Capillary Isotachophoresis/NMR: Extension to Trace Impurity Analysis and Improved Instrumental Coupling

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Building upon its promising initial performance, the on-line coupling of capillary isotachophoresis (cITP) to nuclear magnetic resonance (NMR) is extended to trace impurity analysis. By simultaneously concentrating and separating dilute charged species on the basis of their electrophoretic mobility, cITP greatly facilitates NMR structural elucidation. cITP/NMR appears particularly attractive for identifying trace charged synthetic and natural organic compounds obscured by large excesses of other components. A 9.4 μL injection of 200 μM (1.9 nmol) atenolol in a 1000-fold excess of sucrose (200 mM) is analyzed by cITP/NMR. A microcoil, the most mass sensitive NMR probe, serves as the detector as it provides optimal NMR observation of the capillary-scale separation. cITP successfully isolates the atenolol from the sucrose while concentrating it 200-fold to 40 mM before presentation to the 30 nL observe volume microcoil, thereby enabling rapid 1H NMR spectral acquisition of atenolol (experimental time of 10 s) without obstruction from sucrose. For this particular probe and sample, the stacking efficiency is near the theoretical limit as 67% of the sample occupies the 1 mm long microcoil during peak maximum. A multiple-coil probe with two serial 1 mm long microcoils arranged 1 cm apart has been developed to facilitate peak trapping and sample band positioning during cITP/NMR.

As exemplified in combinatorial bead synthesis,1–3 single-cell studies,4–9 and individual vesicle studies,10–12 classical measure-

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Published on Web 04/17/2002


2306 Analytical Chemistry, Vol. 74, No. 10, May 15, 2002

mment techniques must be refined to analyze extremely small sample quantities, a few microliters or less, with optimal performance. Both sensitivity and sampling become critical when small volumes of dilute solutions are analyzed. To obtain full structural characterization, nuclear magnetic resonance (NMR) spectroscopic data are often required. However, in terms of sensitivity, NMR trails the other primary methods of structural identification, mass spectrometry (MS) and infrared spectroscopy, by orders of magnitude, often precluding its application to mass-limited samples.

Thus, to further extend the utility of NMR to small analyte quantities, its sensitivity needs to be enhanced. As NMR sensitivity is proportional to the 1/4 power of the magnetic field strength (B₀)13 the chief strategy to improve NMR sensitivity has focused on increasing B₀. However, the challenge of producing high-field magnets with both excellent homogeneity and stability at reasonable cost has proven to be difficult. Consequently, efforts to enhance NMR sensitivity by less expensive means such as cryogenic probes14–16 and exotic polarization transfer techniques17–19 have been pursued.

One relatively simple, inexpensive method to improve NMR mass sensitivity (S_m), defined as the signal to noise ratio (S/N) per sample amount, involves using reduced-diameter coil probes.20 21


As predicted theoretically\textsuperscript{13,22} and verified experimentally\textsuperscript{22,23} the $S_m$ of an NMR coil is inversely proportional to its diameter for coil diameters above 100 $\mu$m. Moreover, for a given diameter, solenoidal coils possess a higher $S_m$ than other geometries that are used in NMR probes, such as Helmholtz coils.\textsuperscript{13} To fully capitalize on these principles, microcoils, solenoidal coils possessing a diameter of 1 mm or less, have been developed with observe volumes ($V_{obs}$) from 1 $\mu$L to 5 $\mu$L.\textsuperscript{22-26} Microcoil NMR probes have demonstrated 40-fold enhancement in $S_m$ compared to conventional 5 mm NMR probes (coil diameter, 6.7 mm; $V_{obs} = 220 \mu$L).\textsuperscript{23,24} By wrapping the microcoil directly onto fused silica capillary or polyimide tubing, the sample can be introduced by either direct injection\textsuperscript{27} or on-line coupling to microscale separation techniques. In the latter, analytes in complex matrices can be isolated before NMR analysis by microscale liquid chromatography (HPLC),\textsuperscript{28-32} capillary zone electrophoresis (CZE),\textsuperscript{29,30,33,34} and capillary electrophrochromatography (CEC).\textsuperscript{29,30,35}

