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Ensuring quality in 17OHP mass spectrometry measurement: an international study assessing isomeric steroid interference

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Abstract

Objectives: Interference from isomeric steroids is a potential cause of disparity between mass spectrometry-based 17-hydroxyprogesterone (17OHP) results. We aimed to assess the proficiency of mass spectrometry laboratories to report 17OHP in the presence of known isomeric steroids.

Methods: A series of five samples were prepared using a previously demonstrated commutable approach. These samples included a control (spiked to 15.0 nmol/L 17OHP) and four challenge samples further enriched with equimolar concentrations of 17OHP isomers (11α-hydroxyprogesterone, 11β-hydroxyprogesterone, 16α-hydroxyprogesterone or 21-hydroxyprogesterone). These samples were distributed to 38 participating laboratories that reported serum 17OHP results using mass spectrometry in two external quality assurance programs. The result for each challenge sample was compared to the control sample submitted by each participant.

Results: Twenty-six laboratories (68 % of distribution) across three continents returned results. Twenty-five laboratories used liquid chromatography-tandem mass spectrometry (LC-MS/MS), and one used gas chromatography-tandem mass spectrometry to measure 17OHP. The all-method median of the control sample was 14.3 nmol/L, ranging from 12.4 to 17.6 nmol/L. One laboratory had results that approached the lower limit of tolerance (minus 17.7 % of the control sample), suggesting the isomeric steroid caused an irregular result.

Conclusions: Most participating laboratories demonstrated their ability to reliably measure 17OHP in the presence of the four clinically relevant isomeric steroids. The performance of the 12 (32 %) laboratories that did not engage in this activity remains unclear. We recommend that all laboratories offering LC-MS/MS analysis of 17OHP in serum, plasma, or dried bloodspots determine that the isomeric steroids are appropriately separated.

Keywords: 17α-hydroxyprogesterone; 17OHP; method validation; mass spectrometry; interference; isobars

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Introduction

17-Hydroxyprogesterone (17OHP) is a key steroid for screening, diagnosing, and monitoring congenital adrenal hyperplasia (CAH). To improve the measurement and clinical utility of this analyte, laboratories, including those conducting newborn screening, have continued to move to mass spectrometry-based methods for 17OHP quantification [1]. However, mass spectrometry does not guarantee trueness,

and rigor in method validation is required to ensure the robustness of the analysis [2]. Peer comparison through external quality assurance programs (EQAP) provides objective evidence of agreement or disparity, and in the case of liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement of 17OHP in plasma and dried blood spots, there is evidence of disparity. When disparity in results is not recognized, variation in clinical decisions and altered patient management are potential risks [3]. Thus, the goal is to improve the robustness of 17OHP methods to avoid irregular errors and standardize the measurand across time and space [4] (Table 1).

Interestingly, in our previous global survey, almost 50 % of laboratories did not know about or had not tested for interfering steroids in their mass spectrometry 17OHP methods [3]. Several clinically relevant isomeric steroids to 17OHP have been identified. In 21 hydroxylase deficiency patients, 11β-hydroxyprogesterone (11βOHP) is elevated as well as 16α-hydroxyprogesterone (16αOHP) [5], whereas 21-hydroxyprogesterone (21OHP) (frequently called 11 deoxycorticosterone) elevation is seen in the second most common form of CAH (11β-hydroxylase deficiency) [6]. In addition, very preterm birth is associated with alternations in endogenous progesterone metabolism [7]. Indeed, both 16αOHP and 21OHP have been shown to be present in serum samples by two clinical mass spectrometry laboratories [8].

Laboratories performing these assays usually do so from the paediatric/newborn service delivery demand. EQAP material must be commutable to challenge laboratories appropriately and include potential clinically relevant interferences. However, these isobaric steroids to 17OHP are absent in the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) Endocrine Program material [8]. Fortunately, the potential to make lyophilised and spiked commutable material has previously been demonstrated for plasma steroids [9, 10]. Thus, commutable challenge samples could be made using our standard interference protocol (Supplementary Material).

We, therefore, aimed to assess the proficiency of mass spectrometry laboratories to report serum 17OHP in the presence of known isomeric steroids reliably.

Table 1: Efforts in the standardisation of 17OHP methods. The table shows the significant body of work conducted over recent years to support the standardisation and robustness of 17OHP and steroid analysis in general.

