EUROPEAN COMMISSION

bcr information

REFERENCE MATERIALS

Certification of the 17α-Trenbolone mass concentration in lyophilised bovine liver reference materials

BCR-474 (Trenbolone free) and BCR-475 (Trenbolone positive)

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ABSTRACT

Trenbolone (17ß-hydroxy-19-norandrosta-4,9,11-trien-3-one) acetate is an androgenic anabolizing agent used in meat production. Its use is permitted in a number of third countries including the United States of America but is banned in the European Union (Directive 96/22/EC). Trenbolone (TbOH) residues are controlled in urine, faeces, liver, fat and muscle samples. To harmonize the analytical performances within the EU, the « Standards , Measurements and Testing Programme » has funded the development of a series of Certified Reference Materials for veterinary drugs in animal tissues and fluids.

This contribution describes the production and certification of lyophilised bovine liver materials uncontaminated (BCR-474, trenbolone-free, blank material) or contaminated with trenbolone (TbOH) residues (BCR-475; target value : 5.0 μg of α-TbOH/kg of fresh liver equivalent).

Purified extracts were quantitatively analysed by two methods:

- RIA (radio-immunoassay) using an anti-α-TbOH antiserum (limit of detection = 0.7 μg/kg).
- GC-MS (gas chromatography-mass spectrometry) of MO-TMS (methyloxime-trimethylsilyl) derivatives (limit of detection = 0.5 μg/kg).

A study to determine the homogeneity of TbOH residues in liver from treated and untreated animals indicated that the residues are normally distributed and thus that the materials are homogeneous. A stability study at various temperatures (-50, -18, +4, +20 and +37 °C) indicated that the materials are stable.

The results of the certification study demonstrated on one hand the fact that many laboratories still have significant problems with the analysis of samples of liver for Trenbolone. However, at the same time laboratories that do have a validated method operational showed to be able to produce good results.

The certified values obtained for the CRMs are:

BCR-474 (blank)	< 0.5 μg/kg
BCR-475 (positive)	7.6 ug/kg, uncertainty 2.2 ug/kg

ABBREVIATIONS USED

μg	microgram
σ_D	standard deviation
BCR	Community Bureau of Reference
BSTFA	N,N-bis-trimethylsilyl-tri-fluoroacetamide
CAS	Chemical Abstracts Registry Numbers
CER	Centre d'Economie Rurale
CI	Chemical Ionisation or Confidence Interval
CRL	Community Reference Laboratory
CRM	Certified Reference Material
DAD	Diode Array Detector
dpm	desintegration per minute
EI	Electron Ionisation
EU	European Union
FTIR	Fourier Transform Infra Red
GC-MS	Gas Chromatography - Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IA	Immuno Assay
IAC	Immuno Affinity Chromatography
ISO	International Standardisation Organisation
IRMM	Institute for Reference Materials and Measurements
JECFA	Joined Expert Committee for Food Additives
LC	Liquid Chromatography
LOD	Limit of Detection
LL or LLE	Liquid Liquid Extraction
LOQ	Limit of Quantification
MO-TMS	methyloxime - trimethylsilyl
MRL	Maximal Residue Limit
NMR	Nuclear Magnetic Resonance
NRL	National Reference Laboratory
PCI	Positive Chemical Ionisation
RIA	radio-immunoassay
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RM	Reference Material
rpm	run per minute
TBME	ter-butyl methyl ester
TMS	tri-methyl-silyl
sd or SD	standard deviation
SOP	standard operating procedure
SPE	solid phase extraction
TbOH	Trenbolone
ULg	Université de Liège

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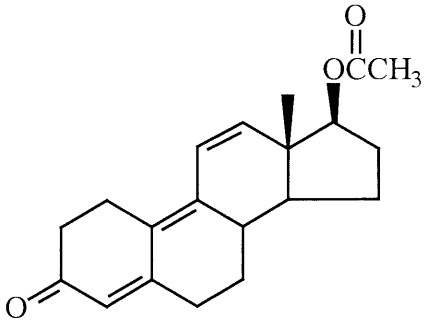
1. INTRODUCTION

1.1 Need for a CRM

Trenbolone (17ß- hydroxy-19-norandrosta-4,9,11-trien-3-one) acetate (CAS: 10161-34-9)(formule 1.1) is an androgenic anabolizing agent used in meat production. Its use is permitted in a number of third countries including United States of America but is banned in the European Union (Directive 96/22/EC).

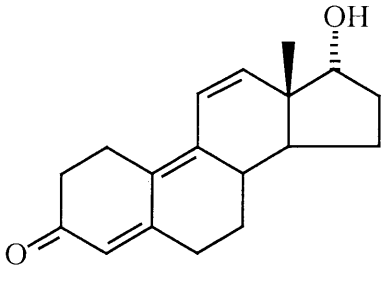


Formule 1.1 - ß-Trenbolone acetate

Trenbolone (CAS: 10161-33-8) residues are controlled in urine, faeces, liver and muscle samples. This control is sometimes difficult owing to the suspected unstability of TbOH residues. Therefore, it is highly desirable to improve the accuracy of TbOH residue measurements, thereby making the comparability of test results possible between different laboratories in all Member States. One of the most important ways of establishing a common basis for accurate measurement is to have readily available reference materials.

It was decided to produce two different reference materials of bovine liver uncontaminated blank material (BCR-474) or contaminated with trenbolone residues (BCR-475).

TbOH residues are present in liver of treated animals mainly as glucuronide or sulphate derivatives of α-TbOH (CAS: 80657-17-6) (also called epi-trenbolone) (17 α-hydroxy-19-norandrosta-4,9,11-trien-3-one) (formule 1.2).



Formule 1.2 - α-Trenbolone

Residue levels were thus determined after ß-glucuronidase-arylsulphatase hydrolysis and the concentrations are expressed in μg of α-TbOH/kg of equivalent fresh liver.

The analytical procedure for TbOH residues determination are either based on GC-MS analysis or on immunochemical assays (IA) (RIA : radio-immunoassays; EIA : enzyme immuno-assays). IA are often used as screening method, GC-MS as confirmatory method.

IA are susceptible to interferences either due to the lack of specificity of the antibody used or due to the sensitivity of the antigen-antibody reaction to physico-chemical conditions such as pH.

Comparison of results of circular analysis in laboratories using GC-MS showed large variations probably due to a lack of standardisation of the analytical procedures used by the different participating laboratories.

It is therefore important to improve the accuracy of TbOH residue measurement by using Certified Reference Materials, thereby making test results comparable independently from the testing site.

1.2 Choice of the material

Liver is generally considered as the target organ for the control of residues of xenobiotics. It is an edible tissue. Muscle and kidney would have also been possible reference materials for the assay of trenbolone residues in animal tissues. Due to the limitation of the budget, it was decided to focus the effort on one type of sample and liver was chosen.

After hydrolysis of TbOH residues, the main metabolite of trenbolone acetate is α-TbOH and the target value of its concentration was fixed : 5 μg/kg fresh liver. Indeed, this value corresponds to that fixed by JECFA and Codex Alimentarius as MRL for α-Trenbolone in liver. Nevertheless, it has to be reminded that the use in EU of trenbolone and other hormones is banned in meat production.

1.3 Design of the project

The certification programme was organised by Dr Leendert van Ginkel, National Institute of Public Health and the Environment, Laboratory for Residue Analysis, PO Box 1, 3720 BA Bilthoven, The Netherlands. Expert laboratories from EU Member States were invited to participate. Participants were free to use their own measurement procedure but were asked to supply a detailed description of the procedure they used by means of a questionnaire provided (with data concerning the validation).

In order to establish a reliable analytical programme for the certification measurements, quality control criteria were set up in advance. The work started with a preliminary study to allow each laboratory to get used with the lyophilised samples and to detect any particular problems. Each laboratory was asked to analyse the test samples and to send the results before starting with the analyses for certification. The results are described in detail under heading 7."Certification campaign".

2. PARTICIPATING LABORATORIES

2.1 Coordination

National Institute of Public Health and the Environment (RIVM), Laboratory for Residue Analysis, PO Box 1, 3720 BA Bilthoven, The Netherlands. Leendert van Ginkel.

2.2 Provision and preparation of materials

- University of Liège, Faculty of Veterinary Medicine, Department of Food Sciences. Laboratory of Analysis of Foodstuffs of Animal Origin, boulevard de Colonster, 20, Sart-Tilman Bat. B43bis, B 4000 Liège, Belgium. Pol Gaspar, Marie-Louise Scippo and Guy Maghuin-Rogister.
- European Commission DG JRC, Institute for Reference Materials and Measurements, B-2440 Geel, Belgium. Gerard N. Kramer.

2.3 Homogeneity studies

- University of Liège, Faculty of Veterinay Medicine, Department of Food Sciences. Laboratory of Analysis of Foodstuffs of Animal Origin, boulevard de Colonster, 20, Sart-Tilman Bat. B43bis, B 4000 Liège, Belgium. Pol Gaspar, Gery Van Vyncht and Guy Maghuin-Rogister.

2.4 Stability studies

- University of Liège, Faculty of Veterinary Medicine, Department of Food Sciences. Laboratory of Analysis of Foodstuffs of Animal Origin, boulevard de Colonster, 20, Sart-Tilman Bat. B43bis, B 4000 Liège, Belgium. Pol Gaspar, Gery Van Vyncht and Guy Maghuin-Rogister.

