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Schemes of metabolic patterns of anabolic androgenic steroids for the estimation of metabolites of designer steroids in human urine

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ABSTRACT

Unified metabolism schemes of anabolic androgenic steroids (AAS) in human urine based on structure classification of parent molecules are presented in this paper. Principal components analysis (PCA) was applied to AAS molecules referred in the World Anti-Doping Agency (WADA) list of prohibited substances, resulting to their classification into six distinct groups related to structure features where metabolic alterations usually occur. The metabolites of the steroids participating to these six groups were treated using the Excel©classification filters showing that common metabolism routes are derived for each of the above PCA classes, leading to the proposed metabolism schemes of the present study. This rule-based approach is proposed for the prediction of the metabolism of unknown, chemically modified steroids, otherwise named as designer steroids. The metabolites of three known, in the literature, AAS are estimated using the proposed metabolism schemes, confirming that their use could be a useful tool for the prediction of metabolic pathways of unknown AAS.

1. Introduction

Anabolic androgenic steroids (AAS) are synthetic substances derived from modifications in testosterone molecule [1], the main natural androgenic and anabolic steroid formed in the interstitial (Leydig) cells of the testes. The misuse of AAS in sports led to a ban by the International Olympic Committee in 1974 and since 2003 by the World Anti-Doping Agency (WADA).

AAS are extensively metabolized peripherally, notably in the liver and their target tissues, where conversion to their active form is sometimes required before they can elicit their biological action [2]. The metabolism of AAS generally follows the metabolic pathways observed for testosterone metabolism [3] and has been comprehensively reviewed [4–6]. The enzymes that convert testosterone to its distinct metabolites also act towards AAS when similar groups and configurations are present [4,7–9]. Metabolism conversions are categorized into Phase I reactions where enzymatically catalyzed alterations are observed (mainly oxidations and reductions), which convert the steroid into more polar compounds in order to inactivate the drug and to facilitate its elimination from the body. Phase II reactions conjugate the steroids or their metabolites mainly with glucuronic acid or sulfate and also help elimination of steroids from the body.

As part of our research interests about the use of Time-of-Flight mass spectrometry (TOF-MS) for the detection of designer steroids and preventive doping control analysis [10], a tool that could predict the Phase I metabolites of designer steroids would be valuable. Not only because the lack of reference materials does not permit the careful elucidation of the metabolic profile of these unknown steroids, but mainly because human studies with such compounds are against the Declaration of Helsinki. Furthermore, it is well known that predictive drug metabolism is based either on models, such as quantitative structure–metabolism/activity relationships (QSM/AR) and molecular modeling of enzymes and ligand docking, or on rule-based expert systems and databases [11–13]. However, none of these methods has been applied to predictive metabolism of AAS.

This paper presents a rule-based approach for the estimation of possible metabolites of unknown steroids in human urine, based on the main metabolic pathways of the known synthetic AAS included in the WADA list of prohibited substances [14]. Parent AAS structures were classified using principal components analysis (PCA) into distinct groups according to their similarities in structural features, which affect metabolism or where metabolism alterations occur. The AAS metabolites of the above PCA classes were treated using the Excel©software classification filters (Microsoft® Office Professional Edition 2003), showing that common metabolic pathways are derived from structurally similar parent molecules. These common metabolic alterations for each PCA class of parent AAS were further grouped into specific metabolism schemes.

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The proposed metabolism schemes categorize the metabolism pathways of Phase I reactions based on the A-ring structure of the parent molecule. Other structural characteristics throughout the rest AAS structure have been also taken into account. Case studies for the estimation of metabolites of three known, in the literature, AAS (6α-methylandrostenedione, androst-4-ene-3,6,17-trione and androsta-1,4,6-triene-3,17-dione) using the proposed metabolism schemes are presented, confirming that their use could be a useful tool for the estimation of metabolic pathways of unknown AAS.

The estimation of Phase II reactions of synthetic, unknown AAS is beyond the scope of this paper, because the preparative procedures followed by the anti-doping laboratories detect currently unconjugated molecules.

2. Method

2.1. Organization of the AAS metabolism data

Prior to the construction of the proposed AAS metabolism schemes, collection of literature data concerning the metabolic alterations of the known AAS included in the WADA list of prohibited substances [14] was carried out. The parent molecules and their metabolites were summarized in Table 1. In order to reveal common metabolic alterations derived from common structural features of the parent molecules from a statistical point of view, the following data sets were constructed:

2.1.1. Data set for AAS parent molecules classification (PCA analysis)

The data set for PCA analysis describes the structures of the parent AAS in terms of their substitution and existence of double bonds at various but specific positions throughout the steroidal structure where metabolic alterations usually occur. The dimensions of the data matrix are $N \times K$ (43×16), where N is the number of observations (parent molecules, shown in Table 1) and K is the number of variables (structure features acceptable of metabolic alterations, see abbreviations in Fig. 2), and was further subjected to PCA analysis.

The detection of outliers and the elucidation of trends, similarities and dissimilarities among the molecular structure of the parent AAS tested were carried out using the PCA method [15]. PCA involves calculating linear combinations of the original descriptors. The data are represented in the K-dimensional space (where K is equal to the number of variables) and are subsequently reduced into a few principal components, which are descriptive dimensions that describe the maximum variation within the data. Any prior knowledge relating to the class membership is not used in the PCA. The first PC is the linear combination of the original variables and explains the maximum amount of variance in the data. There are as many PCs as original variables; however, because of the high correlation between variables in many data sets, a reduced number of PCs is adequate to explain most of the data variance. A plot of samples (analytes in this study) as a function of the first two PCs results in a two-dimensional map providing the maximum information in the data set (scores plot). The scores plot displays patterns or groupings within the data and can also be useful in highlighting outliers. The corresponding loadings plots are used to identify which structure variables contribute to the positioning of the parent AAS on the scores plot and hence the variables that influence any observed separation in the data set. The data set was mean-centered before the performance of the PCA. SIMCA-P 10.5.0 statistical program was used to perform the PCA analysis [16].

2.1.2. Data set for AAS metabolites classification (Excel® software classification)

An Excel® datasheet was used to construct the data set where each of the AAS metabolites was depicted as a row, while the columns represent:

- The atoms of the steroidal nucleus. Given that the steroidal nucleus of the studied AAS molecules comprises 19 carbon atoms (Table 1, top), the datasheet includes 19 columns, one for every carbon atom from C-1 to C-19. The columns were filled out with the functional group that each of the steroidal nucleus atom bears (e.g. hydroxyl or methyl groups). 19-Nor steroids bear the symbol "N" in C-19 column, meaning there is no substitute (methyl group) at this position.
- The bonds of the steroidal nucleus (e.g. the column named as Δ1,2 represents the bond between the carbon atoms C-1 and C-2). The columns were filled out with Y (in case of a double bond) or with space (in case of a single bond).

The Excel® classification filters applied to AAS metabolites isolated the structure alterations at certain sites of the steroidal nucleus compared to that of their parent AAS and contributed significantly to the construction of the AAS metabolism schemes as presented in Section 3.2.

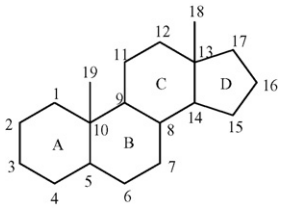
3. Results

3.1. Classification of the AAS parent molecules: PCA analysis

According to PCA, the first two principal components cumulatively accounted for 33.5% of the total data variance. The addition of more PCs increases significantly this percentage without affecting the positioning of the analytes in the PCA ellipse. The application of PCA reveals that the classification of parent AAS can be achieved according to their structural characteristics which are also those structural features which influence metabolism or where metabolic alterations occur. The scores plot (Fig. 1) discriminates six groups of parent AAS (A-1 to A-6 groups), as follows:

- A-1 group: AAS with saturated A-ring structure or 5α-androstan-3-ones. These AAS were positioned at the far right upper and bottom quartiles of the PCA ellipse, e.g. mesterolone, drostanolone, methasterone, mestanolone, oxymetholone and oxandrolone.
- A-2 group: AAS with double bond at C-4,5 position or androst-4-ene-3-ones. Parent AAS of this class were positioned up and down of the centre of the ellipse, e.g. clostebol, nor-clostebol, 4-hydroxy-testosterone, oxabolone, methyl-testosterone, fluoxymesterone, oxymesterone, calusterone, bolasterone and mibolerone.
- A-3 group: AAS with double bond at C-1,2 position or androst-1-ene-3-ones. Parent AAS of this class were positioned at the upper right quartile of the ellipse in proximity with parent AAS of the A-1 group, e.g. 1-testosterone, methenolone, stenbolone and methyl-1-testosterone.
- A-4 group: AAS missing the 3-keto group consist a separate PCA class, the positioning of which in the PCA ellipse is affected by the exact site of double bond (if any) in the A-ring. AAS of this group are positioned at the bottom right quartile of the ellipse due to their structural dissimilarity compared to the majority of the analytes, e.g. desoxymethyltestosterone (madol). The loadings plot (Fig. 2) reveals that the main variable responsible for the positioning of madol is that referred to C-2,3 double bond (symbolized as DB2-3 in Fig. 2), a structural feature which is, indeed, characteristic for many 3-deoxy steroids.

Table 1
General steroidal structure (top), molecular structures, trivial and full scientific names of parent AAS and their metabolites considered for the construction of the metabolism schemes. Carbon atoms of the steroids are numbered according to the standard steroid nomenclature and the cycles designated by letters.