Activity	References
Development of certified reference material by the National Measurement Institute of Australia (Sydney, Australia)	[4]
Addition of reference measurement procedure	[14, 25]
Development of commutable EQA material by Australian Scientific Enterprise (Hornsby, Australia) in conjunction with the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) and the Australasian Association of Clinical Biochemistry and Laboratory Medicine (AACB)	[9, 10]
Development of the European laboratory steroid analysis guideline	[26]
Comparison of mass spectrometry-based reference intervals and units	[3, 27]
Distribution and recommendations from a global questionnaire on serum/plasma 17OHP mass spectrometry assays	[3]
Study investigating the effect of choice of internal standards for 17OHP quantification	[20]

Materials and methods

Laboratories invited to participate in the challenge

The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) and Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek (SKML) participating laboratories reporting MS-based serum 17OHP results were invited for this study. This included RCPAQAP enrolled laboratories (n=22 out of 31 participants running LC-MS/MS), SKML enrolled laboratories (n=15 out of 17 participants running LC-MS/MS), and one European reference laboratory was recruited to provide an orthogonal method, gas chromatography MS/MS (GC-MS/MS). Together, these laboratories spanned Asia, Europe, and Australasia. RCPAQAP and SKML study participants were blinded to the working group organizers. As there was no patient sample exchange between laboratories, the requirement for ethical approval was determined to be exempt for each laboratory according to its local requirements.

Materials

Isomeric interferents were selected based on 1) their potential clinical relevance (i.e., found in patient samples) and 2) they could be purchased readily (i.e., did not require custom synthesis). The following isomeric steroids to 17OHP were selected for inclusion in the study: 11αOHP, 11βOHP, 16αOHP, and 21OHP (Table 2).

Preparation of challenge samples

This study protocol was adapted using the general principles of the RCPAQAP Chemical Pathology Interference Study Protocol (Supplementary Material). Five samples were prepared by Australian Scientific Enterprise (ASE) using the previously demonstrated commutable approach [9, 10]. The baseline serum samples were prepared from discarded post-venesection blood of hemochromatosis patients as part of a sample preparation process for an EQAP. A target 17OHP concentration of 15 nmol/L was determined considering the clinical decision limits related to CAH. Serum baseline 17OHP concentration was determined

Table 2: Isomeric steroids to 17OHP.

Isomer/isobar	CAS	Part number	Supplier	Systematic name
11 Alpha hydroxyprogesterone (11α-OHP)	80-75-1	H952335	Toronto Research Chemicals	21-Hydroxy-pregn-4-ene-3,20-dione
11 Beta hydroxyprogesterone (11β-OHP)	600-57-7	Q3270-000	Steraloids	11b-Hydroxy-pregn-4-ene-3,20-dione
16 Alpha hydroxyprogesterone (16α-OHP)	438-07-3	H952430	Toronto Research Chemicals	16a-Hydroxy-pregn-4-ene-3,20-dione
21 Hydroxyprogesterone (21-OHP) (also known as 11-deoxycorticosterone)	64-85-7	S13406	IsoSciences	21-Hydroxy-pregn-4-ene-3,20-dione

and enriched to approximately[1] 15 nmol/L with 17OHP aqueous standard (product no. H5752; CAS 68-96-2, Sigma Aldrich, St. Louis, MO, USA). This served as the interferent-free control sample.

The four challenge samples were prepared by adding approximately[1] equimolar levels of the selected interfering isomeric steroids to aliquots of the interferent-free sample. A total of five samples were distributed to the participating laboratories: Sample 1 spiked with 11α OHP (product no. H952335; CAS 80-75-1 Toronto Research Chemicals Ontario, Canada); Sample 2 spiked with 11βOHP (product no. Q3270-000; CAS 600-57-7 Steraloids, Inc Newport, U.S.A); Sample 3 spiked with 16αOHP (product no H952430; CAS 438-07-3 Toronto Research Chemicals Ontario, Canada); Sample 4 (the interferent-free control sample); and Sample 5 spiked with 21OHP (product no. S13406; CAS 64-85-7 IsoSciences LLC PA, USA). The samples were then aliquoted, lyophilised, and packed in preparation for shipment.

Distribution and analysis of challenge samples

Samples were distributed to MS laboratories running 17OHP. The RCPAQAP distributed sample sets to enrolled laboratories in the Asia Pacific Region and the central laboratory in Rotterdam (Erasmus MC, University Medical Centre, Rotterdam, The Netherlands). The Rotterdam laboratory then on-distributed to SKML-enrolled laboratories and the European steroid reference laboratory.

On receipt of the sample pack (containing 5 × 1.0 mL vials), participants were asked to store samples immediately as per the individual instructions and follow the reconstitution instructions on the day of analysis (by their routine mass spectrometry-based method). Results were requested to be submitted to the local organiser (RCPAQAP/SKML) via email within two weeks of receipt. All results were requested to be in nmol/L and reported up to one decimal place. Participants were requested to supply additional information on the routine reporting unit and conversion factor used if not routinely reporting in nmol/L.

