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Project identification

1. Defra Project code: VM02143
2. Project title: Approaches to the Control of Boldenone Abuse in Cattle: (2) Confirmatory Method Development and Validation
3. Contractor organisation(s): HFL Ltd
4. Total Defra project costs (agreed fixed price): £ 161,800
5. Project: start date .... 01 January 2004
   end date .... 30 April 2006

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The objectives of the project were two-fold:

1) To develop a residue confirmatory method for the analysis and detection of 17α- and 17β-boldenone as their glucuronic acid conjugates in bovine urine at the minimum required performance limit (MRPL) of $1ngml^{-1}$ and at an agreed action limit of $2ngml^{-1}$.

2) To carry out a multi-site validation exercise consistent with the guidelines laid out in SANCO/1085/2000 Revision 6.

An LC-MS/MS method for the quantitative analysis of 17α- and 17β-boldenone glucuronic acid conjugates in bovine urine was developed. This was based upon the methodology developed for screening of these analytes as part of this project. The methodology was adapted by the inclusion of a step to remove free steroids prior to enzyme hydrolysis.

The ruggedness of the confirmatory methodology was tested using a Youden approach. No loss of performance was observed following the deliberate alteration of the pre-selected critical factors of the procedure. Therefore, no modifications to the methodology were needed. It was shown that the use of the deuterium labelled internal marker successfully accounted for any variation in the absolute recovery of the analyte.

A standard operating procedure (SOP) for the methodology has been produced in a format consistent with the ISO75-2 guidelines.

The stability of 17β-boldenone glucuronic acid conjugate in bovine urine was investigated with both the naturally produced metabolite in an incurred sample and with a reference material spiked into blank urine. In both cases the conjugate was shown to be stable over a twenty week period when samples were stored at the usual freezer operating temperatures of -20ºC and -80ºC.

The methodology was shown to be specific for 17α- and 17β-boldenone in that these compounds were distinguishable from other 17β-boldenone analogues and metabolites.

The working range of the quantitative method was established as $0.5ngml^{-1}$ to $4ngml^{-1}$. High relative recovery (losses accounted for using deuterated internal standard), good precision and good linearity for

calibration point data were obtained over this range.

The decision limit, CCα of the methodology was calculated as 0.9ngml^{-1}, 1.3ngml^{-1}, 0.7ngml^{-1} and 0.9ngml^{-1} for 17α-boldenone and 0.7ngml^{-1}, 0.4ngml^{-1}, 0.3ngml^{-1} and 0.7ngml^{-1} for 17β-boldenone, from results returned by the participating laboratories, CSL, DARD, HFL and LGC, respectively.

The detection capability, CCβ of the methodology was calculated as 1.5ngml^{-1}, 2.2ngml^{-1}, 1.3ngml^{-1} and 1.5ngml^{-1} for 17α-boldenone and 1.3ngml^{-1}, 0.6ngml^{-1}, 0.6ngml^{-1} and 1.2ngml^{-1} for 17β-boldenone, from results returned by the participating laboratories, CSL, DARD, HFL and LGC, respectively.

All positive identifications of 17α-boldenone or 17β-boldenone needed to satisfy criteria that the ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time (RRT), must correspond to that of the system suitability standard at a tolerance of +/- 2.5% and that the relative intensity of two characteristic transitions were within the criteria specified in the SANCO guidelines.

The results obtained from the evaluation indicate that the proposed confirmatory methodology is adequately robust for its intended use. This conclusion is based upon the methodology demonstrating applicability, ruggedness and robustness for the analysis of 17α-boldenone and 17β-boldenone at the reporting level of 2ngml^{-1}. The methodology appears to be reaching the limits of its capability for 17α-boldenone at about 1ngml^{-1}. This is not considered a major issue as current knowledge indicates that the presence of 17β-boldenone is much more indicative of potential abuse of boldenone, its esters or precursors.

The laboratories that collaborated with HFL in the method validation were:

LGC Ltd (residue testing laboratory), Teddington, Middlesex, UK
Department of Agriculture and Rural Development (DARD), Queen's University Belfast, UK
Central Science Laboratory (CSL), Sand Hutton, York, UK

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
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   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

INTRODUCTION

The use of artificial growth promoting agents in food producing animals is currently banned in the European Union (directive 88/146/EEC). 17β-boldenone (alternatively known as 1-dehydrotestosterone) is a potent anabolic steroid and has the potential for abuse in livestock. Therefore, an effective strategy to control potential 17β-boldenone abuse is required.

A recently published review article summarises research carried out in this area {Presence and metabolism of the anabolic steroid boldenone in various animal species: a review: Food Additives and Contaminants, Vol. 21, No.6 (June 2004), pp.515-525}. Any finding of 17β-boldenone glucuronic acid conjugate is considered as confirmation of illegal use, the same, at present, is not true for the 17α-boldenone glucuronic acid conjugate. However, the

presence of 17α-boldenone in the glucuronide fraction would be regarded as a suspicious finding. For a number of reasons, not least the lack of authentic standards, direct analysis of the conjugates is problematic. The approach taken was therefore to analyse the free steroids following enzyme hydrolysis. Researchers have also found unconjugated 17α-boldenone and 17β-boldenone in both dried faeces and urine samples known to have been contaminated with faecal material. It is therefore important to remove any unconjugated analytes before confirming that the presence of 17α and 17β-boldenone in a bovine urine sample is from illicit use.

