Article

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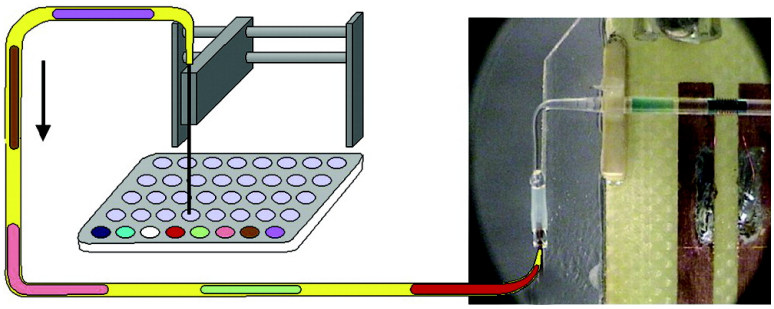

Articles

High-Throughput Microcoil NMR of Compound Libraries Using Zero-Dispersion Segmented Flow Analysis

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An automated system for loading samples into a microcoil NMR probe has been developed using segmented flow analysis. This approach enhanced 2-fold the throughput of the published direct injection and flow injection methods, improved sample utilization 3-fold, and was applicable to high-field NMR facilities with long transfer lines between the sample handler and NMR magnet. Sample volumes of 2 μL (10−30 mM, ~10 μg) were drawn from a 96-well microtiter plate by a sample handler, then pumped to a 0.5-μL microcoil NMR probe as a queue of closely spaced "plugs" separated by an immiscible fluorocarbon fluid. Individual sample plugs were detected by their NMR signal and automatically positioned for stopped-flow data acquisition. The sample in the NMR coil could be changed within 35 s by advancing the queue. The fluorocarbon liquid wetted the wall of the Teflon transfer line, preventing the DMSO samples from contacting the capillary wall and thus reducing sample losses to below 5% after passage through the 3-m transfer line. With a wash plug of solvent between samples, sample-to-sample carryover was <1%. Significantly, the samples did not disperse into the carrier liquid during loading or during acquisitions of several days for trace analysis. For automated high-throughput analysis using a 16-second acquisition time, spectra were recorded at a rate of 1.5 min/sample and total deuterated solvent consumption was <0.5 mL ($1) per 96-well plate.

Introduction

Nuclear magnetic resonance (NMR) analysis is regarded as one of the best means to confirm the identity of synthetic products and to establish purity quantitatively. However, performing routine NMR analyses on a library of 10 000 compounds can require months. Additionally, conventional high-throughput NMR methods using LC/NMR probes typically require 100 μg of analyte, and several milliliters of expensive deuterated solvent. Improvements over existing methods could have a significant impact on the number of samples analyzed per unit time and the cost per analysis.

Flow NMR methods are well-suited to library analysis, offering higher throughput than tube changing devices as well as the ability to draw samples directly from 96-well plates. A variety of high-throughput flow NMR methods have been developed (reviewed in ref 1) which share the common feature of positioning a sample handler outside of the NMR magnet's fringe field and connecting the handler to an NMR flow probe by a transfer line of capillary tubing. The methods differ in how they optimize the necessary steps of clearing, washing, and reloading the NMR probe flow cell through the 2−5-m transfer line while avoiding sample dilution in the dead volumes of the transfer line and NMR probe flow cell (e.g., 300 μL total volume for a 60-μL NMR observed volume of a 3-mm LC/NMR probe).

In direct injection NMR[2] (DI-NMR), samples are injected into an empty (air-filled) flow cell through a 100-μm-i.d. or larger transfer line. Samples can be injected relatively quickly without dilution; however, sample efficiency, defined as the percentage of the injected sample which ultimately resides within the NMR coil observed volume during spectral analysis, can be problematic (e.g., 60 μL of 300 μL injected, or 20%). The need for a wash cycle to reduce sample-to-sample carryover to <1% increases the sample change time. A fully optimized DI-NMR method has successfully demonstrated throughput of 2.5 min/sample using 30 μL of 30 mM analyte,[3] consuming ~1 mL of deuterated DMSO/sample ($150/plate).

In flow injection NMR,[4] the flow lines are maintained filled with solvent. Samples are introduced by means of a sample loop valve and delivered to the probe by a liquid chromatographic pump. Because the sample disperses into

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the carrier solvent during transfer, the final analyte concentration in the NMR coil depends on the sample volume, flow rate, and system dead volume. Sharp gradients of analyte concentration near the NMR coil immediately after injection can cause poor line shape, and an equilibration time of 1−2 min may be required for line shape to sharpen as the analyte diffuses throughout the flow cell. Because the effects of these gradients are more pronounced for dissimilar solvents, the same solvent must be used for both the carrier that sample preparation. On the basis of the injection, equilibration, and wash times reported,[4] FIA-NMR is capable of a throughput of 3.5 min/sample for 100-μg samples, consuming 2 mL/sample of deuterated carrier solvent when washing to reduce carryover to the 1% level.

