CCQM-K69 Key Comparison
Testosterone glucuronide in human urine

Final Report

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1. Abstract

The CCQM-K69 key comparison of testosterone glucuronide in human urine was organized under the auspices of the CCQM Organic Analysis Working group (OAWG). The National Measurement Institute Australia (NMIA) acted as the coordinating laboratory for the comparison. The samples distributed for the key comparison were prepared at NMIA with funding from the World Anti-Doping Agency (WADA). WADA granted the approval for this material to be used for the intercomparison provided the distribution and handling of the material were strictly controlled.

Three National Metrology Institutes (NMIs)/Designated Institutes (DIs) developed reference methods and submitted data for the key comparison along with two other laboratories who participated in the parallel pilot study. A good selection of analytical methods and sample workup procedures was displayed in the results submitted considering the complexities of the matrix involved. The comparability of measurement results was successfully demonstrated by the participating NMIs. Only the key comparison data were used to estimate the Key Comparison Reference Value (KCRV), using the arithmetic mean approach. The reported expanded uncertainties for results ranged from 3.7% to 6.7% at the 95% level of confidence and all results agreed within the expanded uncertainty of the KCRV.

A parallel pilot study, CCQM-P115, was also organized. The same samples used for the key comparison were also used for this pilot study. However, the participants of the parallel study were requested to report both testosterone glucuronide and its analogue epitestosterone glucuronide.

2. Introduction

Anabolic agents represent the class of banned substances within the anti-doping area that are the most commonly reported. The World Anti-Doping Agency (WADA) statistics for 2006 show that 45% of all reported “adverse analytical findings” from their WADA-accredited sports testing laboratories were for anabolic agents, ie 1,966 of a total 4,322 athlete’s samples. Of the 1,966 anabolic agent positives, 1,124 were for the steroid testosterone [1]. Enhanced levels of testosterone with respect to its very close analogue epitestosterone are indicative of testosterone abuse. From 2005 the WADA Prohibited Substances List revised the testosterone/epitestosterone (T/E) ratio from 6/1 to the lower level of 4/1, at which further investigation for potential testosterone abuse must be conducted.

Testosterone exists in human urine mostly as either the 17-glucuronide or 17-sulfate derivative, with the former conjugate generally being the major metabolite. Less than 2% of the total testosterone is normally present as the free steroid in well preserved urine samples. Testosterone glucuronide (17β-3-oxoandrost-4-en-17-yl β-D-glucopyranosiduronic acid, Registry No. 1180-25-2) is thus an important analyte in the detection of steroid abuse. Typically this is measured by anti-doping laboratories using GC-HRMS following solvent extraction of the urine to remove any free steroid, hydrolysis with a selective glucuronidase

enzyme, extraction of the testosterone and then derivatisation [2]. The analyte can also be easily detected without hydrolysis by LC/MS/MS. The important 17α-isomer, epitestosterone glucuronide, must also be measured concurrently when assessing the likelihood of testosterone abuse in sport, and this measurement was part of the parallel pilot study, CCQM-P115.

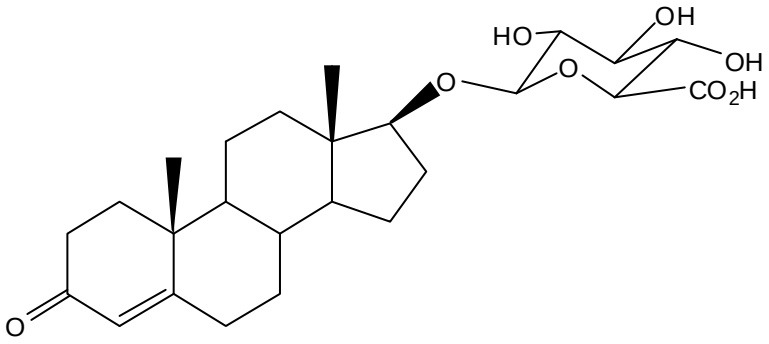

Fig 1. Structure of testosterone glucuronide, the major metabolite of testosterone

As the steroid component of the molecule is the anabolic agent, these steroids are typically reported by drug testing laboratories in terms of the mass concentration of the unconjugated steroid, rather than that of the entire conjugate [3]. For this key comparison the same approach was followed. **The measurand was the mass fraction of testosterone glucuronide expressed as free testosterone.**