For the purposes of the United Kingdom statutory monitoring programme, methods validated to SANCO guidelines are required. It is also desirable that methods are shown to be appropriate and robust by validation across laboratories involved in residue analysis and the statutory testing programme. The aim of this aspect of the project was to develop and validate a confirmatory method for 17α and 17β-boldenone glucuronic acid conjugates with a minimum required performance limit of $1ngml^{-1}$ in bovine urine. The LC-MS/MS methodology developed and validated for screening for 17α- and 17β-boldenone was modified to allow the removal of free steroid prior to enzyme hydrolysis. This was accomplished by initial extraction of the unhydrolysed urine using a C18 solid phase extraction cartridge. The free steroids were then removed by washing the cartridge with diethyl ether. The glucuronic acid conjugates were then eluted from the cartridge, subjected to enzyme hydrolysis using β-glucuronidase from *E Coli*. The resultant sample was then extracted on a second C18 cartridge followed by cleanup on an amine phase cartridge as per the previously developed screening method. Analysis of the resulting extract by LC-MS/MS in MRM mode was also essentially as per the screening method except that the number of transitions monitored was decreased to provide improved signal to noise and scan rate.

HFL has undertaken a full validation of the methodology and has provided CSL, DARD and LGC with blank and augmented urine samples to enable them to participate in the validation exercise. The results returned form the participating laboratories have been used to determine both inter and intra-laboratory variation.

EXPERIMENTAL

Blank matrix

Unless otherwise stated, all analyses were performed using, nominally negative, female bovine control urine, taken from animals at post mortem acquired by the Veterinary Laboratories Agency (VLA) or using negative bovine control urine, taken from 'on farm' animals involved in research studies undertaken by DARD. Prior to pooling the urine, each individual animal's urine was screened for the presence of 17α-boldenone and 17β-boldenone, and only samples found to be 'negative' were used.

Standard Preparation

Where sufficient standard was available, $1mgml^{-1}$ solutions were prepared by accurate weighing and dissolution in methanol. For $^{2}H_3$-17β-boldenone and 17α-boldenone only limited amounts of reference material were available and accurate weighing could not be achieved. For these analytes the weight of analyte claimed by the supplier was assumed to be correct and appropriate dissolution volumes used. Further dilutions were made to produce working solutions at a concentration of $100ngml^{-1}$.

System Suitability Standard

A system suitability standard containing all relevant analytes of interest was prepared by taking 50µl of each working solution, evaporating the solvent and reconstituting in 350µl of 50:50 water:methanol. This standard was analysed prior to each analytical run to check instrument acceptance criteria were met, i.e. retention times, elution order and the signal-to-noise ratio of all analytes.

Method

To satisfy SANCO validation requirements, the methodology is required as an SOP in a format consistent with the ISO75-2 guidelines. An SOP for the method can be found in Appendix I.

Ruggedness Testing

A ruggedness test based on the approach described by Youden and referenced in the SANCO/1805/2000 revision 6 guidelines (pages 30-32) was carried out. This is a fractional factorial design approach. Therefore, interactions between different factors cannot be detected. This type of study deliberately introduces minor

variations into the method. The consequences of these variations upon the detection and quantification of 17α-boldenone and 17β-boldenone glucuronic acid conjugates were observed and determined. The Youden approach is designed not to study one alteration at a time, but to introduce several variations at once. It results in 128 different outcome combinations. Previously, the robustness of the screening methodology had been assessed, for this reason ruggedness testing of the confirmatory method concentrated upon those aspects of the method that were modified, namely the initial extraction, free steroid removal and conjugate elution / hydrolysis.

Ruggedness was tested using bovine urine augmented at a level (4ngml^{-1} 17α-boldenone and 17β-boldenone glucuronide), which was regarded to be high enough to reliably detect any major differences in the response ratios for each analyte. Eight experiments were designed which tested seven different factors. The factors were investigated in duplicate thereby introducing several variations at one time. Raw data were analysed and treated by calculating the ratio of the response of the MRM transition 290>121 for $^{2}H_3$-17β-boldeonone (internal marker) to that of the response of the 287>121 transition for 17α and 17β-boldenone. The average values obtained for each minor variation (factor) were compared to each other to establish if a given factor had any significant influence or effect upon the results obtained. Possible factors that could influence the results were identified in pre-investigative studies. The deviations of these factors usually encountered among and between laboratories were discussed and a level agreed. Full details of the ruggedness testing carried out and the results obtained can be found in a separate report submitted to DEFRA.

Validation

A validation plan identifying the role and requirements for each collaborating partner was produced and agreed prior to commencing validation. This plan included details of the methodology, sample preparation and reporting. The relevant part relating to the approach to validation has been abstracted and is presented in Appendix 2.

RESULTS AND DISCUSSION

Method Development

Sample treatment, extraction and analysis were based upon the screening methodology developed for 17α- and 17β-boldenone as part of this project and based upon that provided by LGC, Appendix 3. In essence, the method consists of the extraction of both free and conjugated steroids using a C18 extraction cartridge. Free steroids were selectively removed by washing the cartridge with diethyl ether. Conjugated steroids were then eluted from the cartridge with methanol to which the internal marker, $^{2}H_3$-17β-boldenone, was added. Following removal of the methanol under nitrogen the extract was reconstituted in phosphate buffer and enzyme hydrolysis using β-glucuronidase from *E Coli* carried out to produce free steroid. Following hydrolysis initial extraction of free steroids was accomplished using a C18 solid phase extraction cartridge. The somewhat complex C18 extract was then cleaned using an NH_2 cartridge before analysis using a triple quadrupole, atmospheric pressure ionisation (API) mass spectrometer operated in electrospray, positive ion mode (ESI+). For 17α- and 17β-boldenone the MRM transition m/z 287 > m/z 121 was used as the quantifier ion, with m/z 287 > m/z 135 used as the qualifier ion; the internal marker was detected using the transition m/z 290 > m/z 121.