Microcoil probes are a recent innovation that offer advantages for high-throughput library analysis.[5−7] The 10−50-fold higher mass sensitivity of these probes both reduces sample consumption and increases analysis speed, and their <5-μL volumes significantly reduce the solvent consumption cost. Higher sample concentrations are required (low- to mid-millimolar), but are typical of stock concentrations for compound libraries. A high-throughput FIA-NMR method ("microVAST") using a commercial microcoil probe with a microfluidic sample loader[5,8] has been introduced. This method has shown 4-min sample changes, consuming 10 μL of sample and 50 μL of deuterated solvent per sample. An interesting alternative approach of loading multiple microcoil flow cells in parallel has also been suggested.[9,10]

Because throughput limitations stem primarily from the long transfer lines, segmented flow analysis (SFA) is potentially a powerful approach for high-throughput NMR. In SFA, samples are moved serially past the detector as a queue of plugs in an immiscible carrier fluid.[11] Samples are changed by advancing the queue a short distance to the next sample, rather than loading each new sample through the entire distance from the sample handler to the NMR probe. In addition, the samples would not disperse into the immiscible carrier, so smaller sample volumes could be loaded without dilution, allowing more rapid NMR spectral acquisition times and further reducing consumption of both analyte and deuterated solvent. While the stability of segmented plugs in the 3-mm vertical flow cells of conventional saddle coil LC/NMR probes is problematic, several preliminary findings with segmented plugs in microcoil NMR probes are promising.

In pioneering work on the optimal sizes of microcoil probes, it was shown that samples sandwiched on both sides by the immiscible fluorocarbon fluid FC 43 could be much smaller than samples sandwiched between air bubbles without degradation of the NMR line shape: only twice the coil size instead of 7 times.[12] The utility of this fluorocarbon-bracketing was demonstrated in obtaining spectra from the 500-ng eluate of a single solid-phase synthesis bead.[13] Although it was noted that the high sample efficiency of fluorocarbon-bracketing could be advantageous to automated NMR, substantial challenges have remained in moving small concentrated samples (1−2 μL, 5−30 mM) through the long transfer lines without loss of sample to a film formed on the capillary wall or without fragmentation of the sample plugs.[9]

This paper demonstrates a robust segmented flow analysis approach to high-throughput automated NMR analysis using a microcoil probe. A queue of small sample plugs in an immiscible carrier fluid were pumped through the transfer line to the NMR probe, stopping flow as each sample plug was centered on the NMR coil. An automated method was implemented by modifying MicroVAST hardware, using the sample handler to form a segmented queue of samples drawn from 96-well plates. A throughput of 1.5 min/sample is shown, and adaptations of the method for trace analysis and for coupling with microseparations were also developed.

Experimental Section

Materials. Polyimide-clad fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ), and the fluoroalkyl silanes were purchased from United Chemical Technologies (Philadelphia, PA). Fluorocarbon FC 43 was from 3M Corp (St. Paul, MN). Teflon capillary and tubing were obtained from Cole-Parmer (Vernon Hills, IL); PEEK capillary, unions, in-line filters, and adapters were from Upchurch (Oak Harbor, WA). The 96-well PCR plates were obtained from Nunc (Rochester, NY). The compounds of the test library (uracil, reserpine, erythromycin, chlorpromazine, tolbutamide, indomethacin, haloperidol, 4-acetamidophenol, indapamide, prilocaine, phenylbutazone, and brucine) were from Sigma. Deuterated solvents and the reference standards tetramethylsilane (TMS) and trimethylsilylproprionate (TMSP) were purchased from Cambridge Isotope Labs (Andover, MA). All other solvents, buffer salts, and dyes were obtained from Fisher Scientific (Pittsburgh, PA) and were used without further purification.

Instrumentation. NMR spectra were acquired on a Varian (Palo Alto, CA) Inova spectrometer with an 11.7-T (500 MHz) actively shielded magnet and a flow NMR package consisting of a Gilson (Middleton, WI) model 215 sample handler and Varian VAST automation software. The Gilson 215 was fitted with a 100-μL syringe, and supplemental VAST automation programming (Tcl scripts) was written, as described below. A sample loader, model HTSL-1100, from Protasis Corp (Marlborough, MA) consisted of a sample loop valve, high-pressure pump, and microprocessor controller. It could either be triggered to deliver a specified volume and rate, or it could be controlled through an RS232 serial connection.

The microcoil NMR probe principally used in these studies was built in-house, as previously described.[14,15] Copper wire (7.5 turns of 50 μm wire) was wrapped on a glass flow cell with 660 μm i.d., 920 μm o.d. (see Figure 1A). The 1.1-mm coil enclosed an observed volume of 0.5 μL of the flow path lumen. The flow cell was ∼0.8 cm long, tapering over an additional 1.5 cm on each side to match the inner diameters of 75/360 and 100/360-μm i.d./o.d. fused-silica inlet and outlet capillaries. All but 5 cm of the 75-μm inlet capillary was replaced with 100-μm-i.d. Teflon tubing. The glass and silica elements of the flow path were coated with perfluorooctyl silane (PFOS), as described below. The circuit was singly tuned to a proton frequency of 500 MHz, and spectra were acquired unlocked.

