Journal of Chromatography A, 1218 (2011) 3559–3564

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Analysis of nucleotides binding to chromatography supports provided by nuclear magnetic resonance spectroscopy

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ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 21 March 2011
Accepted 25 March 2011
Available online 1 April 2011

Keywords:
Nuclear magnetic resonance
Chromatography supports
Nucleotides
Histidine ligand
Molecular interactions

ABSTRACT

The epitope mapping of nucleotides bound to three chromatography supports is accomplished using saturation transfer difference (STD)-NMR spectroscopy. This experiment involves subtracting a spectrum in which the support was selectively saturated from one recorded without support saturation. In the difference spectrum only the signals of the ligands that bind to the support and received saturation transfer remain. The nucleotide protons in closer contact with the support have more intense signals due to a more efficient transfer of saturation. We investigate the effects on the binding to the nucleotides by the introduction of a spacer arm between L-histidine and Sepharose. Our NMR experiments evidence a clear contribution of the spacer to the interaction with all the nucleotides, increasing the mobility of the amino acid and giving different STD responses. This enhanced mobility originates the reinforcement of the interactions with the sugar moiety and phosphate group of 5′-CMP and 5′-TMP or the base of 5′-GMP and 5′-UMP. Hence, with this study we show that by using STD NMR technique on chromatographic systems it is possible to provide a fast, robust and efficient way of screening the atoms involved in the binding to the supports.

1. Introduction

Amino acids were recently used as pseudobiospecific ligands in affinity chromatography matrices to separate and purify plasmid DNA isoforms [1,2]. The high degree of purification required for this separation is still a major obstacle for the large scale development of pDNA vectors for therapeutic use [3]. Therefore, a fundamental knowledge of the main interactions that determine the *in situ* specificity of the affinity chromatography support is the primary requirement for the selection of the optimal support for the purification process. Until now, the molecular interactions that govern the efficiency of amino acid supports for polynucleotides [4,5] or plasmids [6] purification have been explained based on affinity chromatography data and analysis of high-resolution protein–DNA structures [7–9]. A recent study performed by affinity chromatography using different immobilized L-histidine chromatographic matrices (via epoxy moiety or containing a long spacer) suggests that the introduction of a spacer arm between the amino acid and the Sepharose has an influence in the retention of the biomolecules [10]. However, the binding mode of the biomolecules to these supports is not well understood, and is still the subject of active investigation [11].

Several NMR experiments are known for the analysis of biomolecule – ligand interactions, among these the STD-NMR technique has proven its efficacy in detecting the binding of low molecular weight compounds to large biomolecules [12–14]. This method is very useful to map the atoms of the ligand that are in close contact to the biomolecule when the complex is formed [15–17].

In this study we have employed STD-NMR to study the binding of 5′-mononucleotides to three chromatography supports. Our main goal was to test the application of STD-NMR spectroscopy to study support/mononucleotide interaction in order to establish a fast and efficient experimental protocol to identify which atoms of the nucleotide are involved in the binding and that could also be applied to other potential supports for purification of nucleic acids.

Relatively to the supports, we have investigated the effect of the presence of the L-histidine ligand and/or the introduction of a spacer arm in the interaction of the support with 5′-mononucleotides. The schematic representations of the supports

Abbreviations: NMR, nuclear magnetic resonance; STD, saturation transfer difference; His, L-histidine; seph, Sepharose 4B; seph-bisoxy, Sepharose 4B-bisoxyran; Seph-bisoxy-His, Sepharose 4B-bisoxyran-histidine; 5′-GMP, guanosine 5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; 5′-TMP, thymidine 5′-monophosphate; 5′-CMP, cytidine 5′-monophosphate; 5′-UMP, uridine 5′-monophosphate.