In 2005 a CCQM pilot study (CCQM-P68) [4] was co-ordinated for the steroid 19-norandrosterone in human urine. This steroid is the major metabolite of nandrolone, the second most commonly reported steroid of abuse amongst athletes after testosterone. The level of total 19-norandrosterone in the CCQM-P68 sample was 2.1 ng/g, very close to the allowed 2 ng/mL cut off for athletes. There were four participants in the pilot study CCQM-P68 and the agreement between the results from all four institutes was excellent. The expanded measurement uncertainties reported for the four participants ranged from 3.7% – 7.0% and all participants' results overlapped the reference value within their uncertainties. The measurand for CCQM-P68 was the total of all forms of the steroid as this is the WADA requirement for testing for nandrolone abuse. In the case of testosterone, WADA only require the glucuronide form to be tested. This is actually a slightly easier technical challenge as this means that the glucuronide can be measured directly by LC/MS/MS or the low levels of the free steroid can be removed by a simple solvent extraction and then the glucuronide measured by GC-HRMS. There is no need when measuring just the glucuronide to develop separate methodology to identify and quantify the sulfate metabolite.

This key comparison was proposed at the CCQM meeting of April 2007 and approved at the April 2008 meeting. Samples were distributed to the three participants in July 2008 and results were due on 15 October 2008.

3. Participants

Table 1 List of participating NMI's and contact persons

Institute/ Laboratory	Country/Economy	Study	Contact person
NMIA National Measurement Institute, Australia	Australia	CCQM-K69, CCQM-P115	Dr. Lindsey Mackay
LGC Laboratory of the Government Chemist	United Kingdom	CCQM-K69	Dr.Gavin O'Connor
GL HKSAR Government Laboratory of Hong Kong Special Administrative Region	Hong Kong, China	CCQM-K69	Dr. Della Sin

4. Samples

The samples distributed for CCQM-K69 were prepared at NMI Australia (NMIA), in accordance with ISO Guide 34 [5], with funding from the World Anti-Doping Agency (WADA). **WADA gave approval for the use of this material for this comparison on the understanding that any remaining material participants have at the end of the intercomparison will be destroyed (or returned to NMIA) because the distribution of this material is being strictly controlled.** Participating laboratories were required to sign and return statements to this effect.

The material distributed was a lyophilized natural human urine that had been fortified with additional testosterone glucuronide producing a mass fraction of close to 40 ng/g when reconstituted. It contained a natural level of epitestosterone glucuronide of approximately 10 ng/g when reconstituted. The material was stabilised with sodium azide, filtered and then freeze-dried into 20 mL units in 50 mL glass bottles. The material was stored at -20°C. A strict protocol for reconstitution of the sample material with water was supplied to the participants and is outline below.

The material must be reconstituted in the following way:

a) Remove the freeze-dried urine bottle from cold storage and equilibrate to room temperature.
b) Remove the aluminium crimp using a decrimper, but leave the rubber septum in place.
c) Weigh the bottle with septum.

d) Fill a 10 mL or 20 mL syringe fitted with a needle with water, lift the septum to expose the slot in the septum and inject 20.00 g[#] water into the bottle through the slot. If necessary wipe off any drops of water on the exterior of the bottle, for example on the lip of the bottle, and then push the septum down.
e) Reweigh the bottle, septum and water to determine the mass of water added.
f) Seal the bottle with a crimp cap. Invert gently to dissolve all solid material and rinse the sides of the bottle and the rubber stopper.
g) Heat at 40°C for 30 min then equilibrate to room temperature.
h) Take subsamples of the reconstituted material for use within six hours if it is being stored at room temperature.
i) If the reconstituted material is to be stored for longer than six hours it must be ensured that appropriately sterile water has been added and the liquid form should be refrigerated and used within 48 h. The refrigerated material should be warmed to 40°C for 30 min then equilibrated to room temperature before use as above. This process of storing refrigerated and then warming should not be repeated so all of the material should then be used at this point.

[#] The expected value for this material corresponds to the addition of 20.00 g water. If the mass of water added is different then a correction must be applied to take this into account. The reconstitution process has been tested with added water of approximately 20.00 ± 0.20 g mass and thus masses within this range should be aimed for.

5. Reporting

Four bottles of the urine material were provided to each laboratory. A result for three of the individual bottles and an overall, combined result for these bottles were to be reported. A data reporting sheet was supplied for the submission of the results.