Despite possessing high NMR mass sensitivity, microcoils have poorer concentration sensitivity ($S_c$), defined as the S/N per sample concentration, than conventional-scale NMR probes due to the large difference in probe observation volumes. In essence, a several-hundred-fold reduction in volume for the typical NMR probe to a 100 $\mu$L volume produces a probe with approximately 10-fold higher mass sensitivity and approximately 10-fold poorer concentration sensitivity. Moreover, manipulating and loading sample volumes of 1 $\mu$L or less are difficult. To overcome these limitations, microcoil $^1$H NMR was recently integrated on-line with capillary isotachophoresis (cITP),\textsuperscript{36} a powerful capillary electrophoresis (CE) sample stacking technique. Instead of enhancing probe performance, cITP increases the effective $S_c$ of microcoil NMR for dilute charged analytes through on-column sample concentration and alleviates the need to manipulate nanoliter volumes while using the highest mass sensitivity nanoliter-volume probes.


Similar to most other forms of CE, cITP separates charged species on the basis of their electrophoretic mobility ($\mu_e$).\textsuperscript{37-40} As with other types of electrophoresis, isotachophoresis works best at the capillary scale due to efficient Joule heat dissipation.\textsuperscript{41-43} The uniqueness of cITP arises from the insertion of the sample matrix behind a leading electrolyte (LE), which has a higher $\mu_e$ than the sample constituents, and ahead of a trailing electrolyte (TE), which has a lower $\mu_e$. cITP electrolyte systems are available for both cationic and anionic analyses. Upon electric field application, individual components of the sample matrix sort into bands whose relative positions depend on their $\mu_e$ values. Due to the placement of the sample between the LE and TE, analyte bands remain in contact after focusing and travel at constant velocity through the separation channel, adjusting their concentration in proportion to that of the LE. Judicious choice of the LE concentration can achieve sample stacking of dilute charged analytes by 2–3 orders of magnitude.

Consequently, by using cITP in conjunction with microcoil $^1$H NMR, low microliter volumes of dilute charged analytes are injected and concentrated to the nanoliter-volume regime before presentation to the NMR probe. In the initial demonstration, through 100-fold stacking, the on-line coupling of cITP to 30 $\mu$L microcoil probes enabled $^1$H NMR spectra for 4 nmol to 480 pmol of analyte to be acquired in less than 1 min of experimental time.\textsuperscript{36} Moreover, cITP/NMR can successfully separate charged analytes from a mixture while simultaneously concentrating them for microcoil detection. By discontinuing the cITP run to stop the sample band in the microcoil, signal averaging can be employed to improve the S/N or two-dimensional experiments can be performed for structure elucidation.

With these initial promising results as impetus, cITP/NMR has been further refined for trace measurements. Owing to its powerful sample concentration and purification capabilities, cITP has achieved tremendous success in minor component analysis with other detection methods.\textsuperscript{38,44-48} The unique combination of cITP with the structure-rich detection of NMR appears particularly attractive for identification of trace synthetic and natural organic compounds. As prerequisites for cITP trace impurity analysis, the minor component must be charged and must possess a different $\mu_e$ than the main component. The cITP separation will be most efficient when the main component is either charge neutral or the opposite charge of the trace impurity. To satisfy these conditions, the pH can be selectively tuned by choosing an
appropriate LE buffer. By simultaneously concentrating and separating the charged minor component from the main component, cITP/NMR can enable time-efficient structural determination of the minor component without interference from the high-intensity signals of the major component. Without separation and concentration, NMR analysis of a trace species obscured by a large excess of another component is challenging and may be impossible.

As a demonstration of trace impurity measurement, a 9.4 µL injection of 200 µM (1.9 nmol) β-blocker atenolol obscured by the presence of a 1000-fold excess of sucrose is analyzed by cITP/NMR. In addition, enhancements made to the original cITP/NMR instrumental arrangement facilitate operation. Adapting recently developed multiple-coil NMR technology,49 a probe possessing two microcoils in series on the same polyimide sleeve is utilized in this research. By allowing two different regions of the sample capillary to be observed during cITP, this novel NMR probe greatly improves both sample loading and peak trapping. Moreover, a syringe pump added to the outlet end of the cITP separation channel enables easy sample injection and positioning of focused analyte bands.

**EXPERIMENTAL SECTION**

**Chemicals.** All chemicals are used as purchased from the manufacturer without further purification. Glacial acetic acid, sodium acetate, and sucrose are obtained from Fisher Scientific (Fair Lawn, NJ). Atenolol and methyl green are acquired from Sigma Chemical Co. (St. Louis, M O). D2O (99.9%D) is obtained from Cambridge Isotope Laboratories (Andover, MA).