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doi:10.1016/j.chroma.2011.03.055

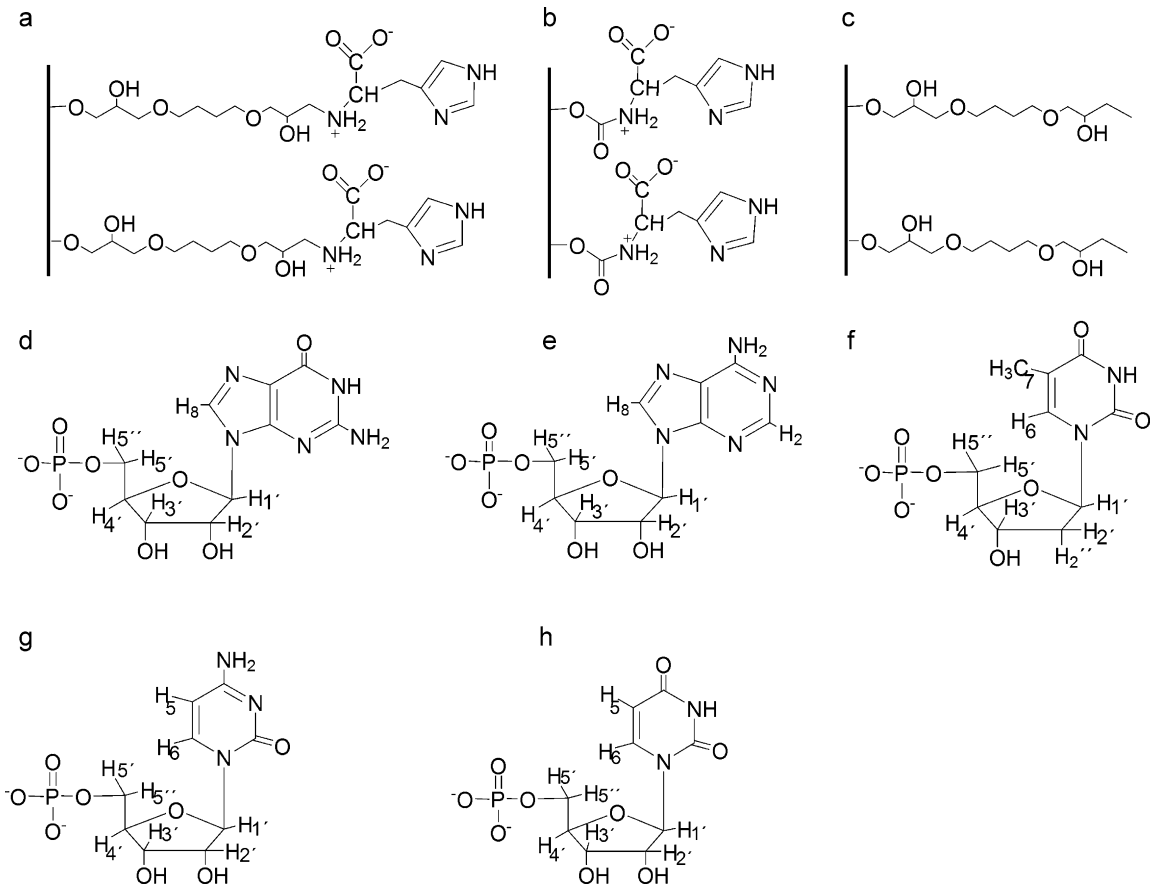

Fig. 1. Schematic structures of the three chromatographic supports (a) seph-bisoxy-His, (b) seph-His, (c) seph-bisoxy and the 5′-mononucleotides (d) 5′-GMP, (e) 5′-AMP, (f) 5′-TMP, (g) 5′-CMP and (h) 5′-UMP.

and the 5′-mononucleotides used in this work are presented in Fig. 1.

As can be depicted from Fig. 1, we have selected one support with L-histidine linked to Sepharose 4B resin through its α-NH_2 group with a ten-carbon 4-butanediol-diglycidyl-ether (bisoxyran) spacer (Fig. 1a). A second support has the L-histidine immobilized in Sepharose 4B resin via epoxy group by the same amino group and without spacer (Fig. 1b). In both supports, the α-carboxyl and ε-amino groups were left free to interact with the mononucleotides. Finally, the third support has only the spacer 4-butanediol-diglycidyl-ether immobilized on Sepharose 4B (Fig. 1c) and was used to study the effect of the spacer, without amino acid ligand in the binding to the chromatography matrices.

