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Determination Using Molecularly Imprinted Solid Phase Extraction (MISPE) Of Derivatized Homocysteine

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ABSTRACT

Homocysteine(HM) is a thiol-containing aminoacid formed from methionine through intermediates S-adenosylmethionine and S-adenosylhomocysteine. High plasma concentrations of this compound have been correlated to several diseases. In this work we reported a potential alternative derivatization procedure to determinate HM via a molecularly imprinted solid phase extraction(MISPE) combined with high pressure liquid chromatography(HPLC). Benzoyl chloride, inexpensive electrophilic reagent and no degradable at room temperature, was used to functionalize HM in order to obtain a detectable adduct. The obtained N-benzoyl-homocysteine(B-HM) was used as template during the preparation of different MIPs.

KEYWORDS

Homocysteine;
Molecularly imprinted polymers;
Solid phase extraction;
UV- detection.

INTRODUCTION

Homocysteine (HM) is a thiol-containing amino acid formed from methionine through the intermediates S-adenosylmethionine and S-adenosylhomo cysteine[1].

The plasma concentration of homocysteine is markedly increased in patients with the genetic disorder called homocystinuria. Furthermore, an elevated plasma homocysteine concentration(hyperhomocysteinemia) is currently considered as an independent risk factor for cardiovascular disease and can be also related to nutrient disturbances in homocysteine metabolism[2,3]. Because of the importance of this aminothiol, several method are available for the measurement of HM concentration in biological samples[4,5].

Every method has its advantages and limitations,

and can be utilized in particular analysis. Some of these methods use techniques, such as, mass spectrometry, electrochemical oxidation or enzyme assay[6]. The remaining methods start with derivatization of the aminothiols with fluorescent reagents. Most of these compounds react with the thiol group[7]. However, some of these adducts are not very stable or their accurate measurement is hampered by the presence of excess reagent and products from side reactions[7]. In order to improve the selectivity, we have developed a new derivatization method for HM that modifies the amino group. The resulting derivative exhibits intense UV absorption and it is very stable. Thus, in this work we reported this alternative and inexpensive derivatization procedure to determinate the homocysteine via a molecularly imprinted solid phase extraction (MISPE) combined with high pressure liquid chromatography (HPLC).

Molecular imprinting is an efficient method to introduce specific molecular recognition sites into a polymeric matrix[8]. The first example of molecularly imprinted polymer (MIPs) was reported half a century ago, however it is only in the last decade that the use of molecular imprinting as a practical tool has become established[9]. The applicability of this technique has led to numerous reports: MIPs were used as stationary phases in chromatographic separation and chiral separation[10]. MIPs were used in immunoassay-like analyses, as synthetic enzymes[11], biosensor and as a tool for the study of molecular recognition receptors[12] and also used in drug delivery systems (DDS)[13]. The technique to produce the MIPs, using the non-covalent approach, involves arranging functional monomers around a templating ligand. This ligand is the selected target substance and it should form a prepolymerization complex with the monomer by non-covalent interactions such as hydrogen bonding, ionic or hydrophobic interactions. The formed complex is subsequently radically copolymerized with a suitable crosslinker. After copolymerization, the template is removed to obtain binding sites specific for the original template[14].

Potential advantages in molecular recognition offered by molecular imprinting include: (1) ability to induce receptor sites with outstanding analyte specificity; (2) ability to produce reusable recognition matrices with good robustness and stability even at extreme physical/chemical conditions[15].

In this work HM was functionalized with benzoyl chloride, inexpensive electrophilic reagent and above all no degradable at room temperature, in order to obtain an UV-detectable adduct of HM.

The so obtained N-benzoyl-homocysteine (B-HM) (U.V. detectable), was used as template during the fabrication of different MIPs (non covalent approach) and considerable differences in the recognition characteristics between imprinted and non imprinted polymers have been observed.

MATERIALS AND METHODS

Materials

Ethyleneglycoldimethacrylate (EGDMA> 99%), dimethylacryliamide(DMAA> 98%), methacrylic acid(MAA> 99%) and 2,2'-azobis(isobutyronitrile) (AIBN>98%), benzoyl chloride (>99%), D,L-homocysteine (HM) were obtained from Aldrich. All solvents were reagent grade or HPLC-grade and used without further purification and they were provided by Fluka Chemie.

