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Molecularly imprinted polymers selective for testosterone; potential application for GC/C/IRMS sample clean-up

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Abstract

The aim was to synthesize a molecularly imprinted polymer (MIP) of sufficient selectivity for testosterone to facilitate sample clean-up for subsequent analysis by gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). A structurally similar analogue, 5α-dihydrotestosterone (DHT), was chosen as the template, rather than testosterone, to avoid the possibility of residual testosterone leaching from the MIP into sample extracts.

A series of non-covalently DHT-imprinted polymers was synthesized using both conventional monomers (methacrylic acid, (trifluoromethyl)acrylic acid) and cross-linkers (ethyleneglycol dimethacrylate, divinylbenzene), and also a novel cross-linker, 5α-androstane-3α,17β-diacryloxy ester (AnDA). The ability of MIPs to discriminate testosterone from epitestosterone was evaluated by HPLC using three different mobile phases: ethyl acetate (EtOAc), dichloromethane:2-butanone (CH_2Cl_2:2-butanone) (90:10) and CH_2Cl_2:acetic acid (AcOH) (99:1). The MIP prepared with DHT/(trifluoromethyl)acrylic acid/divinylbenzene/ AnDA/chloroform ($CHCl_3$) using a using a CH_2Cl_2:AcOH (99:1) mobile phase, showed the greatest selectivity for testosterone compared with epitestosterone. Ongoing work is focused on investigating the application of non-covalently prepared MIPs for solid phase extraction.

Introduction

Molecular imprinting is a process for making a synthetic polymer containing recognition sites for a certain template. An ion, a molecule, or some complex macromolecular assembly (nucleic acid, protein, cell or microorganism) acts as a template, which facilitates the formation of recognition sites in the polymer matrix during the polymerization process (Alexander *et al.*, 2006). The most widespread and most successful approach to molecular

imprinting involves polymerization of vinylic monomers. Apart from the template, a functional monomer, a cross-linker and an initiator take part in the process. All these compounds are dissolved in an appropriate porogenic solvent prior and then polymerization is performed by the addition of an initiator. Monomer molecules are arranged around the template and interact with it through various types of interactions forming the monomer-template complex (Figure 1). The complex is polymerized in the presence of the cross-linker and consequently an insoluble imprinted matrix is formed. Once the polymerization is complete, the removal of the template follows, leaving in the polymer mass the vacant recognition sites, the imprint matching the shape and the size of the template. The imprinted polymer is then able to interact with molecules similar in structure to the template when applied in chemical analysis.

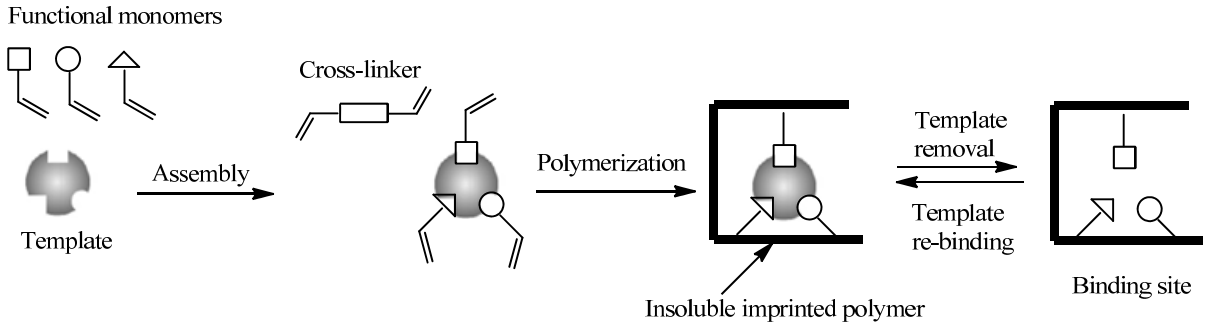


Figure 1: Schematic presentation of the molecular imprinting process. The figure was adapted from Haupt (Haupt, 2001).