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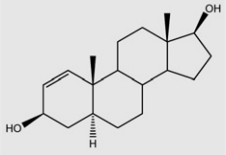
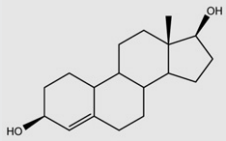
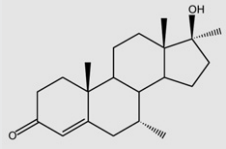
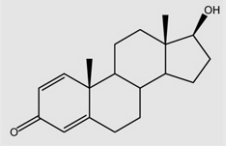
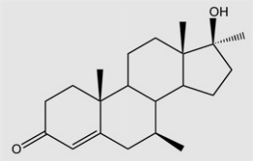
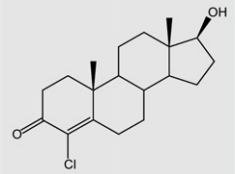
No	Substance	Metabolites
1	1-Androstenediol (5α-androst-1-ene-3β,17β-diol) 	1-androstenedione 5α-androst-1-ene-3α-ol-17-one
2	Bolandiol (19-norandrost-4-ene-3β,17β-diol) 	19-nor-testosterone 19-nor-androsterone 19-nor-etiocholanolone 19-nor-androstenedione
3	Bolasterone (7α,17α-dimethyl-17β-hydroxyandrost-4-ene-3-one) 	7α,17α-dimethyl-5β-androstane-3α,17β-diol 7α,17β-dimethyl-5β-androstane-3α,17α-diol 7α,17,17-trimethyl-5β-androst-13-en-3α-ol
4	Boldenone (Androsta-1,4-dien-17β-ol-3-one) 	5β-androst-1-en-17β-ol-3-one androsta-1,4-diene-3,17-dione 5β-androst-1-ene-3α-17β-diol 5β-androst-1-ene-3,17-dione 5β-androst-1-ene-3α-ol-17-one androsta-1,4-diene-6β,17β-diol-3-one androsta-1,4-diene-6β-ol-3,17-dione 5β-androst-1-en-6β-ol-3,17-dione 5β-androstan-3α-ol-17-one 5β-androstan-3α-,17β-diol
5	Calusterone (7β,17α-dimethyl-17β-hydroxyandrost-4-ene-3-one) 	7β,17α-dimethyl-5α-androstane-3α,17β-diol 7β,17α-dimethyl-5β-androstane-3α,17β-diol 7β,17β-dimethyl-5z-androstane-3α,17α-diol 7β,17α-dimethyl-5β-androstane-3β,17β-diol
6	Clostebol (4-chloro-androst-4-ene-17β-ol-3-one) 	4-chloroandrost-4-en-3α-ol-17-one 4z-chloro-3α-hydroxy-5α-androstan-17-one 4z-chloro-3α-hydroxy-5β-androstan-17-one 4z-chloro-3β-hydroxy-5α-androstan-17-one 4z-chloro-3α,16z-dihydroxy-5z-androstan-17-one

Table 1 (*Continued*)

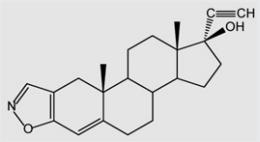
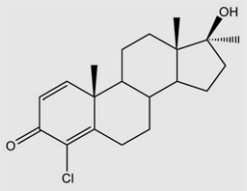
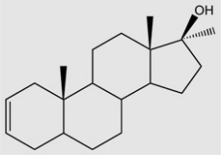
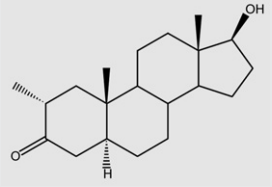
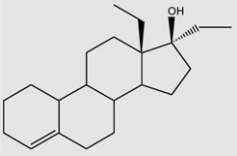
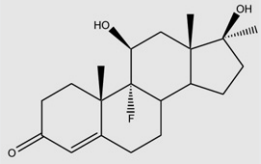
No	Substance	Metabolites
7	Danazol (17α-pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) 	17-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one 17-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one 17-hydroxy-17α-pregn-4-en-20-yn-3-one 6β,17-dihydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one 6β,17-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one 6β-16z-dihydroxy-2z-hydroxymethylethisterone
8	Dehydrochlormethyl-testosterone (4-chloro-1,2-dehydro-17α-methyltestosterone) 	6β-Dehydrochlormethyltestosterone 6β,16β-didydroxy-dehydrochlormethyltestosterone 6β,12-didydroxy-dehydrochlormethyltestosterone 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one
9	Desoxymethyltestosterone (madol) (17α-methyl-5α-androst-2-ene-17β-ol) 	17α-methyl-5α-androst-2z,3z,17β-triol 17α-methyl-5α-androst-2-ene-3,17β-diol 17α-methyl-5α-androst-4z,17β-diol-3-one 17α-methyl-5α-androst-2-ene-2,17β-diol 17α-methyl-5α-androst-2z,3z,6β,17β-tetrol 17α-methyl-5α-androst-3z,6β,17β-triol 17α-methyl-5α-androst-2z,3z,16z,17β- tetrol
10	Drostanolone (2α-methyl-17β-hydroxy-5α-androstan-3-one) 	2α-methyl-5α-androstan-3α,17β-diol 2α-methyl-5α-androstan-3α-ol-17-one 2α-methyl-5α-androstan-3β-ol-17-one
11	Ethylestrenol (17α-ethyl-estr-4-ene-17β-ol) 	See norethandrolone metabolites
12	Fluoxymesterone (11β,17β-dihydroxy-9α-fluoro-17α-methyl-4-androsten-3-one) 	9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol 9α-fluoro-17α-methyl-17β-hydroxy-androst-3,11-dione 9α-fluoro-17α-methyl-11β,17β-dihydroxy-5α-androstan-3-one 9α-fluoro-17α-methyl-5α-androstan-3β,11β,17β-triol 9α-fluoro-17α-methyl-5β-androstan-3β,11β,17β-triol 9α-fluoro-17α-methyl-4-androsten-3β,6β,11β,17β-tetrol 6β-hydroxy-fluoxymesterone 9α-fluoro-17α-methyl-androst-3β,6β,11β,17β-tetrol

Table 1 (*Continued*)

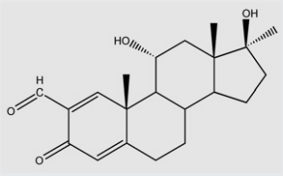
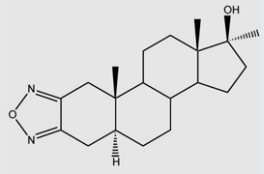
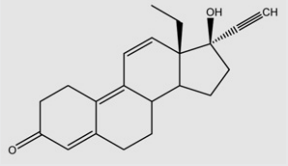
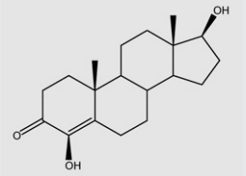
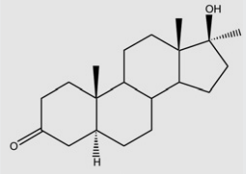
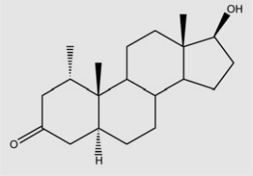
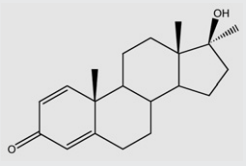
No	Substance	Metabolites
13	Formebolone (2-formyl-17α-methyl-androsta-1,4-diene-11α-17β-diol-3-one) 	2-hydroxymethyl-11α-17β-dihydroxy-17α-methylandrost-1,4-diene-3-one 11α-hydroxymethyltestosterone 11α,17β-dihydroxy-17α-methyl-3-oxo-androsta-1,4-diene-2-carboxylic acid
14	Furazabol (17α-methyl-17β-hydroxy-5α-androstan-[2,3-c]-furazane) 	16z-hydroxyfurazabol
15	Gestrinone (13β-ethyl-17α-ethynyl-17β-hydroxy-4,9,11-estratrien-3-one) 	2z,16β-dihydroxy-gestrinone 6α-hydroxy-gestrinone
16	4-Hydroxytestosterone (4,17β-dihydroxyandrost-4-ene-3-one) 	3α,4β-dihydroxy-5α-androstan-17-one 3β,4β-dihydroxy-5α-androstan-17-one 4β-hydroxy-5α-androstan-3,17-dione 3α-hydroxy-5β-androstan-4,17-dione 3α,17β-dihydroxy-5β-androstan-4-one 5α-androstan-3β,4β,17β-triol 5α-androstan-3α,4α,17β-triol
17	Mestanolone (17α-methyl-5α-androstan-17β-ol-3-one) 	17α-methyl-5α-androstan-3α,17β-diol 17β-methyl-5α-androstane-3α,17α-diol 18-nor-17,17-dimethyl-5α-androst-13-en-3α-ol
18	Mesterolone (1α-methyl-5α-androstan-17β-ol-3-one) 	1α-methyl-5α-androstane-3α,17β-diol 1α-methyl-5α-androstan-3α-ol-17-one 1α-methyl-5α-androstane-3β,17β-diol 1α-methyl-5α-androstan-3β-ol-17-one 18-hydroxy-1α-methyl-5α-androstane-3,17-dione 3α,18-dihydroxy-1α-methyl-5α-androstan-17-one 3β,18-dihydroxy-1α-methyl-5α-androstan-17-one 3α,6ξ,18-trihydroxy-1α-methyl-5α-androstan-17-one
19	Methandienone (17α-methyl-17β-hydroxy-androsta-1,4-diene-3-one) 	18-nor-17,17-dimethyl-androsta-1,4,13(14)-trien-3-one 17α-methyl-5β-androstan-3α,17β-diol 17β-methyl-5β-androst-1-ene-3α,17β-diol 6β-hydroxymethandienone 17-epimethandienone 18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one 18-nor-epimetendiol

Table 1 (*Continued*)