2.5 Statistical Evaluation

- Community Reference Laboratory, National Institute of Public health and the Environment (RIVM), Laboratory for Residue Analysis, PO Box 1, 3720 BA Bilthoven, The Netherlands. Leendert van Ginkel.
- European Commission, DG XII, Standard Measurements and Testing Programme, rue de la Loi 200, B-1040 Bussels, Belgium. Cyrill Dirscherl.
- Norman C. Cunningham, UK

2.6 Certification analyses

Four laboratories participated in the study. In alphabetical order (not corresponding to the laboratory identification used)

- Ecole Nationale Veterinaire de Nantes, Laboratoire National de Reference, (Nantes, F).
- State laboratory (Dublin, IR).
- National Institute of Public Health and the Environment, Laboratory for Residue Analysis. (Bilthoven, NL).
- Staatliches Medizinal-, Lebensmittel- und Veterinäruntersuchungsamt Nordhessen, (Kasser, G).

3. PRELIMINARY STUDY

A preliminary study has been performed to determine the stability, under lyophilisation, of trenbolone residues in liver of trenbolone acetate-treated veal-calves.

3.1 Treatment of animals

One veal-calf (castrated male; ± 300 kg) (ref. Nr 4274) has been administered trenbolone acetate (1/3 implant REVALOR S). It was slaughtered 2 weeks later. Its liver was cut in strips and deepfrozen at –20 °C.

3.2 Lyophilisation

On March 10, 1995, the sample (603 g) was transferred, frozen, under dry ice, in IRMM (Geel, Dr. J. Pauwels and Kramer) for jaw crushing, freeze drying, ball milling and mixing, as well as bottling and labelling.

On April 5, 1995, 20 vials, each containing the equivalent of 10.0 ± 0.1 g of liver, were received by the University of Liège from IRMM (ref. LiLi 1.5 nr 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29)

3.3 Study of the stability of trenbolone residues under lyophilisation

17α-trenbolone was quantitatively determined by RIA and GC-MS (see Annex 1) before and after lyophilisation.

3.3.1 Results

A comparison between RIA and GC-MS measurements on fresh liver and lyophilised material is presented in table 3.1.

Table 3.1 - Comparison between concentrations (in µg/kg) of α-trenbolone in fresh liver and after lyophilisation.

	RIA	*GC-MS*
Liver before lyophilisation	2.1 ± 0.4 (n=23)	1.8 ± 0.3 (n=2)
Liver after lyophilisation	1.6 ± 0.3 (n=12)*	1.8 ± 0.3 (n=2)**

* sample nr: LiLi 1.5/1; LiLi 1.5/2; LiLi 1.5/7; LiLi 1.5/8

** sample nr: LiLi 1.5/23; LiLi 1.5/26

3.4 Discussion

Purification using immunoaffinity columns was preferred to solid phase extraction on C18 columns : precision of the determination was better due to improvement of chromatogram baselines. From the results obtained by RIA, lyophilisation may lead to a loss of 25 % . GC-MS analysis showed no difference between fresh and lyophilised sample.

The number of determinations was higher that that planned in the approved work programme (duplicate for RIA, we performed 23 determinations on fresh liver and 12 on lyophilised material; single for GC-MS, we performed 2 determinations on fresh liver and 2 on lyophilised material).

3.5 Conclusions

1. The veal-calf treated with 1/3 implant of trenbolone contained in its liver about 2 µg/kg of 17 α-trenbolone as determined by RIA and GC-MS. Consequently, the same protocol of treatment will be applied to veal calves for the preparation of the final batch of livers contaminated with trenbolone residues (incurred samples).
2. Taking into account the variability of the concentrations of different samples of the same liver (veal-calf nr 4274) as determined by RIA and the similarity of results between fresh liver and lyophilised material as determined by GC-MS, it can be postulated that the lyophilisation did not affect the content in 17 α -trenbolone.

4. PREPARATION OF THE MATERIALS

The objective was to prepare lyophilised reference materials:

- 500 vials containing the equivalent of 10 g fresh liver contaminated at a concentration level of about 5 µg of α-TbOH / kg fresh liver;
- 500 vials containing the equivalent of 10 g fresh liver non-contaminated by TbOH residues.

Table 4.1 - Flow chart of the project

Start of the project	*Dec. 94*
Preliminary studies:	
- Evaluation of lyophilisation	March - May 94
- optimisation of animal treatment	Jan. 95
Acquiring of materials:	
- Purchase of animals for blank materials	Oct. - Nov. 95
- Treatment of animals	Nov. 95
- Slaughter and collecting of materials	Nov. 95
- Analysis of fresh material	Jan. 96
Preparation of RMs:	
- Blending of fresh material	May - Aug. 96
- Lyophilisation of main batch	«
- Ampouling - sealing and labelling	«
Evaluation of materials:	
- Homogeneity study (initial batch)	Aug. - Sep. 96
- Confirmation 2d lab	Nov. 96
- Homogeneity study (final batch)	Apr. - May 97
- Stability study (final batch)	June 97 - June 98
Certification of reference materials	Apr. 98

4.1 Origin of material

4.1.1 Trenbolone-free material

Liver tissues have been obtained from 10 veal-calves born and kept in the experimental farm of the Faculty of Veterinary Medicine (University of Liège, Belgium). They are certified as animals never treated with anabolic compounds. Untreated veal-calves were slaughtered and dissected in the pathology department of the Faculty under our supervision.

4.1.2 Trenbolone-containing materials

The liver tissues have been obtained from 7 veal calves. Three out of the 7 veal-calves (7769-4274-RUT/1/3) were treated in the Laboratoire d'Hormonologie, CER (Marloie, Belgium) in the framework of a collaborative study on the use of enzyme immunoassay kits of 17□-TbOH in liver. Four veal-calves (1429-5989-4513-1426) were born, kept and treated with trenbolone acetate containing implants (provided by ROUSSEL UCLAF, Romainville, France) in the experimental farm of the Faculty of Veterinary Medicine of the University of Liège.

Both experiments have been performed under our supervision. Experimental conditions are summarised in Table 4.1.

Table 4.2 - Reference number of veal calves and treatment conditions

REFERENCE NR OF VEAL CALVES	*IMPLANTS*	*TREATMENT*	*DURATION OF IMPLANTATION (DAYS)*
7769	Revalor « S »	1 implant	15
4274	Revalor « S »	1 implant	15
RUT/1/3	V-Finaplix H 200	1/3 implant	10
1429	V-Finaplix H 200	1/3 implant	10
5989	V-Finaplix H 200	1 + 1/3 implant	24
4513	V-Finaplix H 200	1 + 1/3 implant	24
1426	V-Finaplix H 200	1 + 1/2 implant	15

4.2 Treatment

The two batches of liver tissue (contaminated with trenbolone and blank material) prepared from veal-calves have been dispatched deepfrozen (-18 °C) in well closed containers to IRMM (Geel, B). The concentration of 17α-TbOH in seven livers (total mass: 10.38 kg) of treated veal-calves varied between 1 and 20 μg/kg. The final concentration should be approximately 5 μg/kg of α-TbOH/kg of fresh liver. The total mass of 10 uncontaminated (blank) livers was about 16.5 kg.

Cryo-crushing, freeze drying, grinding, sieving and homogenisation are described in details in the report established by Kramer et al., in June 1997 (1). A summary of the freeze drying results is given in Table 4.3.

Table 4.3 - Purpose, identification number, description, mass and obtained yield after freeze drying.

Purpose	*Identification No*	*Description*	*Mass after freeze drying (gram)*	*Yield % of dry material*
Trenbolone RMs	LiLi 3.5	Trenbolone-contaminated	2.761	27.4
	LiLi 3.6	Blank	4.123	27.4

Thirty representative samples of 2.8 g powder (corresponding to 10 g fresh liver) were taken from each material for analytical control measurements. The powders were bottled in well cleaned 30 ml brown glass vials and closed with a teflon protected rubber stopper.

4.3 Analytical control

4.3.1 Water determination

Two samples of each material were taken for Karl Fischer moisture determination. The results are presented in Table 4.4.

Table 4.4 - Batch number, water content and mass of the sieved (<125 µm) liver powders

Batch N°.	*Mean water content, %*	*Mass of fraction < 125 µm, kg*
LiLi 3.5	4.98	2.381
LiLi 3.6	4.77	3.239

It must be stressed that these batches represent intermediate materials. The moisture contents of the final batches of lyopilised and bottled candidate reference materials were lower.

4.3.2 Particle size measurements

Particle size measurements have been carried out using a Sympatec particle size analyser with Helos measuring device. The particle size distribution of representative samples of LiLi 3.1 to 3.6 are given in annexes 2 to 7 of the report (Kramer et al, June 1997 (1)). The top particle size for all samples was less than 365 µm.

4.4 Bottling of the candidate reference materials

Finally the lyophilised powders of the remaining batches (LiLi 3.5 and LiLi 3.6) have been bottled in well cleaned amber glass vials of 30 ml using a fully automatic filling machine for powders type Transmatic system « DM » of the company Transmatic Fyllan Limited, Bedford, England. The system is composed of a turn table connected with an indexing belt to the bottom lift and above the load cell a well closed 28 l hopper is mounted. Inside the hopper separately driven stirrers turn continuously during filling of the powder. With the installed hard and software 2.8 g of liver powder was filled into the vials corresponding to 10 g fresh liver (the weight of the powder in each vial is theoretically 2.8 g but it can vary from 2.75 to 2.95 g). Mean production rate 270 vials/h. The vials were closed after evacuation to < 10 Pa (6h) and filling with 99.995 % Argon with teflon faced rubber lids, held on place with aluminium caps.

Table 4.5 - Overview of the produced materials

Batch No	*BCR No*	*Lyophilised bovine liver*	*Nr of vials*
Mixture I	BCR-475	Trenbolone positive	810
LiLi 3.4	BCR-474	Trenbolone free	1041

4.5 Packaging and dispatching

BCR-474 and BCR-475 consist of 801 sets of two samples, one trenbolone-positive and one trenbolone-free packed in a polyethylene bag. Additionally about 200 vials of trenbolone-free material are available.