2. Materials and methods

The supports, Sepharose 4B-epoxy-histidine, Sepharose 4B-bisoxyran-histidine and Sepharose 4B-bisoxyran, and the lyophilized 5′-mononucleotides were purchased from Sigma (St Louis, MO, USA). All spectra were acquired on a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe and processed with the software TOPSPIN 2.0 (Bruker). ^{1}H spectrum of each chromatography support was acquired at 600 MHz with 16 scans and a spectral width of 6009.6 Hz, centered at 2820.93 Hz. The solution of each 5′-mononucleotide was prepared in 90% H_2O and 10% (v/v) D_2O. The final volume was 750 μL adjusted with potassium phosphate buffer 10 mM at pH 8.0. The interaction between 5′-mononucleotides and 4B-epoxy-histidine, Sepharose 4B-bisoxyran-histidine and Sepharose 4B-bisoxyran was studied by STD-NMR. Since the supports are not soluble, the ^{1}H STD-NMR was performed using a suspension of supports and 2-fold molar excess of 5′-mononucleotides in 90% H_2O and 10% (v/v) D_2O. The incubation times of the 5′-mononucleotides with the chromatographic supports was 12 h.

All the spectra were measured at 298 K with 2k scans in a spectral window of 6000 Hz centered at 2824.35 Hz. In the first instance a ^{1}H NMR reference spectrum was acquired. A low power presaturation pulse during the relaxation delay for water suppression was applied. Standard ^{1}H NMR acquisition parameters were loaded and 90° high power pulse was determined [18]. Selective saturation of the support resonances at 1.7 ppm (on-resonance) and 37 ppm (off-resonance) using a series of EBURP shaped pulses (50 ms, 1 ms delay between pulses), for a total saturation time of 2.04 s. Subtraction of saturated spectra from reference spectra was performed by phase cycling. Measurement of enhancement intensities was performed by direct comparison of STD-NMR [18].

Relative STD effects were calculated according to the equation % STD = $(I_0 - I_{sat})/I_0 = I_{STD}/I_0$ by comparing the intensity of the signals in the STD NMR spectrum (I_{STD}) with signal intensities of a reference spectrum (I_0) [13]. The STD NMR signal with the strongest intensity was set to 100% and relative STD NMR effects for all other observable signals were calculated.

3. Results and discussion

Fig. 2 presents the ^{1}H NMR spectrum of 5′-TMP (a) and the STD spectra of 5′-TMP:seph-bisoxy (b), 5′-TMP:seph-His (c), and 5′-TMP:seph-bisoxy-His (d). The STD spectrum was obtained by subtraction of saturated spectra from reference spectra. Reference experiments containing only mononucleotides were performed