The following data was to be included in the report:

a) The mass fraction of testosterone glucuronide in the urine as ng/g expressed as free testosterone
b) Full uncertainty budgets
c) Source and purity of any reference materials used
d) Outline of methodology of analytical method and uncertainty estimation (including a measurement equation)

6. Characterisation of the study material

The study material was characterised in accordance with ISO Guide 35 [6].

6.1 Homogeneity testing

A total of 1,200 bottles of the freeze-dried urine material were produced from approximately 30 L of human urine which were dispensed into 20 mL aliquots and freeze-dried. The dispensing of the liquid urine into the bottles was checked by weighing 33 of the bottles

before and after freeze-drying. The mean residual mass of freeze-dried urine present in each of the 33 bottles of the material was measured as 0.485 g with an RSD of 0.69%. The precision of dispensing was thus very good. The water content of the resulting freeze-dried material was tested in 6 bottles by Karl-Fischer titration and an average of 2.9 % water was observed. This met the desired parameters set for the material.

After freeze-drying of the bulk into 1,200 bottles 31 bottles were subject to homogeneity testing by IDMS using GC-HRMS, with seven bottles subjected to within-bottle testing. Figure 2 shows the between-bottle results for testosterone glucuronide tested in 31 bottles. These results include the within-bottle testing of seven bottles (287, 688, 95, 884, 1091, 1235, 506), with results given in the order of analysis. The error bands graphed for these 7 bottles represent two times the standard deviations of the duplicate results from each bottle.

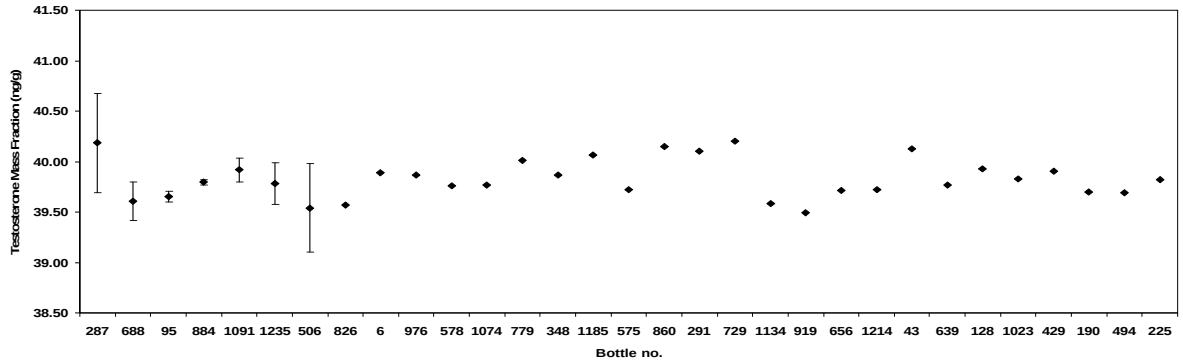

Figure 2 Between-bottle and within-bottle homogeneity testing of testosterone glucuronide in the freeze-dried urine material

The RSD of the mass fraction of the testosterone glucuronide measured by GC-HRMS in each of these 31 bottles was 0.46%. The sample size used for this testing was 5 g.

An analysis of the variance (ANOVA) on the results of these homogeneity tests indicates that at the 95% confidence level, there is no significant difference between the variability of the results within or between bottles for this sample size. The critical value for the ANOVA F statistic at the 95% confidence level is 3.38 for this test. The F value for the measured testosterone glucuronide mass fractions was 1.94. The within-bottle and between-bottle homogeneity of the material appeared to meet the needs of this key comparison.

6.2 Stability testing

The freeze-dried material stored at -20 °C has been stability tested over 12 months. The change in level of testosterone glucuronide over this period has been less than 1%. It should be noted that storage at 40 °C for 6 months resulted in a reduction in level of 50% for testosterone glucuronide, so storage at any elevated temperature should be avoided.

7. Reference Materials Used by Participants

The calibration standards and internal standards used by participants are summarised in Table 2. Only the glucuronide forms were used as the main calibration material and the corresponding D_3-labelled glucuronide form was used as the IDMS internal standard.