5. HOMOGENEITY

After a preliminary study, it was concluded that the α-TbOH concentration of lyophilised powder prepared from liver tissues of TbOH-treated veal-calves is close to the planned (5 μg/kg) TbOH residue contamination of bovine liver reference material and the bulk homogeneity was acceptable.

The vials of lyophilised material are stored at IRMM in Geel. A total of 150 vials was sent to Liège, each containing 2 vials of lyophilised material:

- one vial contains trenbolone-free bovine liver material (reference: BCR-474);
- the second vial contains trenbolone-contaminated liver material (reference: BCR-475).

The reference numbers of the received vials are given in Annex 2.

5.1 Homogeneity studies

As planned in the approved program, samples were taken at regular intervals in the filling sequence as follows:

- trenbolone-free material: 10 samples
- trenbolone-contaminated material: 20 samples

The selected samples were analysed by RIA (duplicate) and GC-MS (single) in such a way that the lowest short-term repeatability was achieved as they were analysed within a single serie.

5.1.1 Trenbolone-free material

Concentration in the ten selected samples were lower than the limit of detection of RIA (LOD: 0.7 μg/kg). This result was confirmed by the GC-MS analyses (LOD: 0.5μg/kg).

The material is thus considered as homogeneous in terms of TbOH residue content, its concentration in α-TbOH being lower than the limits of detection of both RIA and GC-MS.

5.1.2 Trenbolone-containing material

Concentration of α-TbOH in the twenty selected samples are given in table 5.1. They were measured by RIA (in duplicate) and confirmed by GC-MS (single determination).

Table 5.1 - α-TbOH concentrations (µg/kg of corresponding fresh liver) in trenbolone-contaminated material (BCR-475)

Ref. No of the pocket	*RIA* n = 2	*GC-MS* n = 1
8	5.0	4.4
56	5.0	4.5
90	5.1	4.5
131	4.0	5.6
171	4.6	3.8
203	5.6	4.1
255	5.1	5.1
299	5.8	5.0
343	5.3	4.7
381	4.3	5.0
408	5.3	4.1
442	5.1	4.5
482	4.9	5.6
510	4.4	5.7
548	4.9	5.8
569	5.8	4.8
599	6.9	4.8
615	5.4	5.1
767	5.2	4.7
800	5.3	5.2
Mean	***5.1***	***4.8***

Mean results (solid line) for the between-vial homogeneity study for trenbolone-containing material are illustrated in figure 6.1.A and B. The range of C.V. = ± 10% is indicated by interrupted lines.

In fig. 5.1.B, the α-TbOH concentration value of vial nr 131, 171, 203, 408, 482, 510 and 548 fall outside the ± 10 % C.V. range. Nevertheless, they were not statistically considered as outliers.

It has to be reminded that the tolerance ranges of precision in the domain of concentrations relevant with this study (μg/kg) and given in the Decision of the Commission 93/256/EEC (5) are:

for 1 μg/kg	CV = 45% x 1/2 — 45% x 2/3 = 22% — 30%
for 10 μg/kg	CV = 32% x 1/2 — 32% x 2/3 = 16% — 20%

The CV values calculated on the basis of the present study are thus well below the standard values of the EEC Decision. The variations observed in the present homogeneity test are thus considered in agreement with the 93/256/EEC criteria.

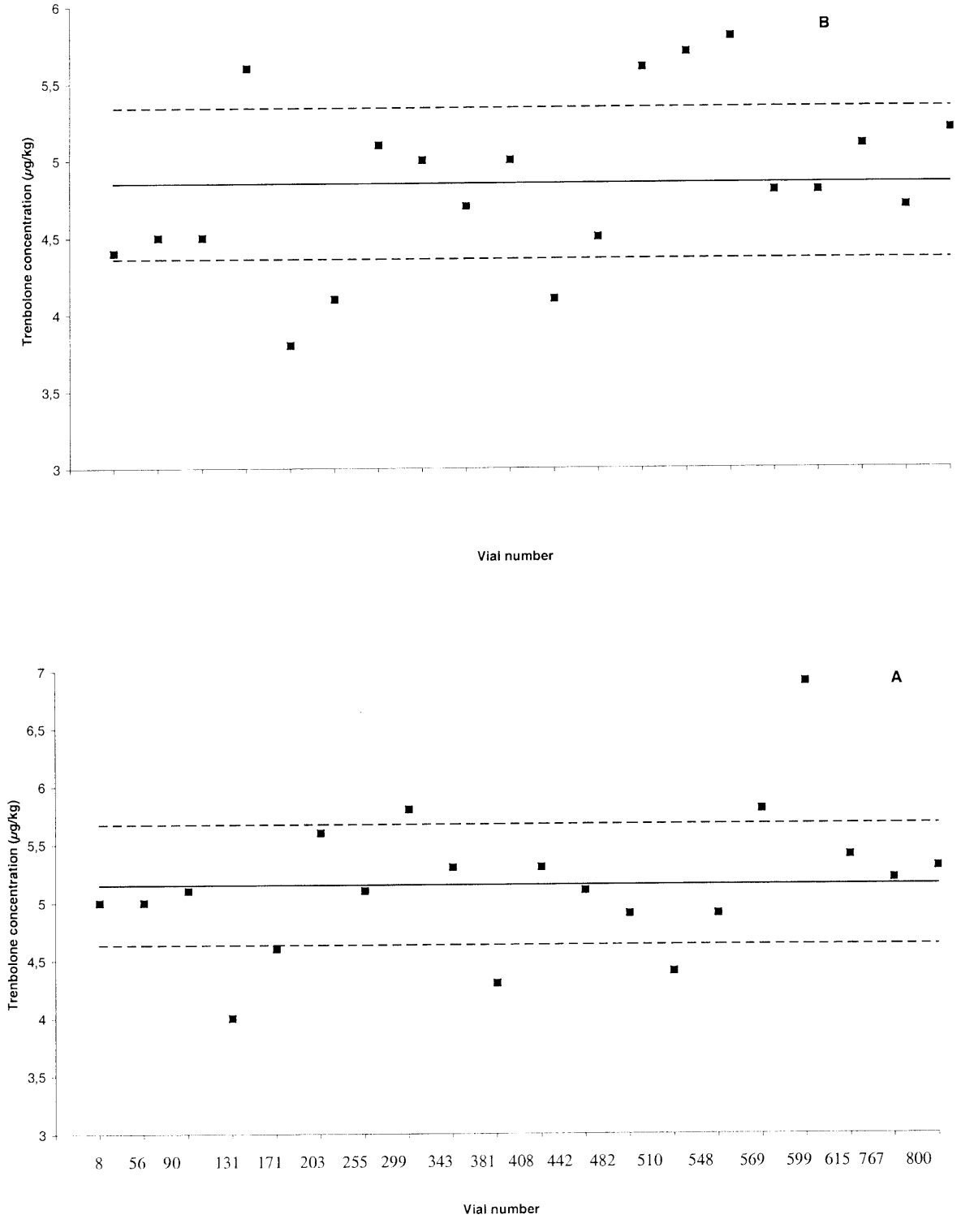


Figure 5.1 -Homogeneity data for vials of trenbolone contaminated material. Alpha-trenbolone concentration in µg/kg of corresponding fresh liver. A. RIA results of table 6.1. B. GC-MS results of table 5.1.

Table 5.2 - Summary of the results of the homogeneity study

	Target conc. µg/kg	*RIA* µg/kg	*GC-MS* µg/kg
TbOH-contaminated Material	5.0	5.1 ± 0.6 (CV = 12 %) (p = 20)	4.8 ± 0.6 (CV = 12.5 %) (p = 20)
TbOH-free Material	-	< LD	< LD

6. STABILITY TESTING

The stability of TbOH residues in the lyophilised materials was determined to assess their storage life and transportability. Vials were stored in the dark at – 50 °C, - 18 °C, + 4 °C, + 20 °C and + 37 °C, analysed in duplicate by RIA and in single by GC-MS at 1 week, 4 weeks, 3, 6 and 12 months.

After incubation during the required period, the samples were kept at – 50 °C until analysis (large series of samples were analysed together in the same run in order to minimise the error of determination).

6.1 TbOH-free material (BCR-474)

The nr of vials that have been selected for the initial 12 month stability study are given in Annex 3. The start of the stability study was June 9, 1997. The end of the twelve month period was June 9, 1998.

The results of the analyses of samples of BCR-474 (TbOH-free material) selected for the stability study showed that the concentrations in α-TbOH were lower than the limits of detection. This material is thus considered as stable in terms of α-TbOH concentration.

6.2 α -TbOH-contaminated material (BCR-475)

The nr of vials that have been selected for the initial 12 month stability study are given in Annex 3. The start of the stability study was June 9, 1997. The end of the twelve month period was June 9, 1998. The results of the initial 12 months stability study are given in table 6.1. These results are also presented under a graphical form in figures 6.1. and 6.2.

Table 6.1 - Stability of BCR-475 (TbOH-contaminated material).