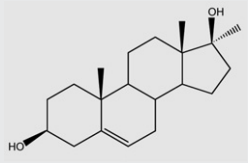
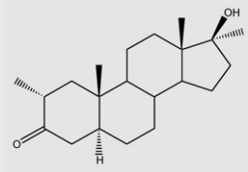
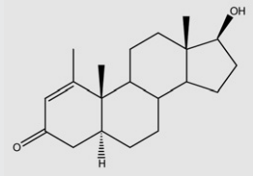
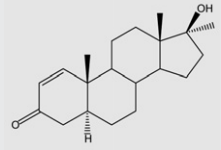
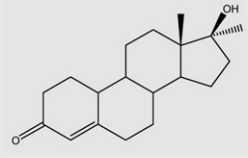
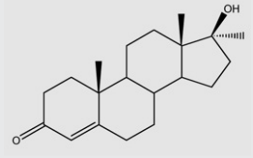
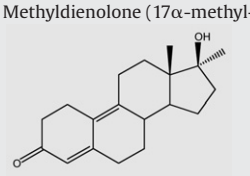
No	Substance	Metabolites
20	Methandriol (17α-methylandrost-5-ene-3β,17β-diol) 	17α-methyl-5β-androstane-3α,17β-diol
21	Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one) 	2α,17α-dimethyl-5α-androstane-3α,17β-diol
22	Methenolone (1α-methyl-5α-androst-1-ene-17β-ol-3-one) 	1-methylen-5α-androstan-3α-ol-17-one 18-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione 3α-hydroxy-1α-methyl-5α-androstan-17-one 17-epimethenolone 1-methyl-5α-androst-1-ene-3,17-dione 3α,6β-dihydroxy-1-methylene-5α-androstan-17-one 6β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione 16α-hydroxyl-1-methyl-5α-androst-1-ene-3,17-dione 16β-hydroxyl-1-methyl-5α-androst-1-ene-3,17-dione 3α,16α-dihydroxy-1-methyl-5α-androst-1-en-17-one 3α,16β-dihydroxy-1-methyl-5α-androst-1-en-17-one 2z-hydroxy-1-methylene-5α-androstan-3,17-dione
23	Methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-androst-1-ene-3-one) 	17α-methyl-5α-androst-1-ene-3α,17β-diol 17α-methyl-5α-androst-3α,17β-diol 17β-hydroxymethyl-17α-methyl-18-nor-5α-androsta-1,13-dien-3-one
24	Methyl-nor-testosterone (17β-hydroxy-17α-methyl-5α-estr-4-ene-3-one) 	No metabolites reported
25	Methyltestosterone (17β-hydroxy-17α-methyl-androst-4-ene-3-one) 	17α-methyl-5α-androstan-3α,17β-diol 17α-methyl-5β-androstan-3α,17β-diol 17-epimethyltestosterone 18-nor-17,17-dimethyl-androsta-4,13(14)-diene-3-one
26	Methyldienolone (17α-methyl-17β-hydroxy-4,9(10)-estradien-3-one) 	No metabolites reported

Table 1 (*Continued*)

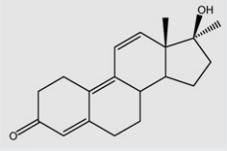
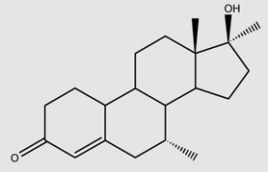
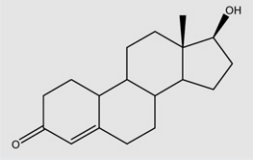
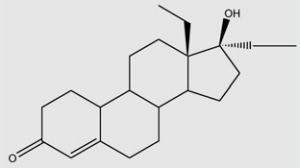
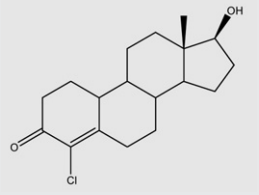
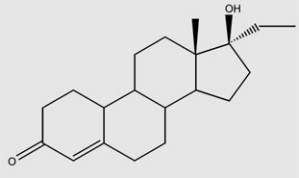
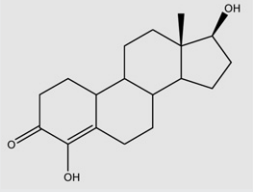
No	Substance	Metabolites
27	Methyltrienolone (17α-methyl-17β-hydroxy-4,9,11-estratrien-3-one) 	No metabolites reported
28	Mibolerone (17β-hydroxy-7α,17α-dimethylestr-4-ene-3-one) 	7α,17α-dimethyl-5β-estrane-3α,17β-diol 7α,17α-dimethyl-5β-estrane-17β-ol-3-one 7α,17α-dimethyl-5β-estrane-6β,16β,17β-triol-3-one
29	Nandrolone (17β-hydroxy-estr-4-ene-3-one) 	19-nor-androsterone 19-nor-etiocholanolone 19-nor-androstenedione 19-nordihydrotestosterone 19-norepiandrosterone
30	Norbolethone (13β,17α-diethyl-17β-hydroxyestr-4-ene-3-one) 	13β,17α-diethyl-5α-gonane-3α,17β-diol 13β,17α-diethyl-5β-gonane-3α,17β-diol
31	Norclostebol (4-chloro-17β-hydroxyestr-4-ene-3-one) 	4-chloro-3α-hydroxyestr-4-en-17-one 4z-chloro-3α-hydroxy-5β-estran-17-one 4z-chloro-3α-hydroxy-5α-estran-17-one 4z-chloro-3α,16z-dihydroxy-5z-androstan-17-one
32	Norethandrolone (17α-ethyl-estr-4-ene-17β-ol-3-one) 	17α-ethyl-5α-estrane-3α,17β-diol 17α-ethyl-5β-estrane-3α,17β,20-triol 17α-ethyl-5β-estrane-3α,17β-diol
33	Oxabolone (19-norandrost-4-ene-4,17β-diol-3-one) 	4-hydroxy-estran-3,17-dione 4-hydroxy-estr-4-an-3,17-dione 3α,4-dihydroxy-estran-17-one 3β,4-dihydroxy-estran-17-one

Table 1 (*Continued*)

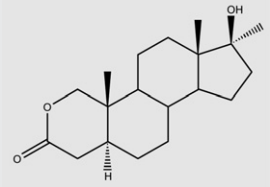
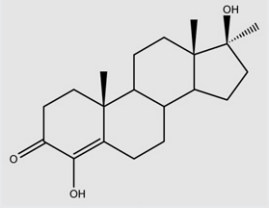
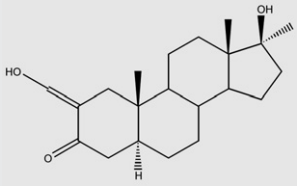
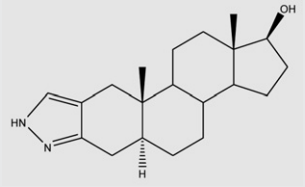
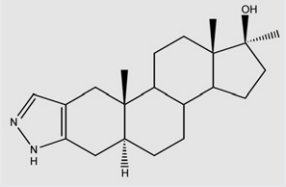
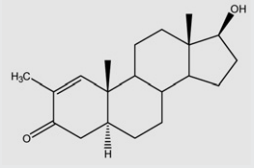
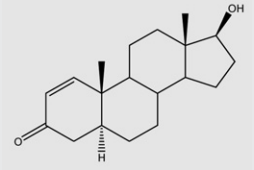
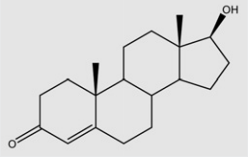
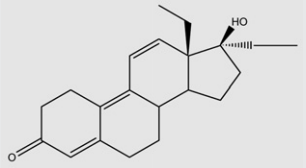
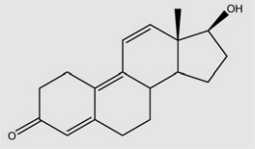
No	Substance	Metabolites
34	Oxandrolone (17α-methyl-17β-hydroxy-2-oxa-5α-androstan-3-one) 	17-epioxandrolone 16α-hydroxy oxandrolone 16β-hydroxy oxandrolone 18-nor-17,17-dimethyl-2-oxa-5α-androsta-13(14)-en-3-one
35	Oxymesterone (17α-methyl-4,17β-dihydroxy-androst-4-ene-3-one) 	No metabolites reported
36	Oxymetholone (2-hydroxymethylene-17α-methyl-17β-hydroxy-5α-androstan-3-one) 	17α-methyl-5α-androstan-3α,17β-diol 2z-hydroxymethyl-17α-methyl-5α-androstan-3z,6z,17β-triol 3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid
37	Prostanozol ([3,2-c]pyrazole-5α-etioallocholane-17β-tetrahydropyranol) 	3′-hydroxy-17-keto-prostanozol 4z-hydroxy-17-keto-prostanozol 3′,6z-dihydroxy-17-keto-prostanozol 17-keto-prostanozol 16β-hydroxy-17-keto-prostanozol
38	Stanozolol (17α-methyl-17β-hydroxy-5α-androstane-[3,2-c]-pyrazole) 	3′-hydroxystanozolol 4β-hydroxystanozolol 16β-hydroxystanozolol 3-hydroxy-17-epistanozolol 16α-hydroxystanozolol 3′,16-dihydroxystanozolol 4α,16-dihydroxystanozolol 4β,16-dihydroxystanozolol 3′-hydroxy-13,14-dehydro-17,17-dimethyl-18-nor-5α-androst-2-eno-(2,3-c)-pyrazole 4α-hydroxy-stanozolol
39	Stenbolone (2-methyl-5α-androst-1-ene-17β-ol-3-one) 	2-methyl-5α-androst-1-ene-16α-ol-3,17-dione 2-methyl-5α-androst-1-ene-16β-ol-3,17-dione 2-methyl-5α-androst-1-ene-3z,16z-diol-17-one 2-methyl-5α-androst-1-ene-3α-ol-17-one 2-methyl-5α-androst-1-ene-16z,17β-ol-3-one 18-hydroxy-2-methyl-5α-androst-1-ene-3,17-dione
40	1-Testosterone (17β-hydroxy-androst-1-ene-3-one) 	5α-dihydrotestosterone 5α-androst-1-ene-3α,17β-diol 5α-androst-1-ene-3,17-dione 5α-androst-1-ene-3α-ol-17-one 1-epitestosterone

Table 1 (*Continued*)

No	Substance	Metabolites
41	Testosterone (17β-hydroxy-androst-4-ene-3-one) 	5α-, 5β-dihydrotestosterone androsterone etiocholanolone androst-4-ene-3,17-dione
42	Tetrahydrogestrinone (13β,17α-diethyl-17β-hydroxy-4,9,11-estratrien-3-one) 	18-hydroxy-tetrahydrogestrinone 16z-hydroxy-tetrahydrogestrinone
43	Trenbolone (17β-hydroxy-4,9,11-estratrien-3-one) 	17-epitrenbolone

- A-5 group: AAS with double bonds at C-1,2 and C-4,5 positions of the A-ring or androsta-1,4-diene-3-ones. These AAS are also positioned right in the ellipse (upper and bottom quartile) between the A-2 and A-1/A-3 groups mentioned above, e.g. boldenone, methandienone, dehydrochlormethyltestosterone (oral turinabol) and formebolone.
- A-6 group: AAS in junction with a heterocyclic ring. Parent AAS of this class were positioned at the bottom right quartile of the ellipse, e.g. prostanozol, furazabol, danazol and stanozolol.

