Investigating the Androgenic Activity of Ozonation Transformation Products of Testosterone and Androstenedione

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Abstract

This study investigates the impact of ozonation on the concentration of two androgen steroid hormones, testosterone and androstenedione, and on androgenic activity as measured using the YAS assay. While these compounds are remarkably similar in chemical structure and showed comparable removal profiles in the presence and absence of a hydroxyl scavenger (isopropanol), large differences between percent removal of target compounds using chemical analysis and percent removal of bioactivity was observed when small ozone doses were applied. The dynamic change and initial increase in bioactivity during ozonation can be attributed to transformation products. These results demonstrate the importance of combining chemical analysis and bioanalytical techniques not only to perform a comprehensive assessment of environmental risks but also as a tool to optimize treatment technologies proposed as a mean to mitigate the impact of contaminants of emerging concern.

Abbreviations

A, androstenedione; AUC, area under dose response curve; CEC's, contaminants of emerging concern; C_i, ozone concentration of stock solution; C_o, initial ozone concentration; DHT, dihydrotestosterone; EC_{50}, median effective concentration; ISO, isopropanol; Ko_3, ozone rate constant; LOD, limits of detection; O_3, ozone; ONPG, ortho-Nitrophenyl-β-galactoside; OTP, ozonation transformation products; T, testosterone; V_x, volume of ozone stock solution; YAS, yeast androgen screen

Keywords

Androgenicity, ozonation, transformation products

1. Introduction

In the recent years, the presence and fate of contaminants of emerging concern (CECs) in the environment has received much attention due to their potential bioactivity and persistence. Although many studies have investigated environmental impacts of CECs there has been less emphasis on transformation products [1, 2]. This is a concern since transformation products formed during natural attenuation or engineered treatment could elicit bioactivity due to the incomplete degradation of important pharmacophores present on the parent compound. For instance, the main metabolites of atrazine (diaminochlorotriazine,

deisopropylatrazine and deethylatrazine) could cause immunotoxicity in larval zebrafish [3]. Recently, it was found that during dehydrogenation process of testosterone by manure-derived bacteria the major transformation product formed was 1-dehydrotestosterone [4], an anabolic steroid with AR binding affinity of 56% greater than testosterone [2, 5]. This study highlighted how slight structural modifications relative to their endogenous analogs can induce drastically higher potency.

Much of the current research focused on defining environmental exposure and persistence of CECs often lacks strong links to biological hazards [2, 6]. Biological hazards may include transgenerational effects, impacts on microevolutionary processes, and immunotoxicity among others [3, 7]. This is often due to heavily relying on chemical analysis techniques for identifying CECs and/or transformation products in environmental samples. Although chemical analyses play a critical role in the characterization of transformation products, our understanding of the ecological implications remains incomplete. Considering these limitations, the use of bioanalytical tools performed in conjunction with chemical analysis is preferred, whereby the biological relevance is examined through inclusive bioactivity-based characterization [2]. This integrated approach will ultimately enable us to understand the bioactivity of complex samples where the identification of compounds responsible for bioactivity in samples cannot be conclusive or would be too time consuming and expensive [8, 9]. The need for integrating bioanalytical tools during routine chemical analysis of environmental samples is reinforced by findings from a recent study involving twenty international laboratories that applied 103 *in-vitro* bioassays to investigate their suitability for benchmark water quality and routine monitoring. It was also concluded that bioassay results could be used to assess and monitor treatment processes and in addition bioassays with specific receptor-mediated modes of action including estrogenic and androgenic effects showed the most promise for routine water quality screening applications [10]. Bioassays selected in the previous study to detect androgenic effects include MDA-kb2, AR-CALUX, and the yeast androgenic screen (YAS) assay among others.