The majority of work was performed using the "standard" non-covalent approach with methacrylic acid (MAA) and ethyleneglycol dimethacrylate (EDMA) as the functional monomer and the cross-linker, respectively. However, there are examples of using novel compounds and methods for molecular imprinting such as cyclodextrins or porous silica (He *et al.*, 2006; Hishiya *et al.*, 1999). Attempts to synthesize molecularly imprinted polymers selective for testosterone have been reported (Cheong *et al.*, 1997; Cheong *et al.*, 1998; Rachkov *et al.*, 1998; Salvador *et al.*, 2007).

The aim of this work was to make a MIP that would selectively bind testosterone (T) from urine samples in order to simplify sample clean-up for subsequent analysis by GC/C/IRMS. Testosterone retained by the MIP and eluted afterwards would be analyzed by GC/C/IRMS. Ideally, the molecularly imprinted polymer selective for testosterone should discriminate

testosterone from epitestosterone, its 17α epimer. These two steroids co-elute under the GC conditions that were developed for GC/C/IRMS analysis. A novel aspect of the current investigation included the use of a derivative of a 17β-androstanediol as a cross-linker, on the basis that its similar structure to testosterone would enhance non-covalent interaction in subsequent analysis. The template employed was 5α-dihydrotestosterone (DHT) to circumvent any potential problems with the polymer leaching if a testosterone template had been used instead.

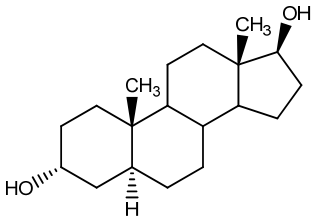
Materials and methods

General

Chemicals used in organic synthesis were obtained from Steraloids (USA), Sigma Aldrich (UK), Acros Organics (UK), Fluka (UK), Alfa Aesar (UK) and Fisher Scientific (UK). Chemicals were used as received unless otherwise stated. All solvents were either laboratory reagent grade or HPLC grade. Dry solvents were obtained from a solvent purification system (Pure Solv, Innovative Technologies, USA). Basic aluminium oxide for column chromatography was purchased from Fluka (Switzerland). Stainless steel chromatography columns (50 mm × 4.6 mm) were purchased from Hichrom (UK).

Organic synthesis

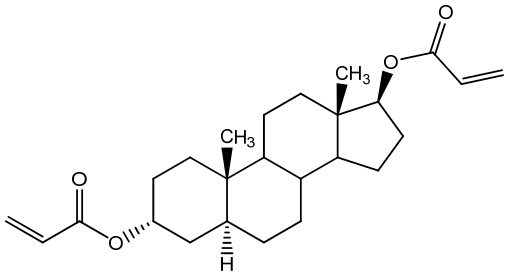
1. 5α-androstane-3α,17β-diol



For reasons of economy, it was decided to synthesize 5α-androstane-3α,17β-diol using DHT as the starting material. DHT (2 g, 6.88 mmol) was added to an oven dried twin-neck round bottom flask. The flask was fitted under argon and dry tetrahydrofuran (THF, 50 mL) was added. When DHT was dissolved, the flask was cooled with a dry ice/acetone bath and K-Selectride® (Potassium tri-*sec*-butylborohydride, 1 M solution in THF, 10.32 mL, 10.32 mmol) added dropwise. The reaction was kept at ~ -80 °C for 8 hours and then left running at room temperature overnight. Deionised water (50 mL) was added to the flask and liquid-liquid extraction performed with $CHCl_3$ (three times). The $CHCl_3$ layers were collected

and purified with deionised water (three times). The purified $CHCl_3$ layer was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum. The white solid 5α-androstane-3α,17β-diol was precipitated from CH_2Cl_2:methanol (CH_3OH) (95:5). The ^{1}H-NMR spectrum of commercially available 5α-androstane-3α,17β-diol matched the ^{1}H-NMR spectrum of our product.

2. 5α-androstane-3α,17β-diacryloxy ester (AnDA) (the novel steroid based cross-linker)



5α-Androstane-3α,17β-diol (1.85 g, 6.325 mmol), N,N-dimethyl-4-amino pyridine (463.63 mg, 3.795 mmol) and triethylamine (4.40 mL, 31.625 mmol) were added to an oven dried twin-neck round bottom flask. The flask was fitted under argon and dry THF (100 mL) was added. When all the compounds were dissolved, the flask was cooled to 0 °C (ice bath) and acryloyl chloride (2.261 mL, 27.83 mmol) was added dropwise very slowly. The flask was kept on ice during the addition of the acryloyl chloride and for an additional 2 hours afterwards. The mixture was then stirred at room temperature for the next 24 hours. Saturated $NaHCO_3$ solution (100 mL) was added to the flask and liquid-liquid extraction performed with CH_2Cl_2 (three times). The CH_2Cl_2 layers were collected and purified with saturated $NaHCO_3$ solution (twice) and saturated NaCl solution (once). CH_2Cl_2 was removed under vacuum giving the darkish yellow solid product. This novel product was sufficiently pure for use without further purification.