It is worth mentioning that the discriminating ability of the PCA analysis is confirmed by the positioning of molecules with conjugated double bonds (trenbolone, tetrahydrogestrinone, methyldienolone, methyltrienolone) at the far left (upper and bottom) quartiles of the ellipse, an evidence for being also different structurally and even metabolically from the "usual" metabolic pathways of the AAS. According to the loadings plot (Fig. 2) the variables referred to C-4,5, C-9,10 and C-11,12 double bonds were, indeed, responsible for the positioning of these AAS with conjugated double bonds.

3.2. Classification and prediction of the AAS metabolites: Excel® software classification

The metabolites of the parent AAS belonging to the above-mentioned PCA classes were treated using the Excel® software classification filters at specific sites of the steroidal structure, in order to examine the existence of common metabolic alterations

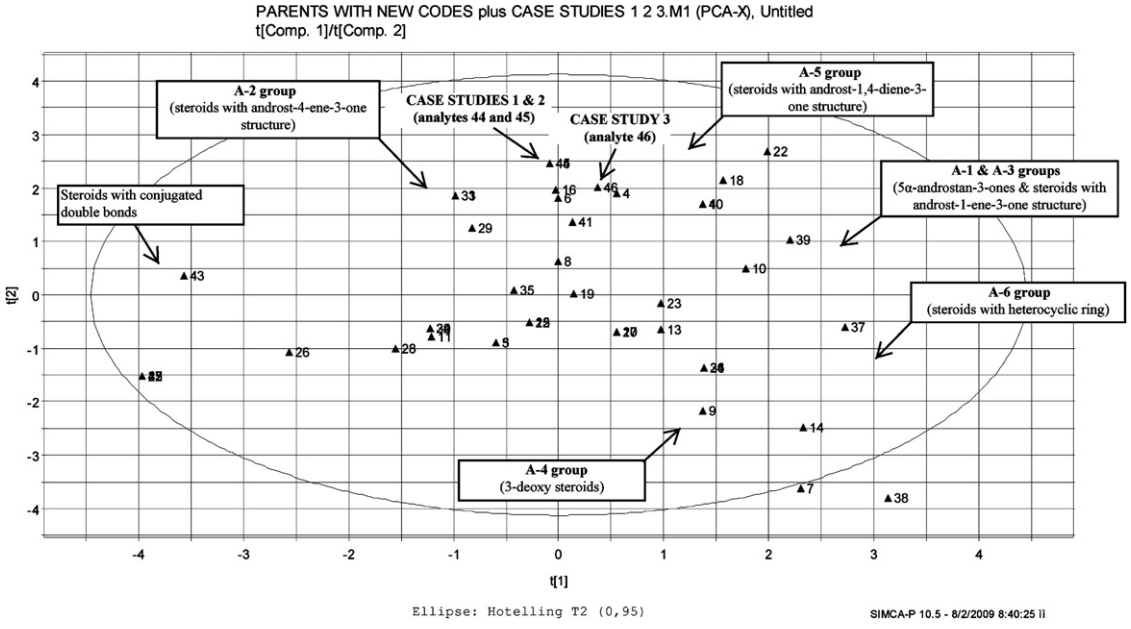


Fig. 1. Scores plot from principal components analysis of the parent AAS molecules. Parent molecules are numbered as in Table 1.

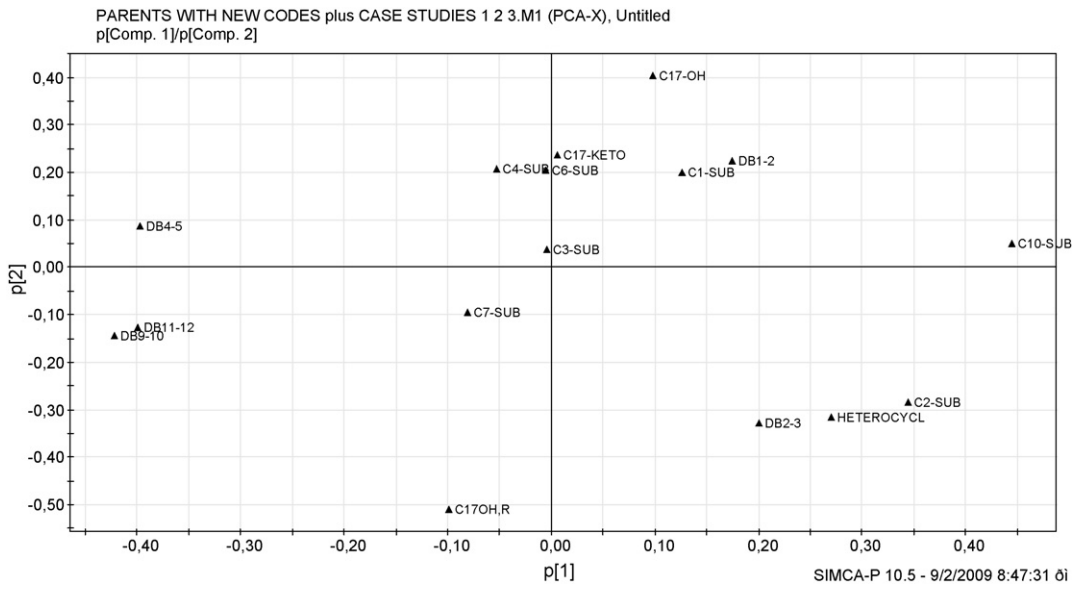


Fig. 2. Loadings plot from principal components analysis of the parent AAS molecules. *Abbreviations*: C1-SUB, C-1 substitution; C2-SUB, C-2 substitution; C3-SUB, C-3 substitution; C4-SUB, C-4 substitution; C6-SUB, C-6 substitution; C7-SUB, C-7 substitution; C10-SUB, C-10 substitution; C-17 KETO, 17-keto group; C17-OH, 17-hydroxylation; C17OH,R, 17β-hydroxylation,17α-alkylation; DB1-2, C-1,2 double bond; DB2-3, C-2,3 double bond; DB4-5, C-4,5 double bond; DB9-10, C-9,10 double bond; DB11-12, C-11,12 double bond; HETEROCYCL, existence of heterocyclic ring.

from common structure features of the parent molecules. The metabolism data were summarized leading to the construction of the metabolism schemes presented in this study, in order to predict all possible combinations of metabolism alterations for unknown AAS molecules.

The main differences between the metabolism data of known AAS and that is predicted from the schemes presented in this study, is that the proposed metabolism schemes carry two crucial characteristics:

(a) generate all possible metabolites from the referred metabolic reactions or from their combinations, regardless of the sequence that they could actually occur as the result of biotransformation processes in the human body,
(b) propose the existence of both isomers after reductions or hydroxylations, e.g. hydroxylations at C-6 and C-16 is proposed to give both α- and β-configurations.

3.2.1. *Proposed metabolism scheme for steroids with 5α-androstan-3-one structure (A-1 group)*

The proposed metabolism scheme for 5α-androstan-3-ones is presented in Fig. 3A. The metabolic reactions (M) for AAS with 5α-androstan-3-one structure are as follows:

- M1 concerns the metabolic alteration of 3-keto group, which give the 3α- and 3β-hydroxyl isomers. This reaction is not applied to steroids with a lactone group (i.e. oxandrolone) and hence to any oxandrolone like molecule.
- M2 concerns the metabolic reactions at position C-17. Depending on the substitution, the scheme discriminates three cases:
  - In case of a 17-keto group, reduction to either 17α- or 17β-hydroxyl group occurs (M2-a).
  - In case of a 17β-hydroxyl group, 17-epimerization and/or oxidation to 17-keto group are proposed (M2-b).
    The oxidation/reduction metabolic reactions of M2-a and M2-b can be considered are reversible ones.
  - In case of 17β-hydroxyl,17α-methyl (or 17α-alkylated, -R) steroids, 17-epimerization and dehydration products (18-nor-17,17-dimethyl-13(14)-ene and 18-nor-17α-methyl,17β-hydroxymethyl-13(14)-ene) are possible metabolic reactions (M2-c).
- M3 is a reaction which leads to hydroxylation at position C-18 and is proposed for steroids with C1- or C2-methyl groups. C-18 Hydroxylation has been observed in mesterolone metabolism which is a C1-methyl substituted steroid [17]. Although C-18 hydroxylation has not been observed in known C2-methyl substituted steroids with 5α-androstan-3-one structure, it is a metabolism pathway proposed in this study in correspondence with C-18 hydroxylation observed in C1- and C2-methyl substituted steroids with androst-1-ene-3-one structure (e.g. methenolone and stenbolone, respectively, Fig. 3C).
- M4 concerns hydroxylation at position C-6 (α- or/and β-isomers).
- M5 concerns hydroxylation at position C-16 (α- or/and β-isomers).

The above-described metabolic reactions (M1–M5) have been observed, in combinations or not, in drostanolone [18], methasterone [19] and mestanolone [4,5] metabolism.

Metabolic reactions M6 and M7 can be assumed as exceptions of the above generally observed metabolic reactions of 5α-androstan-3-ones, and concern steroids with groups which direct or inhibit their metabolism. M6 is referred to steroids with a C2-hydroxymethylene group (as in oxymetholone molecule [4,5]) for which metabolism alterations are mainly focused on C-2 position leading to reduction to C-2 hydroxymethyl group, oxidation to the corresponding C-2 carboxylic acid and then decarboxylation. Reduction of C-3 keto group and hydroxylation at C-6 position are also proposed. M7 concerns steroids with heteroatom at steroidal structure (as in oxandrolone molecule [20,21]) which directs metabolism to C-/D-rings leading to the 17-epimerized, 16-hydroxylated and dehydration products.

3.2.2. *Proposed metabolism scheme for steroids with androst-4-ene-3-one structure (A-2 group)*

The proposed metabolic reactions for steroids with androst-4-ene-3-one structure are depicted in Fig. 3B.