CECs often end up at wastewater treatment plants and are not fully removed as a result of the limitations of conventional treatment methods. In an effort to remove these CECs, advanced treatment technologies such as membranes, UV, and chemical additives are proposed as tertiary treatments. Of these technologies, ozonation has been shown to effectively remove CECs including pharmaceuticals [11-14], personal care products [15], and pesticides [16]. Although studies have shown the removal of estrogen compounds such as 17α-ethinylestradiol during ozonation [1, 17] very few studies have investigated the removal of androgen steroid hormones during ozonation [11], yet these compounds have been reported to disrupt immune function, reproduction and induce masculinization [18]. A possible reason for this trend is the notion that estrogen compounds are more "promiscuous" than androgens as a result of the estrogen receptor containing to activity sites, α and β [19]. However, recent studies have wide structural diversity

among androgen receptor ligands and suggest that a number of exogenous compounds could potentially impact the androgenic receptor[19-21].

The objective of this study was to investigate the removal of androgenic compounds and their associated bioactivity during ozonation, with a particular attention to the effect of slight differences in chemical structure on the efficiency of the treatment and the residual bioactivity associated with ozonation transformation products (OTP). The YAS assay was chosen to monitor the change in androgenic activity during ozonation, both the agonist and antagonist activities. The androgen hormones testosterone and androstenedione were chosen as model compounds considering that these compounds have remarkably similar structures, differing only with respect to a hydroxyl and ketone functional group located on carbon 17 (Fig 1).

Fig 1 Molecular structure of selected androgen steroid hormones a) testosterone and b) androstenedione.

2. Material and Methods

2.1 Chemicals

4-Androstenedione (>98%) was purchased from Sigma-Aldrich. Testosterone (>98%), testosterone-d3 (>99%), and dihydrotestosterone (DHT, 98%) were purchased from Steraloids Inc. Androstenedione-d3 (>99%) was purchased from Toronto Research Chemicals. LC-MS grade methanol (MeOH) and chloroform purchased from Fischer scientific was used to prepare the stock solutions of androgen steroid compounds and perform liquid-liquid extraction of ozonated samples. Stock solutions of testosterone (70 mg/L), androstenedione (100 mg/L), testosterone-d3 (100 ng/L), androstenedione-d3 (100 ng/L), and DHT (5.8mg/L) were prepared in MeOH and all stocks were stored at -20^{o}C until time of experimentation.

2.2 Ozonation experiments

The gaseous ozone (5.1E-04 g/L) was fed into a batch reactor vessel containing 700mL of reverse osmosis water at a constant flow rate of 2.3E-04 L/min until a saturated ozone stock solution was created (target aqueous ozone concentration=19.5 mg/L). Measuring the absorbance with a UV-VIS Spectrophotometer Evolution 220 purchased from Thermo-scientific at the wavelength 260nm and applying the Beer-Lambert law with a molar absorptivity value of 3300 $M^{-1}cm^{-1}$, the ozone concentration in the saturated stock solution was calculated. The saturated ozone concentration within the sealed reactor vessel was measured before and after the addition to samples to ensure no signification change (<5%) in aqueous ozone concentration during the time of use of the ozone stock solution.

Fig 2 Methodology for ozonation experiments of androstenedione and testosterone conducted in reverse osmosis water.

Ozonation of testosterone and androstenedione were conducted in separate and triplicate experiments as shown in Fig 2. Stock solutions, testosterone (70 mg/L) and androstenedione (100 mg/L), were used to prepare working solution free of MeOH in order to eliminate the interaction of MeOH and ozone during the experiment. 0.36 µL of testosterone stock solution and 0.25 µL of androstenedione stock solution were transferred into separate beakers where the MeOH was allowed to evaporate. Once evaporated, the beakers were made up to 50.0 mL of reverse osmosis water to obtain a final concentration of 500 ng/L. The MeOH-free solutions were then sonicated for five minutes and stirred for an additional seven minutes to ensure a homogeneous mixture. 4 mL of the stock solutions were transferred to separate polypropylene tubes to obtain a low initial concentration of 100 ng/L, representative of environmental concentrations considering that androgen hormones have been reported in wastewater effluents at concentrations up to 171 ng/L [18]. These aliquots of the working solution were then diluted with distilled water and treated with different volumes of ozone stock solutions to obtain incremental small doses of ozone in order to obtain various levels of removal of the target contaminants. Ozone was applied as a liquid using the ozone stock solution described above and the final volume, after dilution and addition of the ozone stock, was 20 mL). Each tube was mixed for approximately 30 seconds before and after the addition of ozone stock solution to ensure a homogenous mixture. The resulting applied ozone doses for each sample was calculated by the following equation:

$$Ozone\ Concentration\ (mg/L) = \frac{\left(C_o - C_i/_2\right) \times (V_x)}{20mL}$$

where, C_o is the initial ozone concentration of the stock solution prior to the liquid addition to the samples, C_i is the ozone concentration of the stock solution after the liquid addition to the samples, V_x is the volume (mL) of ozone stock solution added to each sample. The total reaction time for ozone to react with both target compounds 10 minutes, after which no residual ozone was detected.

In order to investigate the degradation mechanism of testosterone and androstenedione, ozonation experiments were repeated using a hydroxyl radical scavenger. The scavenger compound, isopropanol, was added to each tube containing the target compound and water prior to the liquid addition of ozone. Isopropanol has been described as one of the best hydroxyl radical quencher as a result of its high reaction rate constant with the radical (1.9E09 mol/L/s) [22, 23]. The concentration of isopropanol selected for this experiment, 3.5E-07M, was based on Nasuhoglu et al., [22] suggesting the use of an isopropanol molar concentration three orders of magnitude larger than the initial target compound concentration ($C_{o,testosterone}$=3.47E-10M and $C_{o,androstenedione}$=3.49E-10M).

2.3 Sample preparation

A liquid-liquid extraction was performed to prepare the samples for chemical analysis and YAS assay. The organic solvents selected as the dispersion and extraction agents were MeOH and chloroform, respectively. To each 20 mL sample 2 mL of MeOH and 4 mL of chloroform was added. After each addition of solvent the sample was vortexed for approximately one minute. After the addition of both solvents the sample was centrifuged for three minutes at 2500 rpm. Using a syringe needle the organic phase was transferred to a round bottom test tube where the sample was dried under vacuum at 45^{o}C and then re-constituted with 0.5 mL of MeOH. The reconstituted samples were then used for chemical analysis and YAS assay.

2.4 Chemical Analysis

Concentration of testosterone and androstenedione were measured using a Thermo Scientific (Waltham, MA USA) Accela 600 LC system in tandem with A Thermo Scientific LTQ XL mass spectrometer equipped with a high resolution Orbitrap detector. Column configuration consisted of a Thermo in-line filter hardware unit with a 2.1mm ID and 0.2um filter cartridge (Bellefonte, PA. USA) followed by an Agilent UHPLC guard column Zorbax Eclipse plus C18 2.1 x 5mm and 1.8um PN: 821725-901. The initial mobile phase composition was aqueous 2mM ammonium formate 0.1% formic acid buffer-A and methanol 0.1% formic acid buffer-B at a 90:10 with an initial 2min and then increasing the composition on B to 65% in one minute follow by a ramp on B to reach 75% for 4.5min and finally the column was brought to 100% B and held for 1.5min. Column buffer composition was brought immediately to initial conditions for equilibration. 25 µL of sample or its dilution were injected for a total run time of 10min at a constant flow rate of 0.3 mL/min. MS detection was performed by Fourier transform mass spectrometer (FTMS) positive mode, instrument optimization was performed by standard solutions infusion at 10uL/min, while source optimization conditions was done by infusion flow analysis. Nitrogen gas was used for all sheath, auxiliary and sweep gasses, while helium gas was used as the collision gas. Data acquisition analysis was done on the precursor-ions (m/z=287.2000 for androstenedione and m/z=289.2155 for testosterone) for the full scan obtained at 30000 resolution (FTMS) on a mass range from 50-350m/z on Xcalibur Version 2.1 from Thermo Scientific (San Jose, Ca USA).

