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A new derivative for oxosteroid analysis by mass spectrometry

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ABSTRACT

Here we report a new method for oxosteroid identification utilizing "tandem mass tag hydrazine" (TMTH) carbonyl-reactive derivatisation reagent. TMTH is a reagent with a chargeable tertiary amino group attached through a linker to a carbonyl-reactive hydrazine group. Thirty oxosteroids were analysed after derivatisation with TMTH by electrospray ionization mass spectrometry (ESI-MS) and were found to give high ion-currents compared to underivatised molecules. ESI-tandem mass spectrometry (MS/MS) analysis of the derivatives yielded characteristic fragmentation patterns with specific mass reporter ions derived from the TMT group. A shotgun ESI-MS method incorporating TMTH derivatisation was applied to a urine sample.

1. Introduction

Steroid hormones are small molecules synthesized from cholesterol, mainly in cells of the adrenal cortex, the gonads, and the placenta. The initial step in steroid hormone biosynthesis is cleavage of the bond between C-20 and C-22 of the cholesterol side-chain in a reaction catalysed by the enzyme P450scc (CYP11A1) to give pregnenolone. This steroid is subsequently metabolised further by a number of enzymes to the classical steroid hormones. Steroid hormones have important biological functions regulating development and metabolism [1]. The analysis of steroid molecules by mass spectrometry (MS) presents a number of challenges: they are mostly involatile, thermally labile molecules and require derivatisation prior to gas-chromatography (GC)-MS analysis; many are hydrophobic and poorly soluble in aqueous solvents making liquid chromatography (LC) separations difficult; unconjugated steroids are neutral molecules and thus not optimal for atmospheric pressure ionisation (API)-MS; and regulatory steroids may be present in biological samples at very low levels [2]. To overcome some of these challenges we have adopted a strategy where oxosteroids are derivatised with the "tandem mass tag hydrazine" (TMTH) reagent making them suitable for subsequent API-MS analysis (Fig. 1). This strategy is applicable to the analysis of both free and intact steroid conjugates, such as steroid sulphates and glucuronides which require hydrolysis before GC–MS analysis.

Thomson et al. originally developed amine-reactive tandem mass tags (TMT) for labelling of peptides [3]. Here we apply carbonyl-reactive TMTH in the analysis of oxosteroids. The TMTH reagent (Fig. 1) consists of a carbonyl-reactive hydrazine group connected through a linker to a readily protonated tertiary amino group. TMTH has an exact mass 256.19 Da and introduces a mass shift to the derivatised molecule of 238.18 Da (Fig. 1).

The aim of this study was to develop an electrospray ionization (ESI)-MS "shotgun" method for analysis of oxosteroids using the TMTH derivatisation reagent. Thirty oxosteroids including androgens, endogenous and exogenous corticosteroids, estrogens and progestagens, were analysed following derivatisation by direct infusion ESI-MS and ESI-tandem mass spectrometry (MS/MS) using a quadrupole – time-of-flight (Q-TOF) instrument. The method has been applied to profile steroid metabolites in urine.

2. Methods

2.1. Reagents and standards

Steroid standards nandrolone (19-nortestosterone, N), testosterone (T), betamethasone (Bet), prednisolone (Pred), dexamethasone (Dex), flumethasone (Flu), beclomethasone (Bec), cortisol (hydrocortisone, F), cortisone (E), estrone (E1), 5α-dihydrotestosterone (DHT) and pregnenolone (3β-hydroxypregn-5-en-20-one) were

Abbreviations: API, atmospheric pressure ionization; CID, collision-induced dissociation; ESI, electrospray ionization; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry or mass spectrum; MS/MS, tandem mass spectrometry; TMT, tandem mass tag; TMTH, tandem mass tag hydrazine; TOF, time-of-flight; SPE, solid phase extraction.