- M1 concerns the reduction of the C-4,5 double bond leading to both 5α-H and 5β-H isomers. Known steroids which follow this

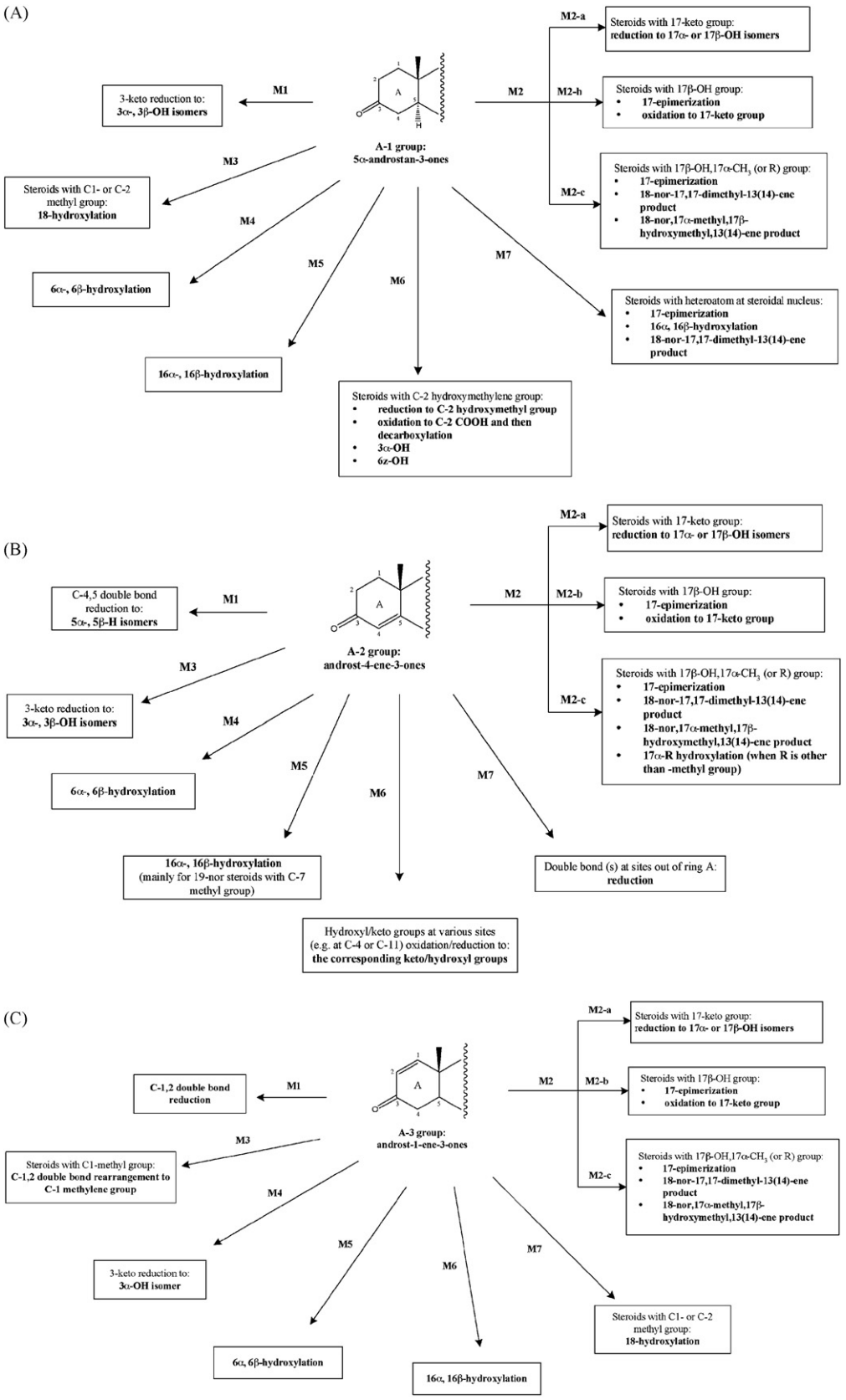


Fig. 3. A. Scheme of metabolism of 5α-androstan-3-ones, B. Scheme of metabolism of androst-4-ene-3-ones, C. Scheme of metabolism of androst-1-ene-3-ones, D. Scheme of metabolism of 3-deoxy androstanes, E. Scheme of metabolism of androsta-1,4-diene-3-ones, F. Scheme of metabolism of AAS with heterocyclic ring and conjugated double bonds.

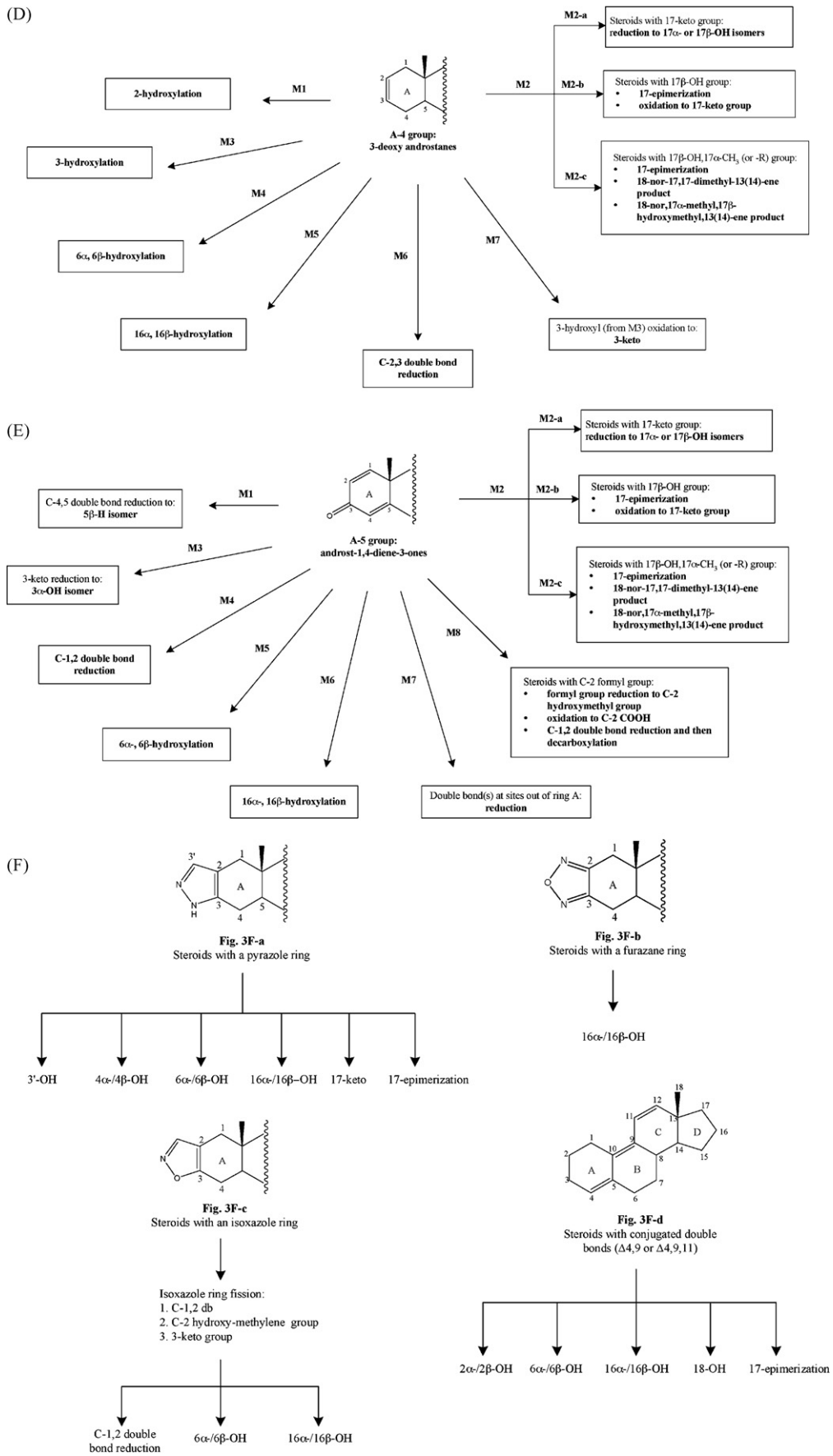


Fig. 3. (*Continued*).

metabolic pathway are methyl-testosterone [22], clostebol [4], 4-hydroxy-testosterone [6] and fluoxymesterone metabolism [23].
- M2 leads to metabolic reactions at position C-17, as those already referred in Section 3.2.1. However, one further metabolic reaction is proposed for androst-4-ene-3-one structure steroids with 17β-hydroxyl,17α-R groups; hydroxylation at 17α-R, when R is other than methyl group, as has been referred in ethylestrenol [4,6] and norethandrolone metabolism [5].
- M3 depicts the reduction of the 3-keto group leading to the 3α-hydroxyl isomer, mainly after formation of a 5β-H steroid from metabolic reaction M1. 3β-Hydroxyl isomer formation is also proposed, although it has not generally been reported, indicating that the reaction does not occur, or that its extent is too small to be detected by the analytical method used [4]. However, in fluoxymesterone metabolism both 3α- and 3β-hydroxyl isomers have been reported as metabolic products. The presence of a C-4 substituent can alter the extent that both the C-4,5 double bond and 3-keto group reductions occur. In the case of clostebol, the chlorine atom at C-4 position inhibits the action of reductases for reduction of the C-4,5 double bond and the 3α-hydroxyl isomer is formed in the presence of the C-4,5 double bond [5,24]. Nevertheless, fully A-ring reduced metabolites have been reported and are of interest as they yield information about the reduction mechanisms of the 5α- and 5β-reductases [5].
- M4 concerns hydroxylation at C-6 position (α- or/and β-isomers).
- M5 has been reported mainly for 19-nor steroids with C-7 methyl group and leads to hydroxylation at C-16 position (α- or/and β-isomers), as occurs in mibolerone metabolism [25,26].
- M6 is referred to possible oxidations of other hydroxyl groups at various positions in the steroidal structure that may be present, e.g. at C-4 position as occurs in 4-hydroxy-testosterone metabolism [27] or at C-11 position as occurs in fluoxymesterone metabolism [23]. The reversible reductions of keto groups to the corresponding hydroxyl groups are also proposed.
- M7 concerns reduction(s) of double bond(s) at sites other than those at ring A of the steroidal structure, e.g. at C-6,7.