2.5 Yeast Androgenic Screen (YAS) Assay

Techniques for yeast culturing and YAS assay procedures were performed as described in [24, 25] with minor adjustments. The YAS assay plates were constructed in a 96-well plate, which contained a triplicate 12 serial dilution of a sample of interest and separate rows consisting of a solvent blank, yeast culture control, and a 12 serial dilution of a positive control. The validity of the YAS assay was confirmed by selecting DHT as a reference compound and positive control, which maintained a median effective concentration (EC_{50}) of 2.35 nM (n=28), in the range of values previous reported [14, 26, 27]. Rows containing samples were

prepared by reconstituting in their appropriate solvents, transferring 10, 25 or 50 µL to assay plate, evaporating, and reconstituting with 200 µL of yeast culture. Rows containing controls were prepared similar to that of samples. The plates were sealed with film and shaken for two minutes and placed in an incubator for 24 hrs. After 24 hrs of exposure 100 µL of buffer containing ONPG was added to the plates. Once color change was observed the plates were transferred to a plate reader where the absorbance was measured. The color intensity observed over sequential dilutions is an indication of the enzyme activity and androgenic potency. The YAS assay was performed for each triplicate ozonation experiment without the presence of isopropanol.

Absorbance values obtained by a microplate reader were corrected for yeast turbidity within the plates (in the yeast control) and normalized to the positive control, DHT, for the determination of endocrine activity. Absorbances were measured at the wavelengths 415 and 595nm, which represent the enzymatic activity and growth control, respectively. The corrected absorbance for the YAS assay was determined by the following equation:

$$\text{YAS Corrected Value} = \text{Sample}_{415nm} - (\text{Sample}_{595nm} - \text{Blank}_{595nm})$$

The androgenic activity of the samples for the YAS assay was determined by dividing the corrected absorbance values of the sample by the corrected absorbance of the positive control that represents 100% activity (highest stable absorbance).

$$\text{Androgenic Activity (\%)} = (\text{Sample}_{\text{corrected absorbance}} / \text{DHT}_{\text{corrected absorbance}}) * 100$$

The antagonist activity using the YAS assay was determined in a similar fashion as the agonist activity. Corrected absorbance of the sample was divided by the positive control; however, the positive control for the antagonist activity is the corrected absorbance value for 10nM DHT.

Using OriginPro software, a sigmoidal dose response curve for each plate was constructed by plotting the androgenic activity percentage over a serial logarithmic dilution. Dose response curves often have variability in shape from one sample to the next in terms of potency and efficacy. To describe a samples potency and efficacy in a single parameter, the term bioactivity is used and refers to the area under the dose response curve (AUC). The percent removal of bioactivity for each ozonated sample of testosterone and androstenedione was calculated using the following equation:

$$Bioactivity\ Removal_{s,ozone\ dose}\ (\%) = \frac{A_{\ s,no\ ozone} - A_{\ s,ozone\ dose}}{A_{\ s,no\ ozone}} \times 100$$

where, $A_{s,no\ ozone}$ is the area under the dose response curve of the sample containing no ozone, $A_{s,ozone\ dose}$ is the area under the dose response curve of the sample exposed to ozone, and $Bioactivity\ Removal_{s,ozone\ dose}$ is the integral inhibition percentage of

bioactivity for each ozone dose applied to testosterone and androstenedione. $A_{s,no\ ozone}$ and $A_{s,ozone\ dose}$ were calculated using the trapezoid rule.

3. Results and Discussion

3.1 Chemical Analysis

Figures 3 represent the removal profiles for testosterone and androstenedione, which were constructed by plotting percent removal with respect to ozone dose.

Fig 3 Percent removal of testosterone and androstenedione with increasing ozone dose. Error bars: standard deviation, n=3.

