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Journal of Chromatography A, 987 (2003) 103–109
JOURNAL OF CHROMATOGRAPHY A
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# Preparation of a molecularly imprinted polymer for the solid-phase extraction of scopolamine with hyoscyamine as a dummy template molecule

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**Abstract**

Molecularly imprinted polymers (MIPs) selective for scopolamine were produced using hyoscyamine (a close structural analogue) as template molecule. The produced polymers were used as media for solid-phase extraction, exhibiting selective binding properties for the analyte from biological samples. Human and calf urine and serum were processed on the MIP under various extraction protocols. The best performance was observed after loading the analyte in aqueous environment facilitating retention on the MIP by non-selective hydrophobic interactions. The MIPs were subsequently washed using an optimised solvent system to enable selective desorption of the analyte. Other related and non-related compounds were accessed to evaluate molecular recognition properties. Recoveries of up to 79% were achieved for the analyte of interest from biological samples.


*Keywords:* Molecular imprinting; Solid-phase extraction; Hyoscyamine; Scopolamine; Tropane alkaloids; Belladonna alkaloids; Alkaloids

## 1. Introduction

Molecular imprinting is now an established method for the production of artificial receptors that can be applied in separation procedures and chemical analyses. Solid-phase extraction (SPE) is one of the major application fields for molecularly imprinted polymers (MIPs) [1–5]. However a significant problem often encountered is the ''bleeding'' of template trapped within the polymer network. As a rule more than 1% of the amount of the template used in the pre-polymer mixture remains bound in the polymer even after thorough washing of the polymer [4]. When the MIP is used for sample preparation even minute quantity leaking from the MIP, will interfere and alter the experimental results to a great extent, especially in the case of trace analysis [4]. An elegant solution to this problem is the utilisation of an analyte analogue, instead of the analyte itself, as template, during polymerisation (''dummy template'') [6–13]. With this approach, any leakage of the ''dummy template'' will not interfere with the analysis, provided that a procedure capable of

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0021-9673/02/$ – see front matter 
doi:10.1016/S0021-9673(02)02048-4

separating the two analogue molecules is applied prior to analyte quantification.

Hyoscyamine and scopolamine are tropane alkaloids known as belladonna drugs, since they are derived from Solanaceae plants. The most known representatives of this plant family are *Atropa belladonna* and *Datura stramonium*, which mainly yield atropine (racemic hyoscyamine). Scopolamine is the major alkaloid in *Scopolia carniolica* and *Hyoscyamus niger*. Numerous cases of intoxication and deaths of animals and humans have been reported following consumption of parts of the above or related plants. Despite the above fact and the repulsive effects in human moral fibre, both alkaloids are still a subject of abuse especially in provincial locations. Furthermore, hyoscyamine and scopolamine represent a group of alkaloids with significant pharmacological interest due to their antimuscarinic action. The compounds have widespread use as anticholinergic, anti-infective, and analgesic pharmaceuticals. Hence there is an interest for the development of analytical methods for their determination in plant and biological samples from diverse fields such as pharmaceutics, forensic toxicology and veterinary medicine.

Quantitative determination of hyoscyamine and scopolamine is conducted mostly by reversed-phase HPLC [14]. However the lack of a strong chromophore in the alkaloids molecule necessitates detection at low UV wavelengths (205–210 nm). At these wavelengths, strong interferences from sample matrix are expected and enhanced purification prior to HPLC analysis becomes essential. Hence, investigation of selective means of alkaloid isolation from biological matrices is of significant interest.

