



Trip Report for
“SMASH Small Molecule NMR Conference”
Burlington, Vermont
September 10-13, 2006

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Abstract: *The SMASH Small Molecule NMR Conference in Burlington, Vermont, on September 10-13th, 2006, combined world class industrial and academic leaders in the field of small molecule NMR with an attendance of about 300 people. Presentations covered a wide range of topics in the field of small molecule NMR.*

Bruker Biospin Meeting (Pre-conference meeting).

A number of lectures were presented by Bruker personnel from Canada, France, Germany and the USA summarizing the advances in high field and Ultra high field magnets as well as advancements made in NMR experiments. Notable points were that the 300 MHz Ultrahigh shield plus magnet is of the same height as unshielded 300 MHz instrument. The 950 MHz ultrasheilded magnet occupies a space of less than 600 square feet and fits in a standard two story building. This translates into a 14 fold reduction in the 5 gauss line compared to a standard 950 MHz instrument. Brukers new cryoprobe covers the temperature range 0-135 °C and the performance for ¹³C, ¹⁹F and ³¹P is enhanced by 4 times over conventional probes. An update was also given on tritium cryoprobe, which will be ideal for use in studying biochemical reactions (using tritium markers). The dual carbon proton (DCH) cryoprobe is available for 400-700 MHz instruments which gives 4 times enhancement of the carbon signal and is good for INADEQUATE experiment.

This year Bruker has introduced Topspin 2.0 which includes new automated sample jet sample changer with high throughput capabilities. It can handle 96 well equivalent of NMR tubes. Topspin 2.0 has manual window adjustments same as XWINNMR. The important feature of Topspin 2.0 is that it can be used to search for coupling constants and electronic signature is also available by typing "esign" and answering the dialog boxes. It can also display the change history.

Quantitative NMR using the ERETIC™ Method

Olivier Assemat, Bruker NMR Application Laboratory, Wissembourg, France.

Dr Assemat described the Electronic Reference Signal (ERETIC™) procedure that can be used as an alternative to internal standards for quantitation by ¹H NMR. The procedure first appeared as a patent by L. Barantin, S. Akika and A. LePape in 1995 (CNRS Patent No. 9506651) and later in other publications. The method originally called ERETIC (Electronic REference To access In vivo Concentrations) provides a reference signal, synthesized by an electronic device, which can be used for the determination of absolute concentrations. The method uses a pseudo-FID similar to an NMR signal which is sent during acquisition. The method requires calibration of the electronic signal using a solution of known concentration:

$$[\text{ERETIC}] = (A_{\text{ERETIC}}/A_{\text{REF}}) * [\text{REF}]$$

Where [ERETIC] is the equivalent concentration of the ERETIC line determined after calibration, A_{ERETIC} is the area of the ERETIC peak, A_{REF} is the area of the calibration peak and [REF] is the concentration of the reference solution. The electronic signal is then used as reference in order to quantify unknown solution concentration:

$$[\text{COMP}] = (A_{\text{COMP}}/A_{\text{ERETIC}}) * [\text{ERETIC}]$$

Where [COMP] is the concentration of the analyte and A_{COMP} is the area of the peak to be quantified. The method requires a special license for automation but can be performed manually without the special license. It is especially useful for potentially hazardous samples and/or for those NMR's that are performed in sealed tubes for which addition of internal standard is not very convenient. Using the software it would take about 10 mins to do the quantitation compared to the internal standard method which would take about an hour (not including sample preparation time). Quantitation on multiple samples can be set up in automation.

Some recommendations for the choice of reference samples was that the reference sample should be as close as possible to the one being quantified and the concentration should be high so that calibration can be done with one scan and fast 90 degree calibration pulse can be used.

Main Conference Presentations.

The Early estimation of Circulating drug Metabolites in Humans

Andrew D Roberts, Drug Metabolism and Pharmacokinetics, GSK, Park Road, Ware, Hertfordshire, SG1120DP, UK.