Table 2 Summary of reference materials employed

Laboratory	Source and purity of calibration standard materials	Source of internal standard materials
NMIA	Testosterone glucuronide, D507c, NMIA, certified as 95.1 ± 1.1%	D_3-Testosterone glucuronide (Na salt), D505, NMIA
	Confirmation standard: Testosterone, M914B, Batch 04-S-01, NMIA, 99.2 ± 1.0%	
LGC	Testosterone glucuronide, Batch 06-S-03, NMIA, certified as 94.7 ± 1.9%	D_3-Testosterone glucuronide, NMIA
GL of HKSAR	Testosterone glucuronide, D507c, Batch 06-S-03, NMIA, certified as 94.7 ± 1.9%	D_3-Testosterone glucuronide, D505, Batch 97-000056, NMIA

8. Methodology

No specific method was prescribed for the analysis. Laboratories were encouraged to use their preferred methodology. Two participants used methods based on GC-HRMS detection and the other used an LC-MS-MS approach. Although other metabolic forms of testosterone are possible, only the glucuronide form was required to be reported (expressed as equivalents of free testosterone). Participants were expected to use an isotope dilution mass spectrometric (IDMS) approach in their analyses and only such results would be incorporated in the Key Comparison Reference Value as these types of methods had been proven in the previous study CCQM-P68 as appropriate for steroid measurements in urine. The methodologies employed by different participants are summarized in Table 3.

All laboratories employed an IDMS technique with deuterated testosterone glucuronide as the internal standard. NMIA and GL of HKSAR employed GC/HRMS as their main detection technique requiring enzymatic hydrolysis to convert glucuronide conjugates to the free steroid and derivatisation of the testosterone to permit efficient gas chromatographic separation. Some differences were observed between the sample workups used. NMIA used a HPLC fractionation to remove coextracted materials from the hydrolysed urine while GL HKSAR employed solid phase extraction (SPE) followed by a liquid-liquid extraction procedure. LGC employed an ethyl acetate liquid:liquid extraction followed by LC/MS/MS detection as the main technique to measure testosterone glucuronide directly.

NMIA and LGC were the only laboratories that reported using a confirmatory method. NMIA's confirmatory method measured free testosterone after hydrolysis of the urine using immuno-affinity clean-up coupled on-line with LC/MS/MS. Extraction of the free steroid

was not carried out for this confirmation as the level of free testosterone was found to be insignificant. The mass fraction of testosterone measured by LC/MS/MS agreed to within 2.1 % of the GC/HRMS result. LGC developed a confirmatory method and measured the actual form of the analyte using LC-FAIMS-MS/MS and excellent agreement was found between their two LC/MS/MS techniques.

Table 3 Summary of methods employed by participants

Laboratory	**Method summary**[†]	**Instrumentation**	**Form of analyte being measured**[†]
NMIA	Addition of mixed D_3-TG/D_3-EG, free steroid extraction, enzyme hydrolysis, solvent extraction, HPLC fractionation, TMS derivatisation, GC/HRMS	GC/HRMS - Finnigan MAT 95 HRMS and Agilent 6890 GC	Free testosterone Free epitestosterone (both from glucuronide forms)
	Confirmation method: TG: Addition of D_3-TG, hydrolysis, immuno-affinity coupled LC/MS/MS	Confirmatory method: Thermo TSQ Vantage LC/MS/MS	Testosterone
LGC	Addition of D_3-TG, solvent extraction, evaporated , reconstituted for LC/MS/MS	Miromass Quattro Ultima LC/MS/MS	Testosterone glucuronide
	Confirmatory method	Thermo Quantum Ultra LC-FAIMS-MS/MS	Testosterone glucuronide
GL of HKSAR	Addition of D_3-TG, free steroid extraction, enzyme hydrolysis, SPE followed by liquid-liquid extraction, TMS derivatisation, GC/HRMS	GC/HRMS – Waters Micromass AutoSpec-Ultima and Agilent 6890N GC	Free testosterone from glucuronide form

[†]Note : TG = Testosterone glucuronide and EG = epitestosterone glucuronide

9. Participant's results and KCRV

There were a limited number of participants in the key comparison largely due to the fact that this analysis requires very specialised expertise in the area of steroid analysis. Two anti-doping laboratories participated in the parallel pilot study, CCQM-P115, in addition to the three NMIs/DIs. Only the key comparison results for testosterone glucuronide are used to estimate the KCRV. In the preliminary report (Draft A), three approaches were used to estimate the candidates for KCRV and their related uncertainties: arithmetic mean, weighted mean and median, as outlined in the document by Steve Ellison titled "Estimation of a consensus KCRV and associated Degrees of Equivalence". The document examined different approaches to the KCRV, its uncertainty and the uncertainty of the degrees of equivalence by taking into account the correlation of the KCRV and the results. Due to the limited number of participants in this study, it was decided that there was no obvious benefit in using these models to calculate the KCRV.

