Quantitative 1H NMR Spectroscopy of Blood Plasma Metabolites

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Synopsis
The absolute quantification of blood plasma metabolites is complicated by the presence of broad resonances originating from serum macromolecules and lipoproteins [1-3]. A method for spectral simplification of proton NMR spectra of blood plasma is presented. Serum macromolecules and metabolites are completely separated by utilizing the large difference in translational diffusion coefficients with diffusion-sensitized proton NMR spectroscopy. The results are compared with those obtained with ultrafiltration, a traditional method for separating macromolecules and metabolites, and demonstrate an excellent correlation between the two methods. The general nature of diffusion-sensitized NMR spectroscopy allows application on a wide range of biological fluids.

Methods
All NMR experiments were performed on a Bruker Avance spectrometer (Bruker Instruments, Billerica, MA) operating at 500.13 MHz for ^{1}H and equipped with a 5 mm triple resonance probe incorporating triple-axis gradient coils. The maximum magnetic field gradient strengths were 500 mT/m for the X and Y directions and 700 mT/m for the Z direction. The NMR sequence is based on a standard stimulated-echo method with an echo-time TE of 8 ms and a repetition time TR = 10,000 ms. Diffusion-sensitization was achieved by placing balanced trapezoidal magnetic field gradients in the TE/2 periods. To achieve sufficient suppression of small metabolites with high diffusion coefficients, the diffusion-factor b was set to 10,200 s/mm^2, with δ = 3.5 ms and Δ = 75 ms. All experiments were performed at 298 K. In order to reduce the viscosity, 250 µl plasma was diluted with 250 µl concentration reference containing 5 mM formic acid in deuterium oxide. Ultrafiltration was performed using Nanosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI) with a 10 kDa molecular weight cutoff. Blood plasma (100 µL) was diluted with 400 µL concentration reference and centrifuged at 14,000 × g and 277 K for up to 3 hours.

Results
Fig. 1 shows a typical result of the application of diffusion NMR to separate the macromolecular baseline from the low molecular weight metabolites. Fig. 1A shows a stimulated echo ^{1}H spectrum acquired with a low diffusion-sensitivity (b = 4.1 s/mm^2) and therefore represents a sum of macromolecules and metabolites. Fig. 1B is acquired under the same experimental conditions, with the exception that the magnetic field gradient strengths have been increased to achieve a high diffusion-sensitivity (b = 10,200 s/mm^2). All low molecular weight metabolites have been suppressed > 100-fold, leaving only resonances from macromolecules. When the macromolecular baseline spectrum is scaled up by circa 20 %, to account for signal loss due to diffusion, and subtracted from the total spectrum (Fig. 1A), the difference spectrum contains only resonances from low molecular weight metabolites. Fig. 2 shows a comparison between the macromolecular baselines obtained by ultrafiltration and diffusion NMR. Both ^{1}H NMR spectra display the same number of macromolecular resonances at approximately the same relative intensities. Note that even after three wash cycles (of 2 hours each), the ultrafiltration method did not result in complete separation of all low molecular weight metabolites from the macromolecules as can be judged from the sharp resonances (arrows). Diffusion NMR is capable of suppressing the metabolite resonances > 100-fold in less than 5 minutes. The correlation of the absolute concentrations obtained with the two methods was excellent with a correlation coefficient of 0.9989 (slope = 0.978).

Discussion
There are several advantages of the presented diffusion NMR approach over more traditional physicochemical separation methods. Firstly, diffusion NMR does not require any significant sample preparation since the metabolite-macromolecule separation is performed during the NMR experiment. This can lead to considerable time savings (several hours versus minutes). Secondly, the minimal sample preparation required for diffusion NMR effectively eliminates contaminants that may be introduced during traditional plasma deproteinization methods, e.g. glycerol in the case of ultrafiltration. Thirdly, intact blood plasma samples allow the measurement of many important physicochemical interactions like compartmentation and exchange and allows the direct study of serum macromolecules and lipoproteins. A potential complication of the diffusion NMR approach is heterogeneity in the translational diffusion coefficients among the various macromolecular resonances. In order to investigate this potential complication, the absolute translational diffusion coefficients of eight macromolecular resonances were measured and averaged to 0.186 ± 0.025 × 10^{-4} mm^2/s with a range of 0.139 to 0.215 × 10^{-4} mm^2/s. With a diffusion b-value of 10,200 s/mm^2, the macromolecular resonances would be suppressed by 13 to 19 %, leading to correction factors of 1.15 to 1.24, in excellent agreement with the average scaling factor used on the plasma samples. The spread of macromolecular diffusion coefficients and associated scaling factors leads to a suppression of macromolecular resonances of > 96 % (A-B spectrum in Fig. 1), which is significantly better than obtained with many of the traditional preparation methods [3]. In contrast to the low macromolecular diffusion coefficients, the average metabolite diffusion coefficient was calculated to be 5.90 ± 1.15 × 10^{-4} mm^2/s with a range of 4.71 to 8.42 × 10^{-4} mm^2/s. Therefore at a b-value of 10,200 s/mm^2 all metabolite resonances are suppressed by > 100-fold.

Acknowledgements
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References
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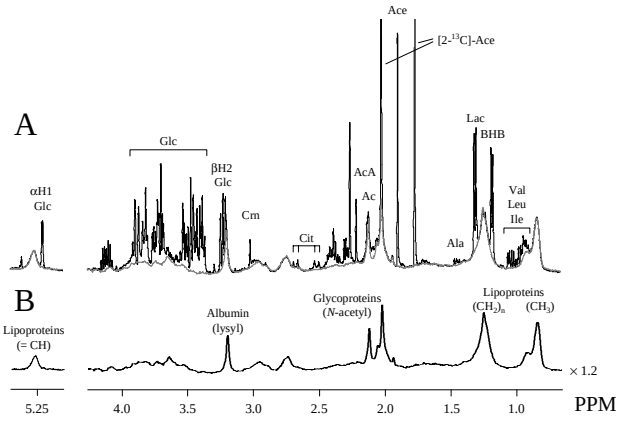

Figure 1: ^{1}H NMR spectra from blood plasma obtained with (A) low (b = 4.1 s/mm^2) and (B) high (b = 10,200 s/mm^2) diffusion-weighting.

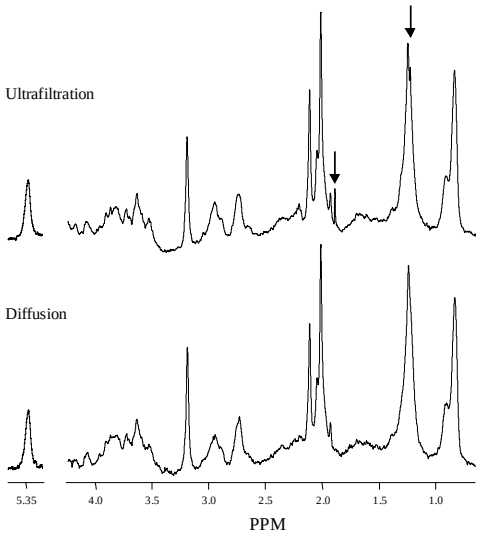

Figure 2: Comparison between the macromolecular baseline obtained by diffusion NMR and ultrafiltration.