In his presentation Professor Roberts summarized that it is important to know about human metabolism and how this information can be obtained and what consequences this information can have on drug development program. Professor Roberts elaborated that appreciating the human metabolism of potential drugs earlier in the development process can have important consequences in the overall assessment of a drugs safety and efficacy. This is important because many drugs on the market have been found to have active metabolites whilst some drugs have potentially

toxic metabolites. He described an approach using cryoprobe-NMR to determine the human systemic exposure to a drug and its metabolites using samples derived from Phase I clinical studies. They were able to gain a reliable estimate of the levels of metabolite relative to parent drug by making use of preparative high performance liquid chromatography together with cryoprobe-NMR technology. To generate sufficient material for NMR they pooled a large volume of plasma, post -PK analysis from highest administered dose and/or therapeutic dose, typically obtaining 30-70 mL pooled plasma. The dried LC fractions were reconstituted in 1:1 ACN:D₂O and ¹H NMR spectra acquired with 256 scans using a dual noesy presaturation with spoil gradients on a 5 mm inverse TXI cryoprobe. The total run time was about 2-2.5 days. Metabolites with concentration of a few hundred nanograms were detected. Examples of selected plasma spectra as well as method validation results were presented. Professor Roberts explained that the data from such a study can be used to assess the need to specifically measure or safety test metabolites, as well as aid in the design of the definitive human radiolabelled study (HRS). He also added that the method can offer an advantage and supplement the data from an HRS if the compound is likely to be cleaved and lose radiolabel from a substantial proportion of drug-related material. In addition, he mentioned that the method is much simpler to use on a repeat dose study than an HRS. One of the limitations of this method is that cryoprobe NMR is still a relatively low sensitivity detector in comparison to other analytical techniques such as LC/MS.

NMR as an Essential Early Discovery Tool for Drug Metabolism Studies

Gregory S. Walker, Pfizer, Pharmacokinetics, Dynamics and Metabolism, Ann Arbor, MI 48015, USA.

Professor Walker summarized that historically structural characterization of metabolites has relied principally on LC/MS/MS technology and on the availability of synthetic standards. While in some cases structurally informative MS/MS fragmentation has sufficed, in others more specific data has been required to assess metabolic sites of modification. In many situations, NMR provides complimentary data that can unambiguously assign structures of metabolites. Traditionally, NMR analysis has required large amounts of sample. However, recent advances in NMR hardware (cryoprobes, LC/MS/NMR and post column SPE) and software have dramatically reduced the time and the amount of material required for NMR structural information on low levels of metabolites. NMR and MS technologies have largely eliminated the need for early chemical synthesis of metabolites, saving resources. He explained by using selected examples of phase reactions such as epoxidation and methylation as well as analysis of urine, bile and plasma as performed for *in vivo* studies.

Recent Pulse Sequences for Heteronuclear Long-Range Correlation and More

Ole W. Sørensen, Institute of Chemistry, Denmark's technical University, 2880 Kgs. Lyngby, Denmark.

The presentation gave an overview of the recent advances in pulse programs for the determination of coupling networks in small molecules. The examples Professor Sørensen used were H2BC and HAT (Homonuclear *J* ATenuated) HMBC which impose COSY information on a heteronuclear long-range correlation spectrum. HAT HMBC is a hybrid of H2BC and HMBC aiming at establishing two-bond correlations absent in H2BC spectra because of vanishing ³J_{HH} coupling constants. Professor Sørensen described that the basic idea is to create an additional π phase difference in the multiplet structure in HMBC peaks with respect to the ⁿ⁺¹J_{HH} coupling constant between the proton(s) attached to a ¹³C and a ¹H separated by n bonds. Thus HMBC peaks associated with small J_{HH} will be the most attenuated in a HAT HMBC spectrum in comparison to a regular HMBC spectrum, i.e. peaks associated with ⁿ⁺¹J_{HH} and ⁿJ_{CH} will for $n > 2$ usually be strongly attenuated. In HMBC there is no distinction between $n=2$ and $n=3$ peaks and there are "missing peaks" because ²J_{CH} in some cases vanishes.