Apparatus

HPLC was performed using a Jasco Model (Tokyo, Japan).

Synthesis of N-benzoyl-D,L-homocysteine (B-HM)

D,L-homocysteine(455 mg, 3.4 mmol) was dissolved in 10 ml sodium hydroxide solution (0.1 M) and benzoyl chloride(500 mg, 1.7 mmol) at 0°C was added in 1 hour . The mixture was stirred at 0°C for 2 hours. Then 10ml of a solution of hydrochloride acid (5 %) were added, achieving the precipitation of N-benzoyl-D,L-homocysteine. The precipitated product was separated by filtration, dried and crystallized from 95% ethanol (0.58 g; yield:, 85%).

IR (KBr) ν 3326; 3060; 2558; 1637 cm^{-1}.

^{1}H-NMR (DMSO): δ_H 7.92(1H,m), 7.65(2H,m), 7.41(2H,m), 4.08(1H, m), 2.67(2H,m), 2.05(2H, m).

Anal. Calc. for $C_{11}H_{13}NO_3S$: C, 55.21; H, 5.48; N, 5.85; Found: C, 55.18; H, 5.70; N, 5.98;

Synthesis of molecularly imprinted polymer

In a typical preparation of MIP, the template (N-benzoyl-D,L homocysteine) was dissolved in a mixture of acetonitrile and DMF(v/v 6/4) containing the functional monomers and EGDMA. Then, AIBN (103 mg) was added and the resultant solution was purged with nitrogen and sonicated for 10 min. The mixture was then incubated under a nitrogen atmosphere at 68°C for 24 h. The amounts of all reagents used are reported in TABLE 1. The resultant bulk rigid polymer was crushed, grounded into powder and sieved through a 63 μm stainless steel sieve. The sieved MIP material was collected and the very fine powder suspended in the supernatant solution was discarded. The resultant MIP material was soxhlet extracted with 200 ml of acetic acid:methanol (1:1) mixture for at least 48 h followed by 200 ml of methanol for another 48 h. The extracted MIP material was dried in an oven at 60°C overnight. The washed MIP material was checked to be free of bound template by HPLC analysis.

The non imprinted polymers (NIPs) (control) were prepared under the same conditions except that no template was used.

Preparation of MISPE columns

The 500 mg amount of dry particles was packed into a 6.0 ml polypropylene SPE column. The column was attached with a stop cock and a reservoir at the bottom end and the top end, respectively. The polymer was rinsed with methanol and then with water.

MISPE procedure and memory effects

To calculate MISPE cartridges loading capacity, N-benzoyl D,L homocysteine was dissolved in a mixture of acetonitrile and methanol 8/2(v/v) to final concentrations of 20 μg/ml. A dry MISPE column was conditioned with 10 ml of chloroform followed by loading of 5 ml of standard solutions. After column drying, 4ml of acetonitrile were applied(whashing step) and finally 5 ml of methanol/acetic acid 9/1 (eluition mixture) to perform the complete extraction of N-benzoyl D,L homocysteine.

HPLC analysis

The liquid chromatography consisted of a Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 254 nm. A 250×4 mm C-18 Hibar® column, particle size 10μm(Merck, Darmstadt, Germany) was employed. The mobile phase was methanol/phosphate buffer 5 mM, pH 4.5(9/1, v/v) and the flow rate was 0.5 ml/min.

RESULTS AND DISCUSSION

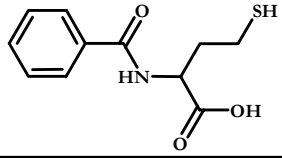
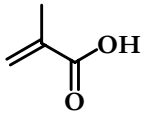
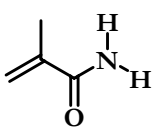
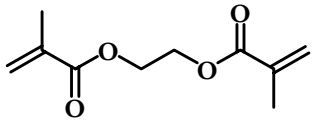
Functionalization of homocysteine

HM was functionalized with benzoyl chloride in order to obtain N-benzoyl-homocysteine (B-HM) is UV detectable at 254 nm (aromatic compounds)[16].

The reaction was carried out, as reported in literature for similar substrates[16], in the presence of benzoyl chloride at 0°C for 2 hours.