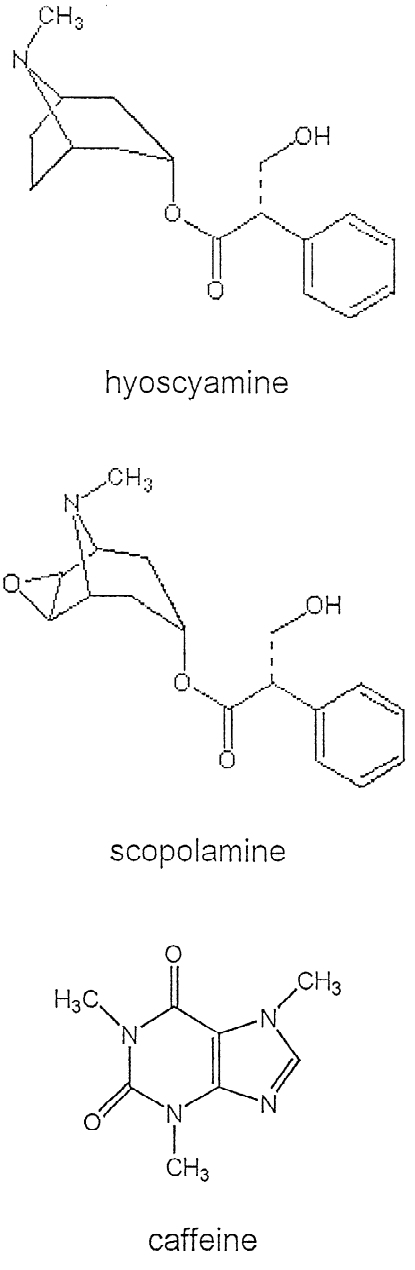


Fig. 1. Structures of hyoscyamine, scopolamine and caffeine.

## 2. Materials and methods

### *2.1. Materials*

Hyoscyamine (**1**), scopolamine **(2),** and caffeine **(3)** were from Sigma (St. Louis, MO, USA) (Fig. 1). Ethylene glycol dimethacrylate (EDMA), methacrylic acid and 2,2′-azobisisobutylnitrile (AIBN) were from Fluka (Buchs, Switzerland). All solvents used (acetonitrile, methanol, ethanol) were of HPLC grade and were obtained from Riedel-de Haen (Seelze, Germany). Water was double deion-

ised and filtered though a 20-μm filter (Schleicher & Schuel, Dassel, Germany).

### 2.2. Polymerisation

The compositions of the pre-polymerisation mixtures are given in Table 1. The procedure from polymer preparation was as follows: the template and the functional monomer (methacrylic acid, MAA) were dissolved in 2 ml of toluene in 20-ml glass tubes. Next the cross linker (EDMA) and the initiator (AIBN) were added to the above solution. The tubes were sparged with $N_2$ for 5 min and subsequently sealed and heated in a water bath at 60 °C for 16 h. The tubes were smashed and the polymers obtained were extracted using the Soxhlet apparatus in order to remove the template from the polymer matrix. The polymer was extracted for 16 h using a mixture of methanol–acetic acid (9:1, v/v), resulting in a sum of 30–35 solvent cycles (one recycle at approximately 30 min). To estimate the effectiveness of the template removal, the concentration of hyoscyamine in the extraction solvents was determined by HPLC. After the extraction the materials were ground using laboratory mortar and pestle and wet sieved within the desired particle size (20–50 μm) using 10-cm diameter laboratory sieves. Fine particles were removed by repeated sedimentation in methanol–water (80:20, v/v).

In order to verify that retention of hyoscyamine was due to molecular recognition and not due to non-specific binding, control non-imprinted polymers (NIPs) were prepared following the same procedure (including extraction), but with the omission of the template (hyoscyamine).

Table 1
Composition of polymerisation mixtures for imprinted (P1–P5) and non-imprinted polymers (B1–B5)

| Polymer | Template (mmol) | MAA (mmol) | EDMA (mmol) |
|---|---|---|---|
| P1 | 0.235 | 2 | 4 |
| B1 | – | 2 | 4 |
| P2 | 0.235 | 2 | 5 |
| B2 | – | 2 | 5 |
| P3 | 0.235 | 2 | 6 |
| B3 | – | 2 | 6 |
| P4 | 0.235 | 2 | 7 |
| B4 | – | 2 | 7 |
| P5 | 0.235 | 2 | 8 |
| B5 | – | 2 | 8 |

### 2.3. HPLC determinations

HPLC analysis was performed using a Merck–Hitachi 655 A-11 liquid chromatograph, a 655 A variable-wavelength UV monitor operating at 210 nm, a 655 processor/integrator, and a Rheodyne (Cotati, CA) 7125 injection valve. The injection volume was 10 μl throughout the study. Separations were carried out on a 15 cm×4.6 mm, 5 μm Supelco (Bellefonte, PA, USA) Discovery $C_{18}$ column. The mobile phase was a mixture of acetonitrile–0.05 *M* aqueous $CH_3COONH_4$ (15:85, v/v) and the flow was maintained at 0.8 ml/min.