The HAT HMBC pulse sequence contains the same number of pulses as regular HMBC and are only a few milliseconds longer. Multiplicity editing is an inherent part of HAT HMBC and the HAT effect only applies for protonated carbons. He used mannose as an example to illustrate the use of HAT HMBC.

In H2BC predominantly $n=2$ peaks because $n=1$ J_{HH} is small or vanishes for $n+1 > 3$. The two-bond peaks "missing" in HMBC show up because ³J_{HH} does not necessarily vanish when ²J_{CH} does. Professor Sorensen used strychnine and cyclosporin A as examples to illustrate the difference and complementarity of HMBC and H2BC. He went further to explain using a 33-residue oligosaccharide. He also discussed the purpose of ¹³C multiplicity editing for determination of multiplicity (¹³C 1D DEPT/SEMUT), resolving overlap (eg HSQC, HMBC, H2BC), purging (eg of residual ¹J correlations in HMBC and HAT HMBC) and making new information visible (eg HAT HMBC).

Application of BIP Pulses to ¹⁹F NMR

Haitao Hu, Discovery Chemistry Research and Technologies, Eli Lilly and Company, Indianapolis, Indiana.

In this presentation the application of broadband inversion pulses (BIPs) to ¹⁹F-¹³C heteronuclear correlation experiments was summarized. Dr. Hu and co-workers took advantage of the fact that fluorine-19 with spin half and

100% natural abundance and a gyromagnetic ratio that is only slightly smaller than that of proton is one of the most sensitive nuclei in NMR. Despite its intrinsic high sensitivity, however, applications of ^{19}F NMR experiments to fluorochemicals are often hampered by the wide dispersion of its chemical shifts, which can extend over 200 ppm. Dr. Hu indicated that substantial improvement in sensitivity was achieved using the BIP pulses when compared to hard pulses. In addition the authors explored the possibility of using heteronuclear TOCSY as an editing/filtering tool, which may find widespread applications in metabolite profiling of fluorine-containing compounds. They take advantage of the fact that the ^1H NMR of a sample of metabolite with biological matrix would be too complex, in comparison the ^{19}F NMR would be very simple.

TINS: New Opportunities for Fragment Based Drug Discovery

Johan G. Hollander, Leiden Institute of Chemistry, Leiden University, Postbus 9502 2300-RA, Leiden, The Netherlands.

Target Immobilized NMR Screening (TINS), is a method that in principle, can be used to find ligands for a broad array of targets including insoluble, integral membrane protein targets obtained in limited quantities said Professor Hollander. He explained that in TINS, the target is immobilized on a solid support and the mixture of compounds to be tested for binding is pumped over the support and binding is detected by 1D ^1H NMR spectroscopy of ligands. The latest results using the instrument was presented, including initial attempts to apply TINS to bacterial membrane proteins and G-protein coupled receptors. The researchers claim that more than 2,000 compounds can be applied to a single sample of the target with no effect on ligand binding, thereby opening the way to screening an entire fragment library using a single sample of the target. Professor Hollander added that an 8 μm flow-injection, triple gradient probehead with a dual-cell sample holder has been developed to enable TINS for medium-throughput ligand screening. Using this hardware and an optimized fragment library, the researchers carried out screening in an automated manner using less than 5 mg of the target. TINS has been successfully applied to a growing number of soluble proteins and nucleic acids.

Latest Advances in Small Molecule Residual Dipolar Couplings (RDCs)

The researchers summarized that elucidation of the relative stereochemistry of asymmetric centers of organic molecules is an important challenge in chemistry since it requires the simultaneous determination of conformation and configuration. While the conventional NMR parameters like NOE and ^3J coupling constants, which provide internuclear distances and dihedral angles, yield the configuration of stereocenters in rigid compounds, this approach is difficult or impossible in cases where the molecule is flexible or the stereocenters are remote in the bonding network. Residual dipolar couplings (RDCs) have proven to be very efficient in the stereochemical assignment of moieties and hold the promise of defining the stereochemistry even in non-rigid molecules. According to the speakers, RDCs rely on the weak alignment of molecules in solution and provide angular as well as distance information that are not contained in NOE's or J couplings. RDCs dipolar coupling measured under partially oriented conditions in an NMR spectrometer, allow the relation of all bond vectors to a common alignment frame, thus facilitating three-dimensional structure elucidation.

