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Dedicated to Prof. Bogdan C. Simionescu on the occasion of his 75th anniversary

NMR PROVEN ALBUMIN INTERACTION WITH METABOLITES IN COMPLEX MIXTURES

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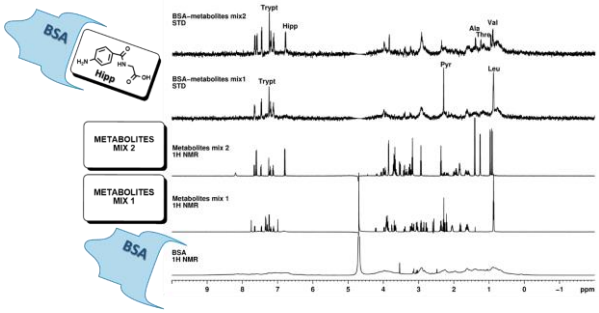
One critical step in new drugs development is the investigation of the interactions between drug candidate and target protein. Nuclear Magnetic Resonance Spectroscopy (NMR) is a well-established technique for studding these interactions.
Due to its availability and structural similarities to human albumin, bovine serum albumin (BSA) is widely accepted as a model for investigating the binding of small molecules to serum albumin. We report here on the evaluation of binding interactions between BSA and 18 metabolites using saturation transfer difference (STD) NMR experiments.
Positive STD signals that indicate metabolite-protein interactions were obtained for leucine, pyruvic acid, valine, threonine, alanine, 4-aminohippuric acid and tryptophan.



INTRODUCTION

The development of new drugs is a relentless preoccupation in medical community. The increase number of bacteria resistant to antibiotics and the advancements in personalized medicine are only two of the driving forces that keep the interest of scientific community in this expensive and time-consuming research direction.

A critical step in new drugs development is the investigation of interactions between drug candidates and target proteins. This molecular recognition process plays a crucial role in understanding the triggered biological processes. It is well known that the biological function of a protein depends on its interactions with other molecules such as ions, small organic compounds, peptides or other proteins.[1-3] Several techniques, including electrophoresis or enzyme-linked immunoadsorbent assay (ELISA),[4,5] spectroscopic,[6] and computational methods,[7,8] are currently used to investigate at atomic level these protein-small molecule interactions.

NMR spectroscopy is a well-established technique for structural studies of organic compounds, polymers or biomolecules such as proteins, peptides or nucleic acids. To date, due to extensive hardware and software

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developments as well as pioneering work of Nobel Prize winner Kurt Wüthrich,[9,10] NMR spectroscopy is the only technique able to study proteins structures in solution.[11] NMR spectroscopy is a versatile technique with hundreds of experiments available for both liquid and solid states, which makes it applicable for structural studies of drug candidates, measurements of binding affinities or high throughput screening of protein-ligand interactions.[12] NMR is also used to identify small metabolites present in biological fluids (such as blood, urine, cerebrospinal fluids and others) that are associated with changes in metabolic pathways caused by specific diseases or drug treatments.

The binding affinity of specific ligands to potential protein receptors can be followed through several NMR parameters. Chemical shift changes, variations in relaxation times or diffusion coefficients are several NMR parameters relatively easy to be obtained in practice and highly sensitive to protein-ligands interactions.[6] Modern NMR methods used for classical structural studies of proteins require site specific isotopic labeling (^{2}H, ^{13}C and ^{15}N), 3D or 4D multinuclear NMR spectra recorded on high magnetic fields instruments, using almost exclusively very sensitive cryogenic probes.[13] Nonetheless, the binding events can be followed through NMR without such extensive sample preparation or technical requirements, using ligand-based NMR methods, in which the resonances of small molecules (ligands, fragments) are observed.[12,14-16] The most common ligand-observed NMR experiments are saturation transfer difference (STD) and water-ligand observed via gradient spectroscopy (WaterLOGSY) that use transferred NOE effect to detect and characterize protein-ligand interactions. While STD experiment is based on magnetization transfer from protein to the bound small molecule, in WaterLOGSY the magnetization originates from water protons (H_2O+D_2O used as NMR solvent). The selectively inverted water magnetization is transferred through dipole-dipole interactions to both water and labile protons (from carboxyl, amino, hydroxyl, imidazole, guanidine or amide groups) from the target protein. From here, via cross-relaxation, the inverted magnetization is transferred to bounded small molecules. In WaterLOGSY experiment, the NMR signals of non-binding small molecules appear with opposite sign.[12,17]