Preparation of polymers

Divinyl benzene (DVB) was first purified by passing through a plug of basic alumina (Al_2O_3), and then distilled under high vacuum to remove polymerization inhibitors. Methacrylic acid (MAA) and ethyeneglycol dimethacrylate (EDMA) were also distilled under high vacuum to remove polymerization inhibitors. Polymers were prepared by bulk free radical polymerization. The chemicals used in the preparation of polymers are presented in Table 1.

Polymer	Template (mmol)	Monomer (mmol)	Cross-linker (mmol)	Solvent (mL)
MIP1	DHT (0.625)	TFMAA (2.5)	EDMA (10)	$CHCl_3$ (2.67)
MIP2	DHT (0.625)	MAA (2.5)	EDMA (10)	$CHCl_3$ (2.80)
MIP3	DHT (0.625)	TFMAA (2.5)	DVB (15.21)	$CHCl_3$ (3.05)
MIP4	DHT (0.625)	TFMAA (2.5)	DVB (13.67) AnDA (1.25)	$CHCl_3$ (3.05)

Table 1: Amounts of template, monomer, cross-linker and solvent used for non-covalent imprinting of DHT.

The template (DHT), functional monomer (MAA, (trifluoromethyl)acrylic acid (TFMAA)) and porogen ($CHCl_3$ dried over molecular sieves) were weighed into a glass vial and the mixture sonicated until all material was dissolved. The addition of the cross-linker (EDMA, DVB or AnDA) followed and the sonication was repeated. The initiator 2,2-dimethoxy-2-phenylacetophenone (25 mg) was then added, the vial sonicated again and purged with argon for 3 minutes. The vial was capped quickly and sealed with Parafilm. The polymerization was performed overnight at 5 °C under UV light at 365 nm. MIP1 and MIP2 were uncapped after the polymerization and ground. MIP3 and MIP4 were heated for 72 hours at 70 °C after polymerization to ensure the whole amount of DVB had polymerized and $CHCl_3$ had evaporated. Non-imprinted polymers (NIPs) as control polymers were prepared the same way as imprinted polymers but without addition of the template.

The vial containing the solid polymer was crushed and the polymer ground in a mortar with a pestle. Ground particles were suspended in approximately 100 mL acetone and left for approximately 30 seconds to form a sediment. Then the acetone layer containing all but the largest particles (> 50 μm) was decanted. This suspension was left for approximately 30 minutes to sediment and, the finest particles (< 5 μm) decanted. The remaining particles were resuspended in acetone and the whole procedure was repeated 3-4 times. This procedure was based on the experience with similar polymers and yielded 5-30 μm size polymer particles (as

measured under an optical microscope). The particles were suspended in CH_2Cl_2 and then packed into chromatography columns (50 mm × 4.6 mm) using a Shandon air-driven column packer. The template was removed from the polymer by flushing the column with CH_3OH:AcOH (8:2, v/v) overnight using an HPLC pump at 0.5 mL/min flow rate. HPLC analysis was performed on a Hewlett Packard 1090 liquid chromatograph with Chemstation operating software. Injections of testosterone (3 mg/mL) and epitestosterone (3 mg/mL) were done manually (5 μL injection volume) and chromatograms developed at 0.5 mL/min flow rate. The elution solvents were EtOAc, CH_2Cl_2:2-butanone (90:10) and CH_2Cl_2:AcOH (99:1).