Multiple urine samples augmented with 17α- and 17β-boldenone, 17β-boldenone glucuronic acid conjugate and internal marker were analysed to check for efficient extraction of conjugated steroids and removal of free steroids. These experiments confirmed the methodology to be effective for the analysis of conjugated steroids in the presence of significant levels of free steroid.

Ruggedness Testing

Ruggedness testing was carried out using a Youden model. Factors investigated were: the pH of the urine, the speed of urine sample application onto the C18 cartridge, the volume of diethyl ether used to remove free steroids, the speed of application of this ether wash, the flow rate of methanol used to elute conjugated steroids, the hydrolysis conditions and the speed of sample application onto the C18 cartridge following hydrolysis. No significant difference in recovery of 17β-boldenone glucuronic acid conjugate was observed for any of the minor changes introduced into the method. From the ruggedness testing results it was evident that there were no critical factors that affected the ruggedness of the methodology. The use of a deuterium labelled internal marker successfully accounted for any variation in the absolute recovery of the analytes.

Stability of 17α-boldenone and 17β-boldenone

The stability of 17β-boldenone glucuronic acid conjugate in urine was assessed using both incurred and augmented urine samples at a concentration of approximately 2ngml^{-1}. The analyte was considered stable if the back-calculated concentration was within 15% of the expected value obtained from the initial experiment. 17β-boldenone glucuronic acid conjugate was found to be stable in urine in the dark at -20C for at least 20 weeks.

Specificity

The specificity of the method was tested by the analysis of twenty 'nominally negative' blank bovine urine samples each extracted and analysed for the presence interferences in the MRM transition m/z 287>121, all four participating laboratories undertook this analysis. For 17α-boldenone, CSL reported seven samples with back-calculated concentrations exceeding 1ngml^{-1}, three of these also exceeded the action limit of 2ngml^{-1}; the values calculated for these samples were 2.0, 2.7 and 3.2ngml^{-1}. For the remaining laboratories none of the samples contained peaks eluting at the expected retention time for 17α-boldenone for which the back-calculated concentration exceeded 1ngml^{-1}, the majority being below 0.2ngml^{-1}. For 17β-boldenone, none of the laboratories reported any of the samples to have a back-calculated concentration exceeding 1ngml^{-1}. CSL reported samples with back-calculated values of 0.4 and 0.6ngml^{-1}. For the remaining analyses only three samples contained peaks eluting at the expected retention time for 17β-boldenone for which the back-calculated concentration exceeded 0.1ngml^{-1}. In all 17α- and 17β-boldenone examinations there was no instance where the presence of the analyte was confirmed by an ion in the qualifier ion chromatogram having the correct retention time and intensity.

Working Range and Calibration Curves

Using the ratio of analyte (17α-boldenone and 17β-boldenone) peak areas to internal marker ($^{2}H_{3}$-17β-boldenone) peak areas gave good linear responses over the calibration range 0.5 to 4ngml^{-1}. Linearity was slightly better for 17β-boldenone than for 17α-boldenone, presumably a result of the internal marker having the same stereochemistry as 17β-boldenone. High recovery, good precision and good linearity were obtained over this range. Quality control standards (QCs) were run alongside in duplicate at low (1ngml^{-1}), medium (2ngml^{-1}) and high (3ngml^{-1}) concentrations. All QC samples analysed were within limits of +/-15% as were the back-calculated values for the calibration curve points (+/-20% in the case of the lowest point on the curve).

Recovery

A summary of the percentage mean recovery results from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit are given in the full report submitted to DEFRA. In the interest of brevity only the results for the reporting level (2ngml^{-1}) are presented here in Table 1.

	Laboratory			
Batch	**CSL**	**DARD**	**HFL**	**LGC**
% Mean Recovery of 17α-boldenone (2SF)				
1	110	75	110	57
2	110	160	100	34
3	72	100	100	35
% Mean Recovery of 17β-boldenone (2SF)				
1	110	81	100	86
2	100	93	110	61
3	86	81	100	67

Table 1: Recovery of 17α-boldenone and 17β-boldenone from bovine urine fortified at 2ngml^{-1}

Repeatability

A summary of the repeatability results from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit is given in the full report submitted to DEFRA. As above, in the interest of brevity the only the results for the reporting level (2ngml^{-1}) are presented here in Table 2. With the exception of results from LGC, the mean values of 17α- and 17β-boldenone obtained from the analysed replicates compare favourably with the

fortified levels. The reason for the low values obtained by LGC is unclear but the same trend was observed at all levels analysed for both analytes. Repeatability (expressed in terms of %CV) for 17α-boldenone and 17β-boldenone at all levels ranged between 3.6% and 31.1% and between 6.7% and 23.5%, respectively. Again, the markedly better repeatability for 17β-boldenone is attributed to the fact the internal standard is a deuterated analogue of this analyte.

	Laboratory			
Batch	**CSL**	**DARD**	**HFL**	**LGC**
Repeatability of 17α-boldenone determination (2SF)				
Mean (ngml^{-1})	1.9	2.3	2.2	0.8
%CV	23	33	3.6	27
Repeatability of 17β-boldenone determination (2SF)				
Mean (ngml^{-1})	2.0	1.7	2.1	1.4
%CV	13	9.0	6.7	16

Table 2: Repeatability of 17α-boldenone and 17β -boldenone determinations in bovine urine fortified at 2ngml^{-1}

Reproducibility

Nine batches of replicates were analysed, three from each laboratory (CSL, HFL and LGC). The batches with the highest %CV's and %RE's for 17β-boldenone at the fortification level of 2ngml^{-1} were chosen to complete the calculation for the reproducibility of the assay. Hence, CSL Batch 3, HFL Batch 2, LGC Batch 3 were chosen as the worst-case examples for this purpose. The results are presented in Table 3.