### 2.4. MIP-SPE

Two hundred mg of each of the corresponding polymers were dry packed in the SPE cartridges. During the extraction experiments the MIP-SPE columns were loaded with 2 ml of a mixture of hyoscyamine and scopolamine of an appropriate concentration (2–50 μg/ml each). Two min after each sample/solvent loading to the MIP-SPE cartridge, vacuum was applied to facilitate the pass of the solvent through the bed. Different protocols were applied (as described later), utilising different solvents during conditioning, loading, washing and eluting of the MIP-SPE column. All the applied fractions were collected and evaporated to dryness (at 45 °C under a stream of $N_2$). The residues were reconstituted in 200 μl of the mobile phase and an aliquot was analysed using HPLC.

#### 2.4.1. Study of breakthrough volume in loading

To investigate the breakthrough volume in loading instead of loading 1 ml×20 μg/ml, experiments of loading 10×1 ml×2 μg/ml were performed. In this experiment 10 succeeding aliquots of 1 ml were loaded on the MIP-SPE cartridge resulting in a total volume of 10 ml. Next, the selected extraction protocol was followed. In all loading, washing and

elution steps the eluate was collected evaporated to dryness and after reconstitution (in the mobile phase) it was analysed by HPLC.

*2.4.2. Study of multiple elution steps*

To investigate the effect of elution volume on MIP-SPE the selected protocol was altered and instead of recovering the analytes in 2×2 ml of the selected solvent, 10 aliquots of 400 μl of the same solvent were applied. Each aliquot was collected evaporated to dryness and after reconstitution (in the mobile phase) analysed using the HPLC.

*2.4.3. Non-related probe*

To verify that the retention of the analytes was due to the molecular imprinting effect, a non-related probe was also used for an extraction simultaneously with the analytes. Caffeine was selected for that purpose (see Section 3) and simultaneous extraction of caffeine and scopolamine was performed in both the MIP and NIP cartridges. One ml of a solution of caffeine and scopolamine (20 μg/ml) was loaded on the SPE cartridges. The selected extraction protocol was applied; eluates from all the extraction steps were collected, analysed in HPLC and respective yields were calculated.

*2.5. Extraction of scopolamine from human serum*

Two hundred μl of human serum were mixed with 44 μl of aqueous scopolamine solution (50 μg/ml) and next 200 μl of cold acetonitrile were added. The mixture was vortex mixed for 1 min and centrifuged at 3000 rpm for 5 min. An aliquot of 200 μl of the supernatant was diluted to 1 ml with the addition of a pH 5 HCl solution; the mixture was applied on the MIP-SPE cartridge. The SPE protocol was applied and the resulting solution was analysed on HPLC.

*2.6. Extraction of scopolamine from human urine*

Two ml of human urine was adjusted to pH 5 with the addition of HCl (1 *M*). An aliquot of the scopolamine stock solution was added to reach concentration of 5, 10 and 20 μg/ml. One ml of the resulting solution was loaded to MIP-SPE cartridge and the selected protocol was followed.

*2.7. Extraction of scopolamine from calf serum and urine*

Calf serum was treated similarly to human serum. Following the addition of an appropriate volume of the scopolamine stock solution, serum proteins were precipitated by addition of cold acetonitrile. The selected SPE protocol was applied and the resulting solution was analysed on HPLC.

Calf urine was treated in a manner similar to human urine. In 2 ml of sample, HCl (1 *M*) was added to adjust the pH to 5. An aliquot of the scopolamine stock solution was added to reach concentration of 5, 10 and 20 μg/ml in the resulting solution. One ml was extracted in MIP-SPE and analysed in HPLC.