Relaxation times (T_1 and T_2) can be also used to monitor these interactions, line-broadening being one of the first indicators for protein-ligand complexes.

We have been using NMR spectroscopy to study metabolites mixtures in complex biological fluids for diagnosing and monitoring metabolic disorders,[18-24] and lipoprotein profiling for cardiovascular conditions as well as pollutants and food induced effects.[25,26] We are currently expanding our interest and NMR approaches to investigate metabolite-protein binding interactions, namely to explore interactions between bovine serum albumin (BSA) and two mixtures of several metabolites.

RESULTS AND DISCUSSION

Bovine serum albumin NMR characterization. Albumin is a single-chain blood protein with approximate 580 amino acids (66.5 kDa) that is involved in binding, transport and delivery of fatty acids, bilirubin, steroids or metallodrugs.[27,28] In this study we used bovine serum albumin (BSA) which, due to its structural similarities to human albumin and availability, is widely accepted as a model for investigating the binding of metabolites and drug candidates to serum albumin.[29]

Before starting the protein-ligand binding experiments, we performed a rapid NMR test to assess BSA purity. The proton NMR spectrum recorded for a BSA aqueous solution is presented in Fig. 1a. As we used a native BSA sample (not isotopically labelled), the proton resonances are very broad and severely overlapped. No precise signals assignments can be made on such proton NMR profile, however several spin systems were identified based on homonuclear couplings obtained in H,H TOCSY spectrum (Fig. 1b). Thus, the following amino acids were recognized: valine/ leucine, alanine/threonine in the aliphatic region 0.5–4.5 ppm and phenylalanine/ tyrosine in the aromatic region 6.5–7.5 ppm. The presence of aspartate was indicated by the AMX spin system from 2.50–2.60 ppm and 4.20 ppm, visible in the TOCSY spectrum. The low intensity aromatic signals from 7.5–8.5 ppm could be assigned to histidine protons. No signals from possible contaminants/preservatives were observed in the proton spectrum, therefore the BSA sample was used without further purification for the binding experiments.

Collecting reference NMR spectra for metabolites mixtures. In this study, we used two aqueous mixtures of the following compounds (**1-18**, Table 1): tryptophan, phenylalanine, glutamine, histidine, leucine, valine, arginine, proline, threonine, alanine, serine, asparagine, pyruvic acid, 4-aminobutyric acid, malic acid, 4-aminohippuric acid, ascorbic acid, 2-oxoglutaric acid. Some of these compounds, like valine, phenylalanine, histidine or leucine, are essential amino acids and are found in human biological fluids such as urine and blood. For example, increased levels of blood and urine phenylalanine is a well-known

marker for phenylketonuria (PKU), a rare inherited disorder which, if left untreated, induces severe brain damage. Other compounds, such as the pyruvic, 4-aminobutyric, malic, 4-aminohippuric, 2-oxoglutaric or ascorbic acids are metabolites found in urine under normal and pathological conditions or as drug biomarkers.