Statistical analysis

The result of the interferent-free sample (Sample 4) was used as the control value. The results of samples enriched with interferent were expressed as both the raw value and a percentage of the respective control value of the laboratory. The within-laboratory imprecision was calculated for the five sample results returned for each laboratory. The average within laboratory imprecision was then calculated and also compared to the EFLM Biological Variation database for optimal analytical imprecision (CVa=7.1 %) and total error (TEa=17.7 %) [11, 12]. Clinically significant interference was considered present if an interferent-enriched sample had a within-laboratory percentage difference greater than the TEa. In addition, the all-laboratory dispersion was assessed using the RCPAQAP allowable performance specifications (APS) of ±20 % against the mean of all returned results for sample 4 and also from the GC-MS/MS orthogonal method results. All data analysis was performed in Microsoft Excel. Stata 17.0 was used to generate the graphs.

Results

Challenge samples

The distributions were part of a special survey jointly conducted by RCPAQAP and SKML in 2022 to MS laboratories running 17OHP. Twenty-six laboratories (68 % of distribution laboratories) across three continents returned results to the organiser, representing 13 out of 22 (i.e., 59 %) RCPAQAP laboratories, 12 out of 15 SKML laboratories (i.e. 80 %), and one out of one GC-MS/MS steroid reference laboratory (Table 3).

Using the GC-MS/MS reference method result for 17OHP, sample 4 had a 90 % recovery, i.e., the target addition was 15.0 nmol/L. The sample 4 all-method median was 14.3 nmol/L, giving a 20 % APS range of 11.5–17.1 nmol/L. Comparison using the target set by the orthogonal GC-MS/MS method showed a target of 13.5 nmol/L, giving a 20 % APS range of 10.8–16.1 nmol/L. The returns for sample 4 results ranged from 12.4 to 17.6 nmol/L, and across all samples (i.e., samples 1–5), the results ranged from 10.7 to 17.6 nmol/L (see Figure 1A).

The within-laboratory CV across the five samples ranged from 0.8 to 10.8 %; the mean CV was 3.1 % (Table 3). One of the 26 laboratories (Lab ID 19) did not return a result for sample 4, and therefore, a percentage difference could not be calculated and was excluded from the difference analysis. One laboratory (Lab ID 4) had results that approached the lower limit of tolerance (minus 17.7 % of sample 4) for the isomeric samples (1, 2 or 3) All 25 laboratories had results for sample 5 (i.e., contained 21OHP as the isomeric steroid) that were

1 The materials available for purchase were not ISO 34 certified, hence the purity cannot be clearly demonstrated. Therefore the materials are not traceable to SI units and concentrations are approximations only.

Table 3: Heatmap of all results returned for 17OHP nmol/L.

Lab ID	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Lab CV %
1	16.0	15.5	14.5	15.6	16.3	4.4
2	15.8	15.9	15.3	15.6	15.9	1.6
3	13.3	13.4	13.0	13.3	13.5	1.5
4	13.0	13.4	13.1	15.7	16.2	10.8
5	14.3	14.0	13.6	14.6	14.4	2.8
6	14.1	13.9	14.6	14.4	14.7	2.4
7	13.7	13.6	13.8	13.5	13.9	1.2
8	13.7	13.1	12.3	13.3	13.3	3.9
9	13	14	13	13	13	3.4
10	14.9	13.4	14.4	14.8	14.4	4.1
11	13.5	14.3	14.1	14.4	13.5	3.3
12	12.8	12.7	12.9	12.5	12.6	1.2
13	16.4	17.0	17.1	17.0	16.3	2.3
14	13.3	13.1	13.6	13.6	13.2	1.7
15	15.7	16.3	15.3	17.6	17.2	5.9
16	12.1	12.0	12.7	13.0	12.7	3.4
17	14.3	14.4	14.2	14.5	14.7	1.3
18	12.9	14.5	14.6	14.1	13.7	5.0
19	11.1	10.7	10.7		10.7	1.9
20	14.2	14.0	14.3	14.2	14.2	0.8
21	14.2	14.4	14.6	14.3	14.3	1.1
22	14.2	13.4	14.6	14.6	14.2	3.5
23	12.1	12.2	13.6	13.0	13.7	5.8
24	12.7	12.7	12.8	12.4	12.7	1.2
25	16.6	15.5	16.4	15.4	16.0	3.3
26	13.5	13.9	13.2	13.5	12.8	3.0

Cells are formatted on their values for 17OHP nmol/L, with the lowest in blue, the midpoint of white and highest in red.

within the acceptance criteria. The differences were not equally distributed around zero on the difference plot, which was unexpected and remains unexplained (see Figure 1B).