The derivatization was specific for amino group[16]; instead many thiol derivatizing reagents, through side-reactions and degradation, could add extra peaks

TABLE 1: Composition of synthesized polymers

Polymer	Template (mmol)	MAA (mmol)	DMAA (mmol)	Crosslinker (mmol)	Solvent
					Acetonitrile/DMF (v/v 6/4)
MIPs1	0.7	4.0	-	20.4	5.0 ml
NIPs1	-	4.0	-	20.4	5.0 ml
MIPs2	0.7	4.0	4.0	20.4	5.0 ml
NIPs2	-	4.0	4.0	20.4	5.0 ml
MIPs3	0.7	4.0	-	20.4	Without solvent
NIPs3	-	4.0	-	20.4	Without solvent

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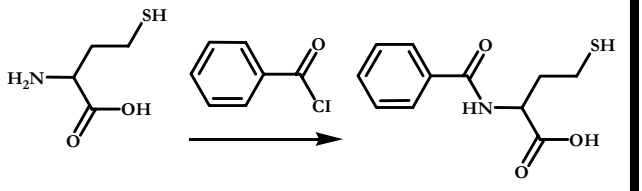

Figure 1: Derivatization of homocysteine

to chromatogram complicating interpretation and quantification[17].

A UV-tagged analyte, in order to be a suitable molecular template for molecular imprinting, must be stable throughout the cross-linking process, which usually involves elevated temperature and/or UV irradiation in the presence of free-radical initiator. Usually, this rule out most of derivatizing agents for homocysteine because of the general instability of their produced adducts. On the contrary, several reports confirm that N-derivatized aminoacids are stable during the crosslinked process[18-19]. Then, this pathway opens up the possibility to realize the stationary phase for solid phase extraction (SPE) based on molecular recognition. Actually, B-HM is a stable adduct and soluble in the organic solvent.

Polymer synthesis, memory effects and chromatographic evaluations.

Different MIPs using B-HM as template were fabricated: using MAA as functional monomer and EGDMA as the cross-linker, but also using MAA and DMAA as functional monomers and always EGDMA as cross-linker. MAA is a common functional monomer for the fabrication of non-covalent type MIPs. The involved functional groups of B-HM in the interactions with MAA, are probably thiol group as well as benzamide group. We decided to use DMAA and MAA as functional monomers, with the hope of an improvement in the recognition effect. EGDMA was used as cross-linker because of its ability to induce good recognition properties. The choice of the polar porogen DMF was dictated by the solubility of the template.

Thus, three kinds of molecularly imprinted polymers were synthesized: using only MAA as functional monomer (MIPs 1), using MAA and DMAA as functional monomers (MIPs 2) and polymerization without solvent (MIPs 3).

TABLE 2: B-HM retained for imprinted and non-imprinted polymers packed in cartridges

Cartridges	Loading step (%retained)	Washing step (% Collected)	Elution step (% collected)
MIPs-1	98	10	88
Nips-1	88	44	40
MIPs-2	92	33	59
NIPs-2	75	37	38

In order to evaluate the loading capacity and the imprinting effect, different extraction solvents were used. The loading solution was always a mixture of acetonitrile and methanol (8:2 v:v) because it is one of the few mixtures able to dissolve B-HM.

The best results were obtained washing the SPE columns with acetonitrile and using methanol/acetic acid (95/5 v/v) as eluate. In TABLE 2 the analyte recovery percentages for any step were reported.

In figure 2 it is possible to note the different behaviour of the polymers compared respectively to the non imprinted ones.

MIPs2 does not show a good imprinting effect, instead MIPs1 shows a better molecular recognition (88% of recovered B-HM).

Probably these different effects depend on the strength interactions between the template and the functional monomers. The expected improvement in the imprinting effect, using also DMAA, was not indeed obtained. Probably DMAA did not play an important role during the pre-polymerization complex and consequently the resultant imprinted polymer offers a limited molecular recognitions.

We did not describe the MIP3 because of absence of selective interaction (No B-HM was retained).

Thus the molecularly imprinted solid phase extraction (MISPE) could allow to remove many unwanted components of samples, to obtain clean chromatograms and to prolong the life of expensive HPLC columns. This MISPE cartridges are able to retain B-HM (88 %) and providing a potential method, combined with HPLC instrument, to monitor in biological fluids levels of HM.

