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RECENT ADVANCES IN DOPING ANALYSIS (8)

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Progress in the Preparation of Anabolic Steroid Reference Materials & Certified Reference Materials

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SUMMARY

This paper describes continuing progress in our program to produce and characterise a collection of anabolic steroid metabolites and deuterates in accordance with the ISO Guidelines for the preparation of reference materials[1]. Recent developments in our laboratory in the following areas will be outlined:

- third-party accreditation for the production of Reference Materials (RMs) and Certified Reference Materials (CRMs)
- progress and problems in the synthesis of steroid metabolites
- synthesis of nandrolone deuterates and 19-d_3-testosterone

INTRODUCTION

One of the factors affecting a testing laboratory's capability to produce reliable reproducible results is the availability of reference materials with property values that can be relied upon by their users. The critical roles of reference materials at various stages in an analytical procedure are shown schematically in Figure 1. The distinction between the broader general category of Reference Materials (RMs) and Certified Reference Materials (CRMs) is that for the latter all property values (eg. chemical purity) are reported with an uncertainty estimate [2]. This typically gives the 95% confidence range for the value. In addition these values are said to be traceable, generally to the International System of Units (SI). Given their key role in ensuring the quality of chemical analyses ISO Guide 34 was published in 1996 and revised in 2000 [3] to outline the general quality system requirements needed to demonstrate the competence of reference material producers. This paper will briefly outline our successful accreditation to the requirements of ISO Guide 34 for the production of anabolic steroid metabolites and

deuterates. In addition progress in completing our initial program for the production of reference materials will be described, as well as the preparation of additional materials.

DISCUSSION

(i) Accreditation requirements for RM & CRM production

ISO Guide 34 was initially developed as specific guidelines for the interpretation of the ISO Guide 17025 [4] (which recently superceded ISO Guide 25) and ISO 9000 [5] series of standards, in the context of reference material production. As for testing laboratories in general, an appropriate quality system for reference material producers has two components: management requirements and technical requirements.

The specific management system requirements are implementing and documenting the procedures in place for:

- Defining the scope for RM and CRM production
- Monitoring the activities of collaborators and contractors
- Documenting the quality system and record storage in a controlled manner
- Auditing operations, both internally and externally
- Controlling non-conformance

The implementation of most of these requirements will be familiar to testing laboratories already operating under ISO Guide 17025 requirements.

The key technical requirements for the production of RMs and CRMs are:

- Staff competency and training
- Protocols for planning production
- Protocols for candidate material preparation
- Validation of all measurement methods
- Calibration, maintenance and quality control (QC) of all measuring equipment
- Procedures for the qualitative characterisation, assignment of property values and estimation of uncertainty budgets for these values
- Protocols for the establishment of the traceability of property values
- Determination of the homogeneity and stability of the materials
- Appropriate secure storage facilities
- Procedures for dispensing, packaging, labelling and dispatch of materials
- Provision of appropriate reports and certificates for endusers

The procedures followed to estimate uncertainty and establish traceability for the property values of CRMs are beyond the scope of this talk and will be reported elsewhere [6].

An accreditation assessment was carried out in November 1999 which included two international experts in CRM production in the inspection team. As a result, third-party accreditation "for the production of Certified Reference Materials and Reference Materials of pure substance organic solids for steroids, steroid metabolites and deuterates, illicit drugs, agrochemicals and agrochemical metabolites" was conferred in January 2000.

(ii) Current status of the steroid RM production program

The requirements for steroid reference material production were divided into two classes. Monitoring endogenous steroid levels required the production of both unlabelled and deuterated metabolites and conjugates for use as internal standards in steroid profiling and for calibration and QC purposes. The synthesis, characterisation and certification of these materials have been undertaken "in-house" by the Pure Substance Reference Material group. Monitoring xenobiotic ("unnatural") steroids needs the production of unlabelled metabolites and key glucuronide conjugates. These materials are required for calibration, to establish limit of detection (LOD) and limit of reporting (LOR) thresholds, for routine QC checks and as positive controls in confirmation assays. Contract laboratories undertook the synthesis and partial characterisation of these materials and, after confirming the characterisation, the final certification was completed by our group.

As reported previously, all materials are characterised by a range of qualitative and quantitative spectroscopic and analytical techniques including GC-MS, GC-FID, LC-MS, NMR, IR, TGA and microanalysis [1].