**cITP.** Since the previous cITP/NMR paper included abbreviated experimental details,56 a full description is provided here, with the differences emphasized. The cationic cITP system employed in this study is similar to the one used previously.56 The LE consists of 160 mM sodium acetate buffered to pH 5.30 with acetic acid in D2O, while the TE is composed of 10 mM acetic acid in D2O. The sample pH is equal to the pH meter reading plus 0.40 for D2O solutions.54 The sample consists of 200 µM β-blocker atenolol in the presence of 200 mM sucrose (1000-fold difference in concentration) dissolved in a 50%TE/D2O solution. Sharing a similar structural motif of one or more aromatic rings and a 3-amino-2-hydroxypropoxy side chain, β-blockers are widely employed as treatment for various cardiovascular diseases including cardiac arrhythmias, hypertension, and angina pectoris.55,56 Possessing a pKa of 9.54, atenolol, like most β-blockers, is cationic at physiological pH.55 The preparation of the sample in 5 mM acetic acid is necessary to ionize the atenolol, provide proper counterion continuity, and increase the conductivity to a sufficiently moderate level.

For this cITP/NMR experiment, the injection protocol consists of an initial TE plug, followed by sample, and then another TE plug. By injecting a TE plug between the LE and sample, the charged trace component, atenolol, is focused away from the uncharged major component, sucrose, in the injected sample band and closer to the boundary between the LE and first TE plug. Ideally, the charged trace component is concentrated and detected without any obstruction from the uncharged major component, which only moves by diffusion. The second TE plug serves to move the sample band toward the NMR microcoil.

All cITP runs are conducted in 200 µm inner diameter (i.d.)/360 µm outer diameter (o.d.) fused silica capillaries internally modified with a covalently bonded poly( vinyl alcohol) (PVA) coating.57 Arising from the charged surface of the bare fused silica walls, electroosmotic (EO) flow tends to degrade boundaries between focused cITP bands. The use of uncharged hydrophilic coatings such as PVA suppresses EO flow, thereby greatly facilitating cITP focusing.

**Microcoil NMR.** Depicted in Figure 1A, the dual-microcoil NMR probe sleeve used in this experiment is patterned after a single-microcoil probe.56 Microcoil probe construction is performed according to standard published procedure.21,26 However, in this case, the two microcoils are manually wrapped around a segment of 370 µm i.d./420 µm o.d. medical-grade polyimide tubing (MicroLumen, Tampa, FL). Each microcoil consists of 16 turns of 50 µm diameter round copper wire with polyurethane coating (63 µm diameter with coating) (California Fine Wire Co., Grover Beach, CA), resulting in a coil length of approximately 1 mm. As observed in Figure 1B, the two microcoils are positioned 1 cm apart on the polyimide tubing.

The polyimide tubing is affixed by epoxy to a printed circuit board specifically designed for two separate impedance matching networks. After the polyurethane coating is stripped from the coil leads, each microcoil is soldered onto its own individual circuit. To tune each coil to 500 MHz, a standard impedance matching network in a balanced tank configuration is employed. Interference between the different coils and their impedance matching circuits is a significant concern in multiple-coil probes.51,52 This leads to the deleterious observation of bleedthrough peaks from samples present in neighboring coils and, in severe cases, problems tuning the coils to the same frequency. In this probe, the two microcoils and their impedance matching networks are so close and in such a geometry that their tunings affect each other.

However, for this application, only one coil is active at a time. Thus, coil interference is greatly diminished by having only the active coil tuned to the 1H Larmor frequency while the inactive one is off resonance. To accomplish this relatively quickly while the probe is situated in the NMR magnet, a high-capacitance bridge is added to each circuit that can be closed (opened) by manually inserting (removing) a plastic rod with a copper metal tip (see Figure 1A). Figure 1C illustrates a schematic of the impedance matching network used for tuning both microcoils.

A network analyzer (HP 8751A network analyzer, Hewlett-Packard, Palo Alto, CA) is used to measure electrical characteristics of the dual-microcoil probe. If the neighboring coil is

detuned, each circuit can achieve a reflectance below -40 dB (0.01% power loss), indicating excellent matching. When both coils are tuned to the same frequency, electrical bleedthrough from one circuit to the other reaches -20 dB (1% bleedthrough loss). By detuning one of the coils, electrical bleedthrough between circuits drops to -30 dB (0.1% bleedthrough loss).