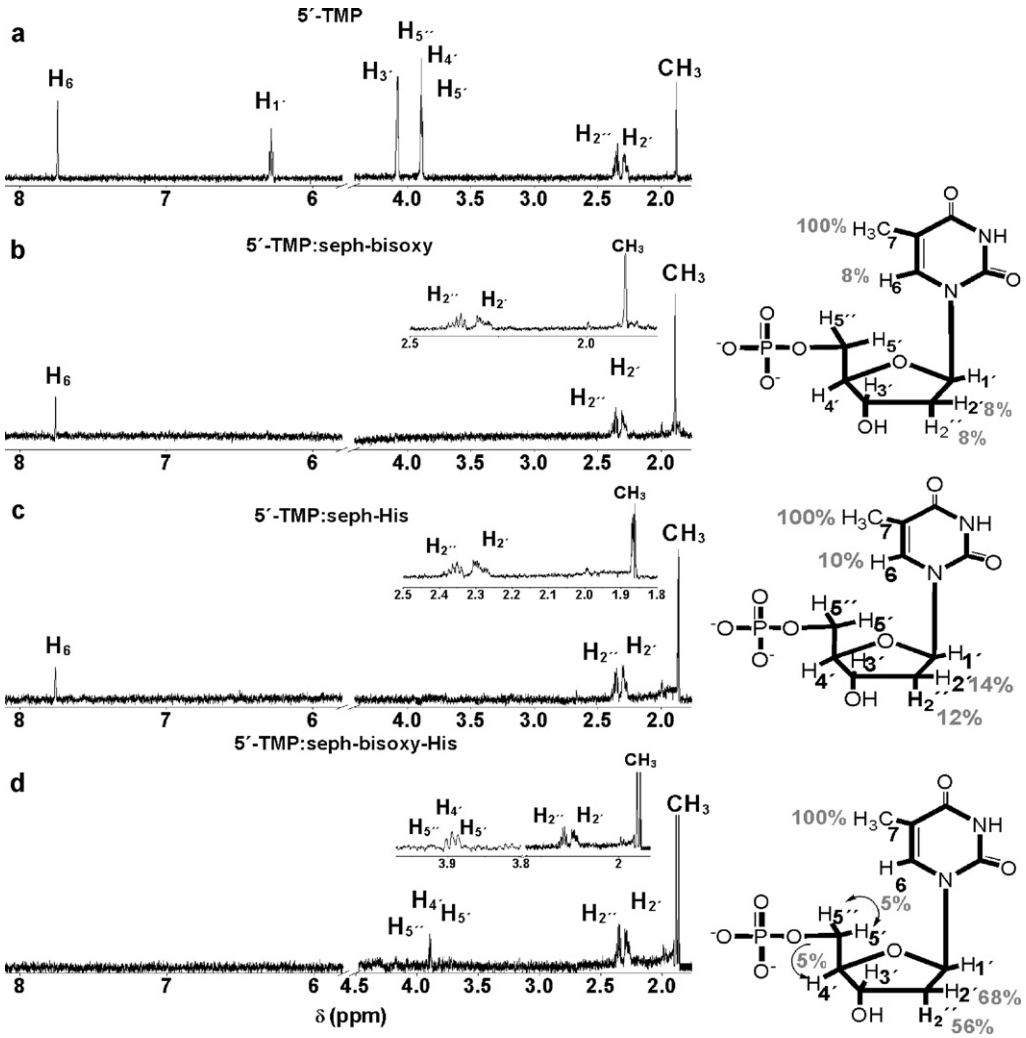

Fig. 2. (a) ^{1}H NMR reference spectrum for free 5′-TMP and STD NMR spectra of: (b) 5′-TMP:seph-bisoxy, (c) 5′-TMP:seph-His, (d) 5′-TMP:seph-bisoxy-His. All spectra were recorded in a Bruker AVANCE III, 600 MHz equipped with a cryoprobe, at 298 K, in H_2O/D_2O (9:1) and potassium phosphate buffer (10 mM, pH 8). The ratios of the intensities I_{STD}/I_0 were normalized using the largest STD effect (methyl protons of the thymine) 100% as a reference. Due to the close overlap between the resonances of $H_{4'}$ $H_{5'}$ and $H_{5''}$ the integration of the signals was difficult to evaluate and the values for these protons are estimated.

under the same experimental conditions to assure that the effects observed in the presence of the supports were due to true saturation transfer, since no signal was present in the STD spectra obtained in the reference experiments.

The relative STD effects were calculated by the individual signal intensities in the STD spectrum (I_{STD}) and in the reference ^{1}H NMR spectrum (I_0). The ratios of the intensities I_{STD}/I_0 were normalized using the largest STD effect (set to 100%).

In the three STD NMR spectra (b), (c) and (d) with 5′-TMP it is clear that the methyl group receives the largest amount of saturation transfer. When considering the interaction with the spacer or the amino acid (spectrum b and c of Fig. 2) alone, there seems to be a preferential interaction with the thymine ring. However, the small increase in the relative STD for $H_{2'}$ and $H_{2''}$ protons of the ribose ring in spectrum c) seems to be related with the presence of the L-histidine and to a different interaction mode due to the presence of the residue. This corroborates with the absence of STD signal for H_6 proton in spectrum d) of Fig. 2 where the interaction with the L-histidine should be maximized due to the conformational mobility introduced in the support by the spacer. Based on the STD responses, it is clear that the spacer between the Sepharose and the L-histidine promotes a stronger interaction between 5′-TMP and support by reinforcing the binding with L-histidine. This affinity of histidine for thymine base was also found in protein–DNA structures solved and is explained by their ability to produce extensive ring-stacking interactions [7].

The epitope mapping of 5′-AMP in the presence of the supports are presented in Fig. 3 and the relative STD intensities of the remaining 5′-mononucleotides are summarized in Fig. 4.