The current status (July 2000) of the program is as follows:

- 90 compounds have been prepared and characterised
- 60 are parent steroids or their Phase I metabolites
- 30 are Phase II metabolites (glucuronides and sulfates)
- 62 have been independently reviewed and are available for issue as reference materials

This is summarised for the endogenous steroid metabolites in Table 1 and for the xenobiotic steroids in Table 2.

(iii) Recent developments in RM synthesis

(a) 3'-Hydroxystanozolol glucuronide

A number of the materials from our initial requirement list have proved to be challenging synthetic targets, particularly for multigram synthesis. For instance we have so far not been

able to prepare at large scale any glucuronides of 17α-methyl steroids which are glucuronidated at the 17β-hydroxyl group. In addition we were unable to obtain the 3'-glucuronide of 3'-hydroxystanozolol (**1**) by chemical synthesis (Fig. 2). However a collaborating laboratory developed an alternative enzymatic synthesis of this compound and has successfully used it to prepare at the required scale the glucuronide (**1**).

(b) Epimetendiol and Normetanol [7]

The synthesis of the key methandienone metabolites epimetendiol (**2**) and 18-normetanol (**3**) has been reported [8], using a selective reduction of the dienone moiety of methandienone as the key step. A contracting laboratory was unable to repeat this synthesis at large scale. A new synthetic scheme which used sequential bromination/debromination of 5β-dihydromethyltestosterone to establish the 1,2-alkene in the A-ring was successful and has been used as a key intermediate in the preparation of both compounds (Fig 3).

(c) Deuterated nandrolone metabolites

Quantitation of the levels of norandrosterone and noretiocholanolone is required to establish whether potential cases of nandrolone doping exceed the current reporting thresholds. The availability of deuterated analogues of both metabolites would greatly facilitate accurate quantitation, and syntheses of both these materials have been completed. They are summarised in Figure 4 for d_4-norandrosterone (**4**) and Figure 5 for d_4-noretiocholanolone (**5**).

(d) 19-d_3-Testosterone

The identification of marker metabolites for the administration of testosterone or, in particular, androstendione, would be helped by the availability of deuterated analogues which have the deuterons in positions resistant to both chemical and enzymatic exchange. Materials labelled at C-19 are expected to maintain the requisite longitudinal stability for metabolism studies. In collaboration with researchers at the University of Sydney we have developed a convenient synthesis of 19-d_3-testosterone[9]. This material is readily converted to 19-d_3-androstendione. Key steps in the synthetic scheme are outlined in Figure 6.

CONCLUSION

The successful implementation of a recognised, accredited Quality System for the production of anabolic steroids, steroid metabolites and deuterates will hopefully make an important contribution to improving the quality and intercomparability of doping analysis worldwide. As a result of our production program we have an extensive collection of materials available, characterised and certified in a fashion that is fit for purpose for current analytical applications and regulatory requirements.

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7. Shorthand names used for convenience. See Ref. 8 for full IUPAC-nomenclature of both these compounds.
8. W. Schänzer and M. Donike, *Anal. Chim. Acta*, 1993, **275**, 23-48.
9. S. Vonwiller and B. Indusegaram, University of Sydney, unpublished results.

