accurately park in the NMR probe samples coming from LC column regardless of the injected concentration. For the future, we can improve our signal enhancement factors by employing a higher magnetic field where the spectral resolution and sensitivity are enhanced. Commercial micro-coil probes are available at 500 and 600 MHz, and we are currently working on a probe that better matches the pre-concentrated sample volume. These changes would enable us to analyze ibuprofen samples with concentrations considerably lower than 0.2 mM.

The higher signal enhancement factors observed in this study over previous work³¹ is mostly due to the employment of a micro-coil NMR probe with an active volume of $\sim 2 \mu\text{L}$. In comparison, Griffiths and Horton used a 4 mm flow probe with an active volume of $\sim 140 \mu\text{L}$. Moreover, the difference in the analytical and trapping columns, ID of the transfer lines, choice of solvents and analytes, and pre-concentration flow rates also likely contributed to our higher enhancement factors.

These results also demonstrate that column trapping is quite competitive with SPE as a signal enhancement technique in HPLC-NMR analyses. The pre-concentration step is faster (e.g. ~ 10 -fold faster than the SPE-NMR method recently reported by Xu et al.²⁹), simpler as it does not require column washing and sample drying, has almost 100% sample recovery, and generates signal enhancement factors that are comparable. In fact, the injection volume of 100 μL used in our studies was 20-fold smaller than the volume at which Xu and co-workers obtained signal increases of >30 -fold, and our signal enhancement was in fact superior to the results of the same authors when they used an injection volume of 500 μL . We anticipate that the use of high magnetic fields will allow further pre-concentration enhancements to be achieved.

CONCLUSION

Our recently developed online HPLC-NMR system is described that successfully separates, pre-concentrates, and analyzes anti-inflammatory drugs such as ibuprofen, naproxen, and phenylbutazone. By employing a commercially available guard column to focus the separated analytes, we obtained concentration enhancement factors above 14-fold. As a result, we were able to obtain NMR spectra of samples with concentrations down to 0.2 mM at 300 MHz with excellent S/N. Our results could be further improved by optimizing sample positioning in the NMR probe, and automation, which would allow us to analyze samples with concentrations below 0.2 mM. This would likely result in further increase in pre-concentration factor as our experiments demonstrated that signal enhancement was inversely proportional to the analyte concentration. Our future plans are focused in two directions: to apply our HPLC-NMR system to drug metabolites studies in urine; and to hyphenate the pre-concentration setting with multiplex micro-coil NMR previously developed in our laboratory.⁴² Preliminary metabolite studies of ibuprofen in human urine suggest that our system can successfully be applied to real biological fluids.

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Figure 1.

Simple schematic of the HPLC-NMR pre-concentration system. After the sample is eluted from the LC column, it is sent through a storage loop to the trapping column for pre-concentration. After solvent switching and back-flushing, the analyte is delivered to the NMR micro-coil probe. The inset represents schematic of the pre-concentration sequence during (A) analyte loading, and (B) sample *back-flushing* from the guard column to the NMR

Figure 2. Schematic drawing of the HPLC-NMR procedure: (a) before a sample appears in Detector 1, (b) during sample parking in the storage loop, (c) during analyte loading on to trapping column, and (d) throughout sample elution from the guard column to the NMR probe. The highlighted regions represent the location of the sample in the system, and the arrows denote direction of the solvent.

Figure 3.

On-flow, stacked 300MHz ^1H NMR spectra of pre-concentrated ibuprofen after injecting a 100 μL , 1mM solution of the analyte. During the acquisition, the solvent composition changed from 90% D_2O /10% d-ACN to 10% D_2O /90% d-ACN, flow rate was 10 $\mu\text{L}/\text{min}$, which caused significant changes in the solvent peak intensities and positions near 4 ppm. The number of transients per spectrum was 1, and the repetition delay and signal acquisition times were 6 and 1 s, respectively.

Figure 4.

^1H NMR spectra of (A) naproxen, (B) ibuprofen, and (C) phenylbutazone acquired after direct injection to the micro-coil probe, following LC without any signal enhancement, and after pre-concentration. Each spectrum was acquired using a Varian Inova spectrometer operating at 299.12 MHz ($nt=512$, $d1=6s$, $at=1s$). The injection volume in each run was 100 μL . The peaks at $\sim 3.2\text{ppm}$, $\sim 4.2\text{ppm}$, and $\sim 4.5\text{ppm}$ are water residue peaks, while the peak seen near 2.1ppm is due to residual acetonitrile.

Figure 5.
Change in pre-concentration factors as the concentration of ibuprofen varies.

Figure 6.

^1H NMR spectra of 0.2 mM ibuprofen (A) after direct sample injection into NMR probe, (B) following optimized LC, and (C) after pre-concentration. Injected volume in each run was 100 μL . The spectra were acquired on a Varian Inova spectrometer operating at 299.12 MHz ($n_t=512$, $d_1=6\text{s}$, $a_t=1\text{s}$). The spectral regions were narrowed in order to focus on the strongest ibuprofen peak. The minor variations in the chemical shift were likely due to slight changes in the pH values.

Chromatographic and pre-concentration conditions used in the HPLC-NMR system for separation and pre-concentration of ibuprofen, naproxen, and phenylbutazone.

Separation			Pre-concentration			
Column	Mobile Phase	Flow Rate (μL/min)	Column	Loading Solvent	Loading Flow Rate (μL/min)	Elution Solvent
Thermo Aquasil C18, 150×50mm	40% H ₂ O 60% ACN 0.1% H ₃ PO ₄	100	Thermo Aquasil C18, 50×1.0mm	90% D ₂ O 10% d-ACN	100	90% D ₂ O 10% d-ACN
						Elution Flow Rate (μL/min)
						10

Table 2

Summary of NMR S/N measurements obtained in three different modes: after directly injecting samples into the micro-coil probe, following optimized chromatographic separation without signal enhancement, and after samples were pre-concentrated through column trapping (N=4).

Analyte ($V_{inj} = 100 \mu\text{L}$)	S/N after direct inj.	S/N after optimized LC	S/N after pre-concentration	Enhancement respect direct inj.	Enhancement respect optimized LC
1.0mM ibuprofen	32.6 ± 1.9	25.8 ± 1.6	222.9 ± 5.0	6.8 ± 0.4	8.6 ± 0.6
1.0mM naproxen	15.5 ± 0.8	12.6 ± 0.7	70.2 ± 3.0	4.5 ± 0.3	5.6 ± 0.4
1.0mM phenylbutazone	50.7 ± 2.7	39.2 ± 2.9	407.8 ± 34.6	8.0 ± 0.8	10.4 ± 1.2
0.2mM ibuprofen	4.8 ± 0.5	3.7 ± 0.5	54.5 ± 3.2	11.4 ± 1.4	14.7 ± 2.2