Improving the Quantification of Amino Acids in Plasma/Serum by 1H-NMR Spectroscopy, Considering their Interaction with Human Serum Albumin

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INTRODUCTION

- Recent clinical trials have shown the important role that some amino acids (AAs) found in serum (phenylalanine, valine, isoleucine, leucine and tyrosine) could play in the early detection of diabetes type 2 and cardiovascular events[1,2]
- High-throughput 1H-NMR serum profiling is a low cost and non sample-destructive method widely used in metabolomics. However, quantitative analysis of these amino acids and other low molecular weight metabolites (LMWMs) in untreated samples is hampered by their interactions with proteins, especially Human Serum Albumin (HSA), making their signals partly invisible[3,4]
- 1D-CPMG pulse is extensively used for quantification of LMWMs because it provides spectral background reduction based on transversal relaxation (T2). Using this pulse in high-throughput 1H-NMR serum profiling assumes that T2 of LMWMs are similar across the samples. However, T2 are sample-dependent as they are modulated by LMWM-HSA interactions and could introduce further error in quantitative analysis by 1D-CPMG
- Two strategies are presented to improve the quantification of LMWMs considering LMWM-HSA interactions. The first is based on competitive binding (by adding exogenous compounds with high affinity to HSA) and the second is based on multivariate curve resolution (MCR) techniques to separate LMWMs signals from macromolecules background signals and correct by T2

NMR EXPERIMENTS

- <u>Experiment nº 1.</u> Standard additions of TSP to a "serum mimic" which consists of 0.6 mM of non-fatted HSA and the 20 most concentrated LMWMs in serum in normal human concentrations. The release of bound LMWMs was promoted by adding several concentrations of TSP (a well-known ligand to HSA) from 0.3 to 12 mM. It was evaluated by monitoring the relaxation time (T2) and the concentration of free LMWMs
- <u>Experiment nº 2.</u> Calibration curves of standard additions of five AAs (phenylalanine, valine, isoleucine, leucine and tyrosine) to real serum samples with and without TSP: evaluating the improvement in the quantification of the clinically-relevant AAs when using TSP in real samples from a healthy volunteer
- The above two experiments were measured by 2D-CPMGpresat (ranging from 0 to 2.5 s) at 310 K

DATA PROCESSING

- Multivariate curve resolution-alternating least squared (MCR-ALS) applied to 2D-CPMG was used for the separation of LMWMs signals from macromocules signals according to their different T2 relaxation times
- The T2 extracted is used to correct the attenuation of LMWMs signal due to T2 relaxation

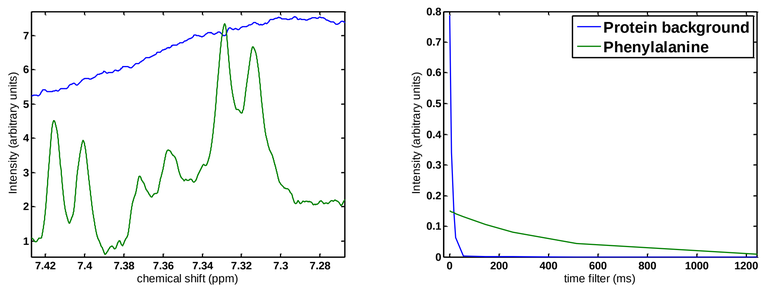

Figure 1. MCR decomposition of 2D-CPMG serum spectra at the aromatic signal of phenylalanine. Left: spectral components. Right: Decay contribution

RESULTS

<u>Experiment nº 1</u>

- Serum mimic experiment reveals different levels of slow-exchange interaction (non-measurable fraction tightly bound to HSA) for each LMWM:
  - Very large (>75%): tryptophan, citrate
  - Large-to-medium (75-10%): phenylalanine, lactate, 3hydroxybutyrate, tyrosine, leucine
  - Small-to-none (<10%): rest (valine, isoleucine, glucose…)
- Complete release of most of the LMWMs bound to HSA is nearly achieved after the addition of 5 mM of TSP
- Even after the complete release of these LMWMs is reached, their T2 (fast-exchange regime) are still increasing towards the reference T2 (LMWMs in aqueous solution)