## 3. Results and discussion

*3.1. HPLC determinations*

In order to validate the properties of the produced polymers, an analytical tool was necessary, capable of determining the alkaloids of interest in treated extracts of biological fluids. The need for the HPLC analysis was 2-fold: first, the method should separate the analyte of interest (scopolamine) from the template–analyte analogue used to prepare the polymers (hyoscyamine). Second, the method should be capable of quantitative determination of scopolamine after the application of the MIP-SPE protocol in various matrices. With the developed HPLC system, elution of hyoscyamine and scopolamine was achieved within 12 min. Detection limits were at 30 ng for hyoscyamine and 20 ng for scopolamine. The method was used for quantitative purposes and the calibration curves obtained showed linearity range ($R^2$=0.9956) from 1 to 100 μg/ml. As can be seen in the corresponding chromatograms (Fig. 2), the method sufficed for the determination of the alkaloids in biological fluids after extraction by MIP-SPE.

*3.2. MIP-SPE*

MIPs are expected to exhibit enhanced selectivity for their template in application environments similar

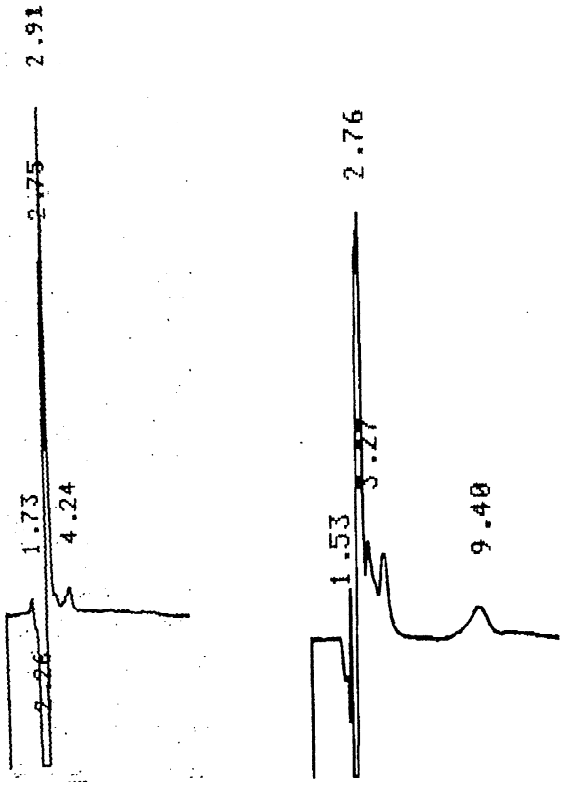


Fig. 2. HPLC analysis of human serum prior (left) and after spiking with scopolamine (right) after MIP-SPE on the MIP with the selected protocol. Conditions in text.

to those of the polymerisation conditions. Therefore in many MIP-SPE protocols the analytes are loaded as solutions in the polymerisation solvent (porogen). It is theorised that the formation of monomer–template complexes will be enhanced, thus selective binding of the template is expected to occur. Subsequently, the sorbent is rinsed with weak solvent to wash out matrix components and finally the analyte of interest is eluted with an appropriate solvent [4].

Another option is to load the sample in conditions of non-selective binding and apply subsequent washing steps to facilitate selective desorption of the analyte of interest. Typically this occurs by loading aqueous samples, where the analytes are retained by hydrophobic, non-selective interactions. At a second stage a solution/solvent is applied to disrupt these interactions and simultaneously enable selective recognition of the analyte by the MIP [4,15]. This mode although requiring more steps, is suitable for the straightforward loading of various aqueous samples such as biofluids, environmental samples, etc.

The MIPs were tested with the second approach of selective desorption, aiming at a straightforward treatment of biological fluids (i.e. loading in aqueous conditions). Initially the several different produced MIPs were validated as media for SPE. The selected MIP (P5) gave the superior performance and was further used for further optimisation. This can be attributed to the high cross-linker molar ratio in the pre-polymerisation mixture (see Table 1). High cross-linker concentrations result in higher rigidity of the polymeric network, an issue necessary for enhanced molecular imprinting effect.