Prior to protein-metabolite interactions experiments, we recorded control proton NMR spectra for the two aqueous metabolite mixtures. The solutions were prepared at 400 μM metabolites concentrations, similar to those used for the interaction studies. At such low concentrations and especially when a mixture of 90% distilled water with 10% deuterated water is used as NMR solvent, the only signal visible in the standard proton spectrum is the water resonance. For the two metabolite mixtures we recorded the proton spectra with simultaneous suppression of the water signal, using an excitation sculpting method that significantly reduces the water resonance so that the metabolites' resonances can be observed (Fig. 2). Because the two mixtures contain 9 and 10 metabolites, most of them with similar structures, some of the resonance signals overlap, especially in the aliphatic region. Nevertheless, we were able to identify at least one separate signal for each metabolite that will be further used as marker in protein-metabolite interaction experiments. Tryptophan was added in both mixtures as reference compound, being previously reported to interact with BSA.[30] The marker signals for each metabolite were identified at the following chemical shifts: triplet at 0.87 ppm for leucine, quintet at 1.81 ppm for 4-aminobutyric acid, multiplet in the interval 2.03–2.07 ppm for glutamine, singlet at 2.29 ppm for pyruvic acid, doublets of doublets at 2.77 and 2.86 ppm for asparagine, doublet of doublets at 3.76 ppm for serine, singlet at 7.00 ppm for histidine, triplet at 7.11 ppm for tryptophan, triplet at 7.34 ppm for phenylalanine, two doublets at 1.00 and 1.06 ppm for valine, doublet at 1.34 ppm for threonine, doublet at 1.50 ppm for alanine, triplet at 2.46 ppm for 2-oxoglutarate, triplet at 3.26 ppm for arginine, triplet at 4.04 ppm for ascorbic acid, doublet of doublets at 4.15 ppm for proline and two doublets at 6.89 and 7.70 ppm for 4-aminohippuric acid.

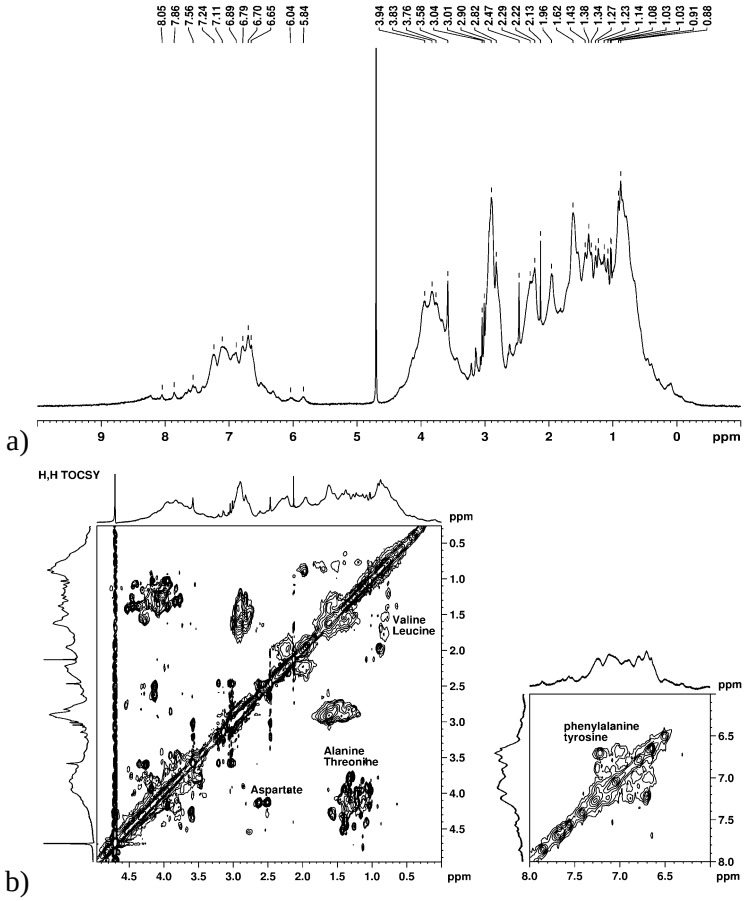
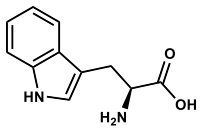
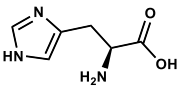
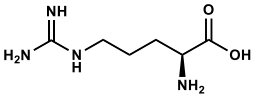
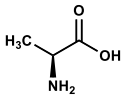
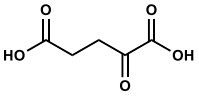
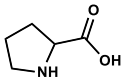
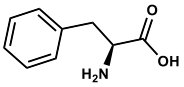
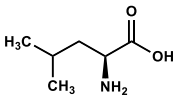
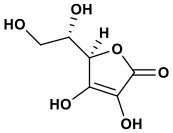
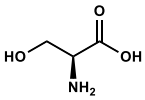
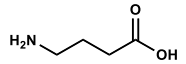
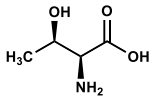
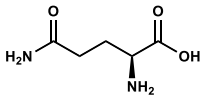
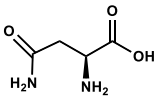
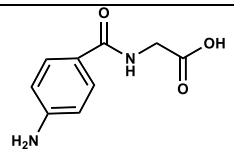
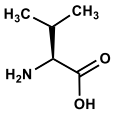
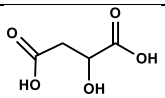
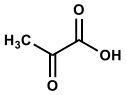