T (° C)	*1 week*		*4 weeks*		*3 months*		*6 months*		*12 months*	
	RIA n=2	GC-MS n=1	RIA n=2	GC-MS n=1	RIA n=2	GC-MS n=1	RIA n=2	GC-MS n=1	RIA n=2	GC-MS n=1
- 50	4.2 ± 0.5	4.4	5.2 ± 1.1	4.0	6.1 ± 0.6	4.2	5.6 ± 0.6	5.1	6.3 ± 0.6	5.0
- 18	5.7 ± 0.1	4.2	5.0 ± 0.5	4.4	4.6 ± 0.4	4.0	4.5± 1.5	4.2	4.3± 0.1	4.6
+ 4	6.5 ± 0.1	4.1	4.9 ± 0.9	4.4	4.5 ± 0.5	3.8	4.9± 0.1	4.5	6.0± 0.1	4.3
+ 20	4.8 ± 0.1	4.4	5.6 ± 0.1	4.4	5.9 ± 0.5	4.1	5.4± 0.1	4.5	4.2± 0.3	3.6
+ 37	4.0 ± 0.4	4.4	5.3 ± 0.5	4.5	4.9 ± 0.2	4.4	5.3± 0.3	4.6	4.9± 0.3	4.3

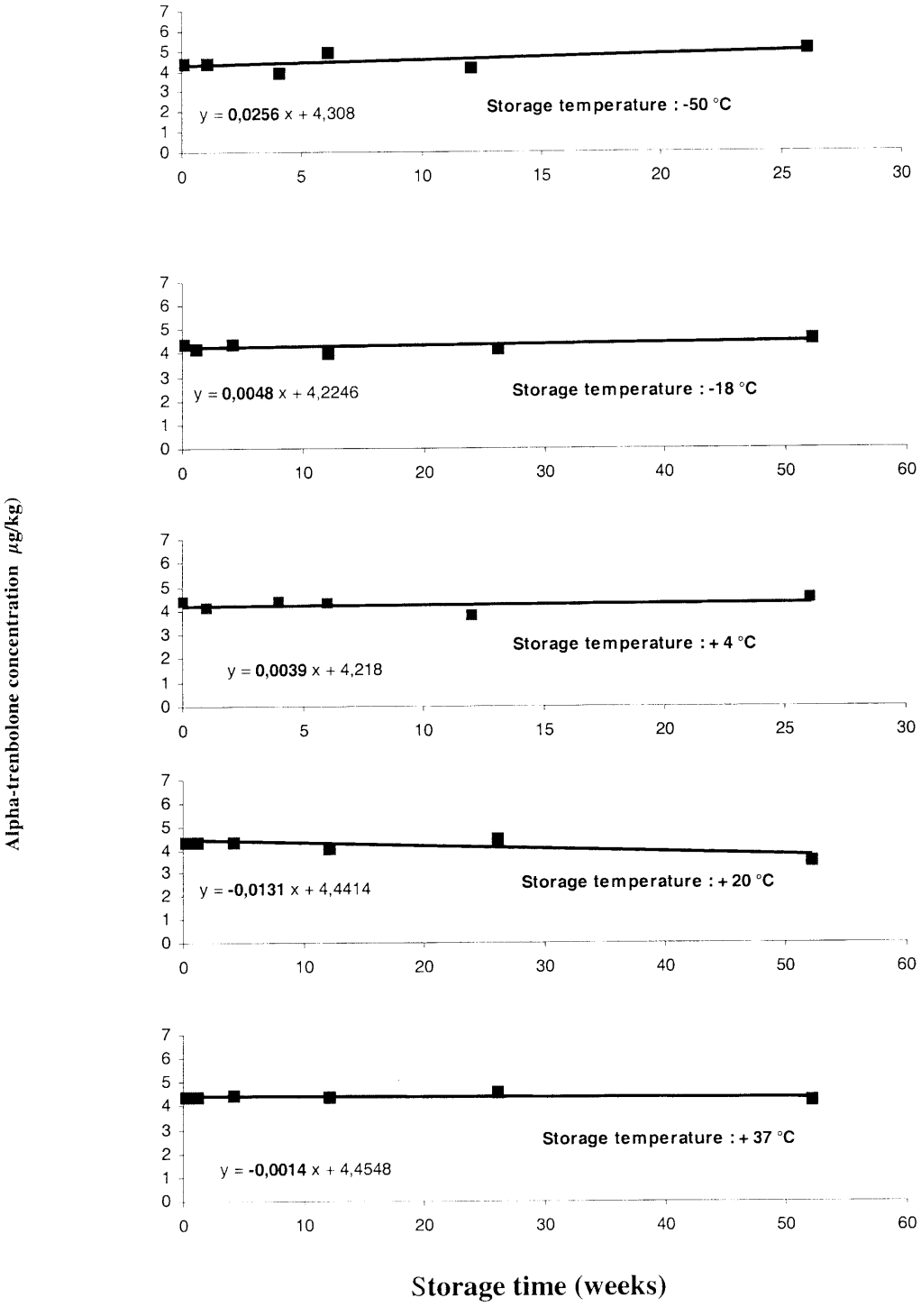


Figure 6.1 - Evolution with time of the alpha-Trenbolone concentration (GC-MS results of table 6.1) in BCR-475 stored at various temperature conditions. We have used the –50 °C result (1 week) as t=0. The slopes of the trend lines are indicated in bold in the equation of the linear regression.

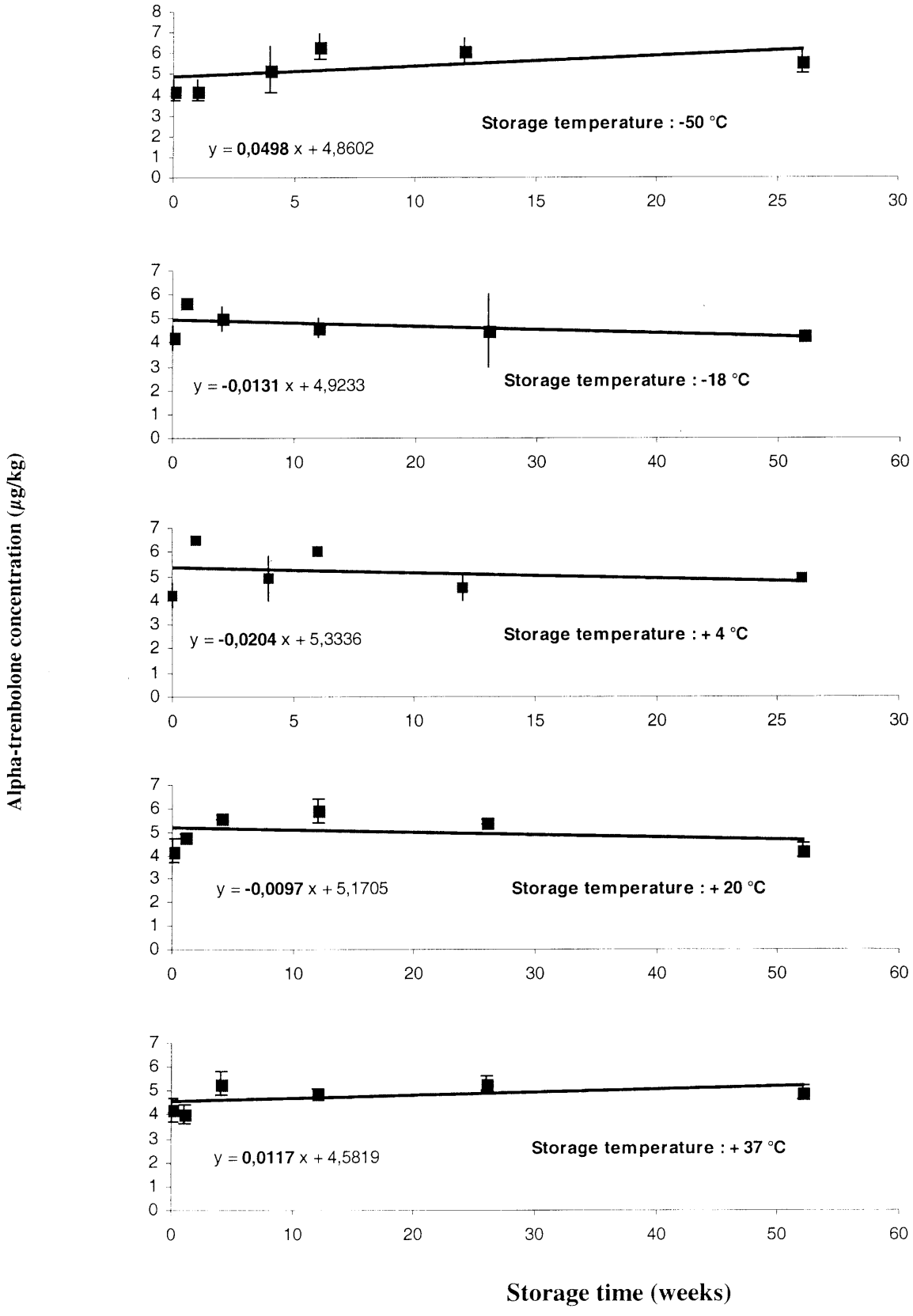


Figure 6.2 - Evolution with time of the alpha-Trenbolone concentration (RIA results of table 6.1, n=2) in BCR-475 stored at various temperature conditions. We have used the –50 °C result (1 week) as t=0. The slopes of the trendlines are indicated in bold in the equation of the linear regression.

Table 6.2 - Stability of the BCR-475 (alpha-trenbolone contaminated material). Salbutamol concentration are expressed in µg/kg. Slopes of the trendlines from the charts reporting the concentrations versus the time of storage at various temperatures (see figures 6.1 and 6.2).

Storage temperature (°C)	*RIA result*	*GC-MS result*
- 50	0,0498	0,0256
- 18	- 0,0131	- 0,0048
+ 4	- 0,0204	0,0039
+ 20	- 0,0097	-0,0131
+ 37	0,0117	- 0,0014

It has to be reminded the tolerance ranges of precision in the domain of concentrations relevant with this study (µg/kg) and given in the Decision of the Commission 93/256/EEC (5) (see p 18). The variations observed in the present stability test are thus considered in agreement with the 93/256/EEC criteria.

All the slopes are in the interval [– 0,05 ; + 0,05].

The effect of time on stability of alpha-TbOH residues in RM475 was assessed by ANOVA (see tables 6.3 and 6.4).