Preliminary data were obtained using a commercial microcoil probe, the ^{1}H capLC microflow probe[5] manufac-

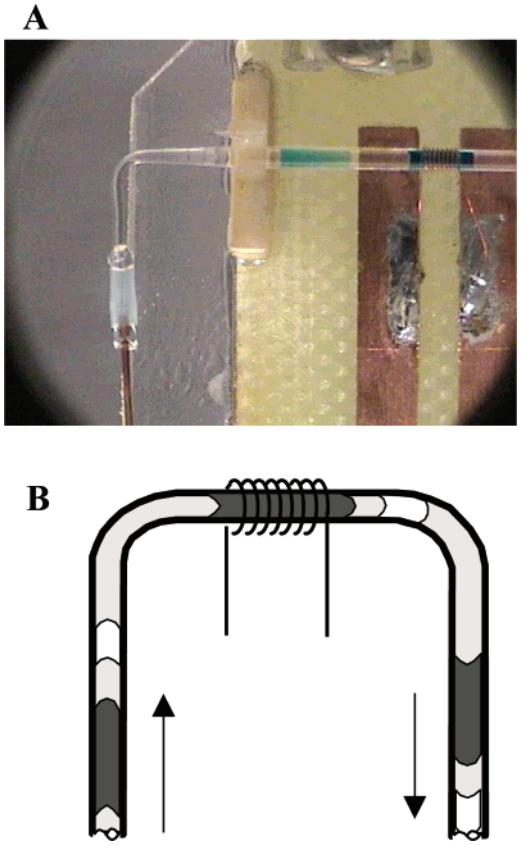

Figure 1. (A) Photograph of the microcoil probe used in these studies containing several sample plugs (DMSO-d_6) separated by a liquid fluorocarbon carrier FC 43. A black 1-μL sample plug is visible in the coil, a blue plug to left, and an undyed plug is in the bend. Plugs were loaded through 1 m of 50-μm-i.d. fused-silica capillary. Inlet capillary and flow cell were coated with perfluorooctyl silane. (B) Segmented flow analysis NMR. Samples (in DMSO, drawn in black) were loaded as a train of "plugs" separated and carried by an immiscible liquid (FC 43, drawn gray). Samples were changed by advancing the queue until the next sample plug was centered in the NMR coil. Wash plugs of solvent (white) were included to reduce carryover.

tured by Magnetic Resonance Microsensors (MRM, Savoy, IL) and distributed by its parent company, Protasis Corp. This probe had a 1.1-μL observed volume (V_{obs}) in a flow cell volume of ~3.5 μL, with 50-μm fused-silica inlet and outlet capillaries. When this probe was used, all connections in the sample loop and transfer lines were made using Upchurch PEEK unions.

The sample handler and loader were connected to the NMR probe as shown in Figure 2 and described in the Results and Discussion Section below. Both the needle line and sample loop were 70 cm of 200/400-μm i.d./o.d. Teflon capillary; the transfer line to the probe was 2 m of 150/400 Teflon capillary; the probe inlet consisted of 1 m of 100/400 Teflon with a residual 5 cm of 75-μm-i.d. fused silica at the connection to the flow cell. To connect the soft Teflon capillary to valves or unions, 2-cm-long pieces of PFOS silica (see below) were butt-jointed to the Teflon using shrink-wrap tubing. The open triangle indicates a bleed valve to facilitate flushing the sample handler path. The bleed valve connected to the sample handler syringe with 1 m of 500-μm-i.d. 1/16-in.-o.d. PEEK tubing, and all remaining connections were made using 250/360-μm i.d./o.d. fused silica. Before each use, the system was flushed with FC 43, freshly degassed under vacuum. Flow rates and system volumes were measured by displacement of a dye plug in a calibrated section of 30-gauge Teflon tubing (~300 μm i.d.) capturing the effluent from the probe outlet capillary.

PFOS Silica. Probes were internally coated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (perfluorooctylsilane, PFOS) monolayer. Surfaces were first activated by washing with 1 N NaOH for 1 h, followed by sequential rinses with water, acetone, and chloroform. A fresh 5% (v/v) solution of PFOS in chloroform was flushed slowly through the probe for 1 h, then the probe was rinsed sequentially with chloroform, acetone, and 10% D_2O in acetone. Surfaces were exhaustively dried with an acetone rinse followed by air flow overnight, then the probe was stored filled with FC 43.

Fused-silica capillaries were coated with a thicker PFOS gel layer using the trimethoxy form of PFOS according to the manufacturer's protocol (United Chemical Technologies). In brief, fused-silica capillaries were washed with peroxide/sulfuric acid, then activated with 1 N NaOH overnight. A solution was first prepared of 95% methanol and 5% water, with acetic acid added to an apparent pH meter reading of 5. Subsequently, 4% (v/v) tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trimethoxysilane was added with vigorous stirring and allowed to react for 1/2 h before introduction to the capillaries for 16 h at room temperature. Capillaries were annealed for 2 h at 80 °C, flushed with the methanol/water/acetate solution, blown dry with helium, then cured and dried at 110 °C with helium flow. The capillaries were stored filled with fluorocarbon FC 43 until use.