3.2.3. Proposed metabolism scheme for steroids with androst-1-ene-3-one structure (A-3 group)

The proposed metabolism scheme for steroids with androst-1-ene-3-one structure is depicted in Fig. 3C. The metabolic reactions are as follows:

- M1 concerns the C-1,2 double bond reduction.
- M2 depicts the metabolic reactions at position C-17, as those already referred in Section 3.2.1.
- M3 is referred for steroids with C1-methyl group which in combination with the C-1,2 double bond leads to rearrangement and formation of a C-1 methylene group, as occurs in methenolone metabolism [5,28].
- M4 depicts the reduction of the 3-keto group leading mainly to the 3α-hydroxyl isomer, as has been reported in metabolism of 1-testosterone [6], stenbolone [6], methyl-1-testosterone [19] and methenolone [28].
- M5 and M6 concern hydroxylations at C-6 and C-16 positions (α- or/and β-isomers), respectively.
- M7 is a metabolic alteration which concerns steroids with C1- or C2-methyl groups and leads to hydroxylation at position C-18, as occurs in stenbolone [6] and methenolone [28] metabolism.

3.2.4. Proposed metabolism scheme for steroids with 3-deoxy androstan structure (A-4 group)

The proposed metabolism scheme for steroids with 3-deoxy androstane structure has been constructed assuming desoxymethyltestosterone (madol) metabolism [29] and is depicted in Fig. 3D. The metabolic reactions concern:

- Hydroxylations at C-2 (M1), C-3 (M3), C-6 (M4) and C-16 (M5) positions.
- Metabolic alterations at position C-17 (M2), as those already referred in Section 3.2.1.
- Reduction of the C-2,3 double bond (if exists) (M6).
- 3-Hydroxyl group oxidation to 3-keto group (M7), mainly after 3-hydroxyl group formation from metabolic reaction M3.

3.2.5. Proposed metabolism scheme for steroids with androst-1,4-diene-3-one structure (A-5 group)

The proposed metabolism scheme for steroids with androst-1,4-diene-3-one structure is depicted in Fig. 3E. The metabolic reactions are as follows:

- M1 concerns the reduction of the C-4,5 double bond leading mainly to the 5β-H isomer. The simultaneous presence of a C-1,2 double bond, as occurs in methandienone [30] and boldenone [31] molecules, inhibits the 5α-reduction. The hydroxyl group as substituent at C-4 position may also hinder the reduction of the C-4,5 double bond, especially of the 17α-methyl,17β-hydroxyl steroids. In oxymesterone molecule, which bears a C-4 hydroxyl group, the reduction of the C-4,5 double bond does not occur while it is rapidly conjugated at the C-4 hydroxyl and excreted in the urine [5]. In the absence of a 17α-methyl group, as in 4-hydroxy-testosterone [6] and oxabolone molecules [6,32] the A-ring reductions normally occur.
- M2 depicts the metabolic reactions at position C-17, as those already referred in Section 3.2.1.
- M3 concerns 3-keto group reduction yielding the 3α-hydroxyl isomer, mainly after formation of a 5β-H steroid from metabolic reaction M1.
- M4 is referred to the C-1,2 double bond reduction mainly after formation of 5β-H/3α-hydroxyl metabolites, as occurs in methandienone [30] and boldenone [31] metabolism.
- M5 concerns hydroxylation at C-6 position (α- or/and β-isomers). Hydroxylation at this position was observed for all metabolites derived from parent molecules with androsta-1,4-diene-3-one structure. C-6β hydroxylation is more pronounced for 17α-methyl,17β-hydroxyl steroids such as methandienone and oral turinabol, where the A-ring reduction is hampered by the presence of a C-1,2 double bond [30,33,34].
- M6 concerns hydroxylation at C-16 position (α- or/and β-isomers).
- M7 concerns reduction(s) of double bond(s) at sites other than those at ring A of the steroidal structure, e.g. at C-6,7.
- M8 can be assumed as exception of the above generally observed metabolic reactions of steroids with androst-1,4-diene-3-one structure, and concerns steroids with a C2-formyl group as in formebolone molecule. The metabolism in formebolone is solely directed to the A-ring, where the reactive formyl group is reduced to the corresponding 2-hydroxymethyl metabolite. Oxidation of the 2-formyl group to β-keto acid, reduction of the C-1,2 double bond and then decarboxylation leads to its metabolite; 11α-hydroxymethyltestosterone. No metabolites from 3-keto group, epimerization at C-17 position or hydroxylation at other positions of the steroid were detected [35].

3.2.6. Proposed metabolism scheme for steroids with heterocyclic rings (A-6 group)

The proposed metabolism schemes for steroids with heterocyclic rings are depicted in Fig. 3F. The metabolic alterations of AAS with a pyrazole ring (Fig. 3F-a) lead to hydroxylations at various positions of the A-, B- and D-rings (e.g. prostanozol [29] and stanozolol [36]). In AAS with a furazane ring (Fig. 3F-b), hydroxylation as metabolic alteration is limited and is yielded mainly at

C-16 position (e.g. furazabol [4,5]). Finally, in AAS with an isoxazole ring (Fig. 3F-c), fission of the heteroxyclic ring is proposed to yield the C-1,2 double bond and 3-keto group formation. Further, reductions of these functions and hydroxylations at C-6 and C-16 positions may be also observed, as occurs in danazol metabolism [37,38].

3.2.7. Proposed metabolism scheme for steroids with conjugated double bonds

AAS with conjugated double bonds, such as gestrinone [6,39] and tetrahydrogestrinone (THG) [6,40], do not undergo extensive biotransformation routes; their metabolic pathways include mainly hydroxylations at C-2, C-6, C-16 or even C-18 positions. In the case of trenbolone, metabolism process is restricted to C-17 epimerization [41]. Other known AAS with conjugated double bonds are methyltrienolone and methyldienolone, for which no metabolism data is available. The proposed metabolism scheme of steroids with conjugated double bonds includes these metabolic reactions and is shown in Fig. 3F-d.

3.3. Guide for the AAS metabolites estimation

The selection of the proposed metabolism schemes (Fig. 3A–F) in order to predict the possible metabolites of a designer steroid, require the use of the flowchart depicted in Fig. 4, which comprises a sequence of answers and replies for the detection of structural features acceptable of metabolic alterations. The sequence of answers and replies of Fig. 4 explores the steroidal core for the presence of groups or sites, starting from the A-ring, which directs the metabolism of steroids according to the metabolic schemes presented in Figs. 3A–F. Special concern has been paid for the presence of a keto group at position C-3 or of a heteroatom that is essential for the functionality of a steroid as anabolic and androgenic [1]. Furthermore, the presence of oxidative groups at position C-2 has been explored because the presence of these groups directs the metabolism of steroids to "unusual" metabolism routes. Following these initial answers and replies, the investigation of the structure of the steroids leads to decision tables to propose which of the Fig. 3A–F to be selected for the prediction of the metabolites of a designer steroid.

4. Discussion

Case studies of three known, in the literature AAS (6α-methylandrostenedione [42], androst-4-ene-3,6,17-trione [43] and androsta-1,4,6-triene-3,17-dione [44]) not included in the WADA list of prohibited substances, are presented as examples of how to use the proposed metabolism schemes. The steps are as follows:

Step 1: Illustrate the parent molecule structure.
Step 2: Answer to the questions of Fig. 4.
Step 3: Follow the metabolic reactions of the figure(s) (Fig. 3A–F) you were directed to. Incompatible metabolic pathways are excluded, e.g. C6-hydroxylation in an already C-6 substituted steroid.

4.1. Case study 1: estimation of metabolites of 6α-methylandrostenedione

The structure of 6α-methylandrostenedione is depicted in Fig. 5 (centre). The flowchart, following the replies to the questions of Fig. 4, leads to the selection of Fig. 3B for the estimation of the metabolites of 6α-methylandrostenedione (combination of the last four replies: YNNN). Following the metabolic reactions depicted in Fig. 3B, 26 metabolites are proposed:

- Metabolites **1** and **2** are derived from metabolic reaction M1 and are the 5α-H and 5β-H isomers from reduction of the C-4,5 double bond of the parent molecule.
- Metabolites **3** and **4** are derived from reduction of the 17-keto group to the 17α- and 17β-hydroxyl isomers from metabolic reaction M2-a.
- Metabolite **5** and **6** are derived from 3-keto reduction to the 3α- and 3β-hydroxyl isomers and occur from metabolic reaction M3.
- Metabolites **7**–**10** are derived from combination of metabolic reactions M1 and M2-a leading to C-4,5 double bond reduction to yield both 5α-H and 5β-H isomers with subsequent reduction of the 17-keto group to the 17α- and 17β-hydroxyl isomers.
- Metabolites **11** and **12** have followed metabolic reactions M1 and M3, which lead to C-4,5 double bond reduction to yield both 5α-H and 5β-H isomers with subsequent reduction of the 3-keto group to the 3α-hydroxyl isomer. The corresponding metabolites from the 3β-hydroxyl isomers (metabolites **13** and **14**, not shown in Fig. 5) are also possible to occur.
- Metabolites **15** and **16** are derived from combination of metabolic reactions M2-a and M3, which lead to reduction of the 3- and 17-keto groups to the 3α-/17β- and 3α-/17α-hydroxyl isomers. The corresponding metabolites from the 3β-hydroxyl isomers (metabolites **17** and **18**, not shown in Fig. 5) are also possible to occur.
- Metabolites **19**–**22** have followed metabolic reactions M1, M2-a and M3, which lead to the C-4,5 double bond reduction to both 5α-H and 5β-H isomers with subsequent reduction of the 3- and 17-keto groups to the 3α-/17β- and 3α-/17α-hydroxyl isomers. The corresponding metabolites from the 3β-hydroxyl isomers (metabolites **23** and **26**, not shown in Fig. 5) are also possible to occur.

