Steroids 76 (2011) 478–483

Contents lists available at ScienceDirect

Steroids

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

A molecularly imprinted receptor for separation of testosterone and epitestosterone, based on a steroidal cross-linker

Ivana Gavrilović[a], Karen Mitchell[b], Alan D. Brailsford[a], David A. Cowan[a], Andrew T. Kicman[a], Richard J. Ansell[b,*]

[a] Drug Control Centre, King's College London, London SE1 9NH, United Kingdom
[b] School of Chemistry, University of Leeds, Leeds LS2 9JT, United Kingdom

ARTICLE INFO

Article history:
Received 22 October 2010
Received in revised form 7 January 2011
Accepted 10 January 2011
Available online 19 January 2011

Keywords:
Molecular imprinting
Molecular recognition
Testosterone
Epitestosterone
5α-Dihydrotestosterone
HPLC

ABSTRACT

A series of molecularly imprinted polymers have been prepared and investigated as stationary phases in high performance liquid chromatography for the separation of testosterone and epitestosterone using non-polar mobile phases. The polymers were imprinted using 5α-dihydrotestosterone as template, and all retain testosterone more strongly than its 17α-OH epimer. The best polymer was prepared using trifluoromethylacrylic acid as functional monomer (interacting with the template via hydrogen bonds), divinylbenzene as 'inert' cross-linker, and chloroform as porogen. It also included a steroid-based cross-linker, which may interact with the template via van der Waals interactions to lend additional 'shape selectivity'. A 250 × 4.6 mm column packed with this polymer gave baseline resolution of testosterone and epitestosterone (15 μg each) in under 20 min. Preparation of the steroid based cross-linker included the selective reduction of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) to the 3α,17β-diol using K-selectride.

1. Introduction

Molecular imprinting is a versatile approach for the preparation of polymeric materials capable of selective recognition of given target molecules. The approach is based on complex formation between a template molecule and polymerisable monomers. The monomer–template interactions may be covalent or non-covalent in nature. After polymerisation, the template is removed, leaving vacant recognition sites, complementary in shape and functional groups to the original template [1].

A number of groups have reported different strategies for the molecular imprinting of testosterone [2–17] and related steroids including methyltestosterone [18] androst-5-ene-3β,17β-diol [19], castasterone [20], nandrolone [21], cortisol [22], 11α-hydroxyprogesterone and corticosterone [23] and have suggested applications for these polymers as protecting groups in functional group transformations, as selective adsorbents in screening approaches, or solid-phase adsorbents for forensic analysis. Molecularly imprinted polymers (MIPs) have also been reported for closely related compounds including estradiol, estrone and ethynylestradiol [24–34], for the analysis of these endocrine-disrupting compounds in environmental samples, and for cholesterol [7,35–42], for selective adsorption of this compound and its detection in bodily fluids.

For a new forensic assay for testosterone it was desirable to develop an adsorbent capable of efficiently resolving testosterone (T) and its 17α-isomer, epitestosterone (E, Fig. 1). Although T has been imprinted using non-conventional approaches such as cyclodextrins [17] or porous silica [16], the majority of works on T-imprinted polymers have employed conventional non-covalent imprinting using T as template, methacrylic acid (MAA) as functional monomer and ethyleneglycol dimethacrylate (EDMA) as cross-linker. The only work which reports selectivity for T over E is the recent one by Bui et al. [2], in which a methyltestosterone (template)–MAA (functional monomer)–EDMA (crosslinker) polymer showed a 20-fold selectivity for T over E in a competitive radioligand binding assay. However, when used in a solid phase extraction (SPE) protocol the polymer retained E almost as strongly as T (reflecting the different conditions used in the two assays – the radioligand binding assay probes only the strongest and most selective binding sites, while SPE uses a much larger number of sites on the polymer, suggesting these weaker sites are much less selective).

Abbreviations: AnDMA, 5α-androstane-3α,17β-dimethacryloxy ester; DHT, 5α-dihydrotestosterone,17β-hydroxy-5α-androstan-3-one; DVB, divinylbenzene; E, epitestosterone; EDMA, ethylene glycol dimethacrylate; HPLC, high performance liquid chromatography; MAA, methacrylic acid; MIP, molecularly imprinted polymer; NIP, non-imprinted polymer; SPE, solid phase extraction; T, testosterone; TFMAA, trifluoromethylacrylic acid.

* Corresponding author. Tel.: +44 113 3436415; fax: +44 113 3436565.
E-mail address: chmrja@leeds.ac.uk (R.J. Ansell).

0039-128X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2011.01.004

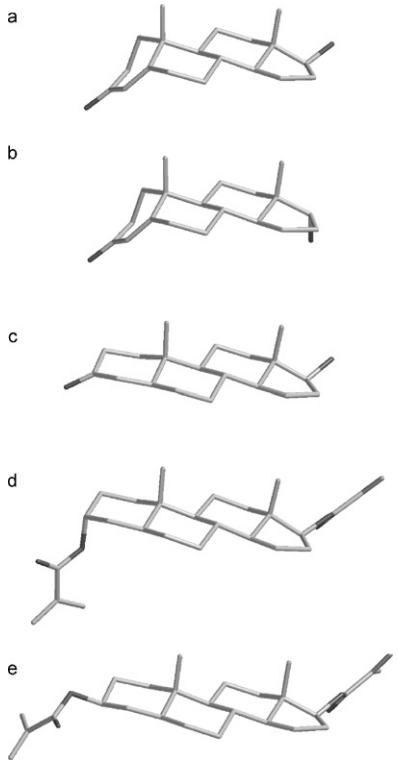

Fig. 1. Structures of (a) testosterone (T), (b) epitestosterone (E), (c) DHT, (d) AnDMA, (e) the AnDMA analogue with 3β-O-methacrylate. Structures calculated using PC Spartan Pro, by semi-empirical minimisation using an AM1 force field.