Comparison of the STD-NMR spectra of 5′-AMP shows subtle differences between the three supports (see spectra b, c and d of Fig. 3). In contrary to 5′-TMP, the introduction of a long spacer between the matrix (Sepharose) and the amino acid does not seem relevant to favor the existence of more contacts. As before, the presence of the amino acid in the support increases the interaction with the sugar moiety. With the support seph-bisoxy-His, the largest STD effect is found for proton $H_{1'}$ (see spectrum d of Fig. 3). The prominent STD signal attributed to H_2 (100% of saturation) in presence of seph-His and (98% saturation) with the support seph-bisoxy-His, should be related with a possible interaction by π–π stacking between the aromatic side chain of histidine and adenine has as been previously discussed [7]. Furthermore, the significant number of contacts with the phosphate group of 5′-AMP at positions 5″ and 5′ both with the supports containing the spacer and L-histidine (spectra b and c of Fig. 3) denotes that the interaction is extended across this nucleotide.

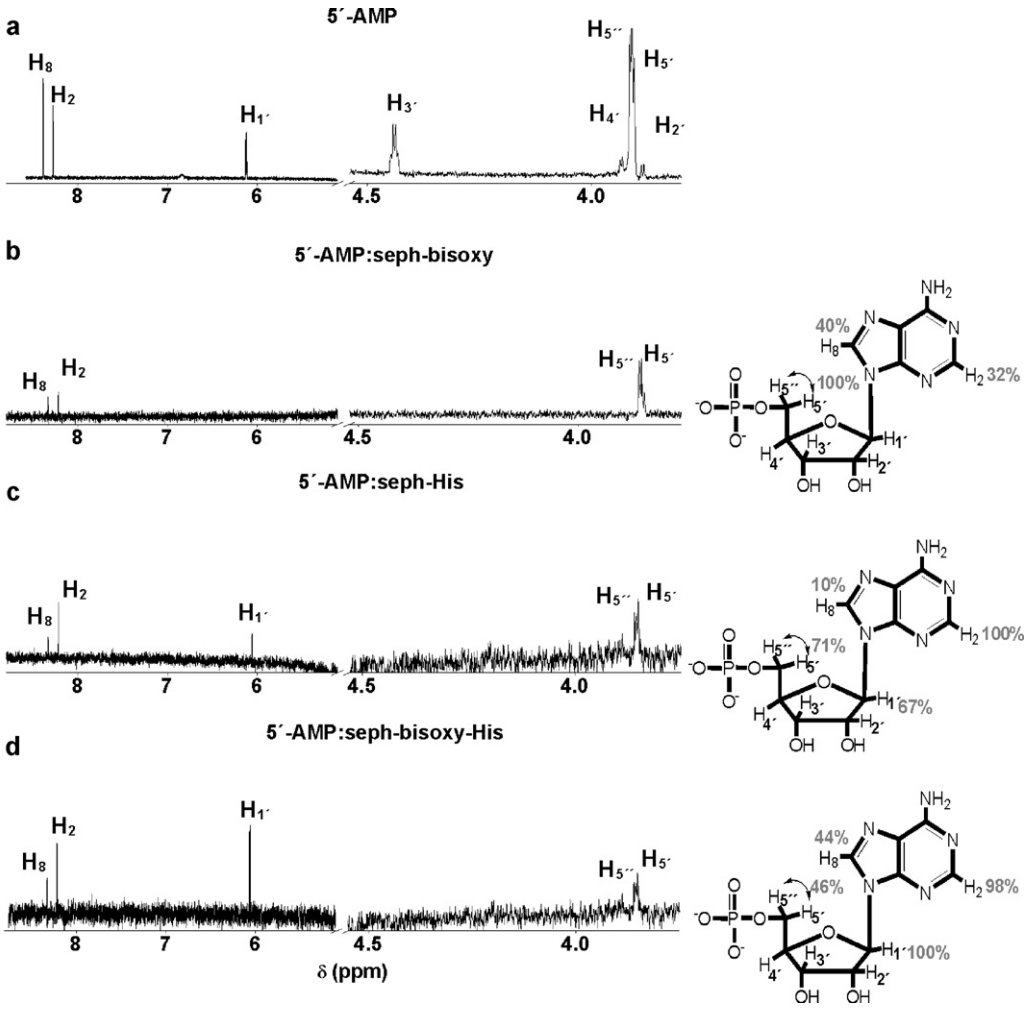

Fig. 3. (a) ^{1}H NMR reference spectrum for free 5′-AMP and ^{1}D STD NMR spectra of: (b) 5′-AMP:seph-bisoxy, (c) 5′-AMP:seph-His, (d) 5′-AMP:seph-bisoxy-His. All spectra were recorded in the same experimental conditions reported in Fig. 2 and the ratios of the intensities I_{STD}/I_0 were normalized using the largest STD effect ($H_{5''}$ to seph-bisoxy-His, H_2 to seph-His and $H_{1'}$ seph-bisoxy) 100% as a reference. Due to the close overlap between the resonances $H_{5'}$ and $H_{5''}$ the integration of the signals was difficult to evaluate and the values for these protons are estimate.

The STD experiments of 5′-GMP in presence of the supports that have L-histidine show the involvement of guanine in the binding (see Fig. 4a). This involvement is clearly related to the strong STD NMR signals of proton H_8 with seph-bisoxy-His (100% of saturation) and seph-His (53% of saturation) and the absence of signals with the support seph-bisoxy (see Fig. 4a). These observations are in accordance with affinity chromatography data [4] and with the analysis of high-resolution protein–DNA structures [7–9], where L-histidine displays binding preference for guanine, followed by adenine and thymidine.