Testosterone and androstenedione follow similar degradation profiles as ozone concentration increases, where both target compounds reached removal leading to concentration below the limits of detection (LOD) ($LOD_{testosterone}$=0.57ppb, $LOD_{androstenedione}$=1.19ppb, determined based on a signal-to-noise ratio of 3:1) when approximately 0.5 mg/L of ozone was applied. By calculating the theoretical oxygen demand (ThOD) of the ozonated solutions, the mg O_3 required to remove one mg ThOD were determined to be 1.80E03 mg O_3/mg ThOD for testosterone and 1.82E03 mg O_3/mg ThOD for androstenedione. Percent removals of the target compounds obtained for the ozone doses of 0.066 mg/L to 0.322 mg/L show testosterone is removed at a higher level than androstenedione. A possible explanation could be the result of ozone having a higher affinity towards the hydroxyl moiety than the ketone moiety on testosterone and androstenedione, respectively. The k_{O3} constant for cyclopentanol and cyclopentanone moiety is 2.0 mol s^{-1} [28] and 0.98 mol s^{-1} [29] present on testosterone and androstenedione, respectively. The difference of k_{O3} constants for cyclopentanol and cyclopentanone is not large but provides a possible explanation for the differences in ozone affinities towards functional groups and moieties on the target compounds.

The results obtained in the presence of a hydroxyl radical scavenger (isopropanol) are presented in Fig 4a and b. In the presence of isopropanol both compounds remained at concentrations above their LOD and a maximum percent removal of approximately 92% was obtained for both compounds at an ozone concentration of 5 mg/L. At an ozone concentration of 0.5 mg/L and in presence of isopropanol, removals of testosterone and androstenedione were 38% and 32%, respectively (compared to almost complete removal in absence of the hydroxyl radical scavenger). The dampened removal profile for both compounds in the presence of a hydroxyl radical scavenger indicates that hydroxyl radicals play a significant role in the degradation of these compounds in the range of ozone dose 0-2 mg/L. Above this ozone dose, the removal profile of both compounds became less dampened in presence of the hydroxyl radical scavenger, indicating that the

hydroxyl degradation mechanism is less prominent in that range of ozone doses, which might be explained by the greater availability of ozone.

Fig 4 Effect of the presence of hydroxyl radicals during ozonation of (a) testosterone (C_o= 100 ng/L) and (b) androstenedione (C_o= 100 ng/L) studied by scavenging the hydroxyl radicals by addition of isopropanol (3.5E-07M). Error bars: standard deviation, n=3.

The chemical structure for testosterone and androstenedione could also provide additional explanation for the reduced percent removal in the presence of isopropanol. A study performed by Segura, et al., [1] investigated the degradation of estrone and identified key ozonation transformation products through structural elucidation and chemical analysis techniques. The study identified electron-rich functional groups (aromatic ring, hydroxyl group, and a ketone) that are more susceptible towards ozone due to its high affinity and selective nature, which results in the cleavage of the aromatic ring [1] (Fig 5).

Fig 5 Molecular structure of estrone.

Similar to estrone, there are key functional groups on both testosterone and androstenedione that may be susceptible for ozone attack (Fig 1). Testosterone has a ketone located at (Carbon #3), alkene at (Carbon#4-5), and a hydroxyl group at (Carbon #17). Androstenedione has a ketone and alkene functional groups located at the same positions as testosterone but has a second ketone located at (Carbon#17) instead of a hydroxyl group. Based on the chemical structures testosterone and androstenedione, which have few functional groups susceptible for ozone attack, these compounds could be relatively more stable during ozonation than estrogen compounds. In addition, the presence of a ketone functional group adjacent to the alkene may cause steric hindrance limiting the degradation pathways . This is confirmed by comparing results reported by Segura, et al., [1] for the removal of estrone. It was found that 0.64 mg O_3/mg ThOD for estrone, corresponding to approximately 2.8E03 times higher removal than testosterone and androstenedione. This significant difference indicates that androstenedione and testosterone are much more resilient than estrone during ozonation.