A summary of the reproducibility results from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit is given in the full report submitted to DEFRA.

Laboratory		17α-boldenone	17β-boldenone
CSL (B3) + HFL (B2) + LGC (B3)	**Mean**	1.4ngml^{-1}	1.8ngml^{-1}
	%CV	44%	23%
	%RE	-28%	-11%

Table 3: Reproducibility of 17α-boldenone and 17β-boldenone determinations in bovine urine fortified at 2ngml^{-1} (worst-case batches) (to 2SF)

Data from DARD was not used to calculate the combined reproducibility because the DARD laboratory, in this instance, was used as the control laboratory. DARD's methodology for sample extraction and analysis differs slightly to the other three participating laboratories.

The best-case scenario for the reproducibility of 17α and17β-boldenone at the fortification level of 2ngml^{-1} was also calculated. The batch with the lowest %CV and %RE for boldenone at the fortification level of 2ngml^{-1} was chosen from each of the three laboratories to complete this reproducibility calculation. These batches were CSL Batch 1, HFL Batch 3 and LGC Batch 1; these results are presented in Table 4. In both instances, the results for 17β-boldenone were significantly better than those for 17α-boldenone, again attributed to the fact that the internal standard was an analogue of 17β-boldenone. Given the required sensitivity the assay and the fact that analysis was carried out across three laboratories the results for 17β-boldenone are entirely acceptable.

Laboratory		17α-boldenone	17β-boldenone
CSL (B1) + HFL (B3) + LGC (B1)	**Mean**	1.8ngml^{-1}	1.9ngml^{-1}
	%CV	29%	10%
	%RE	-11%	-3.7%

Table 4: Reproducibility of 17α-boldenone and 17β-boldenone determinations from bovine urine fortified at 2ngml^{-1} (best-case batches) (to 2SF)

Incurred Matrices

The reported levels of 17β-boldenone found in the incurred samples analysed by each participating laboratory are presented in Table 5. These samples were prepared by first estimating the level of 17β-boldenone glucuronic acid conjugate in a post-administration urine sample and then diluting with 'blank' bovine urine to produce a stock with a nominal value of $2ngml^{-1}$. The best estimate of the true concentration of this stock urine is the mean of the individual values obtained from all laboratories. It is unclear why a 'not detected' result was returned by DARD for all six analyses undertaken (three sample batches analysed in duplicate); these results were excluded from the calculation of the mean level. It is also interesting to note that again the results from LGC were significantly lower than those reported by CSL and HFL. This may be indicative of a systematic error in LGC's analytical procedure, but, as there is no other evidence to support this, the results obtained by LGC have been included in the calculation of the mean concentration. Therefore, the best estimate for the concentration of 17β-boldenone glucuronic acid conjugate in the incurred sample (expressed as the concentration of free 17β-boldenone) is $1.85ngml^{-1}$, The standard deviation of this estimate is $0.33ngml^{-1}$ and fourteen observations were used.

Laboratory	Batch	Concentration 17β-boldenone in incurred sample		Mean	%CV
		A	B		
CSL	1	2.1	2.2	2.1	3.0
	2	2.0	2.1	2.0	4.2
	3	1.9	1.9	1.9	1.1
DARD	1	-ve	-ve		
	2	-ve	-ve		
	3	-ve	-ve		
HFL	1	1.9	1.8	1.8	2.3
	2	2.1	2.2	2.2	3.3
	3	1.9	1.9	1.9	0
LGC	1	Not analysed	Not analysed		
	2	Not analysed	Not analysed		
	3	1.1	1.1	1.1	1.3

Table 5: Amount of 17β-boldenone found in samples prepared from a common incurred urine sample (using a back-calculated approach)

Decision Limit (CCα)

The decision limits for each of the participating laboratories at the required performance limit have been calculated using the back-calculated concentrations of the analytes. Data are presented in Table 6.

Laboratory	CCα 17α-boldenone	CCα 17β-boldenone
CSL	0.9 $ngml^{-1}$	0.7 $ngml^{-1}$
DARD	1.3 $ngml^{-1}$	0.4 $ngml^{-1}$
HFL	0.7 $ngml^{-1}$	0.3 $ngml^{-1}$
LGC	0.9 $ngml^{-1}$	0.7 $ngml^{-1}$

Table 6: CCα values for each analyte and for each laboratory

The results show that for 17α-boldenone with an MRPL of $1ngml^{-1}$ the methodology is operating near the limit of its capability. For 17β-boldenone the capability of the assay appears adequate for an MRPL of $1ngml^{-1}$; again, this improved performance for 17β-boldenone is largely attributed to the internal standard. Whist it is desirable that the assay performs well at the $1ngml^{-1}$ level for both analytes, this is not essential for success. The reported data suggest that the levels of 17α-boldenone produced by faecal contamination are significantly higher than those for 17β-boldenone and that presence of the latter is much more indicative of a case of abuse of boldenone, its esters or precursors.

Detection Capability (CCβ)

Detection capabilities have been calculated from data returned by the participating laboratories. Results for each laboratory (for both analytes) at the required performance limit have been obtained using a back-calculated approach and are presented in Table 7.

Laboratory	CCβ 17α-boldenone	CCβ 17β-boldenone
CSL	1.5 $ngml^{-1}$	1.3 $ngml^{-1}$
DARD	2.2 $ngml^{-1}$	0.6 $ngml^{-1}$
HFL	1.3 $ngml^{-1}$	0.6 $ngml^{-1}$
LGC	1.5 $ngml^{-1}$	1.2 $ngml^{-1}$

Table 7: CCβ values for each analyte and laboratory

Again, the results reflect that the method is operating near the limit of its capability in the case of 17α boldenone but is adequate for 17β-boldenone. CCβ values for 17α-boldenone are around the reporting level of 2$ngml^{-1}$, whereas for 17β-boldenone they are all below this level.