Influence of methanol on the imprinting effect

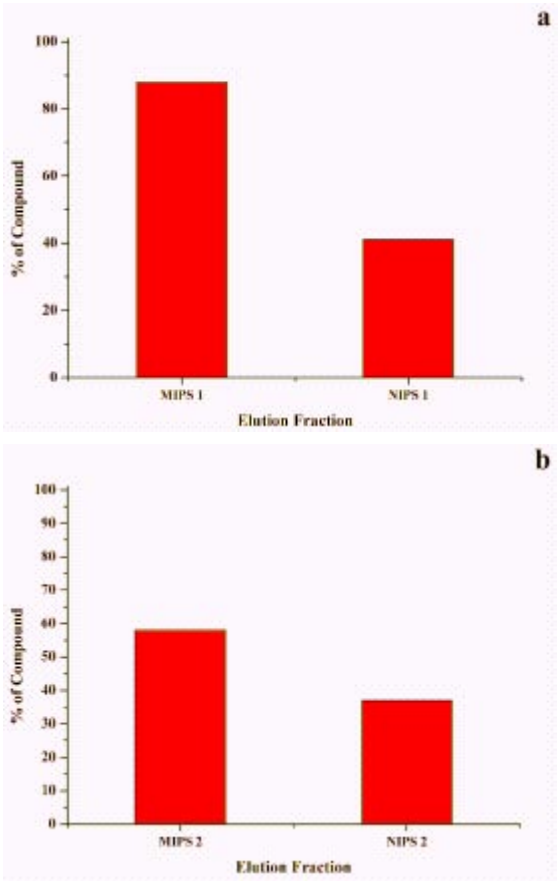

Figure 2: Comparison of recoveries of B-HM in elution fraction related to MIPs1 (a): and MIPs2 columns (b): and respective blank ones after solid phase extraction of standard solution of B-HM

Acetonitrile (such as other common organic solvents) does not dissolve the N-benzoyl-D,L homocysteine (template) and in order to evaluate the memory effects of MIPs, we had to prepare the standard solution in a mixture of acetonitrile and methanol (8:2). Probably the methanol decreases the imprinting effect. To estimate how much the methanol decreases the molecular recognition, we prepared several standard solutions (loading solutions) of the template just increasing the amount of methanol in these ones.

Thus, it has been possible to plot the absorbance (A) of elution fractions versus the different amounts of methanol in the standard solutions in order to extrapolate to amount of methanol=0, the absorbance (intercept) of hypothetical elution fraction deriving from a standard solution of only acetonitrile.

The difference between intercept and absorbance of real standard solution (acetonitrile:methanol 8:2)

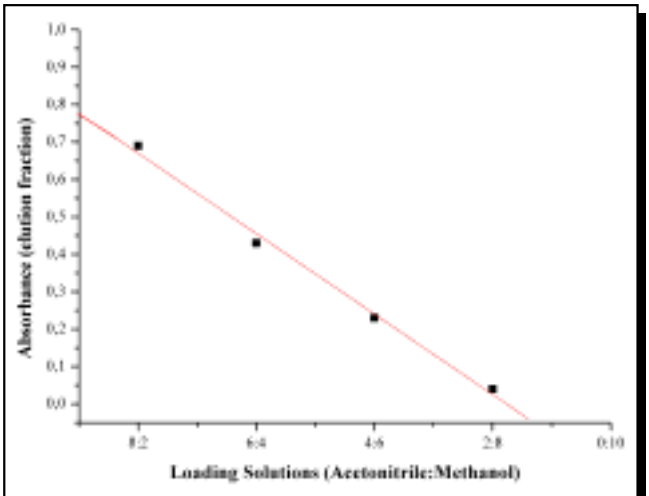

Figure 3: Graph of elution fraction absorbance versus loading solutions

gives us the theoretical influence of methanol in molecular recognition of the template.

In figure 3 is possible to note how the methanol decreased the interaction polymer-template. If methanol was used no more than 20%, we can consider that its influence is not so relevant(Absorbance of a hypothetical solution of B-HM only in Acetonitrile is 0.78 instead of 0.71 in a mix(acetonitrile:methanol 8:2). In fact, the methanol decreases significantly the recognition just when its concentrations is no less than 30 %.

CONCLUSIONS

A new method to determine HM was developed. We synthesized a molecularly imprinted polymer for solid phase extraction (MISPE) of derivatized homocysteine and we evaluated its performance. The present results may provide a useful approach for the development of straight-forward method for the rapid identification and detection of homocysteine.

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