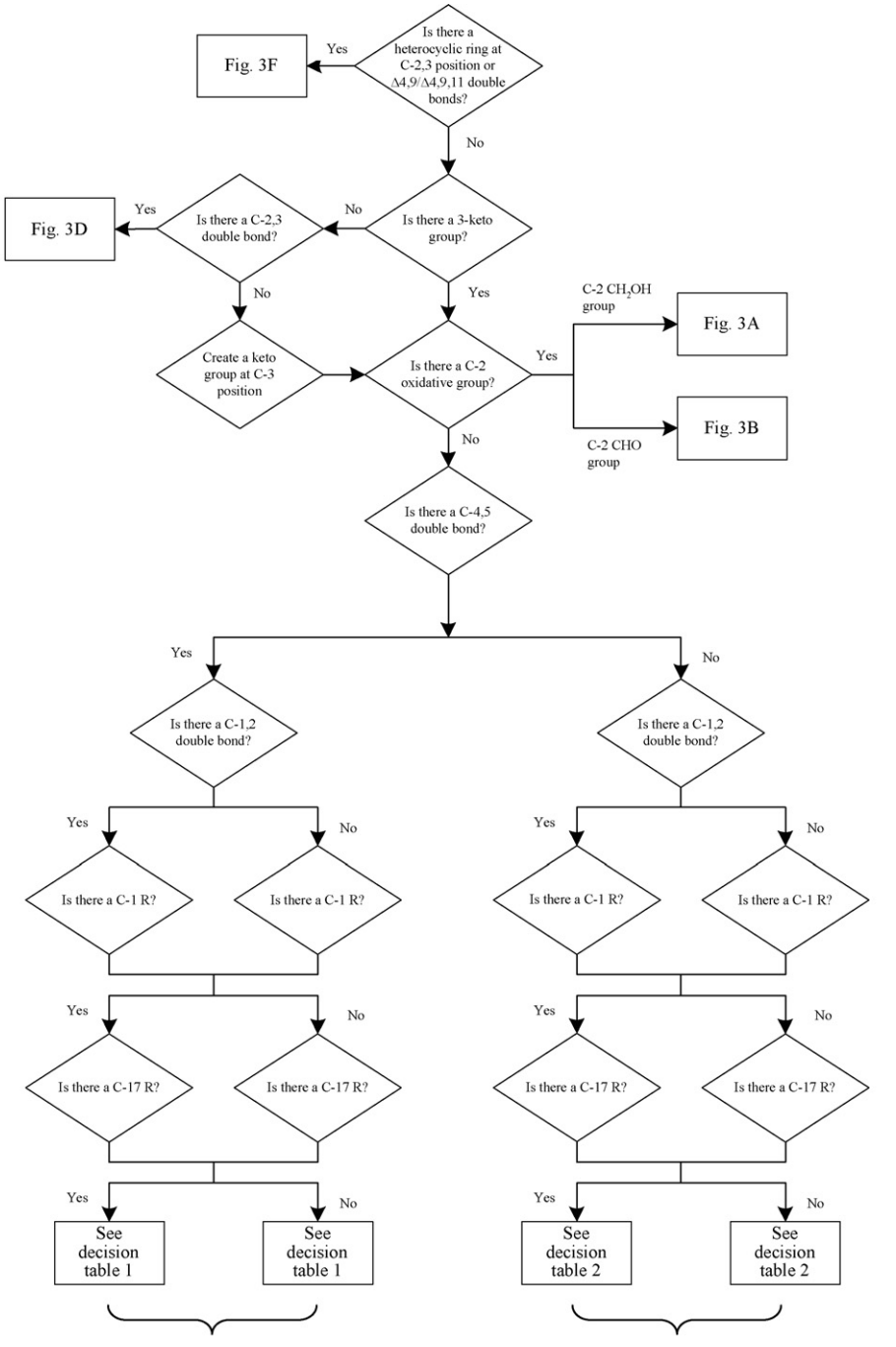
The metabolic reaction M4 (C6-hydroxylation) of Fig. 3B is incompatible for 6α-methylandrostenedione molecule as it is already C6-methyl substituted, while the metabolic reactions M5, M6 and M7 are non-applicable.

The proposed metabolite **12** (3α-hydroxy-6α-methyl-5β-androstan-17-one) is, indeed, referred in literature [42] as the main metabolite of 6α-methylandrostenedione detectable in all urine samples of an administration study, while the proposed metabolite **5** (3α-hydroxy-6α-methylandrost-4-ene-17-one) is referred as a minor one. The proposed metabolite **4** (6α-methyltestosterone) has been also detected but in very low amounts.

4.2. Case study 2: estimation of metabolites of androst-4-ene-3,6,17-trione

The structure of androst-4-ene-3,6,17-trione is depicted in Fig. 6 (centre). The flowchart, following the replies to the questions of Fig. 4, leads to the selection of Fig. 3B for the estimation of the metabolites of androst-4-ene-3,6,17-trione (combination of the last four replies: YNNN). Following the metabolic reactions depicted in Fig. 3B, 72 metabolites are proposed:

- Metabolites **1** and **2** are the 5α- and 5β-H isomers from metabolic reaction M1.
- Metabolites **3** and **4** are the 17α- and 17β-hydroxyl isomers from metabolic reaction M2-a.
- Metabolites **5** and **6** are derived from 3-keto reduction to the 3α- and 3β-hydroxyl isomers from metabolic reaction M3.
- Metabolites **7** and **8** are derived from metabolic reaction M6 yielding the 6α- and 6β-hydroxyl isomers from reduction of the 6-keto group.
- Metabolites **9**–**32** are derived from combinations of two of the above-mentioned metabolic reactions, as those depicted in Fig. 6.



Decision table 1

Combination of the last 4 replies Yes/No	Figure(s) for metabolism
YYYY	Fig. 3E, Fig.3C
YYNY	Fig. 3E
YYYN	Fig. 3E, Fig.3C
YYNN	Fig. 3E
YNYY	Fig. 3B
YNYN	Fig. 3B
YNNY	Fig. 3B
YNNN	Fig. 3B

Decision table 2

Combination of the last 4 replies Yes/No	Figure for metabolism
NYYY	Fig. 3C
NYYN	Fig. 3C
NYNY	Fig. 3C
NYNN	Fig. 3C
NNYY	Fig. 3A
NNYN	Fig. 3A
NNNY	Fig. 3A
NNNN	Fig. 3A

Fig. 4. Flowchart for AAS metabolism schemes selection.

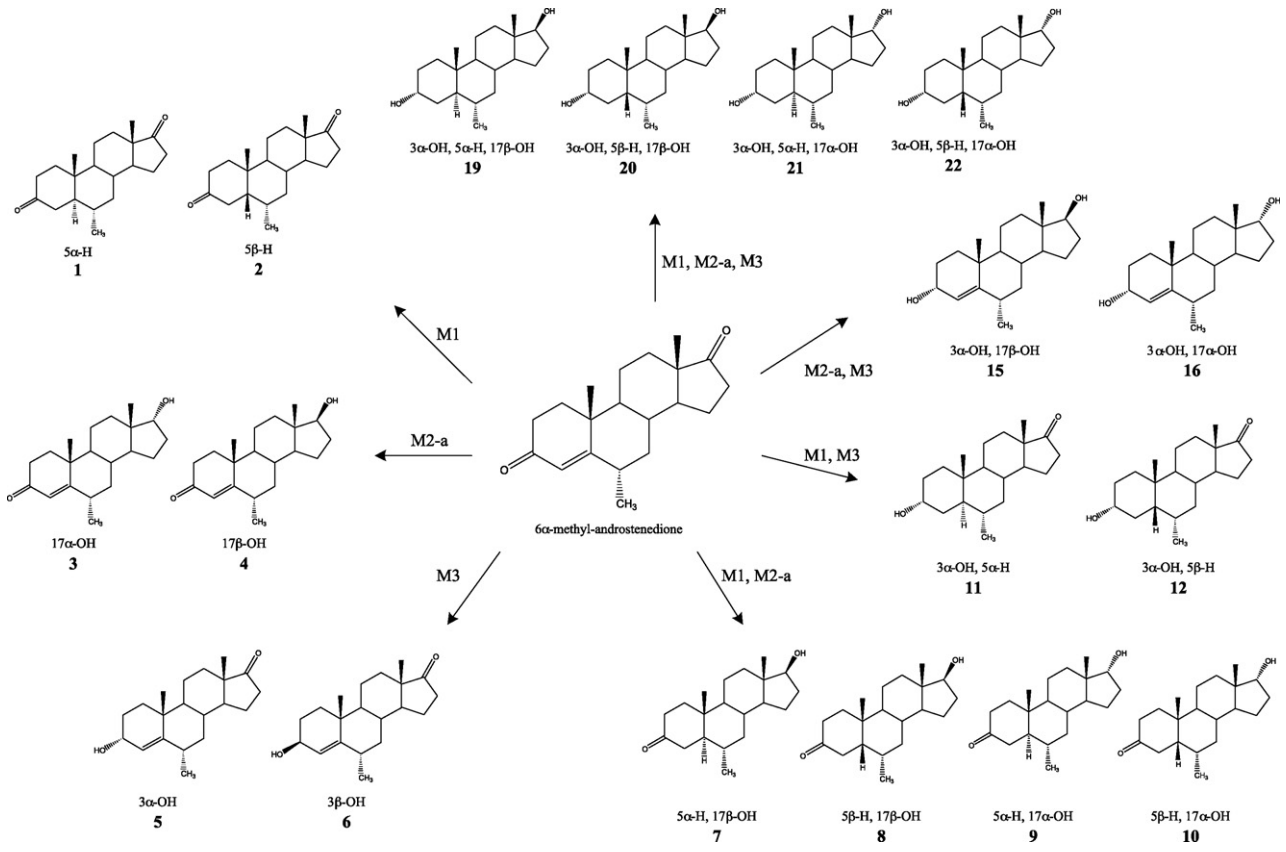


Fig. 5. Scheme of predicted metabolites of 6α-methylandrostenedione.