CONCLUSIONS

An LC-MS/MS methodology for the confirmation of 17α and 17β-boldenone in bovine urine has been optimised and validated. The method is based on the screening procedure reported in VM02143 Screening Method Validation and has been adapted to report on 17α- and 17β-boldenone derived exclusively from glucuronide conjugates. The method has been evaluated across four laboratories according to the guidelines contained in SANCO/1085/2000 Revision 6.

The ruggedness of the proposed methodology was tested prior to the full method validation using a Youden approach. No significant factors affecting the performance of the assay were identified.

17β-boldenone glucuronide from an incurred sample was shown to be stable in the sample matrix, ie bovine urine, for up to 20 weeks after storage at -20°C in the dark. Similarly, 17β-boldenone glucuronide spiked into nominal blank urine was shown to be stable for up to 20 weeks after storage at -20°C in the dark.

The results obtained from the validation exercise indicate that the proposed methodology is adequate for its intended purpose of confirming the presence of 17α- and 17β-boldenone glucuronides in bovine urine. This conclusion is based upon the methodology demonstrating robustness, reliability and sensitivity for the analysis of 17α- and 17β-boldenone glucuronides at a level of 2$ngml^{-1}$. However, it was apparent that the procedure would benefit from the addition of a deuterated 17α-boldenone internal standard to provide greater robustness and reliability for the quantification of 17α-boldenone, should a threshold level for this analyte be defined in the future.

APPENDIX 1

METHOD SOP

1 Title

Validation of a LC-MS/MS Confirmatory Method for the Analysis of 17α- and 17β-Boldenone Glucuronides in Bovine Urine

Warning – Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

2 Scope

This is a liquid chromatographic-mass spectroscopy (LC-MS) assay for the confirmatory analysis of 17α- and 17β-boldenone glucuronides in bovine urine, with a minimum required performance limit of $1ngml^{-1}$.

From research there are no critical factors that should be particularly noted to ensure the ruggedness of this methodology. Although one should note that during solid phase extraction the cartridges should never be subjected to full vacuum (drying a cartridge is the exception to this).

3 Principle

The confirmatory procedure utilises 5ml of bovine urine which is initially subjected to solid phase extraction (SPE) on a C18 cartridge followed by enzyme hydrolysis. After hydrolysis another SPE clean-up is employed, using two different sorbents (C18 and NH_2 phases). The eluent is then evaporated and reconstituted in the reconstitution solvent. Aliquots (20µl) are analysed using a LC-MS/MS system in positive electrospray mode under MRM conditions, acquiring the transitions 290>147, 290>138, 290>121, 287>147, 287>135 and 287>121.

4 Reagents and Materials

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and deionised water.

4.1 Reagents

Methanol
Hexane
Diethylether
Phosphate buffer (disodium hydrogen orthophosphate 2-hydrate ($Na_2HPO_4.2H_20$), 4.44g and sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_20$), 3.44g in 500ml of water, pH6.8 checked)
E-Coli enzyme solution 10 Units/µl
Water:methanol (80:20)
Ethyl acetate:methanol (60:40)
Ammonium formate buffer (25mM pH4.5)

4.2 Materials

Bakerbond C18 e/c 500mg 3ml cartridges, Part no: 7020-03
Phenomenex Strata amine 500mg cartridges, Part no: 8B-S009-HBJ
Phenomenex Luna C18 (2), 150 x 2.0mm, 5µm, Part no: 00F-4252-B0
Phenomenex security guard column system.Holder, 4.0 L mm, Part no.:KJO-4282, cartridge C18 4.0 L mm x 3.0 ID mm, Part no: AJO-4287

5 Apparatus

Liquid-chromatographic-mass spectrometric/mass spectrometric system (LC-MS/MS)
(Specific system used: Waters 1525µ Binary HPLC pump attached to a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer)
Heating block at 70^0C, capable of accepting 3ml vials, 5ml and 10ml glass tubes
Bench-top vortex mixer

Pipette 5ml, air displacement
Pipette 50µl, positive displacement
Pipette 250µl, positive displacement

6 Sampling

Test samples should be stored at -20°C or lower until needed for analysis.

7 Drug Standards

7.1 Stock Solutions

17β-boldenone standard solution: 1mgml^{-1} in methanol
17α-boldenone standard solution: 100µgml^{-1} in methanol
17β-boldenone glucuronide standard solution: 1mgml^{-1} in methanol
$^{2}H_{3}$-17β-boldenone internal marker: nominal 1mgml^{-1} in methanol

7.2 Reference Solutions

17β-boldenone standard solution: 100ngml^{-1} in methanol
17α-boldenone standard solution: 100ngml^{-1} in methanol
17β-boldenone glucuronide standard solution: ~166ngml^{-1} in methanol
$^{2}H_{3}$-17β-boldenone internal marker: nominal 10µgml^{-1} in methanol

8 Procedure

8.1 Sample Preparation

NB: If test sample has been frozen, defrost at room temperature.

Prepare an internal marker working solution by pipetting the volume of drug reference solution detailed in Table 1, into a 10ml volumetric flask and make up to the mark with methanol.