Automation. Automation was controlled using Varian VAST automation programming on the spectrometer host computer (Sparc Ultra 5, Solaris 8, vnmr 6.1C NMR software). NMR acquisition setup macros were written to (1) automatically detect and position an arriving sample and (2) set up a standard spectrum of a sample (16 scans, 1.05-s acquisition time, 16-Kb points). Four sample handler programs (Tcl scripts) were written to (1) form a train of four samples and hold it in the needle line, (2) draw a train from the needle line into the sample loop, (3) change samples by triggering the sample loader to run until stopped by the autodetection macro, and (4) initialize the sample queue by moving a sample train one-half of the distance from the sample loop to the NMR probe. The sample loader was controlled by means of Unix shell scripts, which could be called from either vnmr macros or tcl scripts.

Sample Preparation. The test library of 12 known pharmaceutical entities was prepared as 1-mL aliquots at 30 mM in DMSO-d_6 and stored at 4 °C. For carryover measurements, standard samples were 4% chloroform-*h* in DMSO-d_6 (standard S1) and 2% acetone-h_6, 5% green food color in DMSO-d_6 (standard S2). Ninety-six-well plates were typically alternating columns of S1 and S2, with the third and sixth columns replaced by the 12-member model library. For automated NMR runs, all wells contained 3 μL unless otherwise indicated, and plates were covered with adhesive film. Sample plates were placed on 4-mm foam rubber pads on the sample handler racks, carefully positioned under the

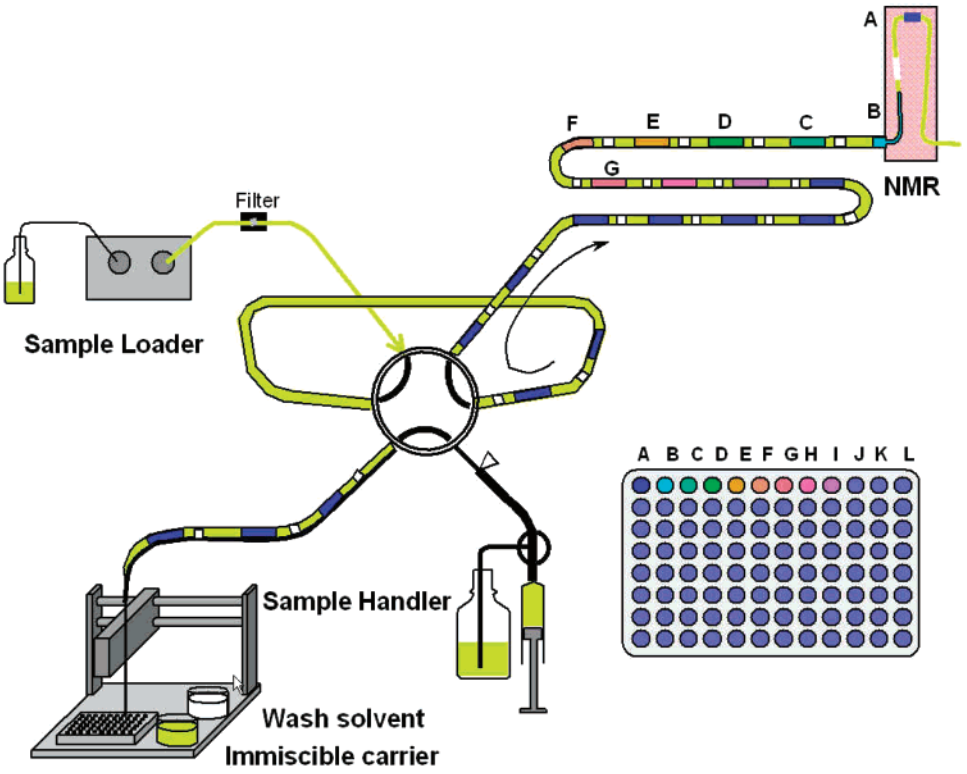

Figure 2. Apparatus for segmented flow analysis NMR. In the center is the sample loop valve of the Protasis HTSL-1100 sample loader. The illustrated "deliver" position placed the sample loop in line between the sample loader pump and the transfer line connected to the NMR probe. In the "fill" position, the sample handler syringe drew sample plugs into the loop via a 200-μm-i.d. capillary threaded through the sample handler needle. The sample plugs were formed by alternately drawing samples in DMSO, the immiscible fluorocarbon FC 43, and wash plugs of clean solvent. The transfer line to the probe was 3 m long (43 μL).

needle, and taped into position. Two additional vials on the sample handler rack supplied 0.5 mL of FC 43 and 0.1 mL of the wash/autodetection solution, 1% TMSP (60 mM) in DMSO-d_6.

NMR Spectroscopy. Sample spectra shown were 16 transients of 16-Kb points, 8000-Hz width, 45° tip angle, auto gain, with no additional relaxation beyond the 1.05-s acquisition time. The spectra were processed by zero-filling to 64-Kb points and Fourier transformed with 1 Hz of exponential line broadening. (Line widths are reported without line broadening.) Autodetection used single scan spectra with a 60° tip angle, fixed gain, and other parameters as above; the region from −0.5 to +0.5 ppm was monitored for a peak with S/N > 10 to detect the TMSP in the wash plugs. The COSY spectrum was a magnitude COSY, 128 increments of 16 transients processed with linear prediction in t1 to 512 points, apodized with sinebell-squared matched to acquisition time in both time domains. Total acquisition time was 10 min.