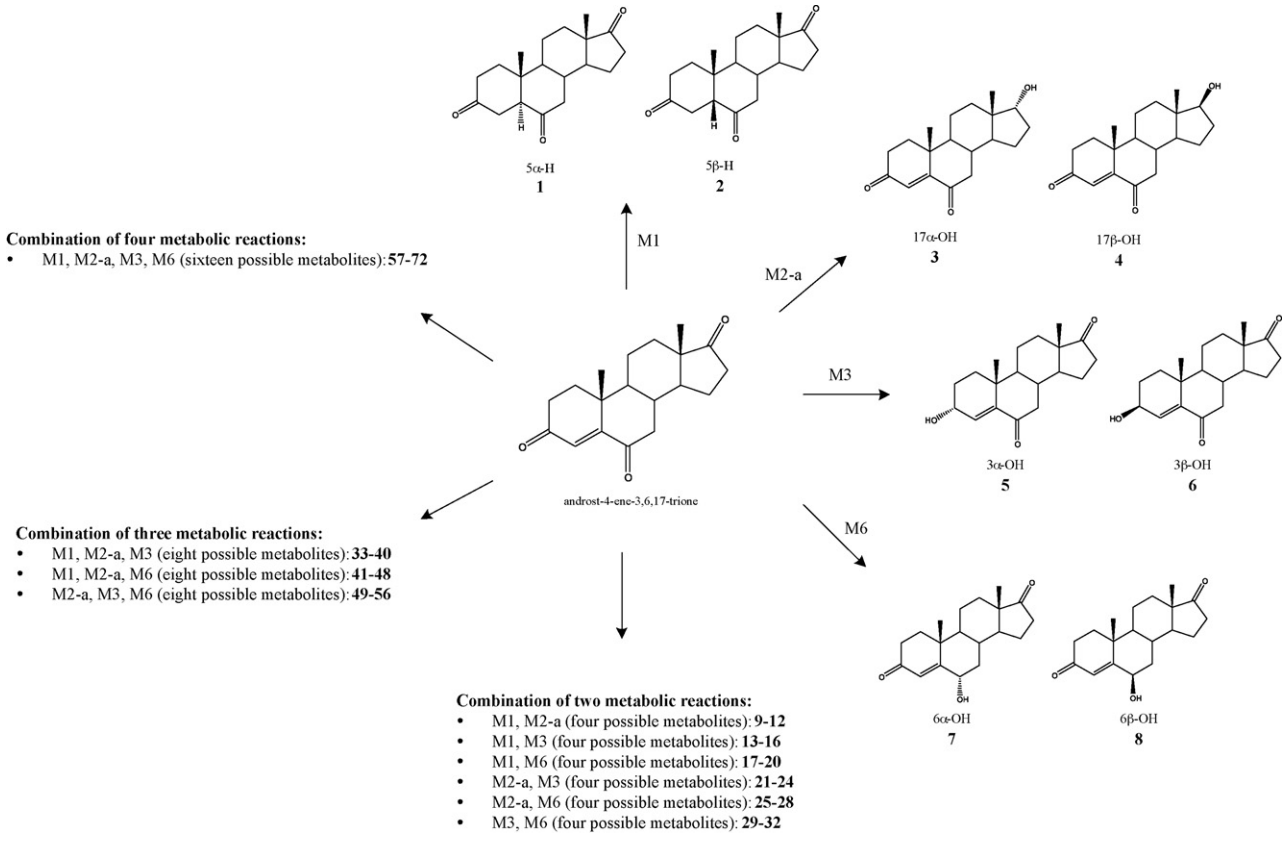


Fig. 6. Scheme of predicted metabolites of androst-4-ene-3,6,17-trione.

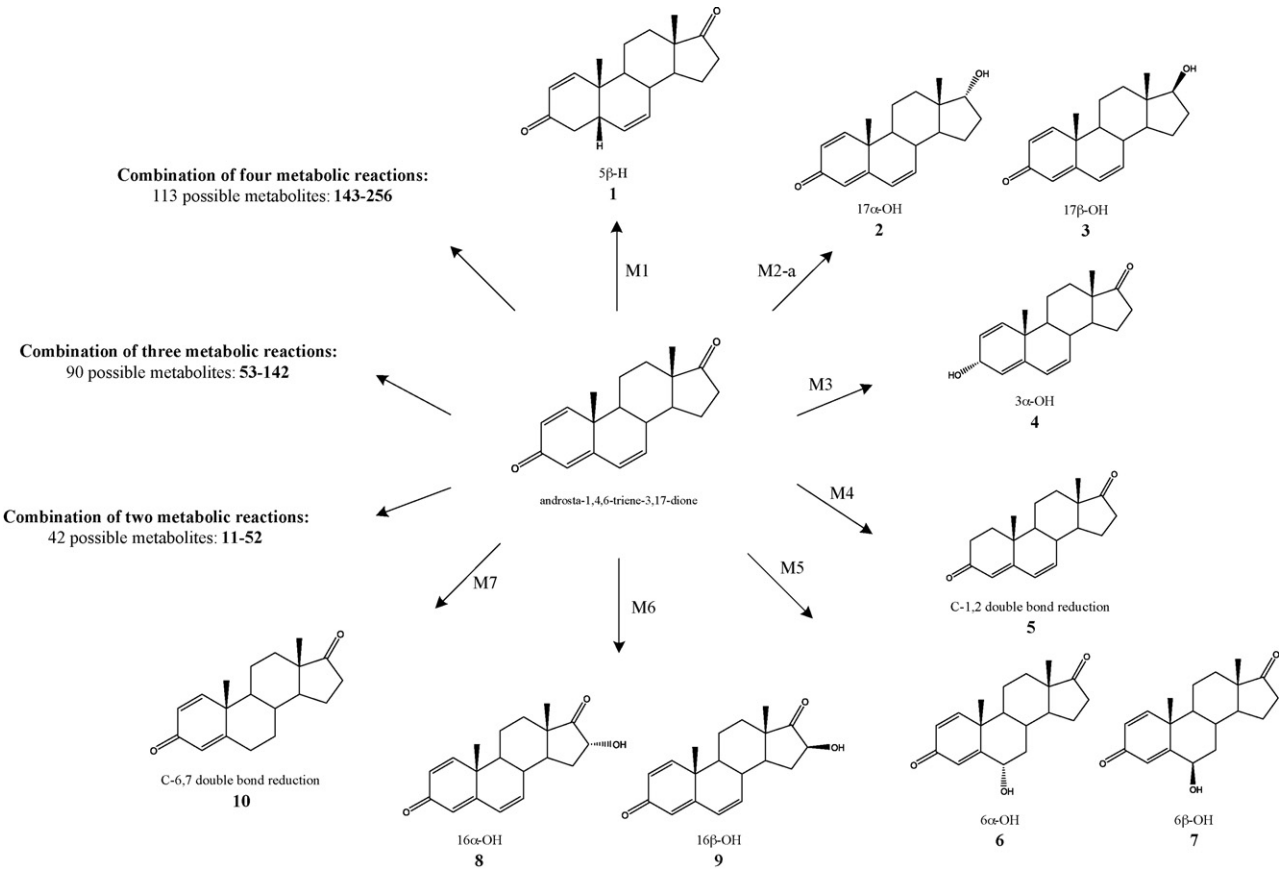


Fig. 7. Scheme of predicted metabolites of androsta-1,4,6-triene-3,17-dione.

- Metabolites **33**–**56** are derived from combinations of three of the above-mentioned metabolic reactions, as those depicted in Fig. 6.
- Metabolites **57**–**72** can derive from combinations of four of the above-mentioned metabolic reactions, as depicted in Fig. 6.

The metabolic reactions M4, M5 and M7 are non-applicable to the androst-4-ene-3,6,17-trione molecule.

The proposed metabolite **7** (6α-hydroxy-androstendione) and the one from combination of metabolic reactions M2-a and M6 (6α-hydroxytestosterone) are, indeed, referred in the literature as metabolic products of androst-4-ene-3,6,17-trione detected in excretion studies [43].

4.3. Case study 3: estimation of metabolites of androsta-1,4,6-triene-3,17-dione

The structure of androsta-1,4,6-triene-3,17-dione is depicted in Fig. 7 (centre). Following the replies to the questions of Fig. 4, the possible metabolites of androsta-1,4,6-triene-3,17-dione are proposed according to Fig. 3E (combination of the last four replies: YYNN). Following the metabolic reactions of Fig. 3E, 256 metabolites are proposed:

- Metabolite **1** can derive from metabolic reaction M1 and is the 5β-H isomer from reduction of the C-4,5 double bond of the parent molecule.
- Metabolites **2** and **3** concern the reduction of the 17-keto group to the 17α- and 17β-hydroxyl isomers from metabolic reaction M2-a.
- Metabolite **4** is derived from the 3-keto reduction to the 3α-hydroxyl isomer from metabolic reaction M3.
- Metabolite **5** concerns the C-1,2 double bond reduction according to metabolic reaction M4.
- Metabolites **6** and **7** are derived from metabolic reaction M5 leading to the 6α- and 6β-hydroxyl isomers.
- Metabolites **8** and **9** from metabolic reaction M6 are the corresponding 16α- and 16β-hydroxyl isomers.
- Metabolite **10** concerns the C-6,7 double bond reduction from metabolic reaction M7.
- Metabolites **11**–**52** can derive from combinations of two of the above-mentioned metabolic reactions.
- Metabolites **53**–**142** can derive from combinations of three of the above-mentioned metabolic reactions.
- Metabolites **143**–**256** can derive from combinations of four of the above-mentioned metabolic reactions.

The metabolic reaction M8 of Fig. 3E is non-applicable in this case study. The proposed metabolites **3** (17β-hydroxyandrosta-1,4,6-triene-3-one) and **5** (androsta-4,6-diene-3,17-dione) are referred in the literature as metabolites detected in human urine after the administration of androsta-1,4,6-triene-3,17-dione. The metabolites from the combination of the metabolic reactions M2-a and M4 (17β-hydroxyandrosta-4,6-diene-3-one), from M2-a and M7 (17β-hydroxyandrosta-1,4-diene-3-one) and from M1, M2-a and M7 (17β-hydroxy-5β-androst-1-ene-3-one) have been also detected in excretion studies [44].

Furthermore, supporting evidence that the above-estimated metabolites of the case studies, 6α-methylandrostenedione, androst-4-ene-3,6,17-trione and androsta-1,4,6-triene-3,17-dione, are in correspondence with those detected from known parent molecules with structural similarities, provides the PCA analysis. The application of PCA analysis, after the insertion of the structural features of the case studies molecules in the parent AAS datasheet

described in Section 2.1.1, positions their spots (Fig. 1, analytes 44, 45 and 46, respectively) in close contact to molecules belonging to the A-2 and A-5 groups, i.e. molecules with androst-4-ene-3-one and androst-1,4-diene-3-one structures.

Overall, metabolism schemes based on the metabolic alterations of known AAS, included in the WADA list of prohibited substances [14], have been presented in this paper for the prediction of biotransformation routes of chemically modified analogues, probably detected by anti-doping laboratories. The construction of the metabolism schemes took into consideration the alteration or influence in biotransformation routes induced by certain structural moieties of the parent molecule. The presented metabolism schemes can be used as guide of a rapid, but valuable, estimation of metabolism pathways when an unknown AAS is detected or found in the literature.

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