Table 6.3 - Trenbolone concentration (µg/kg, RIA results of table 13) and Anova F-values.

	Storage temperature					
Storage time (weeks)	- 50°C	- 18°C	4°C	20°C	37°C	Mean
1	4,2	5,7	6,5	4,8	4	5,04
4	5,2	5	4,9	5,6	5,3	5,2
12	6,1	4,6	4,5	5,9	4,9	5,2
26	5,6	4,5	4,9	5,4	5,3	5,14
52	6,3	4,3	6	4,2	4,9	5,14
Mean	***5,48***	***4,82***	***5,36***	***5,18***	***4,88***	***5,144***
F value : 0,047						
Tabled F value ($\alpha = 0,05$) : 3,01						

In both cases (RIA and GC-MS), $F_{obs} < F_{tabled}$, we can thus conclude that we accept the hypothesis of equality of the means of each storage condition. The measurement uncertainty of the long term stability can be translated into an additional uncertainty term for the overall average value by using the uncertainty in the value for the slope of the trendline t=-18 °C as an estimate. This uncertainty is expressed as standard deviation = 0,4 µg/kg at a level of 4,3 µg/kg (which amounts to 9 %).

Table 6.4 - Trenbolone concentration (µg/kg, GC-MS results of table 13) and Anova F-values.

	Storage temperature					
Storage time (weeks)	- 50°C	- 18°C	4°C	20°C	37°C	Mean
1	4,4	4,2	4,1	4,4	4,4	5,04
4	4	4,4	4,4	4,4	4,5	5,2
12	4,2	4	3,8	4,1	4,4	5,2
26	5,1	4,2	4,5	4,5	4,6	5,14
52	5	4,6	4,3	3,6	4,3	5,14
Mean	***4.54***	***4,28***	***4,22***	***4,20***	***4,44***	***4,336***
F value : 0,018						
Tabled F value (α = 0,05) : 3,01						

6.3 Conclusions on homogeneity and stability

It was concluded that both reference materials are suitable as candidate CRMs and ready to undergo the certification study. The results of the homogeneity study indicated that the materials are homogeneous within the precision of the analytical methods used (%RSD 12). It is realised that the preferred repeatability for the method used for assessing homogeneity of candidate CRMs should not exceed 5%. However, with respect to the determination of trace amounts of veterinary drugs in biological matrices values of 15 % are considered acceptable and in line with the current "state of the art". The uncertainty of the stability indicated an additional uncertainty of 9%. The uncertainty caused by inhomogeneity and instability will be incorporated in the extended uncertainty of the material. The materials will be monitored on a continuous basis during their storage at IRMM.

7. CERTIFICATION CAMPAIGN

7.1 General

The certification was carried out according to the "Guidelines for the production and certification of BCR reference materials", mainly chapters 4 and 8 (3).

In April 1998 samples, calibrants and documentation were dispatched to all the participants by the CRL. Acknowledgement of receipt of material, method performance data and the results of analyses for each participant returned to this laboratory. Participants were given a total of three months from sample dispatch in which to perform the Pilot Study and the Certification Study.

7.2 Requirements for the certification campaign

For certification strict adherence to the following guidelines was necessary. Each participant was allowed to use its own assay methods. It was agreed that all the measurements would be performed with the highest achievable level of accuracy. In particular participants had to check and provide documentary evidence in respect of the following points:

- The work was done under proper reproducibility conditions (i.e. not just under repeatability conditions) which included all sources of within-laboratory variation. This is consistent with the quality requirements for a certification study. Proper reproducibility was assessed by performing 6 replicate measurements of each RM on 2 different days.
- All relevant equipment was properly calibrated (balances, volumetric equipment, etc.)
- The purity of solvents and reagents was verified.
- The linearity of any ratio measuring device was demonstrated.
- Calibration was achieved by means of the calibrants provided.
- All individual results were reported, with sufficient number of significant figures to allow calculation of the standard deviations. Outlying data were not to be removed, and no other form of data reduction or manipulation was to be applied.
- Standardised Operation Protocols were strictly adhered to.
- Reconstitution of the RMs was performed strictly according to the prescribed procedure.
- The responsible persons for the analysis of the RMs had to be able to provide evidence, if requested to do so, that any of the above-mentioned criteria were fulfilled in their laboratory, and that at the time of the certification measurements, the performance of the procedure was in full statistical control.

7.3 Materials provided for the certification analysis

7.3.1 Documentation

Seven documents were supplied to each participant: "Study Protocol", "Calibrant Sheet", "Method Sheet", "Quality Sheet", "Results for Test Vials Sheet", "Results Sheet" and "Acknowledgement Sheet". Participants were asked to return the "Acknowledgment Sheet" to the CRL on receipt of all materials.

7.3.2. Reference Materials

Details of the RMs are shown (Table 7.1).

Table 7.1 - Details of the CRMs dispatched for certification.

CRM	*Mass per vial* [1]	*Number of vials*	*Target concentration range 17α-Trenbolone*
17α-Trenbolone containing liver (BCR-475)	10.0 g	6 (+2)[2]	2 - 10 µg/kg
Blank liver (BCR-474)	10.0 g	6 (+2)[2]	< 0.1µg/kg

[1] : after reconstitution of the lyophilised content

[2] : 2 vials (test vials) to test the analytical procedure on lyophilised material (Pilot study).

7.3.2 Standards

7.3.2.1 Calibrant

One vial containing 17α-Trenbolone. Reference number: EU/CRL: 57 obtained from the RIVM "Bank of Reference Standards". These compounds are under full purity and stability control and have a purity of 99 ± 1%.

7.3.2.2 Isotope enriched internal standard

One vial containing 100 µg 17β-Trenbolone-d2 Reference number: EU/CRL: 59 obtained from the RIVM "Bank of Reference Standards". The isotopic purity is 99 ± 1%, the amount of undeuterated material < 0.01%.

7.4 Practical considerations

7.4.1 Storage of CRMs and calibrants

The samples of RMs had to be stored at –18 °C (the current storage temperature for all RMs). Calibrants were to be stored at +4 °C or lower, in the dark.

7.4.2 Methods to be used

Each laboratory was allowed to use its own analytical procedure to analyse the samples. Using the provided questionnaire ("Method Sheet") they were asked to supply a detailed description of the method to be used for the both the analysis of the test vials (Pilot Study) and the RMs (Certification Study). The "Method Sheet" had to be returned to the CRL with the results of the test vial analysis before the certification study was undertaken.

7.4.3 Preparation of calibrant stock solution

For calibration purposes participants had to use only the trenbolone calibrant which was provided. The stock solution for calibration and spiking purposes was prepared according to the indications given the "Calibrant Sheet". The calibrant stock solution had to be stored at – 20 °C in the dark. Based on this solution, fresh dilutions were to be prepared on each day of analysis.

7.4.4 Calibration

The 17α-Trenbolone calibrant provided was used to construct a calibration curve consisting of 4 to 6 points covering the concentration range which included the expected 17α-Trenbolone concentration and a zero calibrant. The calibration curve was to be established on each day of determination. Linear regression analysis, including the actual signal for the zero calibrant, was to be used for quantification.

7.4.5 Recovery determination

All laboratories used the deuterated internal standard for recovery correction so there was no need for external recovery control based on spiked blank samples.

7.4.6 Quality control

Each participant in the certification exercise was requested to provide evidence that the measurement procedure was in full statistical control during the time of the test vial analysis and certification measurements (see "Quality Sheet").

7.4.7 Determination of RMs (for the Pilot Study and the Certification Study)

The materials to be analysed for both the Pilot Study (test vials) and the Certification Study were to be analysed by the same method. For the Certification Study, six separate measurements of each RM were to be made. For each measurement a separate vial was to be used. For certification only, the six measurements had to be spread over at least two different days in two separate analytical runs (i.e. two series of 2 vials). The simultaneous analysis of two corresponding RMs was possible but not imperative.

7.5 Statistical evaluation

The results accepted on technical grounds were subjected to the following statistical treatment: for each accepted set of results (i), the mean value was calculated as the arithmetic mean of the individual measurements, as :

$$x_i = \frac{1}{n_i} \sum_{j=1}^{ni} x_{ij}$$

n_i being the number of measurements carried out in the i^{th} laboratory (j=1... n_i). The corresponding standard deviation was estimated as:

$$S_i = \sqrt{[\sum_{j=i}^{ni} (x_{ij} - x_i)^2 / (n_i - 1)]}$$

The sets of results found acceptable on technical grounds were submitted to the following statistical tests:

- Kolmogorov-Smirnov-Lilliefors test to assess the conformity of the distributions of individual results and of laboratory means to normal distributions;
- Nalimov test to detect "outlying" values in the population of individual results and in the population of laboratory means;
- Cochran test to detect "outlying" values in the laboratory variances (S_i);
- One-way analysis of variance (F-test) to compare and estimate the between and within-laboratory components of the overall variance of all individual results.

The set mean values (and not the individual results) were used for the calculation of the overall mean of the results.

For Cochran and Nalimov tests, a value is called an "outlier" when the hypothesis that it belongs to the population of results considered, can be rejected with 1% risk of error. A value is termed a "straggler" when the risk of erroneous rejection lies between 1 and 5%.