Various extraction protocols were studied with altering elution and washing steps; the selected protocol exhibited the highest molecular recognition rate, i.e., the highest yield in the MIP and the highest difference between the MIP and the NIP.

The effect of the pH value of loading and washing steps on the extraction recovery was investigated in both basic and acidic range. Loading of aqueous solutions was investigated in a wide pH range: from acidic (pH 3) to alkaline pH (pH 11). A washing step typically with 1 ml of water or acetonitrile was utilised to remove non-retained components. Elution was done with 1 ml of acetonitrile–acetic acid (9:1, v/v).

When loading in neutral pH, scopolamine was eluted from both columns (MIP and NIP) in the washing step of 1 ml with acetonitrile. Changing the pH value of the loading step to alkaline (9 and 11), scopolamine was retained by the polymers and was eluted in the final elution step, but with similar recoveries. Acidic loading (pH 3, 5, and 6) resulted in better recoveries in the imprinted polymer. However in general the yield was not satisfactory ranging from 40 to 60%, and the behaviour of both polymers was similar to a great extent.

To overcome this and reveal the differentiation of the two sorbents, a ‘‘molecular recognition’’ step was added in the alkaline pH protocol. In this step toluene (the polymerisation solvent) was used as a washing solvent after a first washing with 1 ml of water. Using this protocol scopolamine was recovered in the final elution step of acetonitrile–acetic acid (9:1, v/v) in both polymers (MIP and NIP) no matter the loading solution pH value (7, 9, or 11).

Altering the pH value of the washing step, it was observed that washing with ''alkaline'' acetonitrile (pH 9 or 11) resulted in quantitative elution from the NIP and significant elution from the MIP. In contrast scopolamine was still retained on the MIP in slightly acidic pH range. Therefore the final selected MIP-SPE protocol comprised the following steps:

(1) loading of the MIP with 1 ml of an aqueous solution of Sco 10 μg/ml (pH 5),

(2) washing twice with 1 ml of a mixture of acetonitrile–aq. HCl (adjusted to pH 6),

(3) eluting twice with 2 ml of an acetonitrile–acetic acid (9:1, v/v) mixture.

With this extraction protocol the MIP exhibited enhanced retention for scopolamine and elution of it in the final step of ''acidic'' solvent mixture. In contrast, in the NIP most of the analyte was recovered at the washing step, indicating non-selective binding. Results are tabulated in Table 2. With the selected conditions hyoscyamine was also extracted from aqueous solutions, either separately or simultaneously with scopolamine. In the tested concentrations (1, 2, 5, 10 and 20 μg/ml) the MIP provided satisfactory capacity retaining selectively both alkaloids.

### *3.2.1. Breakthrough study*

The results of the breakthrough study are summarised in Table 3. This study revealed a weaker retention of the analytes on the NIP polymer, where breakthrough occurred in the ninth loading step. In contrast on the MIP no breakthrough was observed and the analytes were recovered in the final elution step. Loading a constant quantity of the analyte but in 10-fold sample volume indicates the preconcentration potential of the MIP-SPE protocol.

Table 2
Recovery of scopolamine in the imprinted polymer (MIP) and the non-imprinted polymer (NIP) with the selected SPE protocol

| | Found (μmg) | |
|---|---|---|
| | MIP | NIP |
| Load | 0.00 | 2.20 |
| Wash | 0.15 | 6.86 |
| Elute | 9.49 | 0.65 |
| Total | 9.64 | 9.71 |

Added 10 μg.

Table 3
Results of the breakthrough load study

| | Found (μg) | |
|---|---|---|
| | MIP | NIP |
| Load 1 | 0.0 | 0.0 |
| Load 2 | 0.0 | 0.0 |
| Load 3 | 0.0 | 0.0 |
| Load 4 | 0.0 | 0.0 |
| Load 5 | 0.0 | 0.0 |
| Load 6 | 0.0 | 0.0 |
| Load 7 | 0.0 | 0.0 |
| Load 8 | 0.0 | 0.0 |
| Load 9 | 0.0 | 0.93 |
| Load 10 | 0.0 | 3.71 |
| Wash | 1.29 | 12.55 |
| Elution | 19.15 | 1.55 |
| Total | 20.44 | 18.74 |

Ten loading steps of 1 ml (2 μg/ml) scopolamine solution.