Proton $H_{2'}$ receives the highest amount of saturation (100%) in the presence of supports seph-His and seph-bisoxy, however the combination of seph-bisoxy-His decreases the interaction with ribose, as depicted from the lower STD signals for $H_{2'}$ (7% of saturation). In this case, the spacer linked to the amino acid does not contribute to the increase in the interactions with 5′-GMP.

Fig. 4 also shows the relative STD intensities of 5′-CMP with the different chromatographic supports. The binding profile of 5′-CMP with the three supports is very similar and suggests that 5′-CMP binds strongly through sugar–phosphate backbone. This affirmation finds support in the fact that it is the ribose moiety that establishes more STD contacts. In the absence of the spacer (support seph-His, Fig. 4b) the H_5 proton of the cytosine shows the most intense STD signal. This suggests that C5 of cytosine may form CH···O hydrogen bonds with the carbonyl (O=) and/or carboxyl (O^-) groups of the histidine side-chains, contributing to the specificity of recognition. This result is consistent with previous studies concerning crystal structures analysis of protein–DNA interactions [9].

The analysis of the contact map of 5′-UMP reveals that all its protons contribute to the binding to seph-His (see Fig. 4c), while with seph-bisoxy only a few STD responses are detected. Thus, the binding of 5′-UMP increases in presence of support seph-His and decreases with seph-bisoxy. The detailed analysis of the STD spectra shows that the $H_{1'}$ of ribose is the one with the highest signal (100% of saturation) on all the supports, followed by the uracil proton H_5 with seph-bisoxy and seph-bisoxy-His. The weaker STD intensity of H_6 (≈20% of saturation) is only observed with the L-histidine supports (seph-bisoxy-His and seph-His) (see Fig. 4c). This could be related to stacking interactions and H-bonds established between uracil and imidazole of histidine [7–9]. With seph-bisoxy and seph-bisoxy-His supports, the stronger STD intensities observed for H_5 are attributed to the 4-butanediol-diglycidyl-ether spacer (see Fig. 4c).