Results and Discussion

The goal of this work was to develop an automated segmented flow NMR method that could increase throughput and utilize low-mass samples efficiently and could accept samples in 96-well plates. In current methods, samples are often inefficiently utilized because of either the large dead volume of the transfer line and NMR flow cell in DI-NMR methods or the sample dilution with FIA-NMR methods. Throughput is limited by the time required to empty and rinse the entire flow path or for samples in the flow cell to be diluted to negligible levels if the flow cell is not emptied. Additional equilibration time after sample loading may be required if dilution during injection results in steep analyte concentration gradients near the NMR coil.

The target application here was high-throughput analysis of a "well-characterized compound" library. Products of parallel solution-phase synthesis on the 0.1-mmol scale were purified using preparative scale reversed-phase HPLC, with collection of a single fraction guided by on-line mass spectrometric detection.[16,17] The primary goal of SFA-NMR method development was to achieve the highest possible throughput yielding spectra of sufficient quality to be interpretable by automated spectral analysis software. Because the chemical library constituents are synthesized at the 30-mg scale, sample consumption at the 100-μg level typical for existing flow NMR methods was acceptable; however, gains in sensitivity and sample utilization were desirable to detect, and if possible to identify, contaminants at the 5% level. Because samples would be provided at similar concentrations, carryover of 1% would in most cases not be detectable and could be attributed if necessary to the previous sample analyzed. The high operating cost of deuterated solvent consumption should be reduced as much as possible, and the method should be implementable with commercially available instrumentation.

Zero Dispersion Segmented Flow Analysis. A segmented flow analysis approach to microcoil NMR analysis (SFA-NMR) is illustrated in Figure 1B, a sketch of a microcoil flow cell containing several sample plugs separated by an immiscible carrier fluid. One sample plug is centered on the

NMR detection coil at the moment flow would be stopped, and a wash plug of clean solvent precedes each sample. Because samples do not disperse into the immiscible carrier, the sample plug volume may be the minimum required to obtain high-resolution spectra (typically twice the NMR coil observed volume). In addition, samples are not diluted during transfer to the probe, so comparable NMR data quality may be obtained with shorter acquisition times than when using an FIA-NMR method. In the segmented flow approach to high-throughput NMR, the entire transfer line to the sample handler would be filled with a queue of many such sample plugs. The detection cell is cleared of the old sample, washed, and filled with new sample in rapid succession by advancing the queue through the distance of one sample-to-sample separation. Successful implementation of SFA-NMR requires that sample plugs be moved through several meters of transfer capillary between the sample loader and the NMR probe without the plugs becoming fragmented or analyte adsorbing onto capillary surfaces.

Zero dispersion segmented flow methods[11] have been demonstrated and are based on the principle that if the carrier fluid has a favorable contact energy with the tubing wall, relative to the sample, a film of carrier is maintained between the wall and the sample as sample plugs are moved.[11,18,19] The combination of a fluorocarbon carrier liquid in Teflon tubing was recently demonstrated in continuous flow PCR, a method that is particularly sensitive to carryover.[20] The fluorocarbon FC 43 is used in building microcoil probes to match the magnetic susceptibility of the copper wire of the coil[21] and has been shown to improve line shape when used to "bracket" small aqueous sample plugs in microcoil NMR.[12,22] FC 43 also has a relatively high viscosity (2.8 cs) among fluorocarbons, which favors film formation.[18]

Feasibility Studies and Method Development. Preliminary studies were made, observing the movement of DMSO sample plugs using FC 43 as the carrier fluid in capillaries of several different materials. In the Teflon capillary, FC 43 was the continuous phase: DMSO plugs did not contact the capillary wall and could be moved through several meters with no detectable carryover (<0.1%) or losses at all flow rates tested (0−20 μL/min). In plain fused-silica capillary, DMSO was the continuous phase, retention of a DMSO film depleted sample plugs by 2 μL for each meter of movement (in 200-μm capillary). In PFOS-coated silica capillary, neither phase was continuous: a DMSO film was not retained, so sample losses were negligible at modest flow rates (1−10 μL/min); however, carryover of minute droplets could occur (10−100 nL/m) if imperfections existed in the coating.

It was also found necessary to push the sample train through the NMR probe under positive pressure rather than to pull samples through the system with a syringe or peristaltic pump at the detector outlet, as in a traditional SFA system.[11] The flow rate of FC 43 through the microcoil probe with vacuum applied to the outlet capillary was <1 μL/min; changing samples in 30 s would require a flow rate on the order of 10 μL/min, which could be obtained with modest pressures of 150 psi. We therefore pursued a strategy of pulling sample and FC 43 plugs into a sample loop, then pushing the sample train through the transfer line and microcoil NMR probe by positive displacement. To facilitate adoption of the method, it was implemented by modifying a conventional microVAST installation.

The sample handler and loader were connected with the microcoil NMR probe using Teflon capillary, as shown in Figure 2. Sample plugs were loaded into the sample loop by drawing consecutive plugs of FC 43, wash solution, FC 43, and sample via a 200-μm Teflon capillary threaded through the sample handler needle. The transfer line to the NMR coil held two trains of four samples with a 7-μL gap between them. Samples were automatically positioned in the NMR coil by calibrating a delay between initial detection of their NMR signal (FC 43 has negligible ^{1}H or ^{2}H signal) and stopping the sample loader. The sample handler could operate independently of the NMR spectrometer and sample loader: during the time the sample loop was clearing slowly as four NMR spectra were being acquired from the train in the NMR probe, the sample handler was forming a new sample train from the next four wells of the microtiter plate. To avoid interrupting analysis, this new train was held in the needle line until the sample loop was cleared.