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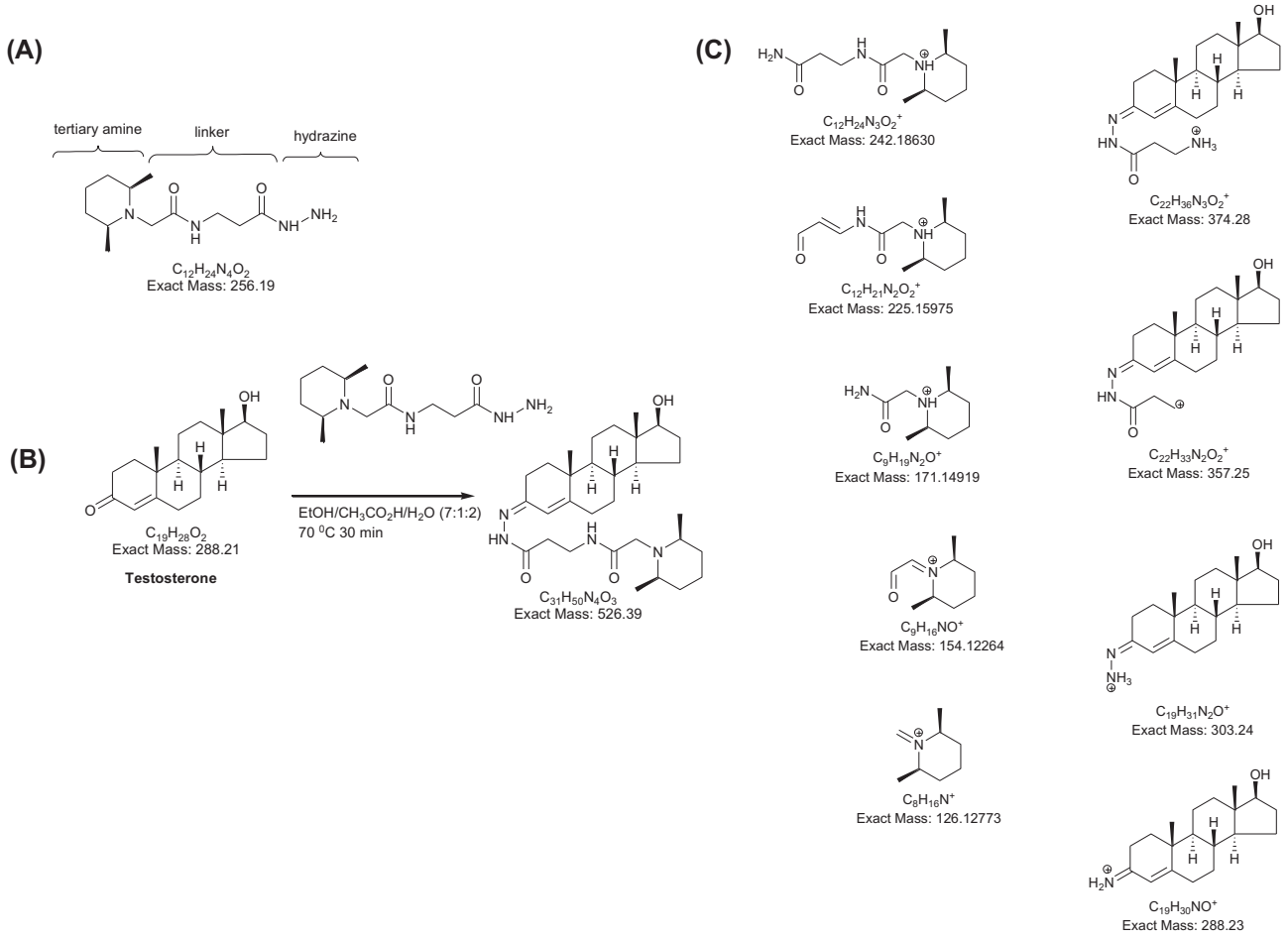

Fig. 1. (A) Generic structure of the TMTH derivatisation reagent. (B) Derivatisation reaction of oxosteroids with TMTH exemplified by testosterone. (C) Structures of the major fragment ions observed in the MS/MS spectrum of the $[M+H]^+$ ion of TMTH derivatised testosterone.