To obtain high-resolution NMR spectra from microcoils constructed from copper wire, the surrounding environment must possess a magnetic susceptibility similar to that of copper. Consequently, the dual-microcoil probe is encased by a plastic bottle that is subsequently filled with M F -1 (Magnetic Resonance M icrosensors Corp., Savoy, IL), a perfluorinated organic liquid possessing a magnetic susceptibility within 3% of copper’s. Earlier microcoil CE/NMR probes consisted of a coil wrapped directly around a fused silica capillary. Hence, for this design, the microcoil probe lifetime is limited by that of the separation capillary. As first demonstrated in the previous cITP/NMR study, sleeve microcoil probes afford easy capillary exchange. However, there are several drawbacks. First, compared to a coil wrapped directly around a given sample capillary, a sleeve probe designed to hold the same sample capillary size possesses a slightly lower Sm due to the small increase in coil diameter. Thus, the predicted Sm for the 420 μm o.d. polyimide sleeve probe is 86% of that expected from a microcoil wrapped directly around a 360 μm o.d. fused silica capillary. Moreover, sleeve microcoils tend to attain lower NMR spectral resolution than standard microcoils. When housed in the NMR probe head, the sample capillary must bend around tight turns since the coil axis is orthogonal to the bore of the magnet. For standard microcoil probes, the sample capillary is glued permanently in place. Consequently, the capillary portion near the microcoil does not experience strain. However, in sleeve probes, the sample capillary is not fixed firmly in place. Furthermore, due to the flexible nature of the thin polyimide sheath, strain on the sample capillary can slightly bend the polyimide sleeve near the microcoil, resulting in significant NMR spectral degradation. Still, high-resolution NMR spectra of 1–2 Hz can be obtained with sleeve probes if proper care is taken to ensure that the sleeve is not distorted when the sample capillary is housed.

cITP/NMR. All cITP/NMR experiments are conducted on a Varian 500 MHz spectrometer with a wide-bore (89 mm diameter) magnet. The instrumental arrangement for coupling cITP on-line to microcoil NMR is depicted in Figure 2. The system is constructed in such a fashion that all cITP manipulations can be executed with the microcoil probe already situated inside the NMR magnet bore. The basic cITP instrumental arrangement is nearly identical to that of CZE. The outlet end of the cITP channel is situated in an LE buffer reservoir, and the inlet end is placed in a TE buffer vial. To conduct cITP, an electric field is applied across the two ends of the channel. For these cITP experiments, a high-voltage power supply (Series 230, Bertan Associates, Hicksville, NY) delivers a potential of 20.0 kV. Platinum electrodes are used at the buffer reservoirs to avoid unwanted electrochemical reactions. Given the cationic cITP system, the electrode at the TE buffer vial is kept at positive potential while the electrode at the LE buffer reservoir is grounded. A digital multimeter monitors the current on the ground side.

For the coupling, a 95 cm long PVA-coated capillary is simply threaded through the microcoil sleeve. The closest microcoil is 86 cm from the inlet. With a 200 μm i.d. sample capillary inserted into the polyimide sleeve, each microcoil possesses a Vobs of approximately 30 nL. The sample capillary is then connected to the outlet LE buffer reservoir by the following Teflon tubing segments: 4 cm of 304 μm i.d./762 μm o.d. tubing, 73 cm of 813 μm i.d./1422 μm o.d. tubing, and 11 cm of 1321 μm i.d./1930 μm o.d. tubing. Tubing connections are sealed with Teflon heat shrink (Small Parts, Inc., Miami Lakes, FL). The relatively large cross-
sectional areas of the Teflon tubes ensure that the majority of the electric field is dropped across the separation capillary. Prior to use, the separation channel is filled with 0.1% Triton X-100 detergent (Fisher Scientific) for 30 min to suppress EO flow in the Teflon tubing during cITP and then flushed with several column volumes of LE.