Results and Discussion

The non-covalent imprinting approach was used in this work to make imprinted polymers. In this approach, non-covalent interactions are formed between the template and the monomer (Alexander *et al.*, 2006). DHT was chosen instead of testosterone as the template to overcome the possibility of template "bleeding". Template bleeding occurs due to the small amount of template remaining strongly bound to the polymer even after applying extensive procedures for template removal. This small amount of template bleeds slowly during the polymer application and interferes with analytical results (Ellwanger *et al.*, 2001), a potential problem of particular relevance to drug control in sport given the high sensitivity required, e.g. minimal required performance level of 2 ng/mL for some anabolic agents. To solve this problem, a close structural analogue of the compound of interest was chosen as the template (e.g. DHT and testosterone). If DHT bleeding still occurred, it would not interfere with the results of GC/C/IRMS analysis since DHT and testosterone do not co-elute under the GC conditions that had been developed.

MAA and TFMAA are commercially available functional monomers; EDMA and DVB are commercially available cross-linkers. Gore *et al.* reported the synthesis of functional monomers and cross-linkers containing covalently linked cholesterol (Gore *et al.*, 2004). Based on the work by Gore *et al.*, Mathivanan *et al.,* Fernández *et al.* and Patil *et al.,* the novel cross-linker AnDA was synthesized for the non-covalent imprinting of DHT (Fernández *et al.*, 2005; Mathivanan *et al.*, 1995; Patil *et al.*, 2008). 5α-Androstane-3α,17β-diol used for AnDA synthesis is commercially available but it was synthesized in-house to reduce cost, by reducing DHT with K-Selectride ®. This route to 5α-androstane-3α,17β-diol has not been reported previously but selective reductions of other 5α-androstane-3-ones to

the 3α-alcohols have been described (Göndös *et al.*, 1988). AnDA is then formed by reaction of 5α-androstane-3α,17β-diol with acryloyl chloride. The structural similarity of the steroid core of AnDA with DHT would enable van der Waals and hydrophobic interactions with DHT. This should create more selective recognition sites in the imprinted polymer. It was expected that this would increase the retention of testosterone on the molecularly imprinted polymer and thereby result in the polymer discriminating testosterone from epitestosterone, where the orientation of the C_{17} hydroxyl groups are opposed.

The re-binding of testosterone and epitestosterone to the different MIPs was tested by employing them as stationary phases in HPLC. HPLC analysis was performed using three different mobile phases: EtOAc, CH_2Cl_2:2-butanone (90:10) and CH_2Cl_2:AcOH (99:1). Different mobile phases were examined, because the future work aims to investigate MIP application for solid phase extraction. The retention of testosterone on all polymers using EtOAc was much weaker than using mobile phases based on CH_2Cl_2. It is known that macroporous imprinted polymers based on cross-linked acrylates swell to different extents in different solvents, hence recognition is usually best when MIPs are used in the same solvent as they were made in. CH_2Cl_2 is very similar to $CHCl_3$, which was used for the imprinting, but less hazardous. AcOH and 2-butanone, mobile phase modifiers, interact with the binding sites in the polymer and with the steroid. These interactions are comparable in strength to the non-specific interactions of the steroids with the polymers. Hence, these modifiers serve to reduce the retention but improve the selectivity. The best separation of steroids was achieved with CH_2Cl_2:AcOH (99:1) and for brevity only these results are presented (Figures 1-5).

Testosterone was retained more on each of the imprinted polymers than it was on the corresponding non-imprinted polymers. The shape and the size of the recognition sites match the three-dimensional structure of the template for imprinting. The structure of DHT, the template molecule in our work, is almost identical to the structure of testosterone; only the delta-4 bond being absent.

The strongest retention of testosterone was observed using MIP1 (DHT/TFMAA/EDMA/$CHCl_3$) (Figure 2). TFMAA interacts with DHT via hydrogen bonds and that interaction is stronger than the interaction of MAA with DHT (based on results from NMR titration experiments, for brevity data not shown). This stronger interaction is supported by MIP1 showing better retention of testosterone, and better discrimination between

testosterone and epitestosterone, than MIP2 (Figure 3). Improved results were obtained with MIP3 (Figure 4), synthesized from DHT, TFMAA, DVB and $CHCl_3$. EDMA has ester groups, which are hydrogen bond acceptors. TFMAA interacts with both DHT and EDMA in the polymerization of MIP1, which makes the recognition less effective. DVB was more suitable as the cross-linker than EDMA, since there are no interactions between TFMAA and DVB.