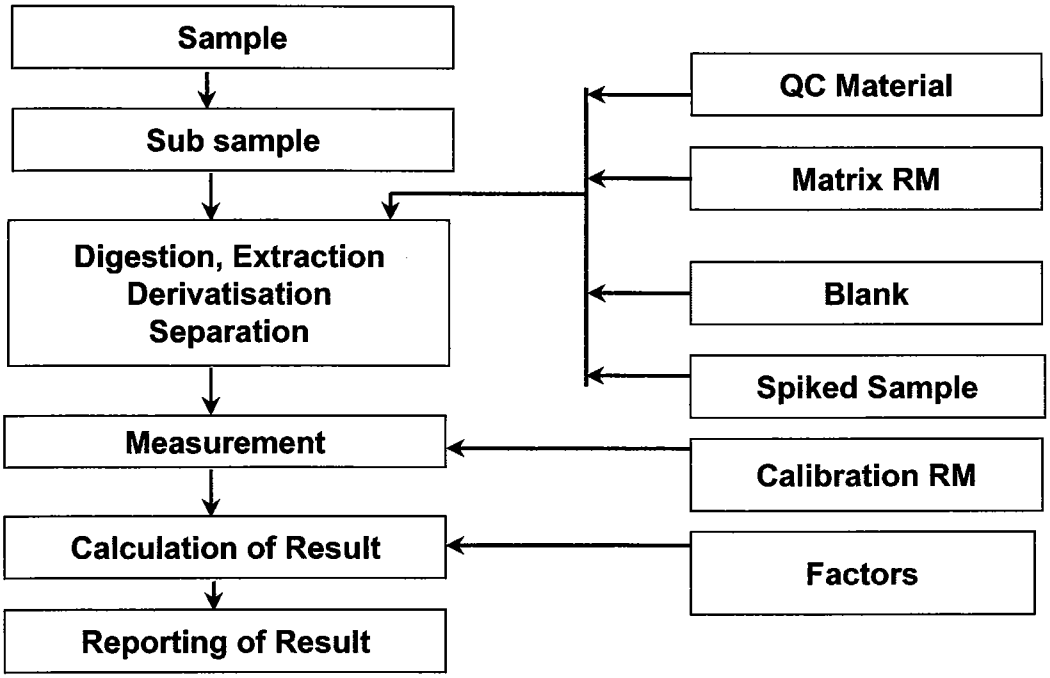

Fig. 1: Roles of reference materials in the analytical process

Steroid Category	*Parent*	*Glucuronide*	*Sulfate*
• **Deuterated endogenous steroids**			
16,16,17-d_3-Testosterone	▲	▲	▲
19,19,19-d_3-Testosterone	✚		
19,19,19-d_3-Androstendione	✚		
d_3-Epitestosterone	▲	▲	▲
d_3-5α-Dihydrotestosterone	▲	▲	▲
d_4-Androsterone	▲	▲	▲
d_3-5β-Dihydrotestosterone	✚		
d_5-Etiocholanolone	▲		▲
d_3-5α-Androstane-3α,17β -diol	▲		
d_3-5α-Androstane-3β,17β -diol	▲		
d_5-5β-Androstane-3α,17β -diol	▲		
d_3-5β-Androstane-3β,17β -diol	▲		
• **Unlabelled endogenous steroids**			
Testosterone	▲	▲	▲
Epitestosterone	▲	▲	▲
5α-Dihydrotestosterone	▲	▲	▲
Androsterone	▲	▲	▲
5β-Dihydrotestosterone	✚		
Etiocholanolone	▲	▲	▲
5α-Androstane-3α,17β-diol	✚	•	
5α-Androstane-3β,17β-diol	✚	✚	
5β-Androstane-3α,17β-diol	✚	✚	
5β-Androstane-3β,17β-diol	✚		

▲ = Synthesis, characterisation and external review complete
✚ = Synthesis and characterisation complete
• = In progress or planned

Table 1

- **Xenobiotic steroids**

	Parent	*Glucuronide*
d_3-Nandrolone	▲	
17α-Nandrolone	▲	
19-Norandrosterone	▲	▲
d_4-19-Norandrosterone	▲	•
19-Noretiocholanolone	▲	▲
d_4-19-Noretiocholanolone	▲	
17α-Ethyl-5α-estrane-3α,17β-diol	▲	+
17α-Ethyl-5β-estrane-3α,17β-diol	▲	+
17α-Methyl-5α-androstane-3α,17β-diol	▲	▲
17α-Methyl-5β-androstane-3α,17β-diol	▲	
7α,17α-Dimethyl-5β-androstane-3α,17β-diol	▲	+
Calusterone	▲	
7β,17α-Dimethyl-5β-androstane-3α,17β-diol	▲	+
2-Hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one	▲	
17-Epioxandrolone	▲	
17-Epimethandienone	▲	
6β-Hydroxymethandienone	▲	
17-Epimetendiol	▲	
17,17-Dimethyl-18-norandrosta-1,13-dien-3α-ol (18-Normetanol)	▲	
17,17-Dimethyl-18-norandrosta-1, 4, 13-trien-3-one	▲	
3'-Hydroxystanozolol	▲	▲
4α-Hydroxystanozolol	▲	
4β-Hydroxystanozolol	▲	
16β-Hydroxystanozolol	▲	
16β-Hydroxyfurazabol	▲	
1-Methylene-5α-androstan-3α-ol-17-one	▲	+
1α-Methyl-5α-androstan-3α-ol-17-one	▲	+
1α-Methyl-5α-androstane-3α,17β-diol	▲	+
d_3-Boldenone	▲	
17-Epiboldenone	▲	
5β-Androst-1-en-17β-ol-3-one	▲	+
2α-Methyl-5α-androstan-3α-ol-17-one	▲	+
4-Chloroandrost-4-en-3-α-ol-17-one	▲	
Turinabol	▲	
6β-Hydroxy-4-chloro-dehydromethyltestosterone	▲	
9α-Fluoro-17,17-dimethyl-18-norandrostadien-11β-ol-3-one	▲	
9α-Fluoro-17α-methylandrosta-4-ene-3α, 6β,11β,17β-tetrol	▲	
6β-Hydroxyfluoxymesterone	▲	