While the inlet TE buffer reservoir consists of a simple plastic vial open to the atmosphere, the outlet LE buffer reservoir is comprised of a three-way plastic junction with a platinum wire at one end for grounding. Since currents are typically low in cITP and the volume of the outlet LE buffer reservoir is relatively large, electrolytic gas formation at the ground connection does not interfere with the cITP run. A three-way plastic port with a manual valve is connected in sequence after the outlet LE buffer reservoir. Loaded with LE, a plastic syringe attached to one end of the three-way port can be used for easy flushing of the cITP separation channel. When cITP is conducted, the syringe is closed to the capillary system.

The most important alteration to the cITP/NMR instrumental arrangement concerns the addition of a syringe pump (PhD 2000, Harvard Apparatus, Holliston, MA), which can accurately deliver/load target volumes and flow rates in both infuse and refill modes. In the previous incarnation, sample injection and positioning of focused analyte bands had to be achieved through hydrodynamic flow. With the syringe pump, these operations can be performed much more easily. Moreover, the cITP channel in the new arrangement is hydrodynamically closed, thereby eliminating hydrodynamic flow and reducing EO flow, both of which tend to degrade cITP focusing. The syringe pump is attached to the outlet end of the three-way port by 5 m of 304 μm i.d./762 μm o.d. Teflon tubing filled with LE. The long length of Teflon tubing is necessary to keep the syringe pump sufficiently far away from the NMR magnet. For this experiment, the syringe pump manipulates a 1.0 mL glass syringe (Hamilton Co., Reno, NV) loaded with LE.

In the previous cITP/NMR arrangement, the ground point was after a valve used for discontinuing flow. The valve worked well for trapping focused sample peaks in the microcoil for stopped-flow NMR experiments. However, if the voltage generator was not turned off before the valve was thrown, the electrolyte circuit would arc and usually result in gas bubble formation in the Teflon tubing connecting the valve to the separation capillary. Once electrolyte fluid continuity is broken between electrodes, the cITP run cannot be continued. With the new cITP/NMR configuration, flow can be halted without experiencing such problems.

Prior to the cITP/NMR experiment, benchtop cITP runs are conducted to determine an appropriate injection protocol. For these benchtop cITP trials, in substitution of the colorless atenolol in injected sample plugs, the visible dye methyl green is used at the same concentration. All other reagents are kept the same. The cITP/NMR injection consists of sequential 9.4 μL aliquots (30 cm band length) of TE, sample, and TE. Hence, only 1.9 nmol of charged analyte is injected for cITP.

For the cITP/NMR experiment, only the active microcoil is tuned to the 1H Larmor frequency. Moreover, the shims are set to optimized values for the active coil. Aside from the brief delay to switch the coil observation, successive spectra are acquired every 10 s for the duration of the run. Standard 1H NMR data acquisition and processing parameters are used: spectral width (SW) 5000 Hz, number of data points (NP) 12800, acquisition time (AT) 1.28 s, relaxation delay (RD) 0 s, pulse width (PW) 55°, number of acquisitions (NA) 8, line broadening (LB) 2, no zero-fill (ZF).

Five Millimeter NMR. All 5 mm NMR experiments are conducted using a Varian Unity-Inova 500 MHz NMR spectrometer with a narrow-bore magnet. With standard 5 mm tubes, the NMR probe Vobs is 220 μL. However, to attain optimal spectral resolution, 600 μL of sample is required. Standard 1H NMR data acquisition and processing parameters are used: SW = 6000.2 Hz, NP = 15360, AT = 1.28 s, RD = 0 s, PW = 55°, NA = 8, LB = 0.3, no ZF.

Data Processing. NMR spectra are processed using either NUTS or Varian software.
RESULTS AND DISCUSSION

Improved cITP/NMR Coupling. NMR detection is enhanced using the dual serial microcoil probe. If only one region of the capillary is observed during cITP, both loading and trapping of focused sample bands in the coil are difficult and time-consuming. With the greatly reduced electroosmotic flow in the coated capillaries, focused analyte zones migrate very slowly, typically at a rate of less than 1 mm/min. While sample bands can be introduced more quickly into the detector by applying slight pressure, the focused cITP zones broaden with pressure-driven flow, resulting in lower analyte concentration. Moreover, for optimal detection, the migrating sample band must be stopped in the coil during peak maximum. However, with only one detector, optimal peak trapping is problematic. This particular challenge is not confined to cITP/NMR but is general to any on-line NMR detection of separations, including HPLC, CZE, and CEC. While the band can be moved backward if it is missed, reversing the flow direction by applying pressure substantially degrades cITP focusing.