Once assembled, the system was calibrated, and flow rates, plug volumes, and automation timing were optimized. A flow rate of 7 μL/min did not overpressure the 200/400-μm i.d./o.d. Teflon capillary sample loop. Plugs of FC 43 as small as 0.3 μL were equally as effective as larger plugs in separating DMSO plugs through the probe, as measured by carryover. Plugs of FC 43 of 0.7 μL or larger provided several seconds with no NMR signal during on-flow NMR, which facilitated autodetection. In calibrating the positioning delay after automatic detection, it was found that variability in signal strength among samples caused variation in positioning, so 1% TMSP was added to the wash plugs to provide a consistent signal for detection. The variability of automatic sample plug positioning was $\pm$0.2 μL, due primarily to the 1-s intervals used for initial detection. The 2-μL sample plugs provided NMR line widths below 1.5 Hz without reshimming over a 0.5-μL window, and no equilibration time was required after stopping flow to observe good line shape.

Performance of the Automated SFA-NMR System. The performance of the system was evaluated by loading samples and acquiring spectra from 96-well plates with 3 μL/well of test library compounds (30 mM in DMSO-d_6) interspersed with standards for assessing carryover and line shape. Automated analysis completed in 2.5 h/plate, plus 24 min to initialize the queue in which four trains were drawn and two were injected. This initialization time is reported separately because it applied to the first plate but not to subsequent plates of continuous high-throughput operation. Spectra, as shown in Figure 3 and discussed below, were output at rates of 1/min along each train of four samples. The sample change and wash was completed in 35 s, NMR acquisition was set to 16 s, and the automation software required 10 s of execution and dead time. Sustainable throughput was 1.5 min/sample due to the time required to draw a new train from the needle line into the sample loop and to advance the queue through the gap between trains (105 s).

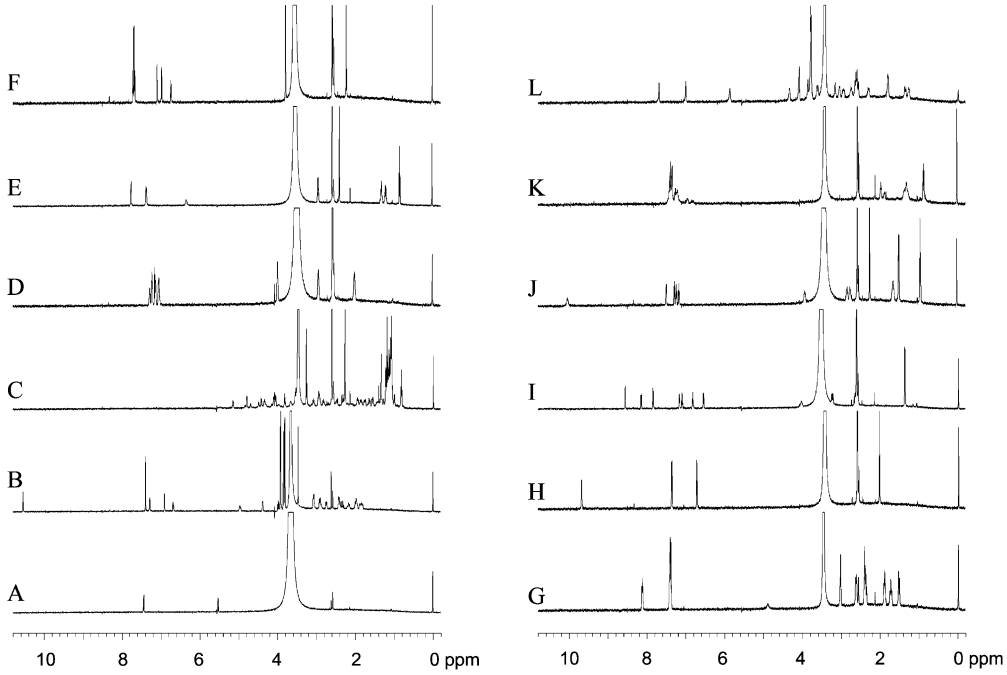

Figure 3. NMR spectra of a model compound library acquired using segmented flow analysis NMR. The average throughput was 1.5 min/spectrum, using 2 μL of 30 mM analyte. Each spectrum was the sum of 16 1-s transients. Compounds: uracil (A), reserpine (B), erythromycin (C), chlorpromazine (D), tolbutamide (E), indomethacin (F), haloperidol (G), 4-acetamidophenol (H), indapamide (I), prilocaine (J), phenylbutazone (K), and brucine (L).