The certified values were calculated as the arithmetic means of the x_i's as :

$$x = \frac{1}{p} \sum_{i=1}^{p} x_i$$

Where p is the number of sets accepted for certification after both technical and statistical scrutiny. The uncertainty of this estimate is the 95% confidence interval of the mean x with the following limits :

$$x - t_{1-\alpha/2} . s/\sqrt{p} \qquad \text{and} \qquad x + t_{1-\alpha/2} . s/\sqrt{p}$$

$t_{1-\alpha/2}$ is the value of the student's distribution for t = p-1 degree of freedom and a significance level $\alpha = 0.05$; S is the standard deviation of the distribution of laboratory mean values. It is estimated as :

$$S = \sqrt{[\sum_{i=1}^{p} (x_i - x)^2 / (p-1)}$$

8. PHASES OF THE CERTIFICATION CAMPAIGN

8.1 Preliminary phase

Previous certification campaigns organised through the BCR have commenced with a discrete pilot study to monitor the performance of the participating laboratories prior to

commencement of the certification study. In the current study, the pilot study was carried out contemporaneously by all the laboratories.

8.2 Main phase

8.2.1 Measurement techniques

The analytical methods used by each of the laboratories participating in the certification study are shown (Table 8.1).

Table 8.1 - Measurement techniques used by participating laboratories

Lab.	*IS*	*Extraction*	*Cleanup*	*Derivatization*	*Detection*
1	17α-trenbolone-d2	TBME after incubation with HP (overnight, 37°C)	SPE on C18 and NH_2 cartridges	MOX and BSTFA 1% TMCS	GC-MS: CI (isobutane) m/z 372 and 374
2	17α-trenbolone-d2	centrifugation after incubation with HP (overnight, 37°C)	SPE on C18, LL 1 Mol/l NaOH, SPE on SiOH	I_2 with MSTFA	GC-MS: m/z 449, 444
3	17α-trenbolone-d2	TBME after incubation with HP (overnight, 37°C)	IAC		LC-MS: m/z 271, 272
4	17α-trenbolone-d2	Heitzmann Cy 1.2 (1994)		BSTFA 1% TMCS	GC-MS: m/z 342, 344

8.3 Results and Discussion

In total 8 laboratories expressed an interest in participation in the certification study. Unfortunately 4 laboratories experienced serious problems during the experimental work and were not able to produce results, which they did regard as acceptable during the preliminary study. The problems were not associated with the lyophilised material supplied but were of a general nature. Subsequently these laboratories withdrew from the study. From the final set of data the results obtained by lab 4 on day two were removed on the basis of an observed abnormality in the peak shape.

8.3.1 Results

8.3.1.1 17α-Trenbolone-free liver

The results obtained for the blank material and the relevant LOD and LOQ values are shown in Table 8.2.

Table 8.2 - Individual laboratory results

Lab.n°	*LOD (µg/kg)*	*LOQ (µg/kg)*	*Content of 17α-Trenbolone in liver (µg/kg)*
1	1.5	4.5	< LOD
2	0.5 - 1	2	< LOD
3	0.5	1.0	< LOD
4	0.5	0.8	< LOD

8.3.1.2 17α-Trenbolone-containing liver

Individual laboratory results are shown (Table 8.3). A bar graph showing laboratory means +/- 95% confidence interval is shown in Fig. 8.1. The results of statistical analyses are shown (Table 8.4) and summarised (Table 8.5).

Table 8.3 - Individual laboratory results for 17α-trenbolone incurred liver (µg/kg) (BCR-475)

N° Lab	*Number of replicates*						*Mean*	*SD*	*SD mean*	*95% CI*
L1	7.56	8.07	8.21	7.14	6.79	7.56	7.55	0.539	0.220	0.565
L2	7.21	8.86	7.50	9.29	9.36	8.79	8.50	0.921	0.376	0.967
L3	5.71	7.43	7.00	7.00	7.14	7.57	6.98	0.662	0.270	0.694
L4	7.86	7.14	7.14	~~10.0~~	*	~~12.1~~	7.38	0.416	0.240	1.033

8.3.2 Statistical evaluation

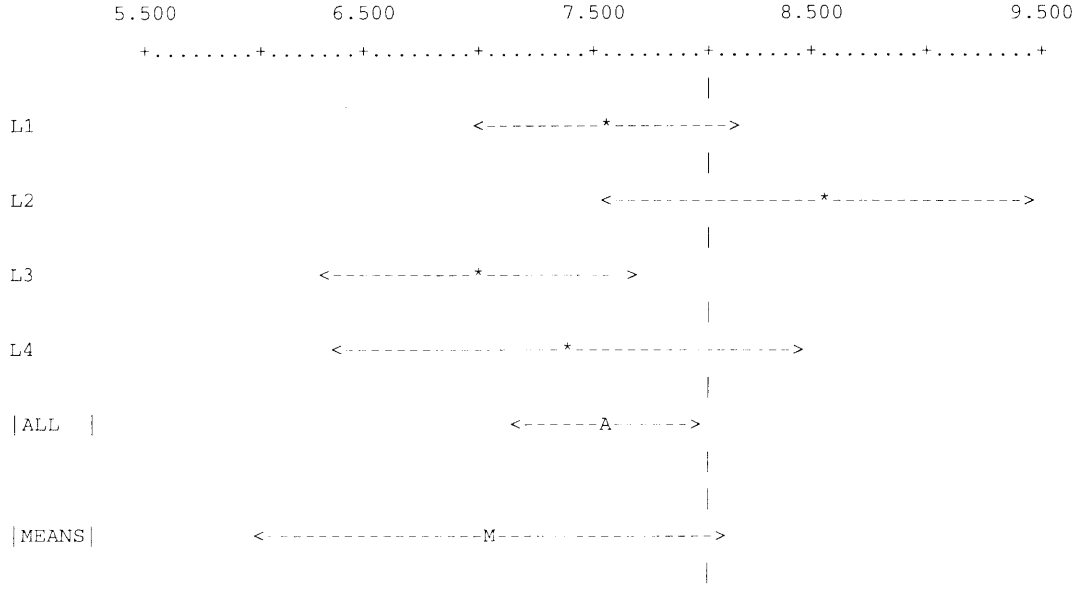


Figure 8.1 - Bar-graphs for laboratory means and 95% CI

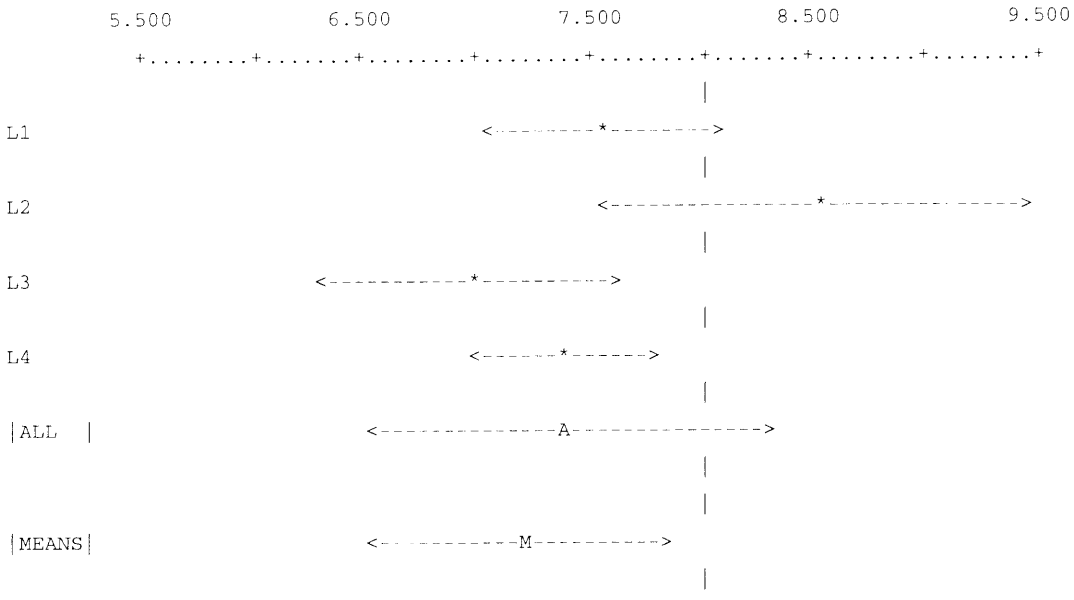


Figure 8.2 - Bar-graphs for laboratory means and standard deviations

Table 8.4 - One way analysis of variances for samples with a constant or variable number of observations

Source of variance	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*Snedecor f*
Between tr.	7.35374	3	2.45125	5.06421
Within tr.	8.22858	17	0.48403	
Total	15.58232	20		
If Bartlett's test is positive then repeatability = 0.69573				
If the systematic errors are random then sigma between = 0.61213				
In that case the reproducibility = 0.92668				

Table 8.5 - Summary of statistical evaluation based on laboratory means (Liver)

Number of accepted sets of results (P):	4
Number of accepted replicates (N):	21
Outlying variance (Cochran test):	no outliers, no stragglers
Homogeneity of variances (Bartlett test):	homogeneous
Normality of distribution of mean values:	normal
(Kolmogorov-Smirnov-Lilliefors)	
Outlying mean values (Nalimov test):	None
Mean of means:	7.60 µg/kg
Std.dev.of distribution of means (S):	0.645 µg/kg
Within-labs std.deviation (Sw):	0.695 µg/kg
Between-labs std.deviation (Sb):	0.612 µg/kg
0.95 confidence interval of the mean:	7.60 ± 1.02 µg/kg

8.3.3 Combined uncertainties

A further calculation was made on the basis of the results obtained from the homogeneity study (see Section 5) and the stability study (see section 6) to see whether the uncertainty of the certified values should take into account a possible (quantified) material inhomogeneity or instability (6). The uncertainty as a result of the certification study is 13.4%, a value in the same order of magnitude as the uncertainty observed during the homogeneity study (12.5%) and slightly larger than the uncertainty of the stability study (9,0%). These values must be compared with the within laboratory repeatability (measurement error) as applicable at the time the homogeneity and stability study were performed (7.5%). The results are shown in Table 8.6.