The dual serial microcoil probe effectively addresses these problems. By situating two microcoils on the same sleeve, the first coil can serve as a scout for the second one. Thus, a migrating sample band can be observed at the first coil and then judiciously trapped at the second coil during peak maximum. Furthermore, to hasten sample loading, a slight precise flow rate can be applied with the syringe pump until the focused analyte zone reaches the scout microcoil, at which point the pressure-driven flow can be halted. Even though the sample band will be dispersed at the scout microcoil, by the time it reaches the second microcoil by electrophoretic flow it will be fully refocused. These advantages have been realized in both benchtop cITP runs of visible dyes and cITP/NMR experiments.59

With this serial arrangement, only one microcoil is active for an extended time period. Consequently, NMR signal isolation between the two microcoils is greatly enhanced by selectively detuning the inactive one. Spectra acquired from a microcoil with the neighboring coil tuned or detuned are shown in parts A and B, respectively, of Figure 3. Both spectra are obtained from the same microcoil loaded with the same 1% H2O/99% D2O sample with identical data acquisition and processing parameters. When the neighboring coil is tuned, a large bleedthrough peak is visible upfield of the expected sharp, native HOD resonance. This broad, out-of-phase peak arises from the HOD present in the neighboring coil. In contrast, when the neighboring coil is detuned, no bleedthrough peak is observed. Moreover, the S/N and peak area of the native HOD resonance are greater when the neighboring coil is detuned as bleedthrough to the other circuit is significantly decreased.

Five Millimeter NMR Analysis of the Trace Impurity Sample. The trace impurity test sample (200 μM atenolol in the presence of 200 mM sucrose in a 50%TE/D2O solution) is added to a 5 mm NMR tube for 500 MHz 1H NMR analysis with a conventional probe to illustrate the performance benefits of cITP/NMR. Figure 4 depicts the NMR spectrum of the sample obtained with data acquisition and processing parameters similar to those employed in cITP/NMR. As illustrated in Figure 4A, sucrose peaks dominate the NMR spectrum; no atenolol peaks are observed at this scale. The atenolol peaks are smaller in intensity than the sucrose 13C satellite peaks. The atenolol resonances can be observed only by raising the scale 500-fold, as in Figure 4B. Due to the concentration difference between the two components, the 5 mm NMR tube cannot be spun to improve NMR spectral resolution and increase S/N because spinning sidebands from sucrose would appear at an intensity similar to that of the atenolol peaks. Because of the limited dynamic range of the analog-to-digital converter, the NMR spectrometer receiver gain cannot be optimized for atenolol, leading to smaller S/N for the trace impurity peaks. Pulse sequences saturating the major component peaks can be conducted to alleviate this problem. However, any minor component peak overlapped with a major component peak will also be saturated in the process. At this large concentration difference, overlapping peaks cannot be resolved by NMR diffusion techniques unless the major component possesses a much greater diffusion coefficient than the trace species. Given these difficulties, NMR structural identification of dilute analytes obscured by large excesses of other components is challenging and often requires an off-line separation to concentrate and purify the analytes.

![Figure 3](image-url). Effect of detuning an inactive, neighboring coil on NMR signal isolation in a multiple-coil probe. 1H NMR spectra obtained from the same microcoil loaded with the same 1% H2O/99% D2O sample. Identical data acquisition and processing parameters for the two spectra (NA = 1, PW = 90°, LB = 0). Both spectra plotted to the same scale. (A) Neighboring coil tuned. Bleedthrough peak observed at 4.4 ppm. S/N of HOD peak at 4.8 ppm, 140. (B) Neighboring coil detuned. No bleedthrough peak observed. S/N of HOD peak at 4.8 ppm, 186.

the capillary is filled with LE, a voltage of 0.5 kV gives a current.