Apart from the functional monomer TFMAA, the new cross-linker, AnDA, contributes to the retention of testosterone on MIP4. AnDA and testosterone interact via van der Waals forces. Hydrogen bonds and van der Waals forces both act to retain testosterone and distinguish it from epitestosterone. The results obtained with MIP4 were the best to date, with near baseline resolution of epitestosterone and testosterone in our HPLC experiment (Figure 5).

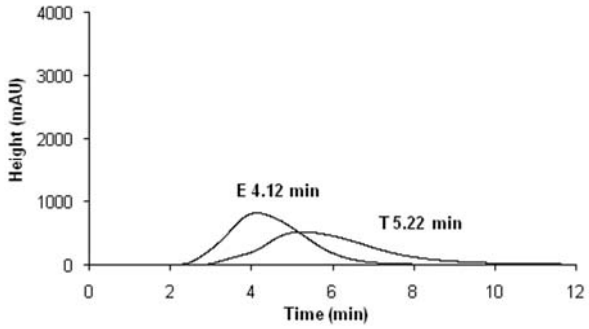


Figure 2: Separation of testosterone (T) and epitestosterone (E) using MIP1 with CH_2Cl_2:AcOH (99:1).

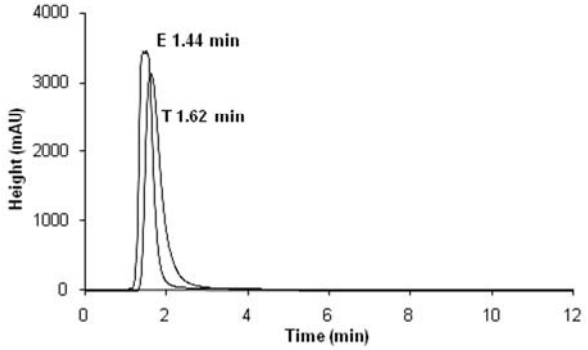


Figure 3: Separation of testosterone (T) and epitestosterone (E) using MIP2 with CH_2Cl_2:AcOH (99:1).

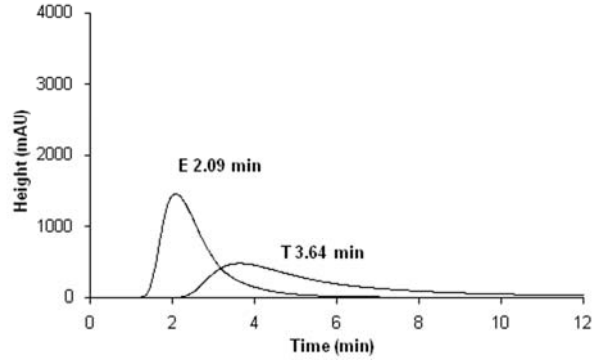


Figure 4: Separation of testosterone (T) and epitestosterone (E) using MIP3 with CH_2Cl_2:AcOH (99:1).

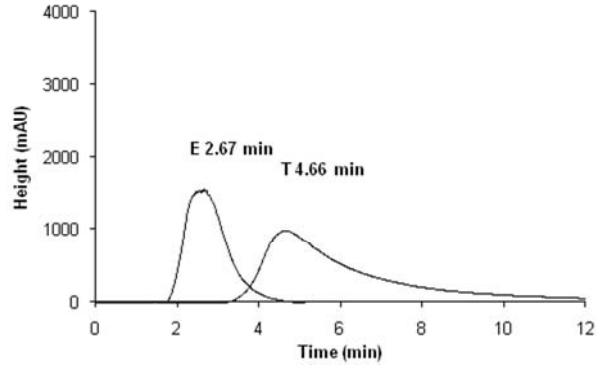


Figure 5: Separation of testosterone (T) and epitestosterone (E) using MIP4 with CH_2Cl_2:AcOH (99:1).

The application of MIPs for drug testing in sport has its potential, as recently discussed in the publication of Bui *et al.* (Bui *et al.*, 2010). Here we have demonstrated the potential of using an androgen derivative as a novel cross-linker to enhance specificity. Our future work focuses on testing the synthesized non-covalently DHT imprinted polymers for solid phase extraction of testosterone from urine in particular as a "clean-up" approach for GC/C/IRMS.

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