At the OAWG meeting held in April 2009, the arithmetic mean was chosen as a reasonable approach to best estimate the true mass fraction of testosterone glucuronide (as testosterone)

in the study material. As each laboratory reported an average of three different bottles of the study material and had assessed the various contributions to their uncertainties, any inhomogeneity in the production of this material would have been captured in the proposed reference value. This approach, however does not take into account the correlation between the standards used by the participants, as they were all supplied by NMIA. Under these circumstances, the uncertainty quoted for the KCRV could be underestimated.

Table 4. Summary of CCQM-K69 results for the mass fraction of testosterone glucuronide reported as equivalents of the free steroid

Participant	Reported Value (ng/g)	Standard uncertainty (ng/g)	Expanded Uncertainty (ng/g)	Expanded Uncertainty (%)	No. of samples	k
NMIA (Australia)	39.94	0.66	1.49	3.7	3	2.26
LGC (UK)	40.3	1.3	2.7	6.7	3	2
GL (HKSAR)	41.34	0.809	1.618	3.9	3	2
Mean (KCRV)	**40.5**	**0.42**	**1.8**	**4.5**		**4.3**

Values reported in Table 4 are exactly as reported by the participants.

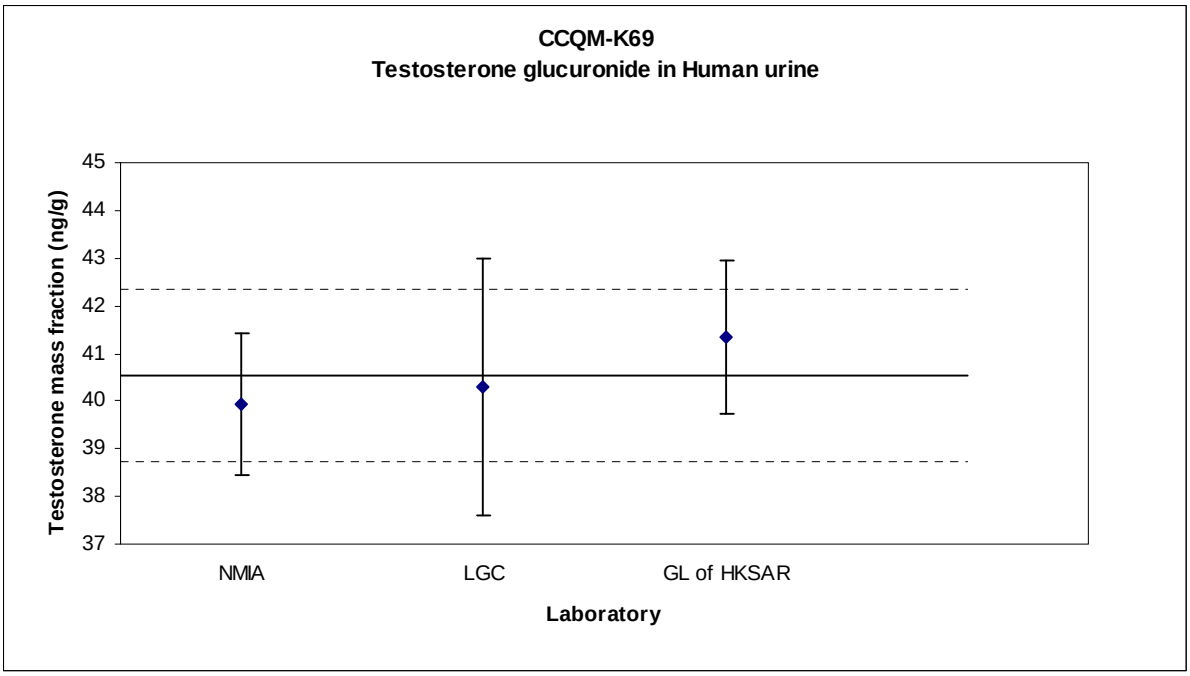

Figure 3. Results for testosterone glucuronide mass fraction (as testosterone) shown with KCRV reference line and expanded measurement uncertainty at 95% confidence level.