3.2 YAS Assay Results

The YAS assay was selected to examine the androgenic activity of testosterone and androstenedione during ozonation. Both the androgenic and anti-androgenic activities for each sample were examined, however there was no anti-androgenic activity observed and these results are not shown. Dose response curves for androgenic activity were generated for each ozonated sample of the target compounds by plotting the percent activity with respect to dilution in logarithmic scale (Fig 6).

Fig 6 Change of androgenic activity during ozonation of testosterone and androstenedione.

Figure 6 shows a dynamic change of androgenic activity during the degradation of testosterone and androstenedione and by using the AUC parameter, which takes into consideration both potency and efficacy, the bioactivity of the ozonated sample can be determined. The percent removal of bioactivity for both compounds were calculated and plotted with respect to each applied ozone dose and compared to percent removals determined by chemical analysis (Fig 7).

Fig 7 Percent removal of testosterone and androstenedione using chemical analysis and percent removal of bioactivity using AUC parameter to describe YAS assay results. T is testosterone and A is androstenedione.

Despite having very similar chemical structures (Fig 1) and comparable removal profiles in the absence and presence of isopropanol (Fig 4) there are notable differences in bioactivity percent removals (Fig 7). These differences are highlighted within the ozone concentration range of 0.066-0.332 mg/L. The use of low ozone doses and testing at incremental doses allowed for the monitoring of changes in bioactivity using the YAS assay. At ozone dose of 0.066 mg/L the bioactivity of testosterone sample is removed by approximately 30% while the bioactivity of androstenedione sample follows a completely different trend and increases by approximately 60%. This trend is continued with the second ozone dose of 0.133 mg/L in which a difference of 50% bioactivity removal is observed. At ozone dose of 0.199 mg/L the bioactivity of testosterone sample is removed by approximately 15% (25% increase of bioactivity from previous ozone dose) while the bioactivity of androstenedione sample decreases by approximately 35%. At an ozone dose of 0.332 mg/L and above the bioactivity removal percentages of testosterone and androstenedione become similar and are approximately >80%.

The dynamic change of bioactivity removal for testosterone and androstenedione as well as the difference between their bioactivity removals are quite unexpected. A plausible explanation for the increase in bioactivities at low applied ozone doses (<0.332 mg/L) could be caused by the bioactivities of transformation products. The potential bioactivity of ozonation transformation products emphasizes the importance of considering structure-activity relationships of compounds. The structure-activity relationship for steroidal androgens has been well documented and it was found that the 3-ketone group (Fig 1a, b) enhances androgenic activity of steroidal AR ligands, while the 17β-hydroxyl group (Fig 1a) is essential for ligand-receptor interactions [20]. These findings suggest that the presence of the 17β-hydroxyl and/or 3-ketone group on transformation products could be responsible for increased bioactivities.

4. Conclusion

The results from the present study emphasize the need for bioanalytical techniques to be used in parallel with routine chemical analysis for assessing the performance of oxidative treatments. Testosterone and androstenedione were selected as target compounds for this study as a result of their similar chemical structure, varying in one functional group (Fig 1a,b) and the lack of information on their fate during ozonation. Although the removal profiles (Fig 4) were comparable in the presences and absence of a hydroxyl scavenger (isopropanol), the ozonated samples produced dynamic and unexpected activities using the YAS assay (Fig 6). By comparing the bioactivity of ozonated samples it was demonstrated that while compounds may slightly differ in chemical structure the difference of bioactivities can be significant (approximately 90% when 0.066 mg/L of O_3 was applied) (Fig 7). The change of bioactivity during ozonation can be attributed to transformation products and it was found that bioactivities were removed >80% at an ozone dose of 0.332 mg/L. Without the use of bioanalytical tools such as the YAS assay these bioactivities would not be revealed and thus result in a limited and incomplete assessment of potential risks posed by the discharge of treated wastewater effluent. The addition of bioanalytical tools as a complementary method to chemical analysis will not only enhance monitoring strategies for assessing potential risks of wastewater containing known and unknown contaminants but will also provide valuable information for selecting and optimizing treatment strategies.

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