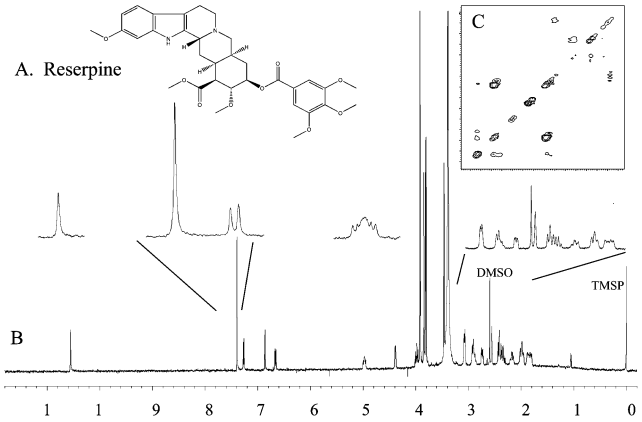

Figure 4. (A) Structure of reserpine. (B) NMR spectrum of reserpine, from Figure 3B, with details of indicated regions. (C) Region of COSY spectrum corresponding to inset below.

Spectra of the 12 test library compounds are presented in Figure 3, and one representative spectrum (reserpine, B) is shown in more detail in Figure 4. The sensitivity of all spectra was sufficient to unambiguously confirm their structures. The weakest signals of these 12 compounds were the multiplet at 5 ppm in reserpine (shown in the inset in Figure 4) and the 18-Hz-wide amide of tolbutamide at 6.2 ppm in Figure 3E, both with S/N = 15. The intensity of most signals were within the range of the aromatic signals shown in the inset of Figure 4, with S/N ratios of 130 and 23, respectively (the 2-proton singlet at 7.4 ppm and the weaker 1-proton doublet at 6.65 ppm). Thus, even with the moderate sensitivity of this home-built probe, it should be possible to identify compounds at 3-fold lower concentration (10 mM) or to detect impurities at the 5% level. For example, minor resonances visible in Figure 3K (phenylbutazone) at 6.8 ppm were 13% of the net aromatic intensity.

Compound identification may at times require advanced 2D spectra, or it may be desirable to analyze samples in protonated solvents requiring solvent suppression. Consequently, care was taken in programming the method so that existing macros for setting up established methods could be inserted, such as for gradient shimming, scout-scan solvent suppression, or 2D spectra. For example, a macro to acquire a magnitude COSY spectrum was added as a single line to one automation queue, and Figure 4C shows the region of the resulting spectrum that corresponds to the 1D inset below it. This flexibility was made a priority in order to enable 2D spectra to be acquired in a data-dependent manner, that is, to acquire a COSY or TOCSY spectrum if automated analysis of a 1D spectrum fails to confirm an expected product.

Importantly, the analyte in immiscible sample plugs was not found to disperse with time, so extended stopped flow acquisitions were possible without loss of signal strength due to dilution of the sample in the NMR coil. For example, in a 72-h acquisition of a trace sample (data not shown), the first 8-h block of data acquisition was identical to the last 8-h block. This stability also made it possible to interrupt long high-throughput analyses. One automation run of a 96-well plate was suspended in software, and the microcoil probe was removed from the magnet without disconnecting the transfer line. After using the spectrometer with a different probe for several hours, the microcoil probe was reinstalled, the automation queue was restarted, and analysis of the plate completed without any problems.

Sample carryover was below 1%, determined by comparing integrals of solvent peaks between alternating samples of 2% acetone and 4% chloroform, with one wash plug between them. A dye test showed that carryover to the wash plugs in the inlet capillary was in the range of 20−30 nL

(<1%), suggesting most of the 5% sample-to-wash plug carryover observed at the NMR coil occurred in the residual 75-μm fused-silica segment of the inlet. The line widths obtained when automatically positioning sample plugs, evaluated from single-scan spectra, were between 1.0 and 1.6 Hz, close to the routine line width obtained with this probe (1.2 Hz). Importantly, the cost of deuterated solvent consumption was negligible: 0.4 mL of DMSO-d_6/plate at $2/mL, including 100 μL of DMSO-d_6 supplied as wash solvent in addition to the 96 3-μL samples.

The efficiency of sample utilization of the SFA-NMR method of this paper was comparable to that attained using capillary isotachophoresis (cITP),[15,23] the most sensitive applied NMR method to date. Moreover, SFA-NMR can relatively quickly load analytes with unknown or zero electrophoretic mobility, which is important for de novo analysis of trace amounts of isolated natural products or drug metabolites. Similarly, SFA-NMR permits microseparations and microconcentration to be performed using optimal techniques and instrumentation if a 1-μL fraction can be collected for subsequent transfer to the NMR microcoil.

SFA-NMR, as demonstrated above, doubled the throughput, quadrupled the sample efficiency, and reduced deuterated solvent consumption over 20-fold as compared to the commercially supported high-throughput flow NMR methods. Nonetheless, a number of straightforward improvements may still be envisioned. For example, lengthening the transfer line to hold 3 trains without gaps would increase throughput by eliminating the longer sample change time between trains. A larger i.d. sample loop could double the rate of loading the sample loop and could hold more samples in longer trains. Using a 10-port sample loop valve to switch between two loops would eliminate the delay to draw new trains into the sample loop. These and other improvements would increase the throughput of SFA-NMR to over one 96-well plate/h. Using segmented flow to alternately load multiple flow cells[9,10] could additionally increase throughput, reaching essentially continuous NMR data acquisition.