The overall agreement between the results is excellent, with a RSD of 1.8%. The arithmetic mean, 40.5 ng/g, has been assigned as the KCRV and is represented in Figure 3 by a solid line across the participants results. The uncertainty of the mean was calculated from standard deviation of the mean results multiplied by a coverage factor of 4.3 (for n=3 laboratories) to give 95% level of confidence and a value of 1.8 ng/g, or 4.5% relative to the mean. The

dashed, horizontal lines indicate the range of the expanded uncertainty of the arithmetic mean. All the three results are well within the expanded uncertainty of the KCRV.

10. Equivalence Statements

The degree of equivalence (DOE) and its uncertainty between an NMI result and the KCRV has been calculated within CCQM according to the following equations:

$D_i = (x_i - x_R)$

$U_i^2 = (k_i^2 u_i^2 + k_R^2 u_R^2)$

where *Di* is the degree of equivalence between the NMI result x_i and the KCRV x_R, and *Ui* is the expanded uncertainty of *Di* calculated by combining the expanded uncertainty $k_i u_i$ of x_i and the expanded uncertainty $k_R u_R$ of x_R. Equivalence statements both in the units of measurement and relative to the KCRV are given in Table 5 and Figure 4.

Table 5. Degree of equivalence for testosterone glucuronide using the arithmetic mean as KCRV

Participant	**Reported Value (ng/g)**	**Expanded Uncertainty (ng/g)**	**D_i (ng/g)**	**U_i (ng/g)**	**D_i (%)**	**U_i (%)**
KCRV (mean)	40.5	1.8				
NMIA (Australia)	39.9	1.5	-0.59	2.3	-1.4	5.8
LGC (UK)	40.3	2.7	-0.23	3.2	-0.56	8.0
GL (HKSAR)	41.3	1.6	0.81	2.4	2.0	6.0

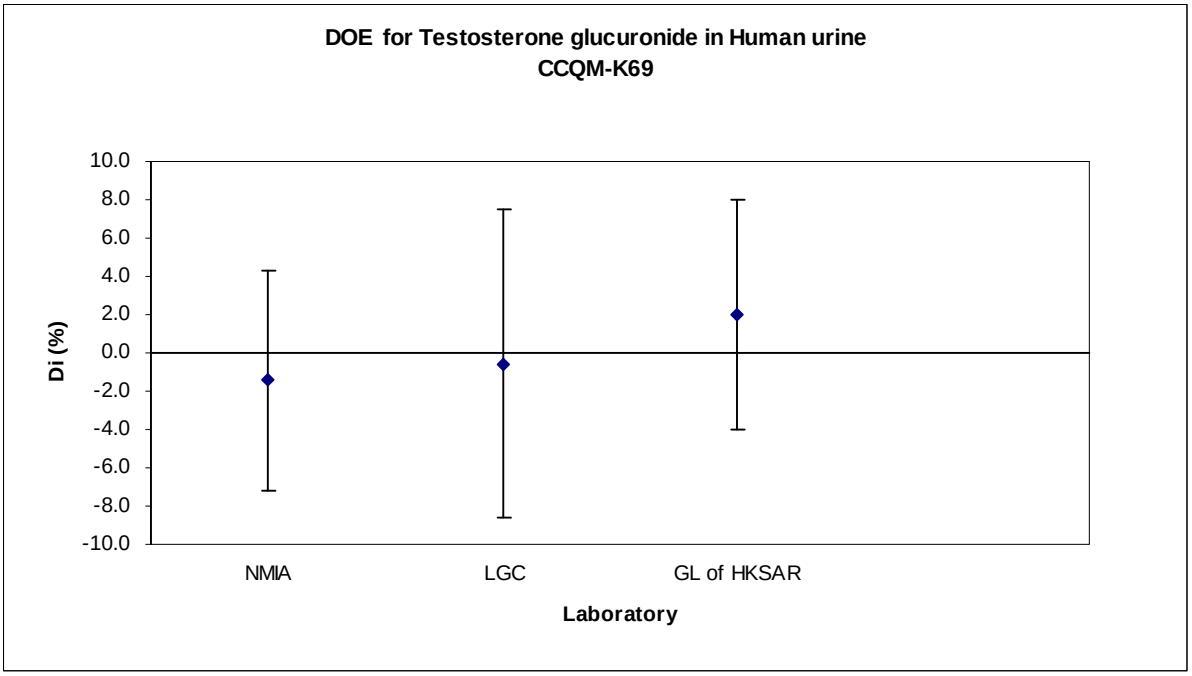

Figure 4. Degree of Equivalence for results of testosterone glucuronide mass fraction (as testosterone) using the arithmetic mean as the KCRV

11. Uncertainty Budgets for Testosterone glucuronide

The uncertainty budgets for testosterone glucuronide reported by each laboratory are given in Tables 6-8.