When the magnet is shimmed while the capillary is filled with LE. The atenolol band traverses the 1 mm long microcoil in 5 min and 40 s, yielding an approximate velocity of 0.44 mm/s. Once the focused atenolol zone migrates past the scout coil, NMR observation is switched to the second coil. Again, only concentrated atenolol peaks are observed in the NMR spectra. The atenolol S/N is the same at peak maximum for both coils. Interestingly, even though both coils possess identical lengths, the atenolol band traverses through the second microcoil in 9.5 min (estimated velocity of 0.26 mm/s). Because the atenolol band is concentrated to the same extent at both coils, the observed difference in migration times stems from a change in velocity, which is not anticipated. During the passage of the atenolol band through the two coils, the current falls from 3.5 to 3.4 μA. The change in current does not explain the observed difference in migration times. The change in velocity may be due to the electrolyte system not yet achieving steady-state conditions or transient EO and/or pressure flow. If a focused analyte zone is not entirely contained within the detector "V_{obs}" quantification of its initial concentration in the injected sample plug is problematic if the velocity varies during the cITP run. Such unexpected observations cannot be learned from single-coil NMR probes.

Figure 5A depicts an on-flow cITP/NMR spectrum acquired from the second microcoil at atenolol peak maximum. No sucrose peaks are observed in this spectrum. For comparison, Figure 5B illustrates a static NMR spectrum of 25 mM atenolol in D_2O obtained from the same microcoil with data acquisition and processing parameters identical to those of the cITP/NMR spectrum. The slight shifts in some of the resonances between the two NMR spectra are caused by a difference in pD (atenolol is cationic in Figure 5A and charge neutral in Figure 5B). By comparing S/N and peak areas in the two spectra, the atenolol concentration in the focused sample band is approximately 40 mM. Thus, in this particular run, cITP stacks the atenolol 200-fold. This corresponds to 67% of the injected 1.9 nmol of sample simultaneously occupying the NMR V_{obs} during peak maximum. In comparison to the same microcoil probe operating without the benefit of sample stacking, cITP/NMR reduces experimental time by a factor of 40000. As a vivid demonstration of the high S_m of microcoils, the atenolol S/N in Figure 5A is comparable to that in Figure 4 despite the 64-fold difference in injected sample volume (9.4 μL for cITP/NMR to 600 μL for 5 mm NMR). Through cITP, the effective S_c of microcoil NMR for dilute charged analytes now approaches that of 5 mm NMR probes.

Examining NMR spectral resolution in Figure 5A, B, the cITP/NMR peaks are broadened by 1 Hz compared to those in the static spectrum, an extent that cannot be solely attributed to electrophoretic current. The degradation in NMR spectral resolution probably arises from magnetic susceptibility differences between the focused sample band and the surrounding electrolyte buffers.
near the microcoil. Because additional contraction of the microcoil leads to other detection methods such as conductivity detectors, potential gradient detectors, ultraviolet/visible absorption diode arrays, and M.S. Larger diameter sample capillaries may benefit cITP/NMR by further increasing S/N through greater V/obs.

To accurately quantify the amount of analyte initially present in the injected sample plug by NMR, a multiple serial coil probe possessing one coil with a V/obs large enough to fully contain the analyte band can be constructed and implemented. To confirm that the entire sample zone rests within the NMR coil, spatially selective excitation experiments can be conducted by orienting a gradient coil along the sample capillary direction. Moreover, by either installing another transmittance coil or altering one of the NMR circuits to enable double tuning of a coil, heteronuclear experiments can be performed to facilitate structural identification.

Enabling efficient peak trapping during on-flow experiments, multiple serial coil probes will benefit the on-line coupling of NMR to HPLC, CZE, and CEC as well as cITP. By properly adding diodes to NMR circuits, selective detuning to improve coil isolation in multiple-coil probes can be automatically controlled on a fast time scale. This technique is not limited to multiple-coil probes with a serial arrangement and will greatly enhance the performance of multiple parallel coil probes used for increasing sample throughput. Flexible fluidic arrangements and multiple-microcoil probes will expand the performance capabilities of on-flow NMR.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Michael E. Lacey and Dr. Raju Subramanian for helpful discussions, Dr. Roger A. Kautz and Professor Barry L. Karger (Northeastern University Department of Chemistry) for supplying PVA-coated capillaries and the impetus to study cITP/NMR, and Professor Andrew G. Webb (University of Illinois Department of Electrical and Computer Engineering) for use of his network analyzer and useful discussions. We greatly appreciate the valuable technical assistance of Dr. Paul F. Molitor and the Varian Oxford Instruments Center for Excellence in NMR (VOICE Lab) in the School of Chemical Sciences at the University of Illinois. We gratefully acknowledge financial support from the National Institutes of Health (Grant GM53030).

Received for review December 19, 2001. Accepted March 3, 2002.

AC015744P