▲ = Synthesis, characterisation and external review complete
\+ = Synthesis and characterisation complete
• = In progress or planned

Table 2

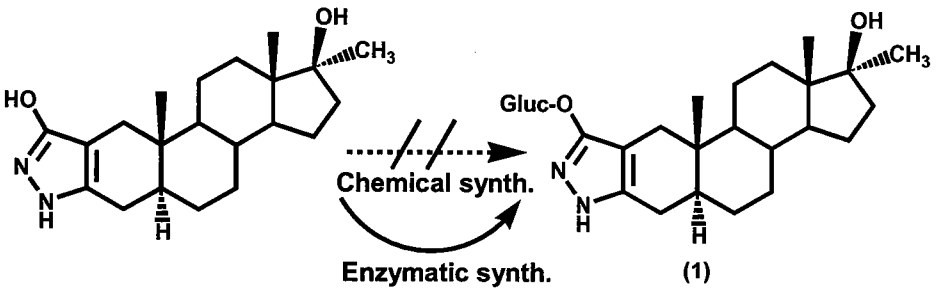
OH
CH3
HO
N
N
H
H
Chemical synth.
Enzymatic synth.
Gluc-O
OH
CH3
N
N
H
H
(1)

Fig. 2

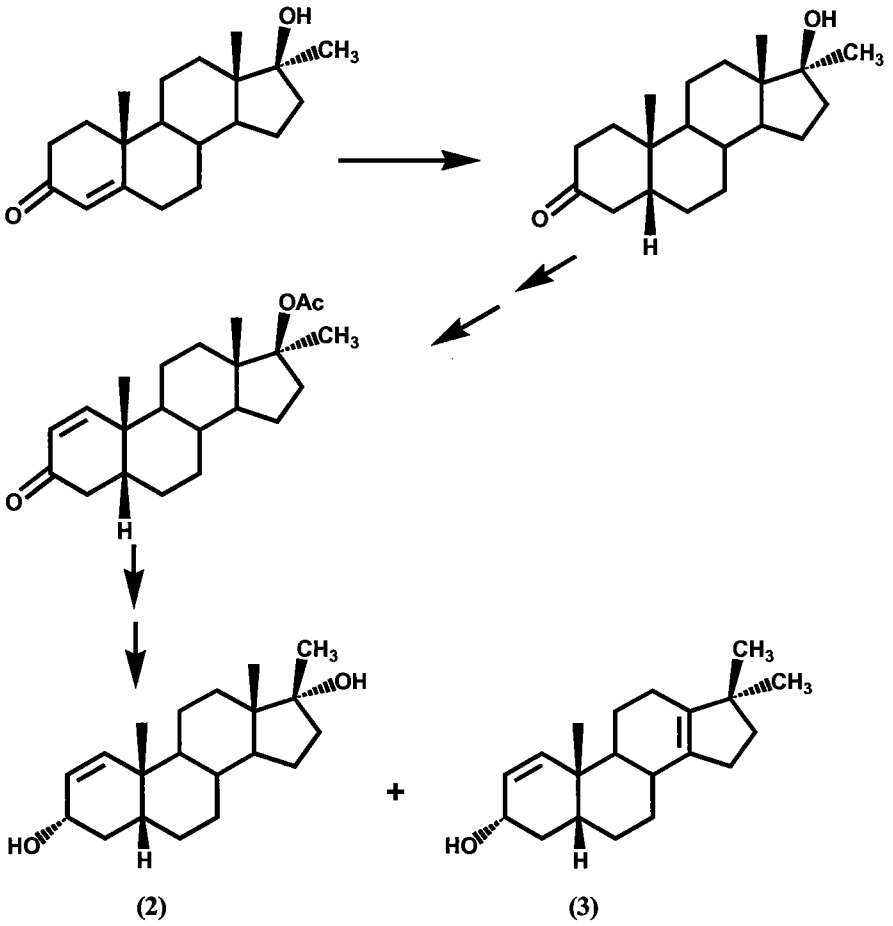
OH
CH3
O
OH
CH3
O
H
OAc
CH3
O
H
CH3
OH
HO
H
+
CH3
CH3
HO
H
(2)
(3)

