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RECENT ADVANCES IN DOPING ANALYSIS (9)

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Stability trials of anabolic steroid Reference Materials

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INTRODUCTION

The National Analytical Reference Laboratory - Pure Substance Reference Materials team (NARL-PSRM) is an accredited Reference Material producer operating in accordance with ISO Guide 34 requirements. It produces and supplies a range of anabolic steroid Reference Materials (RMs) and Certified Reference Materials (CRMs) to IOC accredited laboratories for use as calibration standards, surrogates and internal standards. These materials are supplied as dried solids in amber glass ampoules accompanied by a certificate of analysis. Stability testing is one of the key technical requirements for the production of CRMs and as such NARL has undertaken a pilot study involving the stability testing of a range of ampouled RMs under various storage conditions. Currently we have over 80 steroid RMs and CRMs in our range and 64 have been ampouled to date and these materials are the subject of this poster.

AMPOULING PROCEDURE [3]

The ampouling operation consists of :-

- Thorough cleaning and deactivation (pre-silanisation) of amber glass ampoules.
- The preparation of an accurately weighed RM stock solution (5.0 mg/ml) in a volatile solvent (typically methanol).
- Transfer of 200 µl of this solution by calibrated pipette to individual ampoules.
- Gentle evaporation under vacuum of the solvent leaving the material as a solid residue.
- Gravimetric checking of the sample weight per ampoule on 20% of the batch population
- Argon flushing of each ampoule immediately prior to sealing
- Automated flame sealing of each ampoule
- Appropriate labelling of the ampoules
- Confirmation of the identity of the material for each batch

STABILITY PROTOCOL

The stability testing involved taking duplicate ampoules and subjecting them to storage for six months out of direct sunlight under the following conditions :-

- cool storage (-5 °C): designated ***Amp -5 °C***
- room temperature(~ 20 °C): designated ***Amp RT***
- storage at 40 °C: designated ***Amp 40 °C***

Solutions of a limited number of these RMs were also prepared in duplicate in methanol and stored for six months under the following conditions:

- re-sealed as solutions in amber glass ampoules and stored in the dark at 40 °C: designated ***MeOH 40 °C***
- stored in 2 ml screw cap clear glass GC vials, exposed to indirect sunlight at room temperature: designated ***MeOH RT & light***.

After six months storage under the various stability regimes one sample was taken and analysed by either GC or HPLC along with a sample from the bulk material from which the ampoules were prepared. Each sample was injected in duplicate and the results tabulated and compared to the original purity data obtained. In the event of a suspicious set of results the second duplicate sample was checked to confirm the earlier result.

Assumptions regarding stability data and the resultant conclusions

It is a well-established practice in the pharmaceutical industry to subject formulations to accelerated stability trials to more rapidly assess potential shelf life stability properties.[4] This is based on the assumption that the rate of degradation doubles for every 10 °C increase in storage temperature and hence:-

- 6 months storage at 40 °C is equivalent to 2 years storage at room temperature.
- 6 months storage at 20 °C is equivalent to 2 years storage at or below 0 °C.

The materials under test are categorised in the summary table by the dominant functional group they contain.

Compound	Coll'n No.	Amp -5 °C	Amp RT	Amp 40°C	MeOH RT & light	MeOH 40 °C
4-En-3-one Steroids						
d_3-Testosterone	D546	✓	✓	✓	✓	+
19-d_3-Testosterone	D644	✓	✓	✓		✓
Epitestosterone	D547	✓	✓	✓		✓
d_3-Epitestosterone	D548	✓	✓	✓	✓	+
d_3-Nandrolone	D583	✓	x	x		+
5β-Androst-1-en-17β-ol-3-one (Boldenone metab.)	D564	✓	✓	✓		✓
6β-Hydroxyfluoxymesterone	D617	✓	✓	✓		x
Calusterone	D618	✓	✓	✓		✓
19-d_3-Androstendione	D645	✓	✓	✓		+
1,4-Dien-3-one Steroids						
17-Epimetandienone	D562	✓	✓	✓	+	✓
6β-Hydroxymetandienone	D565	✓	✓	✓		✓
17,17-Dimethyl-18-norandrosta-1,4,13-triene-3-one	D576	x	x	x	x	x
9α-Fluoro-18-nor-17,17-dimethyl-androstadiene-11β-ol-3-one	D571	✓	✓	+	✓	✓
d_3-Boldenone	D581	✓	✓	✓	x	+
17α-Boldenone	D582	✓	✓	✓		+
Turinabol	D613	✓	✓	✓	+	✓
6β-Hydroxyturinabol	D615	✓	✓	✓		✓
2-Hydroxymethyl-formebolone metabolite	D622	✓	✓	✓	+	✓
Keto steroids						
5α-Dihydrotestosterone	D553	✓	✓	✓		x
d_3-5α-Dihydrotestosterone	D552	✓	✓	✓	x	x
d_5-Etiocholanolone	D528	✓	✓	✓	✓	✓
d_4-Androsterone	D549	✓	✓	✓		✓
19-Norandrosterone	D555	✓	✓	✓		✓
d_4-19-Norandrosterone	D584	✓	✓	✓	✓	✓
19-Noretiocholanolone	D554	✓	✓	✓		✓
d_4-19-Noretiocholanolone	D623	✓	✓	✓	✓	✓
1α-Methyl-5α-androstan-3α-ol-17-one (Mestanolone metab. 1)	D557	✓	✓	✓		✓
1-Methylene-5α-androstan-3α-ol-17-one (Metenolone metab.)	D619	✓	✓	✓	✓	✓
2α-Methyl-5α-androstan-3α-ol-17-one (Drostanolone metab.)	D567	✓	✓	✓		✓
4-Chloro-4-androsten-3α-ol-17-one (Clostebol metab.)	D563	✓	✓	✓	✓	✓
Hydroxy steroids						
d_3-5α-Androstane-3α,17β-diol	D593	✓	✓	✓	✓	✓
d_3-5α-Androstane-3β,17β-diol	D594	✓	✓	✓		+
d_5-5β-Androstane-3α,17β-diol	D580	✓	✓	✓	✓	✓
d_3-5β-Androstane-3β,17β-diol	D626	✓	✓	✓		✓
17 α-Ethyl-5α-estrane-3α,17β-diol (Norethandrolone metab.)	D558	✓	✓	✓		✓
17 α-Ethyl-5β-estrane-3α,17β-diol (" " ")	D559	✓	✓	✓		✓
17α-Methyl-5α-androstane-3α,17β-diol (Methyl Testos metab.)	D560	✓	✓	✓	✓	✓
17α-Methyl-5β-androstane-3α,17β-diol (" " "	D561	✓	✓	✓	✓	✓
7α,17α-Dimethyl-5β-androstane-3α,17β-diol (Bolasterone	D614	✓	✓	✓		✓
7β,17α-Dimethyl-5β-androstane-3α,17β-diol (Calusterone	D624	✓	✓	✓		✓
1α-Methyl-5α-androstan-3α,17β-diol (Mestanolone metab.2)	D556	✓	✓	✓		✓
9α-Fluoro-17a-methylandrosta-4-ene-3α, 6β,11β,17β-tetrol	D616	✓	✓	✓		✓
17-Epioxandrolone	D620	✓	✓	✓		✓
17β-Methyl-5β-androst-1-ene-3α,17α-diol (EMD,	D638	✓	✓	x	✓	✓
17,17-Dimethyl-18-norandrosta-1,13-diene-3α-ol (18-	D639	✓	✓	✓		✓