### *3.2.2. Multiple elution*

In the study of multiple elution steps, breakthrough of the NIP was already observed in the wash fraction as can be seen in Table 4. In contrast, on the MIP no breakthrough was observed during washing. Elution of the analytes occurred with the elution solvent and mainly in fractions 1–5. This study was used to optimise the conditions of the elution step. Thus, further selectivity of the polymer can be obtained by applying several elution steps but with sacrifice in simplicity and time consumption.

Table 4
Results of the multiple elution study

| | Found (μg) | |
|---|---|---|
| | MIP | NIP |
| Wash | 0.38 | 20.20 |
| Elution 1 | 12.39 | 3.65 |
| Elution 2 | 6.10 | 0.99 |
| Elution 3 | 2.55 | 0.60 |
| Elution 4 | 1.94 | 0.0 |
| Elution 5 | 1.46 | 0.0 |
| Elution 6 | 0.99 | 0.0 |
| Elution 7 | 0.46 | 0.0 |
| Elution 8 | 0.0 | 0.0 |
| Elution 9 | 0.0 | 0.0 |
| Elution 10 | 0.0 | 0.0 |
| Total | 26.27 | 25.44 |

Loading 1 ml (30 μg/ml) scopolamine solution (eluting solvent–acetonitrile–acetic acid, 9:1, v/v).

### 3.2.3. Simultaneous extraction of scopolamine and caffeine (as a non-related probe)

As a non-related prove the selected compound should not be a belladonna alkaloid but at the same time it should not differ greatly in terms of functionality and molecular polarity. Caffeine was chosen to serve as a non-related probe. Caffeine is also an alkaloid but of different molecular structure, shape and size. Comparing the retention behaviour of caffeine and the belladonna alkaloids could provide further evidence of molecular recognition in the MIP-SPE procedure. From the performed experiments (Table 5) it can be seen that, while the NIP does not retain either caffeine or scopolamine, the MIP exhibits selective retention only for the template ''analogue'', scopolamine. This indicates a definite molecular imprinting effect.

### 3.3. Application in real samples

For the pre-treatment of human urine MIP-SPE, provided good clean-up but with lower recoveries ranging from 46 to 59%. Similar results were obtained from the treatment of calf urine. In general the yield from urine samples was not satisfactory necessitating further studies.

In contrast, for serum samples (either human or calf) the recovery was acceptable. The use of MIPs as media for the sample pre-treatment of human serum provided enhanced sample clean up. The recognition properties of the polymer improved the purification of the sample and provided adequate recovery, ranging from 60 to 79% depending on concentration.

Table 5
Extraction recoveries of scopolamine and caffeine (as a non-related probe) after MIP-SPE with the selected protocol

| | Found (μg) | | | |
|---|---|---|---|---|
| | Caffeine | | Scopolamine | |
| | MIP | NIP | MIP | NIP |
| Load | 3.32 | 2.48 | 0.00 | 0.14 |
| Wash | 15.98 | 16.35 | 0.00 | 19.32 |
| Elute | 0.48 | 1.25 | 20.00 | 0.34 |
| Total | 19.75 | 20.08 | 20.00 | 19.80 |

Added 20 μg of each alkaloid.

## 4. Conclusions

The results obtained in this work indicate the feasibility of utilising polymers imprinted with a structural analogue for the selective extraction of scopolamine. The produced MIP recognised and retained selectively hyoscyamine (the template) and scopolamine the analyte of interest during SPE applications. Other alkaloids used as non-related probes were not retained from the MIP-SPE cartridge. Breakthrough during loading aqueous solutions was observed only in the NIP. The MIP retained the analyte from large sample volumes (10 ml) indicating the pre-concentration potential of the method. The elution profile from both MIP and NIP cartridge was used to optimise the elution conditions. This study indicated further differentiations in the behaviour of the two polymers. The developed MIP-SPE protocol provided enhanced sample clean-up and adequate recovery from biological samples.

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