The calculations are based on the following parameters:

- The "true" standard deviation between bottle (named s_{bb}, bb for "between-bottle")
- u^*_{bb}, the "detection limit" for the inhomogeneity
- The certified uncertainty if the inhomogeneity was included.

The equations used are:

$$s_{bb} = \sqrt{u^2_{c,bb} - s^2_{meas}}$$

$$u^*_{bb} = s_{meas} \cdot \sqrt[4]{\frac{2}{\nu_{smeas}}}$$

$u_{bb} = s_{bb}$ or u^*_{bb}, depending what is larger (to be on the safe side)

$$U_{CRM}[\%] = k\sqrt{\frac{s^2}{n} + u^2_{bb} + u^2_{lts}}$$

s_{bb}	variation between-bottles "inhomogeneity"
u_{lts}	uncertainty of long term stability [9% in this study]
$u_{c,bb}$	CV between-bottle [12.5% in this study]
s_{meas}	measurement variation (corresponding to CV within-bottle) [7.5% in this study]
ν_{smeas}	degrees of freedom for s_{meas} (for 10 replicates: 9) [20 in this study]
u_{bb}	uncertainty of inhomogeneity
s	relative standard deviation of the means in the collaborative study [8.4% in this study]
n	number of accepted datasets in the collaborative study [4 in this study]
k	coverage factor (2 for all calculations)

Table 8.6 - Calculations of the combined uncertainties taking material inhomogeneity into account

$u_{c,bb}$ [%]	u^{*}_{bb} [%]	s_{meas} [%]	S_{bb} [%]	$u_{c,bb}$ [%]	n	$U_{stability}$ [%]	U_{CRM} (k=2) [%]	U_{CRM} (k=2)
17α-Trenbolone	4,3	7.5	10	12.5	4	9	28.2	2.2

8.3.4 Discussion

The results described within this report demonstrate the fact that many laboratories still have serious problems with the analysis of samples of liver for the presence of residues of trenbolone. However, at the same time those laboratories that do have a validated method operational, demonstrate the fact that good results can be obtained.

Based on these conclusions the further dissemination of analytical procedures into National Reference Laboratories will have to take place during the next years. The responsible CRL will take the necessary initiatives. The materials prepared within this project will be highly useful for this purpose.

The analytical methodology used by the four laboratories producing results can be considered as "state of the art". Due to the intrinsic characteristics of these methods, the final result can be considered representative for all methods suitable for the determination of Trenbolone.

Taking the results described within this report into account, as well as the fact that the number of accepted participants exceeds the minimum of 3, it is concluded that the materials can be certified.

9. CERTIFIED α-TRENBOLONE CONCENTRATIONS

The proposed values obtained for the BCR-CRMs are summarised in Table 10.1.

Table 9.1 - Mass concentrations for 17α-trenbolone in BCR-474 and 475 (µg/kg).

BCR-CRM		*mass concentration and uncertainty (µg/kg)*	*Status of value*
474	Blank	< 0.5	Certified
475	Positive	7.6 +/- 2.2 (k=2)	Certified

10. INSTRUCTIONS FOR USE

10.1 Transport and storage

The reference materials are supplied in lyophilised form sealed under vacuum in brown glass vials. The materials may be shipped by postal services or carrier at ambient temperatures. On receipt, the materials should be stored in the dark at –18 °C.

10.2 Reconstitution of the CRMs

- Allow the vial to warm up (keep in the dark at room temperature for at least 30 min.).
- Add a known volume of de-ionised water into each vial (don't carry out the weighing under extreme temperature conditions). The quantity of water which must be added is as follows (Table 10.1):

Table 10.1 - Details for reconstitution of CRMs.

Matrix	*Mass of de-ionised water (g)*
17α-Trenbolone containing liver (BCR-475)	7.20 (± 0.02)
Blank liver (BCR-474)	7.20 (± 0.02)

Following addition of water, each vial is swirled thoroughly and when applicable, the necessary amount of internal standard is added.

10.3 Use of the certified values

These materials may be used to check the precision and the trueness of the laboratory measurement process according to ISO Guide 33 (4).

10.3.1 Assessment of precision

The precision of the measurement process is assessed by comparing the within-laboratory standard deviation (Sw) determined during the certification step. All necessary equations are listed in detail in ISO Guide 33. The within-laboratory standard deviation is: 0.7 µg/kg.

10.3.2 Assessment of trueness

The trueness of the measurement process is checked by comparing the average $\bar{x}$ of n measurement results with the certified value, μ. The criterion for acceptance of the results is as follows:

$$a_2 - 2\,\sigma_D \leq \bar{x} - \mu \leq a_1 + 2\,\sigma_D$$

- a_1 and a_2 are adjustment values chosen by the experimenter according to economical or technical limitations or stipulations.
- $2\,\sigma_D$ is the long term within-laboratory standard deviation of the user's method.

10.4 Intended use of the blank CRMs

The blank materials can serve several purposes:

1. To establish recovery-values for a method of analysis at various levels of contamination, through spiking of the material. Spiking can be accomplished by adding an accurately known amount of 17α-trenbolone to a portion of the CRM.
2. To check the specificity of a method for 17α-trenbolone by providing an effective blank.
3. To investigate limits of detection.

11. REFERENCES

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6. Linsinger, TPJ, Pauwels J, van der Veer, A, Schimmel H, Lamberty A (2000). Homogeneity and Stability of reference materials. Accred. and Qual. Assur., 6, 20-25.

12. ANNEX 1 - ANALYTICAL METHODS

12.1 RIA METHODS - Analysis 17ß-TbOH in liver

12.1.1 Material

All the solvents are of analytical-reagent grade from Merck (Darmstadt, Germany). ß-glucuronidase-arylsulphtase from *Helix Pomatia* was obtained from Boehringer (Manheim, Germany).

Tritiated 17ß-trenbolone (RU2341 - x 10304A) and pure (non radioactive) 17ß-trenbolone (n° 3A0096B) were from ROUSSEL-UCLAF (Romainville, France). The antiserum (anti 17ß-trenbolone, rabbit R 16) was from CER (Marloie, Belgium) (cross reactivity: 17 ß-trenbolone:

100 %; trenbolone acetate : 0.45 %; trendione : 0.14 %; testosterone : 0.17 %; nortestosterone : 0.16 %; 17ß-estradiol : 0.85 %, oestriol : 0.09 %; methyltestosterone, 17α-oestradiol, oestrone, progesterone, diethylstilbestrol and cortisol showed no cross reactions).

12.1.2 Method

10 g liver, or the equivalent of lyophilised material, were mixed and 0.2 M acetate buffer pH 4.6 was added to reach a total weight of 30g. This suspension was incubated with 100 µl ß-glucuronidase-arylsulphatase (Helix pomatia juice) during 10 min at 55°C followed by an overnight incubation at 37° C . An aliquot of the suspension, corresponding to 0.1 g of liver, was extracted with diethylether. After evaporation of the solvent, the extract was solubilised in a mixture of methanol-water (55 : 45, v/v). Hormone residues were successively purified by liquid-liquid extraction with hexane and solid phase extraction on C18 (BAKER) columns. After washing of the column and elution of trenbolone residues, the yield of purification was determined by radioactivity measurement and 17ß-trenbolone was assayed by RIA.

12.1.3 Radioimmunoassay (RIA)

A standard curve (logit {[B/B_0] x 100} vs log{hormone concentration}) ranging from 0 to 200 pg of ß-TbOH per 100 µl was established.

Tubes containing:

- 100 µl of standard solution (concentration : 0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 pg of ß-TbOH per tube, in duplicate),
- 100 µl diluted tritiated 17ß-trenbolone (8000 dpm),
- 100 µl anti- ß-trenbolone antiserum (dilution: 70 µl serum in 5 ml phosphate gelatine buffer),

were mixed with vortex and incubated at 37°C during 30 min followed by an overnight incubation at 4°C.

Bound and free fractions were separated by addition of 0.5 ml of a dextran-coated charcoal suspension at 4°C (200 mg of a mixture of 5 g charcoal and 0.5 g Dextran T70, suspended in 50 ml 0.2 M phosphate gelatine buffer , pH 7.4). The tubes were vortexed, incubated at 4°C

for 15 min. The incubation was followed by a centrifugation at 4°C during 10 min at 3000 rpm.

An aliquot of 500 µl of supernatant was taken in each tube and diluted in a scintillation vial with 4 ml scintillation cocktail (Ecoscint A). The radioactivity in the vial, corresponding to the amount of tritiated ß-trenbolone bound to the antibody, was counted during 5 min.

12.1.4 Limit of detection

Estimated limit of detection (L.D.) : (mean of 28 blank values + 3 sd) = 0.4 µg ß-TbOH /kg fresh liver.

12.2 Analysis 17α-TbOH in liver (SOP : MB/8/205)

12.2.1 Materials

All the solvents are of analytical-reagent grade from Merck (Darmstadt, Germany). ß-glucuronidase-arylsulphtase from *Helix Pomatia* was obtained from Boehringer (Manheim, Germany).

Tritiated 17α-trenbolone (RU27423 - x 10262A)(1.85 TBq/mmol) and pure (non radioactive) 17α-trenbolone (n° 7A0290) were from ROUSSEL-UCLAF (Romainville, France). The antiserum (anti 17α-trenbolone, rabbit R3) was from CER (Marloie, Belgium) (cross reactivity : 17α-trenbolone : 100 %; 17β-trenbolone : 0.03 %; trendione : 0.12 %; trenbolone acetate, testosterone, methyltestosterone, nortestosterone, 17β-estradiol; 17α-estradiol, estrone, estriol, progesterone, diethylstilbestrol and cortisol showed no cross reactions).