Fig. 3

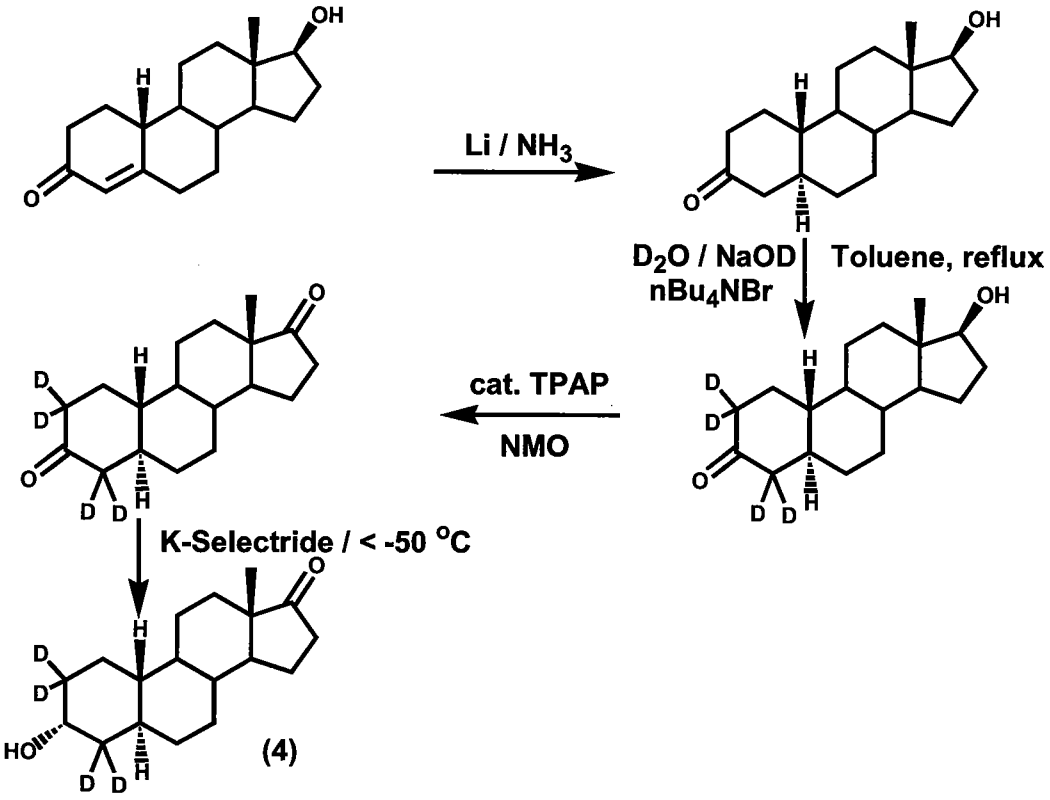
Li / NH3
D2O / NaOD
nBu4NBr
Toluene, reflux
cat. TPAP
NMO
K-Selectride / < -50 °C
(4)