Compound	Coll'n No.	Amp -5 °C	Amp RT	Amp 40°C	MeOH RT & light	MeOH 40 °C
Heterocyclic steroids						
16β-Hydroxyfurazabol	**D602**	✓	✓	+		✓
4β-Hydroxystanozolol	**D641**	✓	✓	✓		+
3'-Hydroxystanozolol	**D577**	✓	✓	✓	✓	x
16β -Hydroxystanozolol	**D621**	✓	✓	✓		✓
Glucuronides						
Testosterone glucuronide	**D507**	✓	✓	✓	✓	✓
d_3-Testosterone glucuronide	**D505**	✓	✓	✓		x
Epitestosterone glucuronide	**D603**	✓	✓	✓	✓	✓
d_3-Epitestosterone glucuronide	**D604**	✓	✓	✓		✓
Androsterone glucuronide	**D572**	✓	✓	✓		✓
d_4-Androsterone glucuronide	**D575**	✓	✓	✓	✓	✓
Etiocholanolone glucuronide	**D607**	✓	✓	✓		✓
19-Noretiocholanolone glucuronide	**D595**	✓	✓	✓		✓
19-Norandrosterone glucuronide	**D596**	✓	✓	✓		✓
3'-Hydroxystanozolol glucuronide	**D640**	✓	x	x	x	x
Sulfates						
d_3-Testosterone Sulfate	**D508**	✓	✓	+		x
d_3-Epitestosterone Sulfate	**D506**	✓	✓	✓		✓
Epitestosterone Sulfate	**D605**	✓	✓	✓		✓
Testosterone Sulfate	**D591**	✓	✓	✓	✓	✓

LEGEND	% purity decrease
✓	< 2%
+	2-5%
x	>5%

CONCLUSIONS

- Good storage stability was observed for all but one ampouled solid using the recommended storage temperature (at or below 4 °C).
- The exception is a trienone which is an equine metabolite of methandienone.
- The certification of the purity values can be extended in most cases to two years from the certification date (current practice is one year).
- Steroids containing a 4-en-3-one or 1,4-dien-3-one functionality are more prone to breakdown in solution than steroids lacking enone groups.
- 19-Nor-4-en-3-one systems (e.g. nandrolone) are significantly more susceptible to breakdown than 19-methyl-4-en-3-one steroids.
- Hydroxy steroids, even those containing a 17-methyl 17-hydroxy (tertiary hydroxyl) group were found to be stable under all storage conditions (both in ampoules and in methanol).

- Keto steroids and heterocyclic steroids generally show good stability when ampouled in the solid form and moderate solution stability.
- Conjugated steroids (sulfates and glucuronides) all showed good stability under the recommended storage conditions (at or below 4 °C). 3′-Hydroxystanozolol glucuronide and to a lesser extent d_3-testosterone glucuronide did however show that they are susceptible to breakdown at higher storage temperatures.
- It is recommended as good laboratory practice that working solutions of steroid reference materials be stored at 4 °C out of direct sunlight and monitored at regular intervals for potential breakdown.

REFERENCES

1. Steven Westwood, David Hancock, Christie Moule, Bruce Noble and Scott Starling; Progress in the Preparation of Anabolic Steroid Reference Materials and Certified Reference Materials. In: *Proceedings of the 18th Cologne Workshop on Dope Analysis*, Sport und Buch Strauß Köln, (2001), 109-118.
2. ISO Guide 34, (2000): General requirements for the competence of reference material producers.
3. Ampoule Sealing of Reference Materials, ver. 1.0, NARL Pure Substance Reference Materials Team Method Manual.
4. Kenneth Connors, *Chemical Stability of Pharmaceuticals*, 2nd ed, Wiley (1986).