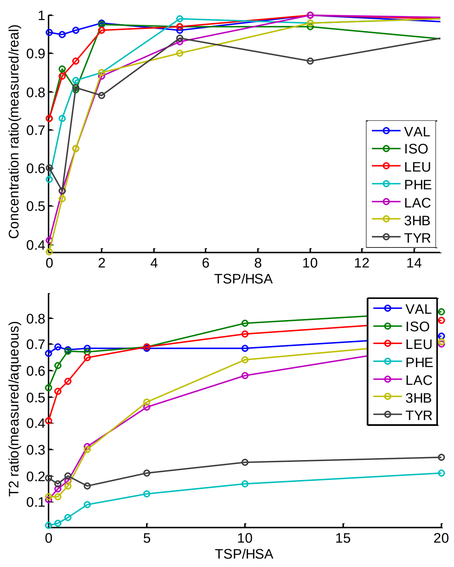

Figure 2. Top: ratio between LMWMs concentration calculated with MCR and real concentration in serum mimic as a function of the ratio TSP:HSA. Bottom: ratio between LMWMs measured T2 and their reference T2 as a function of the ratio TSP:HSA

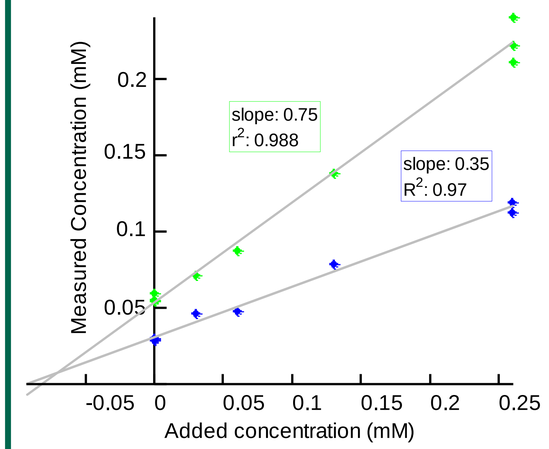

Figure 5. Calibration curves of phenylalanine in the serum sample with (green) and without (blue) 5 mM of TSP

<u>Experiment nº 2</u>

- In the calibration curves of the five AAs (phenylalanine, valine, isoleucine, leucine and tyrosine), the slope is proportional to the percentage of AAs in the free state
- Phenylalanine and leucine show significant improvement after adding 5 mM of TSP, but phenylalanine was not completely released despite the results obtained in the serum mimic. Tyrosine was not affected by TSP

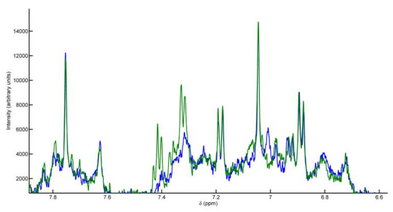

Figure 3. Aromatic region of a CPMG of 120 ms T2 filter for serum sample with (green) and without (blue) 5 mM of TSP

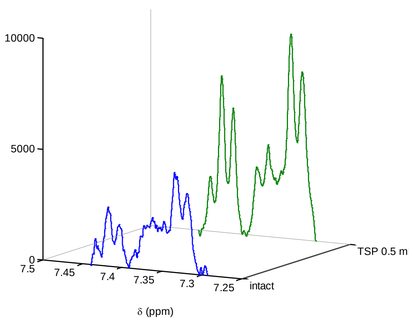

Figure 4. Phenylalanine signals obtained using MCR and 2D-CPMG with (green) and without (blue) 5 mM of TSP

	% free (untreated)	% free (+ 5 mM of TSP)	p-value
VAL	93	101	0.142
ISO	93	98	0.054
LEU	86	97	0.024*
TYR	58	58	0.89
PHE	35	75	4e-04*

Table 1. Comparison between the free fraction of 5 AAs before and after addition of 5 mM of TSP. P-value was evaluated between the repetitions at maximum addition point for each condition (asterisk: significant at p<0.05)

CONCLUSIONS

- The addition of TSP in serum samples reduces the interactions between HSA and LMWMs and improves the quantification of the evaluated AAs
- MCR was found a valuable technique to separate components in a quick an unsupervised way. For more accurate quantifications, the sample-dependent T2 correction is required and MCR provides a T2-corrected quantification
- Some LMWMs completely released in the mimic model after the addition of TSP were no longer completely released in real serum experiments. This result suggests further binding interactions with other macromolecules present in serum than human serum albumin

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