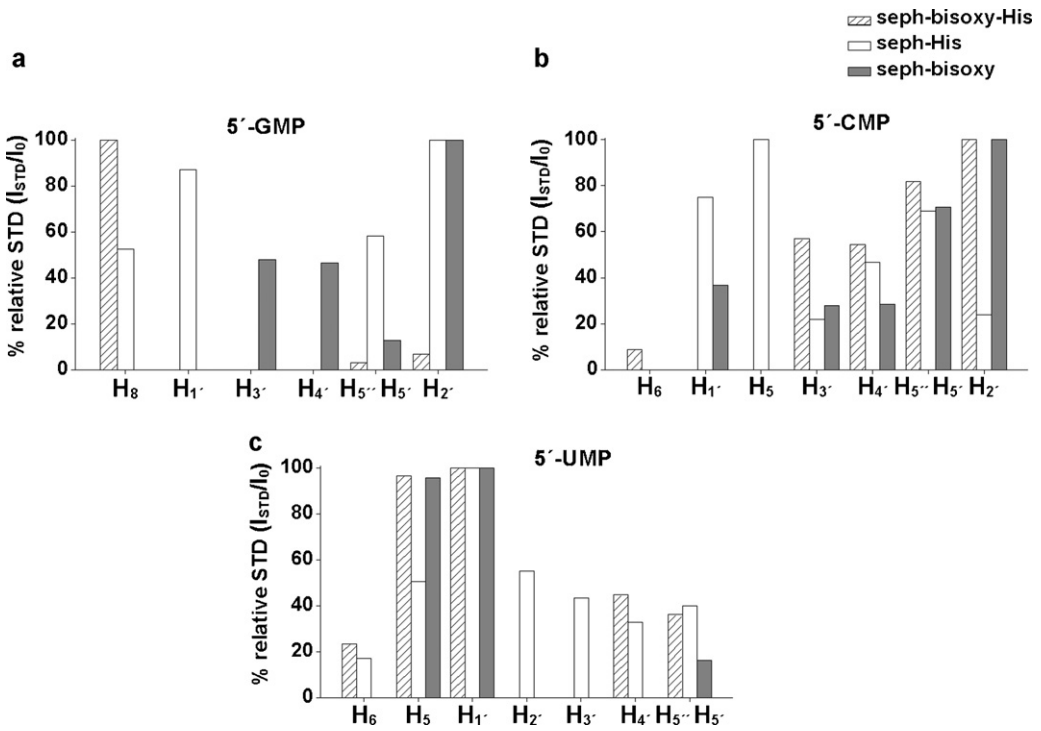

Fig. 4. Relative STD intensities for (a) 5′-GMP, (b) 5′-CMP, (c) 5′-UMP bound to the supports seph-bisoxy-His (dashed), seph-His (white) and seph-bisoxy (gray). Due to the close overlap between the resonances $H_{5'}$ and $H_{5''}$ the integration of the signals was difficult to evaluate and the values for these protons are estimated.

4. Conclusions

In summary, the experimental protocol proposed is swift and highlights the binding characterization of 5′-mononucleotides to three chromatographic supports in solution. Their ability to bind L-histidine immobilized on Sepharose resin was thoroughly investigated, as well as, the effects on the binding of the introduction of the long spacer arm between L-histidine and Sepharose. Our NMR experiments evidence a clear contribution from the spacer to the interaction and show that all mononucleotides exhibit a response in the STD spectra, indicating that all are in direct contact with the supports. The introduction of a spacer between the Sepharose and the immobilized L-histidine increases the mobility of the amino acid; however this effect in the interaction with the different 5′-mononucleotides is not the same. With the exception of 5′-GMP, the increased mobility has a reflection in an overall increase in the number of detected STD contacts, either through the reinforcement of the interactions with the sugar moiety (5′-CMP, 5′-TMP) or the base (5′-GMP, 5′-UMP). From 5′-TMP, the CH_3 of thymine leads to the most prominent STD signals, suggesting similar binding profile with all the supports. Also, 5′-UMP binds L-histidine immobilized and bisoxyran spacer mainly through the uracil moiety and sugar backbone. In the case of 5′-GMP, the supports that are coupled to L-histidine show the involvement of guanine in the binding. 5′-AMP exhibits similar binding epitopes to all the supports, while 5′-CMP interacts preferentially through H_5 of cytosine with seph- His. The backbone of 5′-CMP binds significantly the others supports, namely, at positions $H_{5'}$ and $H_{5''}$ and the sugar proton $H_{2'}$. The experiments proposed could be exploited to study others affinity ligands in order to improve the specificity of binding to complex DNA or RNA sequences. Therefore, we are currently applying this method to screen others chromatographic supports with these nucleotides to avoid the time consumption required by the chromatography technique.

Acknowledgements

Carla Cruz acknowledges the post-doctoral grant from FCT (SFRH/BPD/46934/2008). The NMR spectrometers are part of the Portuguese National NMR Network (RNRMN) and were purchased in the framework of the National Program for Scientific Reequipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and Fundação para a Ciência e Tecnologia. This work was partially supported by Portuguese Foundation for Science and Technology (PTDC/EQU-EQU/65492/2006 and PTDC/EBB-BIO/114320/2009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.03.055.

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