from Sigma–Aldrich (Poole, Dorset, UK). Androstenedione (AD) was from Fluka (Germany). Tetrahydrodeoxycortisol (THS), tetrahydrodeoxycorticosterone (THDOC), tetrahydrocorticosterone (THB) and tetrahydrocortisol (THF), were from Steraloids Inc (Newport, RI, USA). 5α-Androsterone (A), estrone sodium sulphate (E1-S) and 17-hydroxypregnenolone (3β,17α-dihydroxypregn-5-en-20-one) were purchased from Makaira Ltd (London, England). Dehydroepiandrosterone (DHEA) was from Avanti Polar Lipids (Alabaster, AL, USA). Sodium DHEA-3-sulphate (DHEA-S) was from TCI (Tokyo, Japan). 5α-Androsterone glucuronide (A-GlcA) was from LGC Standards GmbH (Wesel, Germany). Other steroids, 3α-hydroxy-5β-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one, 3α-hydroxy-5α-pregnan-20-one, [11,11-$^2H_2$]-3β-hydroxy-5α-pregnan-20-one, 3β-hydroxypregn-4-en-20-one, 3β-hydroxypregn-5,16-dien-20-one, [3,4-$^{13}C_2$]-progesterone (pregn-4-ene-3,20-dione) were from previous studies [4]. For steroid structures see Supplementary Fig. S1.

Solid phase extraction (SPE) cartridges, certified Sep-Pak C18, 3cc, 200 mg were from Waters Inc. (Milford, Massachusetts, USA), Leur Lock syringes were from BD Biosciences, (Oxford, UK). Solvents were from Fisher-Scientific (Loughborough, Leicestershire, UK) of HPLC or Analytical grade. Acetic acid 100% AnalaR Normapur was from VWR International S.A.S, (Baire, France). The derivatisation reagent TMTH (Fig. 1) was supplied by Proteome Sciences plc. (Surrey, UK).

2.2. Derivatisation of oxosteroids with TMTH

1 mg of each steroid was dissolved in 1 mL ethanol and vortex mixed, then diluted with ethanol to give working solutions of 0.1 and 0.01 μg/μL. The derivatisation procedure was as follows: 5 mg of TMTH (0.02 mmole) was dissolved in 60 μL of ethanol. 10 μL of steroid working solution (about 3.5 or 0.35 nmole) was added, followed by 20 μL of water (HPLC grade) and 10 μL of glacial acetic acid. The mixture was vortex mixed and warmed at 70 °C for 30 min on block heater. The sample mixture was dried using Scanspeed vacuum concentrator (Coolsafe, West Sussex, UK) and reconstituted in 1 mL of 10% methanol.

A Sep-Pak C18 cartridge was attached to Agilent Manifold Processing Station (Agilent Technologies, Inc., Wilmington, USA) and vacuum applied. The sorbent was washed with 6 mL 100% methanol followed by an equilibration rinse with 6 mL of 10% methanol. Derivatised steroids reconstituted in 1 mL of 10% methanol were applied to the column. The column was washed with 6 mL of 10% methanol (to remove excess of derivatisation reagent) and steroid-hydrazones eluted using 3× or 4 × 1 mL of 100% methanol (depending on the steroid), producing fractions 1–4. Mono-derivatives were present predominantly in the first and second fractions. Double derivatives were present also in the third and fourth fractions.

2.3. ESI-MS and -MS/MS analysis of derivatised oxosteroids

ESI-MS and ESI-MS/MS analysis was performed on a Q-TOF (Waters, UK) using nano-ESI needles (EconoTips12, Presearch Ltd.). The ESI-MS and MS/MS conditions were as follows: spray voltage, 1.8 kV; collision gas, argon; collision voltage from 5 V for MS, 30–40 V for MS/MS; LM from 10 to 15; HM from 10 to 15; ion polarity, positive. The Q-TOF was calibrated externally by