Fig. 1 – a) The ^{1}H NMR spectrum of native bovine serum albumin, recorded in PBS buffer with 10% D_2O, at 600 MHz; b) H,H TOCSY spectrum recorded for BSA, with the identified amino acids spin systems annotated on the 2D plot. Both experiments were performed on a standard 5 mm inverse detection room temperature probe.

Table 1

Chemical structures for the compounds used in binding study

 Tryptophan (**1**)	 Histidine (**4**)	 Arginine (**7**)	 Alanine (**10**)	 2-oxoglutaric acid (**13**)	 Proline (**16**)
 Phenylalanine (**2**)	 Leucine (**5**)	 Ascorbic acid (**8**)	 Serine (**11**)	 4-aminobutyric acid (**14**)	 Threonine (**17**)
 Glutamine (**3**)	 Asparagine (**6**)	 4-aminohippuric acid (**9**)	 Valine (**12**)	 Malic acid (**15**)	 Pyruvic acid (**18**)

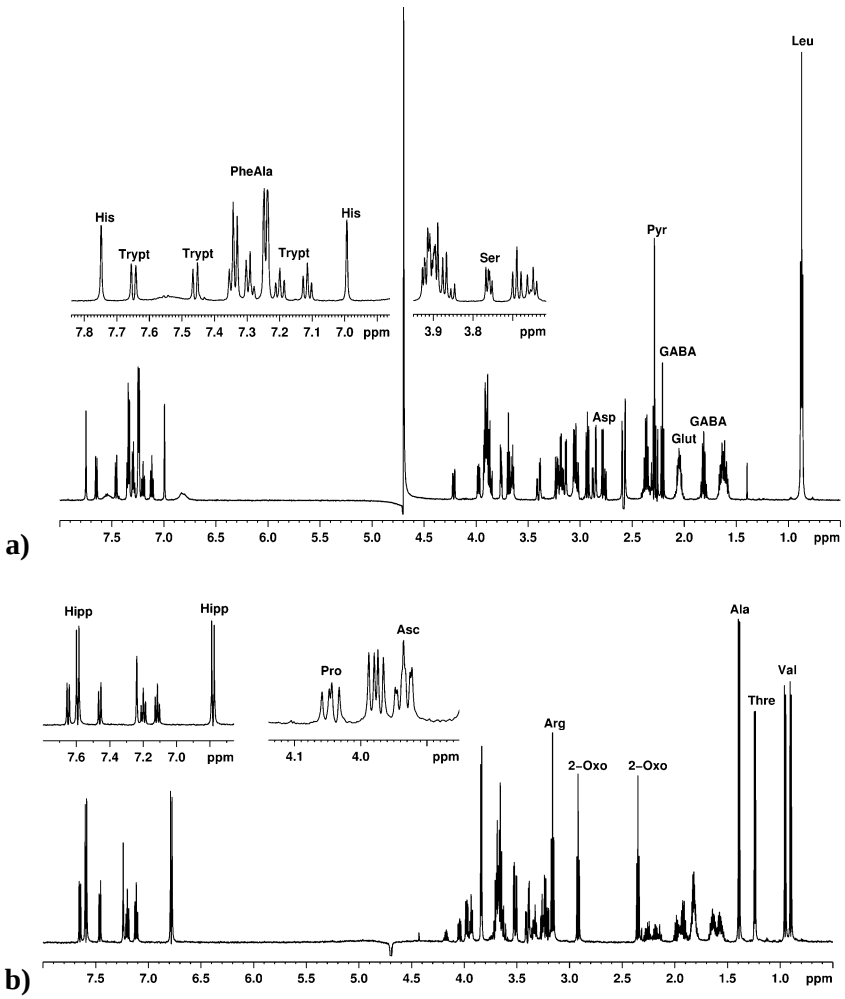


Fig. 2 – ^{1}H NMR spectra recorded for: a) aqueous solution 1 (10 metabolites mixture); b) solution 2 (9 metabolites mixture), with suppression of water resonance, at 600 MHz. Assignment abbreviations, Leu: leucine, GABA: 4-aminobutyric acid, Glut: glutamine, Pyr: pyruvic acid, Asp: asparagine, Ser: serine, His: histidine, Trypt: tryptophan, PheAla: phenylalanine, Val: valine, Thre: threonine, Ala: alanine, 2-Oxo: 2-oxoglutaric acid, Arg: arginine, Asc: ascorbic acid, Pro: proline, Hipp: 4-aminohippuric acid.

Metabolite observed STD NMR experiment. The interactions between the studied metabolites and BSA protein were followed through Saturation Transfer Difference (STD) spectra, an NOE-based NMR experiment. This is a useful NMR experiment in fragment-based drug design studies, used for screening and validation of protein-small molecule interactions. For this experiment, dilute solutions are normally used, the protein concentration being in the micromole range with 5 to 20-fold excess concentration of small molecules mixture. Since the NMR signals of the small molecules are observed, no isotopic (^{2}H, ^{13}C or ^{15}N) labelling of the protein is required and there are no restrictions on the protein's size, high-molecular weight proteins being also suitable.[16]

The proton NMR spectra recorded for the two BSA-metabolites solutions are presented in Fig. 3. In order to visualize the relevant resonance signals, water suppression was again applied. Compared with the initial proton spectra of the two metabolite mixtures (Fig. 2), the BSA presence in quite low concentration (40 μM) is only indicated by a broad background signal resembling a distorted baseline. In these standard proton spectra, the metabolites' signals are unaffected by the BSA presence, their multiplicities and chemical shifts values being similar with those of the initial mixtures.