Table 6 Uncertainty budget for Testosterone glucuronide-NMIA

Parameter	x_i	$u(x_i)$	**Percent contribution to total uncertainty**
Method precision related to ratio measurements, mass measurements and sample variation	1.0000	0.0014	0.73%
Mass of calibration solution added to calibration blend (g)	0.48196	0.000049	0.004%
Mass of internal standard added to sample blend (g)	0.49699	0.000049	0.004%
Mass of internal standard added to calibration blend (g)	0.49160	0.000049	0.004%
Mass of sample added to sample blend (g)	5.09038	0.000049	<0.001%
Mass fraction of testosterone glucuronide expressed as free in calibration solution (ng/g)	417.822	3.07	19.8%
Ratio of testosterone to D_3-testosterone in internal standard material, R_y	0.0068	0.00049	<0.001%
Ratio of testosterone to D_3-testosterone in the sample/standard, R_x, R_z	36.73	1.03	<0.001%
Observed difference between different analytical techniques	1.0000	0.015	79.5%

Combined standard uncertainty	1.7%
Combined expanded uncertainty	3.7%

The two main contributors to the NMIA uncertainty budget for testosterone glucuronide were the analytical bias and calibration standard mass fraction, contributing 79.5% and 19.8% respectively to the total variance.

Table 7 Uncertainty budget for Testosterone glucuronide- LGC

Parameter	**Percent contribution to total uncertainty**
Measured isotope amount ratio of sample blend/ Measured isotope amount ratio of calibration blend	39%
Mass fraction of analyte in primary standard	23%
Blend to blend precision	38%
Uncertainty associated with gravimetric preparation	0.01%
Conversion factor for testosterone glucuronide to testosterone	<0.01%
Combined standard uncertainty	3.2%
Combined expanded uncertainty	6.7%

The major contributor to the LGC uncertainty budget was the measured ratios of sample and calibration blends which contributed 39% to the total variance. The second largest contributor was the blend to blend precision.

Table 8 Uncertainty budget for Testosterone glucuronide - GL of HKSAR

Parameter	**$u(x_i)/ x_i$**	**Percent contribution to total uncertainty**
Mass concentration of primary standard	0.010071	34.54%
Mass of sample matrix in sample blend	0.000064	0.22%
Mass of labelled standard in sample blend	0.000056	0.19%
Mass of primary standard in calibration blend	0.000081	0.28%
Mass of labeled standard in calibration blend	0.000079	0.27%
Precision of isotope ratios in sample blend	0.002158	7.4%
Precision of isotope ratios in calibration blend	0.016653	57.11%
Combined standard uncertainty	1.96%	
Combined expanded uncertainty	3.9%	

The three main contributors to the GL of HKSAR uncertainty budget for testosterone glucuronide were the precision of the isotope ratios in calibration solution, mass concentration of the calibration standard solution and the precision of isotope ratios of the sample blend.

12. Scope of this Key Comparison

The effective analysis of ng/g levels of steroids such as testosterone in human urine should demonstrate a capability to measure similar anabolic agents in urine at levels from ng/g upwards. Additionally, at a broader level, this key comparison demonstrates the capability for the selective measurement of a low-level analyte in a complex biological matrix containing many structurally similar analytes. It demonstrates the effective utilisation of IDMS at the ng/g level including the preparation and preservation of calibration solutions, effective extraction and clean-up of an analyte from a complex matrix.

13. Conclusions

The comparability for testosterone glucuronide measurements was successfully demonstrated by all of the CCQM-K69 participants. Considering the complexities involved in measuring these analytes in urine matrices this is an impressive result. All three participants used different analytical approaches and both GC/MS and LC/MS/MS were employed. The mean approach was used to assign the KCRV for testosterone glucuronide in the study sample. However, this approach does not take into account the correlation between results due to use of the same pure standard reference material. The KCRV is assigned as 40.5 ng/g, with expanded uncertainties reported between 3.7% to 6.7%.

14. Acknowledgment

The funding by the World Anti-Doping Agency (WADA) for the production of the material used in this key comparison at NMIA is gratefully acknowledged, as is their permission to use the material in this comparison and to publish these results.

15. References

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