Drug	Reference solution Conc.	Volume Working soln.
$^{2}H_{3}$-17β-boldenone	10µgml^{-1}	1000µl

Table 1: Preparation of internal marker working solution

8.1.1 Remove a 5ml portion of the test sample to a 20ml capped plastic vial
8.1.2 Spike sample with 17β-boldenone glucuronide at appropriate levels as required
8.1.3 Extraction: SPE conditions
C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the unhydrolysed urine sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of hexane
Dry cartridge for approximately 5 minutes using the vacuum manifold
Elute the free analytes from the C18 cartridge with 5ml of diethyl ether
Elute the conjugated analytes from the C18 cartridge with 5ml of methanol

8.1.4 Add 50µl internal marker of working solution to the methanol eluant and mix
8.1.5 Spike sample with 17α- and/or 17β-boldenone at appropriate levels as required
8.1.6 Evaporate the eluate to dryness under Oxygen Free Nitrogen (OFN) on a heating block at 70°C
8.1.7 Add 1ml of phosphate buffer and 200µl of E Coli enzyme to the test sample and mix
8.1.8 Heat overnight in an oven set at 37°C

8.2 Preparation of Calibration Line

NB: Calibration line and controls must be prepared by different analysts.

Pipette the following volumes of drug standards into a 10ml volumetric flask (see Table 2) and make up to the mark with methanol. One mixed standard is known as A1 the other is known as A2.

Drug	Ref solution concentration	Volume
17α-boldenone	20μgml^{-1}	50μl
17β-boldenone	20μgml^{-1}	50μl

Table 2: Preparation of mixed standard working solution

8.2.1 Remove a 5ml portion of the test sample to a 20ml capped plastic vial
8.2.2 Follow step 8.1.3
8.2.3 Add 50μl internal marker of working solution to the test sample and mix as 8.1.4
8.2.4 Spike sample with A1 as shown in Table 3

STD/ Calibration level		Vol of A1 (μl)	Equivalent Conc. in Urine (ngml^{-1})	
			17α-boldenone	**17β-boldenone**
BLANK	A	0	0	0
	B			
1(1/4 MRPL*)	A	25	0.5	0.5
	B			
2 (1/2MRPL)	A	50	1	1
	B			
3 (MRPL)	A	100	2	2
	B			
4 (1.5MRPL)	A	150	3	3
	B			
5 (2MRPL)	A	200	4	4
	B			

Table 3: Preparation of Calibration Line

8.2.4 Follow steps 8.1.6 to 8.1.8
8.2.5 Follow steps 8.4.1 to 8.4.6

8.3 Preparation of Quality Controls

8.3.1 Dispense six 5ml portions of blank urine
8.3.2 Follow step 8.1.3
8.3.3 Add 50μl internal marker of working solution to the test sample and mix as 8.1.4
8.3.3 Spike sample with A2 as shown in Table 4

Control		Vol of A2 (μl)	Equivalent Conc in Urine (ngml^{-1})	
			17α-boldenone	**17β-boldenone**
Low	A	50	1	1
	B			
Medium	A	100	2	2
	B			
High	A	150	3	3
	B			

Table 4: Preparation of Quality Control Samples

8.3.4 Follow steps 8.1.6 to 8.1.8
8.3.5 Follow steps 8.4.1 to 8.4.6

8.4 Extraction

SPE Conditions:

8.4.1 C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the hydrolysed sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of water:methanol (80:20 v/v)
Dry cartridge for approximately 15 minutes using the vacuum manifold
Elute from the C18 cartridge with 5ml of ethyl acetate:methanol (60:40v/v)

8.4.2 NH_2 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml ethyl acetate:methanol (60:40 v/v)
Load sample extract from C18 cartridge in step 8.2.1 and collect eluate

8.4.3 Evaporate the eluate to dryness under OFN on a heating block at 70°C
8.4.4 Reconstitute the dried extract in 350µl of water:methanol (50:50 v/v)
8.4.5 Whirlimix, transfer to a labelled LC-MS vial and cap
8.4.6 Analyse the samples by LC-MS/MS

8.5 Preparation of a System Suitability Standard

8.5.1 Pipette 50µl of 17α- and 17β-boldenone and 50µl of internal marker ($^{2}H_3$-17β-boldenone) into a 3ml glass vial
8.5.2 Evaporate the solvent under OFN on a heating block at 70°C
8.5.3 Reconstitute the dried extract in 350µl of water:methanol (50:50 v/v)
8.5.4 Transfer to a labelled LC-MS vial, cap and submit to LC-MS/MS

9 Analysis

9.1 LC-MS Conditions

Autosampler instrument: Waters 2777 Sample Manager
Pump: 1525µ binary HPLC pump and column oven
Software: MassLynx 4.0 SP2

9.2 LC Conditions

LC instrument: Waters 1525µ Binary HPLC pump
Column: Phenomenex Luna C18(2), 150 x 2.0mm, 5µm
Part no: 00F-4252-B0
Guard Column: Phenomenex security guard column system.
Holder, 4.0 L mm, Part no.:KJO-4282
Cartridge C18 4.0 L mm x 3.0 ID mm, Part no: AJO-4287
Column temp.: Ambient
Mobile phase: Solvent A: Ammonium formate, 25mM, pH4.5
Solvent B: Methanol
Injection volume: 20µl
Run time: 22 min

Time (min)	% Solvent A	% Solvent B	Flow (ml/min)
0	40	60	0.2
1	40	60	0.2
15	10	90	0.2
16	10	90	0.2
16.5	40	60	0.2
22	40	60	0.2

Table 5: Gradient Elution Timetable (linear gradient)

9.3 MS Conditions

MS instrument: Waters Micromass Quattro Premier tandem quadrupole mass spectrometer or equivalent. Note: The optimum conditions for the generation and transmission of ions will vary somewhat from instrument to instrument. Therefore, these values should be used only as a guide.