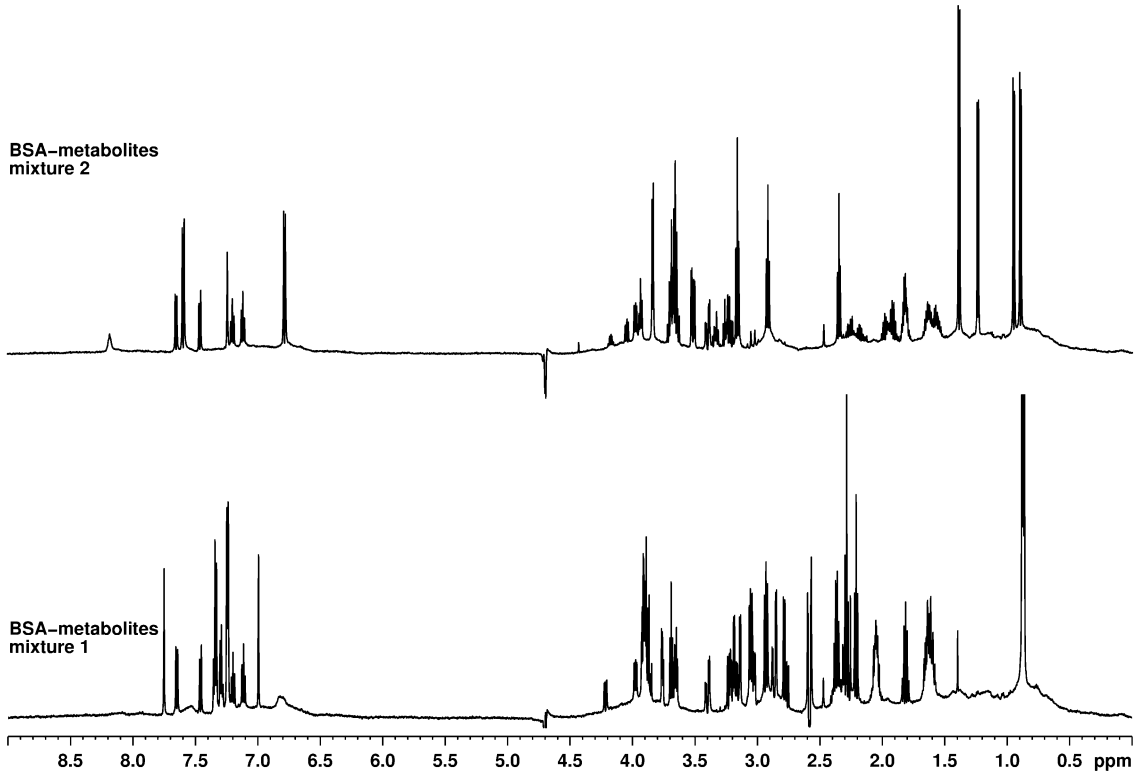


Fig. 3 – Overlapped ^{1}H NMR spectra recorder for the two BSA-metabolites mixtures in PBS buffer with 10% D_2O, at 600 MHz. Both experiments were performed on a standard 5 mm inverse detection room temperature probe. Water suppression was performed during the acquisition to reduce the water signal and visualize the metabolites resonances.

The saturation transfer difference experiment is based on magnetization transfer from protein to the bounded small molecule. In the STD experiment, a low power shape pulse selectively saturates the protein signals. This can be obtained by irradiating the aliphatic region of the proton spectrum, around 0-0.5 ppm, where protein's methyl groups usually resonate. No small molecules signals should be present in the irradiation region. Spin diffusion transfers this saturation to the rest of the protein and to any bounded small molecule. In practice, two 1D spectra are recorded, with and without protein irradiation, the effect of protein binding being visible in the difference spectrum.[12] The STD spectra recorded for the two BSA-metabolites mixtures are presented in Fig. 4. Positive signals that indicate metabolite-protein interactions were obtained for leucine and pyruvic acid, in metabolites mixture 1 and for valine, threonine, alanine and 4-aminohippuric acid, in metabolites mixture 2. For both BSA-metabolites mixtures, tryptophan shows positive signals,

confirming previously reported data.[30] Pyruvic acid has been also previously reported to interact with BSA.[31] To our knowledge, this is the first time when interactions of BSA with leucine, valine, threonine, alanine and 4-aminohippuric acid are demonstrated. Further implemented together with computational methods, the ligand-based NMR experiments could provide details on protein binding sites and structure of protein-ligand complexes. However, such studies are not within the scope of the present study which was aiming to discriminate in a fast way between the compounds which are bonding and those which are not binding with BSA.