The lectures on RDCs provided some history and theoretical background to RDCs as well as covered determination of conformation and configuration via NMR. Introduction to aligning media and alignment of organic solutes was also given. The examples presented in the lectures included assignment of all diastereotopic protons in strychnine and determination of the relative configuration of a five-membered ring compound, α -methylene- ψ -butyrolactone. They focused on the NMR restraints relevant for conformation and configuration determination such as angular dependence of ^3J (Karplus), distances out of the NOE, projection angle restraints from CCR of DQ and ZQ and distances as well as angles out of RDCs. RDCs are necessary for determination of the alignment tensor (A). The speakers explained that the properties desired of liquid crystal were low degree of molecular orientation, that is should dissolve any organic substance and should give homogeneous solution of low viscosity at room temperature. The presenters also described a new pulse sequence that significantly improves the measurement of RDCs of ^1H - ^{13}C pairs that are connected by one bond in liquid crystalline media like poly- γ -benzyl-L-glutamate (PBLG) via the removal of the ^1H - ^1H RDCs. The proof of principle was illustrated using strychnine as an example. The speakers mentioned that the concentration of alignment material is generally determined by trial and error to obtain isotropic solution. A brief overview of polymer gels as alignment media and sample preparation techniques was given in a workshop. According to one presenter it takes about a day to prepare the sample. If using polymer gels as aligning medium the choice of the polymer gel is very critical. Examples of various polymer/solvent combinations were also given.

Although RDC's have been successfully used for a number of compounds, at present it's application is very limited (there are less than ten research groups worldwide who use this technique). Knowledge of different aligning media and sample preparation techniques as well as an understanding of the software used for calculations is required before an increase in the application of RDCs will be seen.

The above summary was based on presentations:

“Introduction to the use of RDCs for the structure determination of organic molecules”

Christina M. Thiele, Technische Universität Darmstadt/Clemens-Schöpf Institut Petersenstr. 22, Darmstadt, 64287, Germany.

“Alignment of Media and Measurement of Anisotropic NMR-Parameters in Various Solvents”

Burkard Luy, Technische Universität München, Lehrstuhl Organische Chemie II, Lichtenbergstr. 4, 85747 Garching, Germany.

NMR in the Forensic Sciences

There were three presentations in this session. All the speakers stressed that the identification of trace amounts of known drugs as well as unknown substances played a key role in the work of forensic science. The law enforcement agencies typically use NMR for the analysis of drug evidence in support of enforcement and intelligence operations, to provide expert witness testimony in courts of law and provide technical support at crime scenes. Examples of some of the applications given were to differentiate different sources of the same substance by quantitative deuterium NMR to link or distinguish precursors of illicit synthetic drugs. It was also illustrated that deuterium NMR was utilized to differentiate drug precursors synthesized from natural products from those made by petrochemicals. At the US EPA's national enforcement investigations center researchers have used NMR to provide quantitative analysis of samples, assisted in analysis of unknowns and for secondary confirmation of results by comparison with other techniques. Researchers at the US Drug Enforcement Administration use NMR for accurate identification and quantitation of both controlled and non-controlled substances found in complex drug. For example, comparison of ^1H -NMR spectra were provided for several drugs such as methylenedioxymethamphetamine (MDMA) in comparison with methamphetamine as well as caffeine. Spectra were also provided for the heroin alkaloids and adulterated heroin with highlights of the resonances characteristic to substances commonly mixed with heroin such as procaine, thiamine, quinine and sugars (eg. mannitol, lactose, sucrose). Speakers also presented the use of NMR for quantitation and structure elucidation of trace amounts of illegal drugs. The three presentations demonstrated the value of NMR as an analytical method in the field of forensic science.

The above summary was from presentations:

“Quantitative Deuterium-NMR on Synthetic Drugs”

Thomas Schaefer, Bundeskriminalamt (BKA), Germany

“NMR Chemical Forensics at the EPA-National Enforcement Investigations Center”

Mathew Rees, US Environmental Protection Agency, USA.