12.2.2 Method

10 g liver, or the equivalent of lyophilised material, were mixed and 0.2 M acetate buffer pH 4.6 was added to reach a total weight of 30 g. This suspension was incubated with 100 µl ß-glucuronidase-arylsulphatase (Helix pomatia juice) during 10 min at 55°C followed by an overnight incubation at 37° C . An aliquot of the suspension, corresponding to 0.025 g of liver, was extracted with diethylether. After evaporation of the solvent, the extract was solubilised in a mixture methanol-water (55 : 45, v/v). Hormone residues were successively purified by liquid-liquid extraction with hexane and solid phase extraction on C 18 (BAKER) columns. After washing of the column and elution of TbOH residues, the yield of purification was determined by radioactivity measurement and 17α-TbOH was assayed by RIA .

12.2.3 Radioimmunoassay (RIA)

A standard curve (logit {[B/B_0] x 100} vs log{hormone concentration}) ranging from 0 to 200 pg of α-TbOH per 100 µl was established.

Tubes containing:

- 100 µl of standard solution (concentration : 0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 pg of α-TbOH per tube, in duplicate),

- 100 µl diluted tritiated 17α-trenbolone (8000 dpm),

- 100 µl anti- α-trenbolone antiserum (dilution: 70 µl serum in 5ml phosphate gelatine buffer),

were mixed with vortex and incubated at 37°C during 30 min followed by an overnight incubation at 4°C.

Bound and free fractions were separated by addition of 0.5 ml of a dextran-coated charcoal suspension at 4°C (200 mg of a mixture of 5 g charcoal and 0.5 g Dextran T70, suspended in 50 ml 0.2 M phosphate gelatine buffer , pH 7.4). The tubes were vortexed, incubated at 4°C for 15 min. The incubation was followed by a centrifugation at 4°C during 10 min at 3000 rpm.

An aliquot of 500 µl of supernatant was taken in each tube and diluted in a scintillation vial with 4 ml scintillation cocktail (Ecoscint A). The radioactivity in the vial, corresponding to the amount of tritiated α-trenbolone bound to the antibody, was counted during 5 min.

12.2.4 Characteristics of the RIA method

Blank value (liver from untreated veal-calf) : 23.4 ± 2.1 pg (n = 27)

Recovery : 86 ± 8 % (n =70)

Estimated limit of detection (mean of 18 blank values + 3 sd) = 0.7 µg/kg

12.3 GC-MS METHOD - Analysis 17α-TbOH and 17β-TbOH in liver

12.3.1 Material

All the solvents are of analytical-reagent grade from Merck (Darmstadt, Germany). b-glucuronidase-arylsulphtase from *Helix Pomatia* was obtained from Boehringer (Manheim, Germany), N,N-bistrimethyl-silyltrifluoroacetamide (BSTFA), dry pyridine from Macherey-Nagel (Düren, Germany) and methoxylamine hydrochloride from Supelco (Bellefonte, PA, USA).

α-trenbolone (n°7A0290) and β-trenbolone (n°3A0096B) were gifts from Roussel Uclaf (Romainville, France).

Deuterated 17β-trenbolone was from the Community Reference Laboratory (CRL) (RIVM, Bilthoven, The Netherlands) (code 91M4366)

Immunoaffinity columns (TT04TB95) were from CER (Marloie, Belgium).

12.3.2 Apparatus

An HP-5890 Series II gas chromatograph and an HP-7673 automatic injector were supplied by Hewlett-Packard. The mass spectrometer used was a VG AutoSpecQ (VG Analytical, Manchester, UK).

12.3.3 Method

10 g liver, or the equivalent of lyophilised material, were mixed and 0.2 M acetate buffer pH 4.6 was added to reach a total weight of 30 g. This suspension was incubated with 100 µl ß-glucuronidase-arylsulphatase (Helix pomatia juice) during 10 min at 55°C followed by an overnight incubation at 37° C. An aliquot, corresponding to 4 g liver, was mixed with a known amount of deuterated β-trenbolone in acetate buffer.

The digest was extracted with diethylether (2 x 10 ml). The ether extract was evaporated to dryness. The dry residue was solubilised in 20 ml of phosphate buffer saline (PBS). The aqueous solution was washed with 20 ml hexane and centrifuged. After centrifugation, the extract was passed through the immuno-affinity column used as recommended by the manufacturer.

The purified extract was evaporated to dryness at 40° C under a nitrogen stream and derivatized with 100 µl of methoxylamine hydrochloride in dry pyridine solution (20 mg/ml) at 60° C for 1 hour. Pyridine was evaporated to dryness under a nitrogen stream (40°C) and trimethylsilylation was performed with 50 µl of BSTFA (60°C, 30 min).

12.3.4 Gas chromatography

The temperature of the GC column oven was initially 120°C for 1 min, then increased at 15°C/min to 240°C and subsequently at 5°C/min to 300°C, the final temperature being maintained for 9 min. The carrier gas was helium (grade N60) with a column head pressure of 100 mbar and a flow-rate of 1 ml/min. A 1-µl volume of the derivatisation mixture was injected in the splitless mode; the injector and transfer line temperature were 300°C.

12.3.5 Mass spectrometry

The positive electron impact mode (EI^+) was applied with an electron energy of 70 eV and a trap current of 200 µA. The source temperature was 190°C. The resolution (at 10 % of valley) of the sector part of the hybrid instrument (VG-AutoSpecQ) for the MRM mode was 100. The dwell time was 80 ms.

Estimated limit of detection :

Signal to noise ratio (S/N) = 3 corresponding to 0.5 µg trenbolone/kg liver.

13. ANNEX 2 - REFERENCE NUMBERS OF THE VIALS USED IN THE HOMOGENEITY STUDY

The vials of lyophilised material are stored at IRMM in Geel. A total of 150 pockets was sent to Liège, each containing 2 vials of lyophilised material :

- one vial contains trenbolone-free bovine liver material (reference : BCR-474);
- the second vial contains trenbolone-contaminated liver material (reference : BCR-475).

The reference numbers of the received pockets were the following :

8-26-56-67-72-75-81-85-90-96-101-104-115-120-124-131-134-138-139-142
145-148-149-152-154-157-160-163-165-168-171-174-177-179-182-185-188
202-203-223-233-243-249-255-257-263-266-268-279-282-283-292-299-302
308-312-314-315-319-324-327-330-332-343-347-352-356-365-371-375-378
379-381-385-388-391-394-398-401-404-408-412-415-418-421-424-428-431
435-439-442-445-449-452-455-459-463-466-469-473-476-479-482-487-490
493-496-499-503-506-510-514-517-520-524-527-530-534-538-542-545-548
551-554-557-560-563-566-569-572-575-579-580-583-587-591-595-599-604
607-612-615-767-771-777-783-788-793-796-800

14. ANNEX3 - REFERENCE NUMBERS OF THE VIALS USED IN THE STABILITY STUDY

Table 14.1 - Vials of BCR-474 selected for the initial 12 month stability study

T (° C)	*MONTHS*		
	3	*6*	*12*
	08/09/97	08/12/97	09/06/98
- 50	138 - 75	415 - 421	67 - 81
- 18	101 - 120	490 - 496	104 - 124
+ 4	177 - 185	554 - 560	179 - 188

Table 14.2 - Vials of BCR-475 selected for the initial 12 month stability study

T (° C)	*WEEKS*		*MONTHS*		
	1	*4*	*3*	*6*	*12*
	16/06/97	07/07/97	08/09/97	08/12/97	09/06/98
- 50	26 - 72	412 - 418	138 - 75	415 - 421	67 - 81
- 18	96 - 115	487 - 493	101 - 120	490 - 496	104 - 124
+ 4	174 - 182	551 - 557	177 - 185	554 - 560	179 - 188
+ 20	257 - 268	604 - 612	263 - 279	607 - 563	266 - 282
+ 37	347 - 365	771 - 783	352 - 371	777 - 788	356 - 375

European Commission

EUR 20488 — Certification of the 17α-trenbolone mass concentration in lyophilised bovine liver rererence materials, BCR-474 (trenbolone free) and BCR-475 (trenbolone politive).

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Trenbolone (17ß-hydroxy-19-norandrosta-4,9,11-trien-3-one) acetate is an androgenic anabolizing agent used in meat production. Its use is permitted in a number of third countries including the United States of America but is banned in the European Union (Directive 96/22/EC). Trenbolone (TbOH) residues are controlled in urine, faeces, liver, fat and muscle samples. To harmonize the analytical performances within the EU, the « Standards , Measurements and Testing Programme » has funded the development of a series of Certified Reference Materials for veterinary drugs in animal tissues and fluids.

This contribution describes the production and certification of lyophilised bovine liver materials uncontaminated (BCR-474, trenbolone-free, blank material) or contaminated with trenbolone (TbOH) residues (BCR-475; target value : 5.0 μg of α-TbOH/kg of fresh liver equivalent).

Purified extracts were quantitatively analysed by two methods:

- RIA (radio-immunoassay) using an anti- α-TbOH antiserum (limit of detection = 0.7 μg/kg).
- GC-MS (gas chromatography-mass spectrometry) of MO-TMS (methyloxime-trimethylsilyl) derivatives (limit of detection = 0.5 μg/kg).

A study to determine the homogeneity of TbOH residues in liver from treated and untreated animals indicated that the residues are normally distributed and thus that the materials are homogeneous. A stability study at various temperatures (-50, -18, +4, +20 and +37 °C) indicated that the materials are stable.

The results of the certification study demonstrated on one hand the fact that many laboratories still have significant problems with the analysis of samples of liver for Trenbolone. However, at the same time laboratories that do have a validated method operational showed to be able to produce good results.

The certified values obtained for the CRMs are:

BCR-474 (blank) < 0.5 μg/kg

BCR-475 (positive) 7.6 ug/kg, uncertainty 2.2 ug/kg