Divert Valve

Time (min)	0.00 - 6.00	6.00 - 16.00	16.00 - 22.00
Position	Waste	Detector	Waste

Table 6: Divert valve conditions

Source settings: **ESI+**
Capillary (kV): 3.50
Cone: 50
RF Lens 1: 33
Aperture: 0.2
RF Lens 2 0.2

Source temp: 120°C
Desolvation temp: 450°C
Cone gas flow: Approximately 90 L/Hr Nitrogen
Desolvation gas flow: Approximately 500 L/Hr Nitrogen

Channel	Parent (Da)	Daughter (Da)	Dwell (sec)	Cone (V)	Collision (eV)
1	287.15	120.80	0.2	20	22
2	287.15	134.85	0.2	20	15
3	287.15	146.85	0.2	20	18
4	290.15	120.80	0.2	30	22
5	290.15	137.85	0.2	30	15
6	290.15	146.85	0.2	30	18

Table 7: MRM of 6 mass pairs (time 0.5 to 15 min; ESI+)

10 Data Interpretation

17α- and 17β-boldenone and their internal marker, $^{2}H_{3}$-17β-boldenone are detected by LC-MS/MS. The expected retention times and monitored ions are detailed in Table 8. $^{2}H_{3}$-17β-boldenone should be indicated in all test portions by the presence of a peak at the correct retention time and by the required ion transitions being present under positive ESI conditions.

Drug	RT (min)	RRT	Precursor ion	Product ions
$^{2}H_{3}$-boldenone (IM)	9.95	1	290	138, 121
17β-boldenone	10.00	1.01	287	135, 121
17α-boldenone	11.92	1.20	287	135, 121

Table 8: Retention times and monitored ions

For confirmatory purposes, when MRM analysis is employed, four identification points must be secured. These comprise the analyte RT and three mass spectral ions (one precursor ion and two daughter ions). The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, must correspond to those of the suitability standard with the tolerances shown in Table 9.

Relative Intensity (% of base peak)	LCMS Tolerance (relative)
>50%	+/- 20%
>20% - 50%	+/- 25%
>10% - 20%	+/- 30%
</=10%	+/- 50%

Table 9: Maximum permitted tolerances for relative ion intensities using a mass spectrometric technique

Interfering ions in the mass spectra should be less than 20% of the intensity of the most intense ion.

10.1 Integrate and ratio the peak areas of the analyte and internal marker in extracted curve and samples for determination. The ion ratios obtained in samples for determination should correspond to those obtained from the extracted curve samples.

10.2 Plot the calculated ratios against spiked concentration. The value for a sample for determination can then be obtained from the curve.

10.3 Ensure that the ratios of the chromatographic retention times of 17α-boldenone and 17β-boldenone to that of the internal standard, i.e. the relative retention times (RRTs) obtained for the samples for determination are within +/- 0.5% of those obtained for the extracted sample and system suitability standard.

10.4 Any ratio of analyte:internal marker obtained from a sample for determination that is at or above the value obtained for an extracted standard of concentration 0.5 x MRPL should be submitted for confirmatory analysis.

APPENDIX 2

METHOD VALIDATION PLAN

The method will be validated in the range of 0.5ng/ml to 4ng/ml for 17α- and 17β-boldenone. It is intended to demonstrate adequate capability at a concentration of 1ngml^{-1} in urine.

At HFL, analysis will be carried out using a Waters Quattro Premier series, triple quadrupole LCMS system or equivalent as stated in Appendix 2. Analysis of samples by LGC, DARD and CSL will employ systems already used for the statutory testing programme.

Assay validation will comprise assessment of the following:

- Applicability/Ruggedness
- Stability
- Selectivity/Specificity
- Working range and calibration curve
- Trueness/Recovery
- Repeatability
- Reproducibility
- Decision limit

All positive identifications of 17α- or 17β-boldenone must satisfy the following criterion:

The ratio of the chromatographic retention time of the free analyte to that of the internal standard, ie the relative retention time (RRT), must correspond to that of the system suitability standard at a tolerance of +/- 2.5%.

Applicability/Ruggedness (minor changes)

Ruggedness testing has been performed prior to validation. Minor changes in the analysis were deliberately introduced and their consequences upon the quantification and detection of 17α- and 17β-boldenone at a level of 4ngml^{-1} determined. A Youden approach was applied to key elements from the pre-treatment, clean-up and analysis phases of the method, thereby demonstrating the overall applicability and ruggedness of the methodology.

Stability of 17α- and 17β-boldenone in urine

A pooled incurred sample with an estimated concentration of conjugated analyte of approximately 2ngml^{-1} (equivalent. to 3.23ng/ml of 17β-boldenone glucuronide) will be subjected to the following testing regime. The stability of 17β-boldenone glucuronide in urine at -20°C and -80°C will be investigated during validation of the confirmatory methodology. The incurred sample will be divided into at least 20 aliquots at a volume appropriate for the analysis. Two aliquots will be analysed immediately as replicates; the others will be frozen and analysed as replicate pairs (-20°C and -80°C experiments) after 1, 2, 4 and 20 weeks. The remaining aliquots will be kept frozen (at both temperatures) in case further stability testing is required.

Analysis will be carried out at 0, 1, 2, 3, 4 and 20 weeks or until degradation phenomena are observable.

Specificity

An evaluation of specificity will include two parts.

Firstly, an evaluation of a limited number of substances that might give rise to an interfering signal will be carried out. Compounds with similar structures or molecular weights and substances likely to be used in combination with the analyte will be examined, for example other steroids, both exogenous and endogenous. Other steroids will include testosterone, nandrolone, androstenedione, etc. The assessment will be carried out by comparison of chromatograms for the presence of peaks likely to interfere with analysis of 17α- and 17β-boldenone or the internal marker.