“NMR in the Fight against Illicit Drugs”

Robert Thompson, US Drug Enforcement Administration, USA.

Posters

Water Suppression with 2D/2D Hindsight

Bao D. Nguyen, Xi Meng, Kevin J. Donovan and A. J. Shaka, Chemistry Department, University of California, Irvine, CA 92697-2025.

The authors presented a new water suppression technique Solvent-Optimized Double Gradient spectroscopy (SOGGY) which according to them gives extremely high suppression water resonance, flat baseline and excellent signal retention across the spectrum. The need to suppress the water resonance in the aqueous solution has been a focus of attention in the NMR community for many years. Whereas water concentration is about 10⁴-10⁵ times more than the millimolar concentration of the solute, the principle goal of water suppression is therefore to dramatically attenuate the enormous intensity of water resonance in order to be able to observe the solute resonances. Many water suppression techniques with pros and cons have been proposed and reviewed in the literature. The most effective approach to date to suppress the water is the destruction of water magnetization by pulse field gradient (PFG) in combination with the use of spin echo resulting in zero net rotation of solvent

resonance and a 180° inversion to all other resonances. The researchers explained that the SOGGY sequence is based on the simple scheme of DPFGE, G1-S-G1-G2-S-G2, where S is any pulse sequence, and G1 and G2 are the gradients. Gradient strength of G2 is different from G1 to avoid refocusing of previously dephased magnetization by G1. They employ the refocusing element $S = [\text{soft } 180^\circ(x) \text{ composite } 180^\circ(-x)]$, where a soft 180° pulse is applied at the water resonance frequency and a hard composite 180° pulse consists of a computerized optimization of 4- pulses: $81^\circ(x)81^\circ(-x)342^\circ(x)162^\circ(-x)$. According to the researchers this new computer-optimized composite 180° pulse provides a much better inversion bandwidth over the conventional 180° pulse which has a narrow resonance offset bandwidth (95% inversion of $\pm 0.2B_1$) and has a better B1 field compensation. The authors presented ¹H NMR spectra of 2mM sucrose in 90 % water obtained using three most popular water suppression techniques, 3-9-19, WATERGATE and PURGE, compared with SOGGY. In their study they found that SOGGY gave the best water suppression and also the best retention of the solute signals as measured by the anomeric doublets. All of experimental excitation profiles of NMR pulse sequences of 3-9-19, PURGE and SOGGY were collected on the VarianUnityPlus 500MHz spectrometer, equipped with triple resonances probe and Triax PFG, using a ‘Doped 2Hz’ sample, 0.1 mg/mL, 0.1% DSS, and 1% H₂O in D₂O, at 25°C.

¹H NMR Method for the Routine Spectroscopic Determination of Enantiomeric Purity of Active Pharmaceutical Ingredients Fenfluramine, Sertraline, and Paroxetine

Romila D. Charan, Jonathon S. Salsbury and Paul K. Isbester, Analytical Quality Services, Albany Molecular Research, Inc., Albany, NY 12212-5089, USA.

Enantiomeric purity of three active pharmaceutical ingredients (API's) were determined using NMR and the chiral solvating agent (CSA) 1,1-Bi-2-naphthyl (BINOL). The technique described is the first successful application of using CSA's for enantiomeric purity determination for fenfluramine HCl, sertraline HCl, and paroxetine HCl. We found the CSA technique described herein to provide comparable results to traditional chiral HPLC methods and other published procedures for evaluation of enantiomeric purity for these compounds. The procedure is commonly used in our laboratory as a complementary/alternative method to chiral HPLC or optical rotation measurements for routine determination of enantiomeric purity. Enantiomeric purity determination by ¹H NMR utilizing chiral solvating agents does not require special instrumental techniques, chemical derivatization, or standards and is therefore ideally suited for rapid analysis of routine samples such as in-process and release testing.

(The work presented was done by Jonathan S. Salsbury and Paul K. Isbester and published in *Magnetic Resonance in Chemistry*; **2005**; 43; 910-917.)