17β/17α selectivity has been observed between β-estradiol and α-estradiol on MIPs made from MAA/EDMA, both in competitive radioligand binding assays [25,34] and when the polymers were used as stationary phases in high performance liquid chromatography (HPLC) [32], although improved separation in HPLC was obtained with a MIP containing trifluoromethacrylic acid (TFMAA) in place of MAA [33].

In this work, a series of MIPs have been prepared and evaluated for the separation of T and E in HPLC using a non-polar organic mobile phase. The mobile phase was chosen to mimic the wash step in the envisaged application of the MIPs in SPE: this wash should elute E while T remains bound to the polymer. DHT (5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one) was chosen instead of T as the template to avoid false results in SPE due to 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 may interfere with analytical results [43]. To solve this problem, a close structural analogue to the compound of interest is chosen as the template. Bleeding of DHT from the polymer would not interfere with the subsequent quantitative measurement of T. In addition to evaluating different functional monomers and 'inert' cross-linkers, a functional cross-linker based on DHT (5α-androstane-3α,17β-dimethacryloxy ester, AnDMA, Fig. 1) has been investigated in the expectation that van der Waals stacking interactions might lend additional selectivity to the imprinted sites as has been demonstrated by others in the case of cholesterol [40,44].

2. Experimental

2.1. 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 and silica gel for column chromatography were purchased from Sigma Aldrich. Empty stainless steel chromatography columns were purchased from Hichrom (UK).

2.2. Organic synthesis

5α-Androstane-3α,17β-diol (**2**): DHT (2.00 g, 6.88 mmol) was added to an oven dried two-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 h and then left running at room temperature overnight. Deionised water (50 mL) was added to the flask and liquid–liquid extraction performed with $CHCl_3$ (3× 100 mL). The $CHCl_3$ layers were collected and purified with deionised water (3× 50 mL). The purified $CHCl_3$ layer was dried with anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The white solid **2** was purified by recrystallisation from CH_2Cl_2:CH_3OH (95:5) (^{1}H NMR consistent with the commercially available compound, see Supplementary information)

5α-Androstane-3α,17β-dimethacryloxy ester (AnDMA): **2** (2.92 g, 10 mmol), triethylamine (4.58 mL, 50 mmol), and 4-dimethylaminopyridine (0.1752 g, 6 mmol) were stirred in dry THF (150 mL) under argon and cooled to 0 °C using an ice bath. Methacryloyl chloride (4.3 mL, 44 mmol) was added dropwise and stirring continued at 0 °C for a further 2 h, then at room temperature for another 24 h. The mixture was added to saturated $NaHCO_3$ solution (150 mL) and liquid–liquid extraction performed with DCM (3× 150 mL). The DCM layers were collected, combined and washed with saturated $NaHCO_3$ solution (2× 150 mL) and saturated NaCl solution (1× 150 mL). The DCM fraction was then dried with $MgSO_4$, filtered and 4-methoxyphenol (40 mg) was added. Solvent was removed *in vacuo* to produce viscous yellow oil (2.00 g). Hexane (300 mL) was added to the oil with shaking and sonication, decanted into another flask and removed *in vacuo* to produce a viscous yellow oil, which crystallised on drying and storing at 5 °C to give a very pale yellow/white solid (2.45 g, 83.9%) m.p. 66–69 °C; found C 74.30%, H 9.20% ($C_{27}H_{40}O_4$ requires C 75.66%, H 9.41%); ^{1}H NMR δ (500 MHz; $CDCl_3$) 6.03 (2H, d, 2× =CH_2), 5.47 (2H, d, 2× =CH_2), 5.02 (1H, s, –CH at C3), 4.57 (1H, t, J 8.3 Hz, –CH at C17) 1.88 (3H, 2 s, 2× methacryl CH_3), 0.76 (3H, 2s, –CH_3 at C10 and C13) 2.25–0.67 (22H, m, –CH and –CH_2, remaining protons of steroid backbone); m/z (ESI) 446.3252 (M+NH_4^+ requires 446.3265); α_D = +15.9° ($CHCl_3$)

2.3. Preparation of polymers

Divinylbenzene (DVB) was purified by passing through a plug of basic alumina (Al_2O_3), then distilled under reduced pressure to remove polymerisation inhibitors. MAA and EDMA were also distilled under reduced pressure. Polymers were prepared by bulk free radical polymerisation. Amounts of chemicals used in the preparation of polymers are presented in Table 1.

Template (DHT), functional monomer (MAA or TFMAA) and porogen ($CHCl_3$ dried over molecular sieves) were weighed out into a glass vial and the mixture sonicated until all material was dissolved. Cross-linker (EDMA, DVB, AnDMA) was added and sonication was repeated until all material was dissolved. The initiator 2,2-dimethoxy-2-phenylacetophenone (25 mg) was added, the vial was sonicated again and purged with argon for 3 min. The vial was capped quickly and sealed with parafilm. Polymerisation