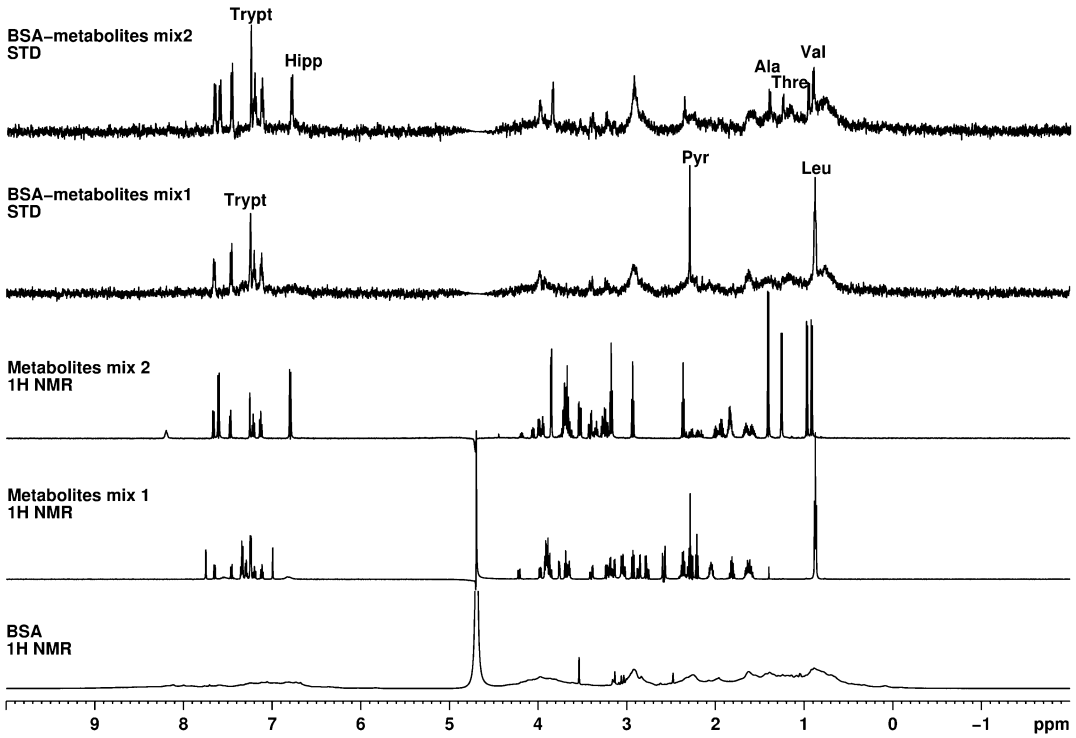


Fig. 4 – Overlay of the standard proton spectra for BSA, metabolites mixture 1, mixture 2 and STD spectra recorded for the two BSA-metabolites mixtures. All spectra were recorded at 600 MHz, in PBS buffer with 10% D_2O. The STD spectra were obtained by subtracting the on- from off-resonance spectra emphasizing thus only the signals of the metabolites interacting with BSA.

EXPERIMENTAL

The NMR spectra have been recorded on a Bruker Avance NEO 600 MHz spectrometer equipped with a 5 mm inverse-detection z-gradient multinuclear probe. For all the experiments performed, Bruker pulse programs included in TopSpin 4.0.8, acquisition and processing software have been used.

All metabolites and bovine serum albumin used in this study were purchased from Sigma-Aldrich and were used without further purification. In the two BSA-metabolite mixture solutions, the final protein and metabolites concentrations were 40 and 400 micromolar respectively. Phosphate-buffered saline (PBS) with pH 7.4 was prepared in a mixture of 90% distilled water and 10% deuterated water and further used as NMR solvent in all the experiments.

CONCLUSIONS

The interaction between bovine serum albumin and two metabolites' mixtures have been studied through NMR spectroscopy, using a 5 mm room temperature probe. Binding interactions have been proven for BSA with leucine, pyruvic acid, valine, threonine, alanine, 4-aminohippuric acid and tryptophan. Even though the vast majority of protein NMR experiments are performed on highly sensitive cryogenic probes, ligand-based NMR studies can be successfully implemented on room temperature probes, as we demonstrated in this study. Saturation transfer difference experiment is a suitable NMR method to describe the recognition of a ligand by its receptor.

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