Secondly, potential interferences from the sample matrix will be investigated. This will be achieved by the analysis of a minimum of 20 blank bovine samples taken from animals not known to have been administered any drug

substances. The samples will be fortified with internal marker alone and then analysed for any interferences in the region of the chromatogram where 17α- and 17β-boldenone elute. Additional chromatographic peaks in the region of the analytes under investigation will be further assessed for: 1) the possibility of producing false positives; 2) any masking of responses that may lead to false negatives; 3) any influence upon quantification.

The chromatographic system will be checked for injection carry-over.

Working range and calibration curve

At least two independent sets of analyte standards at 0, 0.5, 1, 2, 3 and 4ngml^{-1} will be prepared and analysed.

All subsequent quantitative work will be carried out using appropriate standard curves using a minimum of 5 levels, i.e. 0.5, 1, 2, 3 and 4ngml^{-1} in duplicate. The analytical result should fall within the range of the curve.

Recovery

Recovery will be determined by analysis of 6 fortified blank replicates each at 1.0, 1.5 and 2.0ngml^{-1}. The percentage recovery, standard deviation and coefficient of variation will be determined at each level.

Repeatability

Repeatability will be determined by four separate laboratories. Each laboratory will analyse 6 fortified blank replicates each at 1.0, 1.5 and 2.0ngml^{-1} on three separate occasions (54 samples in total in each participating laboratory). The mean concentration, standard deviation and coefficient of variation within each laboratory will be determined at each level and on each occasion.

Reproducibility

Reproducibility will be determined by analysis of 6 fortified blank replicates each at 1.0, 1.5 and 2.0ngml^{-1} on three separate occasions at the four separate laboratories. The data collected from the repeatability test will be used for calculating the reproducibility of the method. The overall mean concentration, standard deviation and coefficient of variation will be determined at each level and on each occasion.

Decision Limit (CCα)

The decision limit, CCα will be determined by the calibration curve procedure as outlined in the SANCO guidelines. Data collected from the repeatability determinations will be used for this purpose. The corresponding concentration at the y-intercept plus 1.64 times the standard deviation of the reproducibility of the intercept will provide a value for the decision limit (α - 5%).

Detection Capability (CCβ)

The detection capability, CCβ, will be determined from the analysis of blanks used in the specificity test, fortified at a decision limit derived from results received from the participating laboratories. HFL will issue this decision limit value to the participating laboratories once the inter laboratory CCα value for the confirmatory procedure has been determined.

APPENDIX 3

Comparison of LGC screening methodology with modified confirmatory procedure

<u>LGC screening method</u>

Sample preparation

Remove a 5ml portion of the test sample to a plastic vial
Add 50µl internal marker of working solution to the test sample and mix
Add 2ml sodium acetate buffer
Check the pH manually and add more buffer if needed to bring the pH of the urine to pH 5
Add 50µl helix pomatia, enzyme to the test sample and mix
Heat for 1 hour in an oven set at 37°C
Centrifuge urine samples for 10 minutes @ 2000rpm prior to extraction

SPE Conditions:

C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the hydrolysed urine sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of water:methanol (80:20 v/v)
Dry cartridge for approximately 15 minutes using the vacuum manifold
Elute from the C18 cartridge with 5ml of ethyl acetate:methanol (60:40v/v)
NH_2 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml ethyl acetate : methanol (60:40 v/v)
Load sample extract from C18 cartridge in step 8.2.1 and collect eluate
Evaporate the eluate to dryness under Oxygen Free Nitrogen (OFN) on a heating block at 70°C
Reconstitute the dried extract in 350µl of 50:50 water:methanol
Whirlimix, transfer to a labelled LC-MS vial and cap
Analyse the samples by LC-MS/MS

<u>Confirmatory analysis procedure</u> (following modifications to LGC method by HFL)

Sample preparation

Remove a 5ml portion of the test sample to a plastic vial
Extraction: Separation of free and conjugated fractions

C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the unhydrolysed urine sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of hexane
Dry cartridge for approximately 5 minutes using the vacuum manifold
Elute the free analytes from the C18 cartridge with 5ml of diethyl ether
Elute the conjugated analytes from the C18 cartridge with 5ml of methanol

Add 50µl internal marker of working solution to the methanol eluant and mix
Spike sample with 17α- and/or 17β-boldenone at appropriate levels as required
Evaporate the eluate to dryness under Oxygen Free Nitrogen (OFN) on a heating block at 70°C
Add 1ml of phosphate buffer and 200µl of E Coli enzyme to the test sample and mix
Heat overnight in an oven set at 37°C (=hydrolysed urine sample)

SPE Conditions:

C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the hydrolysed urine sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of water:methanol (80:20 v/v)

Dry cartridge for approximately 15 minutes using the vacuum manifold
Elute from the C18 cartridge with 5ml of ethyl acetate:methanol (60:40v/v)

NH_2 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml ethyl acetate : methanol (60:40 v/v)
Load sample extract from C18 cartridge in step 8.2.1 and collect eluate

Evaporate the eluate to dryness under Oxygen Free Nitrogen (OFN) on a heating block at 70°C
Reconstitute the dried extract in 350µl of 50:50 water:methanol
Whirlimix, transfer to a labelled LC-MS vial and cap
Analyse the samples by LC-MS/MS

Summary explanation of procedures:
The LGC screening methodology uses direct hydrolysis of the urine test sample prior to extraction. Following solid phase extraction, the resultant extract represents a combined free and glucuronic acid conjugated fraction.

The modified procedure suggested for the confirmatory analysis involves an initial pre extraction of the unhydrolysed urine test sample to remove the free fraction. The conjugated fraction generated from this procedure is then hydrolysed. The conjugated fraction is then extracted using the same SPE procedure as that for the screening analysis.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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