Fig. 4

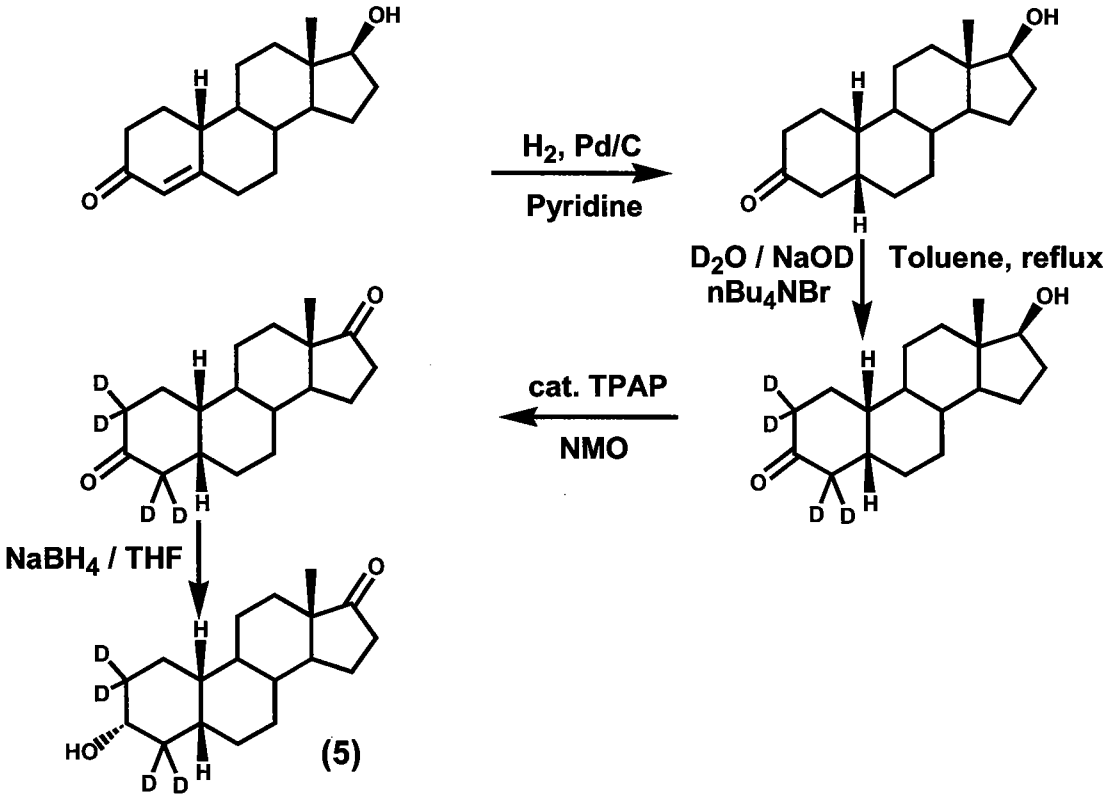
H2, Pd/C
Pyridine
D2O / NaOD
nBu4NBr
Toluene, reflux
cat. TPAP
NMO
NaBH4 / THF
(5)

Fig. 5

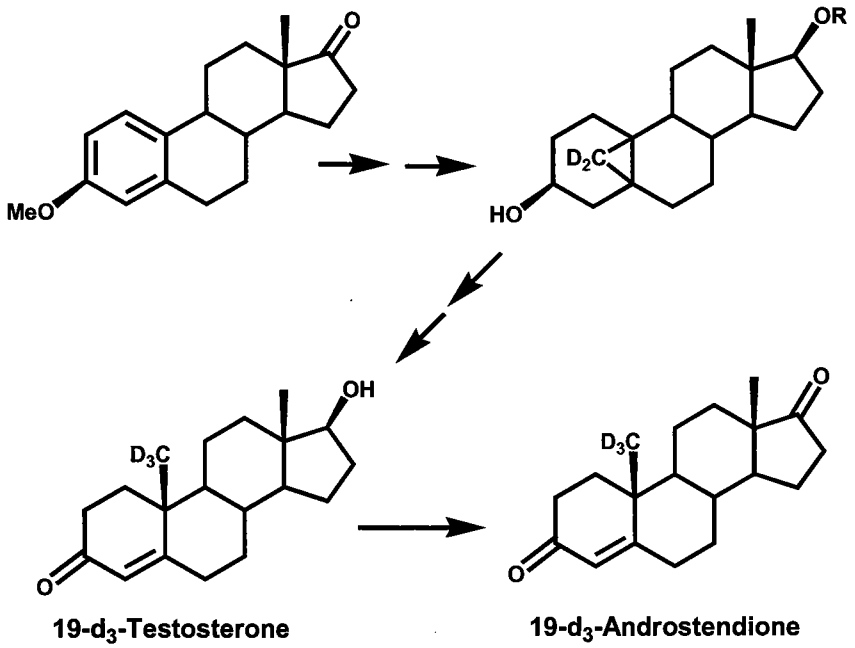
MeO
O
HO
OR
D2C
D3C
OH
O
D3C
O
19-d3-Testosterone
19-d3-Androstendione

Fig. 6