Conclusions

A method for high-throughput NMR has been demonstrated using segmented flow analysis of 2-μL samples into a 0.5-μL microcoil probe. A semicontinuous train of sample plugs separated by plugs of an immiscible carrier fluid were queued in the transfer lines between a sample handler and the NMR probe. Samples were detected in the probe and automatically positioned. Wash plugs of clean solvent with a chemical shift reference standard between the sample plugs reduced sample-to-sample carryover to below 1%. Sample change times of 35 s were realized. Total deuterated solvent consumption was below 0.5 mL ($1)/plate, including the sample volumes, a significant reduction from conventional DI-NMR[2] (100 mL, $150) or microcoil FIA-NMR[8] (20 mL, $30). The immiscible plugs remained fixed and are, therefore, amenable to long acquisition times and facilitate sample recovery after analysis. The method may be implemented using the commercial (MRM) microcoil probe, which has three times the sensitivity of the home-built probe demonstrated above and has recently been made available with Teflon inlet capillaries. Immiscible plug injection provides a general means to efficiently transfer submicroliter samples over distances of several meters and may be applied to coupling offline microscale separation and concentration methods to smaller and more sensitive microcoil probes than are commercially available at this time.[12]

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References and Notes

(1) Keifer, P. A. *Curr. Opin. Chem. Biol.* **2003**, *7*, 388−394.
(2) Keifer, P. A.; Smallcombe, S. H.; Williams, E. H.; Salomon, K. E.; Mendez, G.; Belletire, J. L.; Moore, C. D. *J. Comb. Chem.* **2000**, *2*, 151−171.
(3) Wheeler, P. Presentation at SMASH Conference, Verona, Italy, 2003.
(4) Keifer, P. A. *Magn. Reson. Chem.* **2003**, *41*, 509−516.
(5) Olson, D. L.; Norcross, J. A.; O'Neil-Johnson, M.; Molitor, P. F.; Detlefsen, D. J.; Wilson, A. G.; Peck, T. L. *Anal. Chem.* **2004**, *76*, 2966−2974.
(6) Wolters, A. M.; Jayawickrama, D. A.; Sweedler, J. V. *Curr. Opin. Chem. Biol.* **2002**, *6*, 711−716.
(7) Eldridge, G. R.; Vervoort, H. C.; Lee, C. M.; Cremin, P. A.; Williams, C. T.; Hart, S. M.; Goering, M. G.; O'Neil-Johnson, M.; Zeng, L. *Anal. Chem.* **2002**, *74*, 3963−3971.
(8) Protasis 2003. Application note: HT-NMR with Sample Management http://www.microNMR.com/download/files/MicroVAST%20Application%20Note.pdf; mirrored at http://www.barnett.neu.edu/NMR.
(9) Macnaughtan, M. A.; Hou, T.; Xu, J.; Raftery, D. *Anal. Chem.* **2003**, *75*, 5116−5123.
(10) Wolters, A. M.; Jayawickrama, D. A.; Webb, A. G.; Sweedler, J. V. *Anal. Chem.* **2002**, *74*, 5550−5555.
(11) Patton, C. J.; Wade, A. P. In *Analytical Instrumentation Handbook*, 2nd ed. rev.; Ewing, G. W., Ed.; Marcel Dekker: New York, 1997; pp 153−155.
(12) Behnia, B.; Webb, A. G. *Anal. Chem.* **1998**, *70*, 5326−5331.
(13) Lacey, M. E.; Sweedler, J. V.; Larive, C. K.; Pipe, A. J.; Farrant, R. D. *J. Magn. Reson* **2001**, *153*, 215−222.
(14) Olson, D. L.; Lacey, M. E.; Webb, A. G.; Sweedler, J. V. *Anal. Chem.* **1999**, *71*, 3070−3076.
(15) Kautz, R. A.; Lacey, M. E.; Wolters, A. M.; Foret, F.; Webb, A. G.; Karger, B. L.; Sweedler, J. V. *J. Am. Chem. Soc.* **2001**, *123*, 3159−3160.
(16) Cai, H.; Kiplinger, J. P.; Goetzinger, W. K.; Cole, R. O.; Laws, K. A.; Foster, M.; Schrock, A. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 544−554.
(17) Baldino, C. M.; Casebier, D. S.; Caserta., J.; Slobodkin, G.; Tu, C.; Coffen, D. L. *Synlett* **1997**, 488.
(18) Nord, L.; Karlberg, B. *Anal. Chim. Acta* **1984**, *164*, 233−249.
(19) Adler, H.; Burns, D. A.; Snyder, L. R. In *Advances in Automated Analysis, Technicon International Congress*; Mediad Inc.: Tarrytown, NY, 1973; Vol. 9, pp 81−85.
(20) Curcio, M.; Roeraade, J. *Anal. Chem.* **2003**, *75*, 1−7.
(21) Olson, D. L.; Peck, T. L.; Webb, A. G.; Magin, R. L.; Sweedler, J. V. *Science* **1995**, *270*, 1967−1970.
(22) Webb, A. G.; Grant, S. C. *J. Magn. Reson., Ser. B* **1996**, *113*, 83−87.
(23) Wolters, A. M.; Jayawickrama, D. A.; Larive, C. K.; Sweedler, J. V. *Anal. Chem.* **2002**, *74*, 2306−2313.

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