The equimolar concentration of the five steroids did not give equivalent ionization, indicating that for the same concentration, some isomeric steroids may have more influence than others on producing irregular results, but this will depend on the concentration present in the individual patient sample (Figure 2).

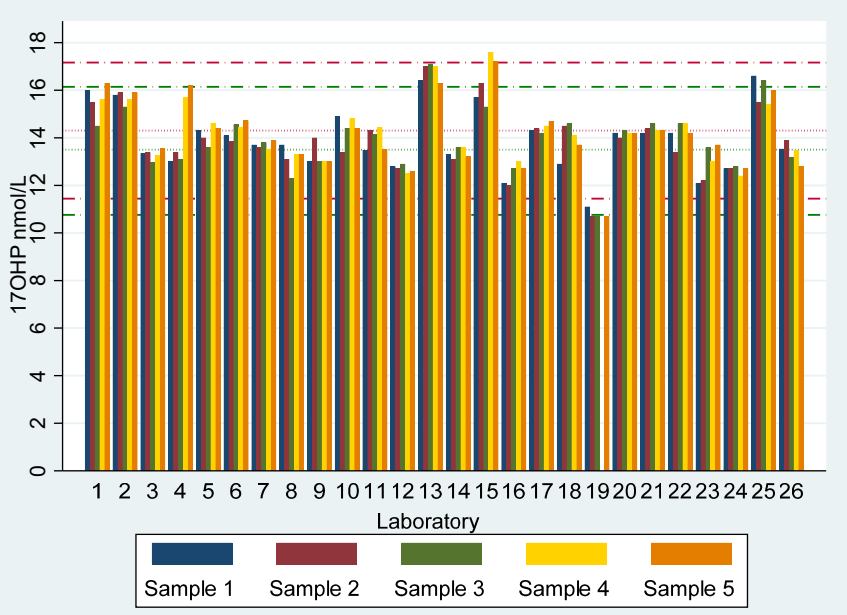
Discussion

This study has demonstrated the robustness of the 17OHP MS-based methods offered by 25 of the 26 responding laboratories for their ability to adequately separate steroid isomers from 17OHP. Importantly, at the time of analysis, laboratories did not know the samples contained interfering steroids: 11αOHP, 11βOHP, 16αOHP, and 21OHP. The mean analytical CV achieved in this study of 3.1 % and all but one laboratory's CV (Lab ID 4) was in the optimal range for 17OHP based on the EFLM biological variation database [11]. The study also exemplifies the invaluable role of EQAP in supporting interlaboratory harmonisation and identifying continuous quality improvements [13].

A number of laboratories (32 %) did not engage in this educational challenge, nine of 22 enrolled RCPAQAP laboratories and three of 15 SKML enrolled laboratories, so we cannot ascertain if these laboratories are separating their isomeric steroids appropriately. Furthermore, because the laboratories were deidentified for us and spanned two EQA programs, information on whether the individual laboratories used in-house or commercial LC-MS/MS methods was not available to us.

The reason laboratories have moved from immunoassay to mass spectrometry-based analysis or its inclusion as a second-tier method for quantification of 17OHP over the last decade is its improved specificity. The measurement of steroids is sometimes described as "an art" as it requires a high level of both analytical and clinical expertise [14]. Analytically, there is often a balance between the separation and speed of methods, and all method validation studies need to include documented experimental evidence of interference studies [15]. However, what should be included in such studies is not explicitly described and is left to the individual laboratory to determine. Whilst many positional isomeric steroids are easily separated chromatographically, it is necessary to ensure the robustness of the assay as it serves neonatal, children, adolescents, and adult patients [16]. This is where the importance of clinical knowledge supports appropriate analytical methods, including for the analysis of steroids such as 17OHP analysis.

As well as analytical standards for method validation, clinical guidelines recommend that methods be able to separate isomers to ensure accurate 17OHP measurement to avoid spurious elevation above the cutoff for late-onset CAH [17]. In newborns, a different panel of steroids is present due to the maternal-placental-foetal unit and also to the persistence of the foetal adrenal gland resulting in 16 hydroxylated (e.g., 16OHP) and sulphated (e.g., pregnenolone sulphate) steroids [18, 19]. The foetal adrenal zone is maintained until at least the equivalent of the term; therefore, extra caution is needed in measuring preterm baby samples [19]. Our recent survey of 44 laboratories (globally) performing 17OHP analysis by LC-MS/MS found that approximately 50 % of the survey laboratories did not know or did not assess for all potentially relevant interfering steroids. The findings showed that most laboratories knew about 21OHP as a potential interferent, but few experimentally determined the impact of other potential steroid isobars on 17OHP [20].



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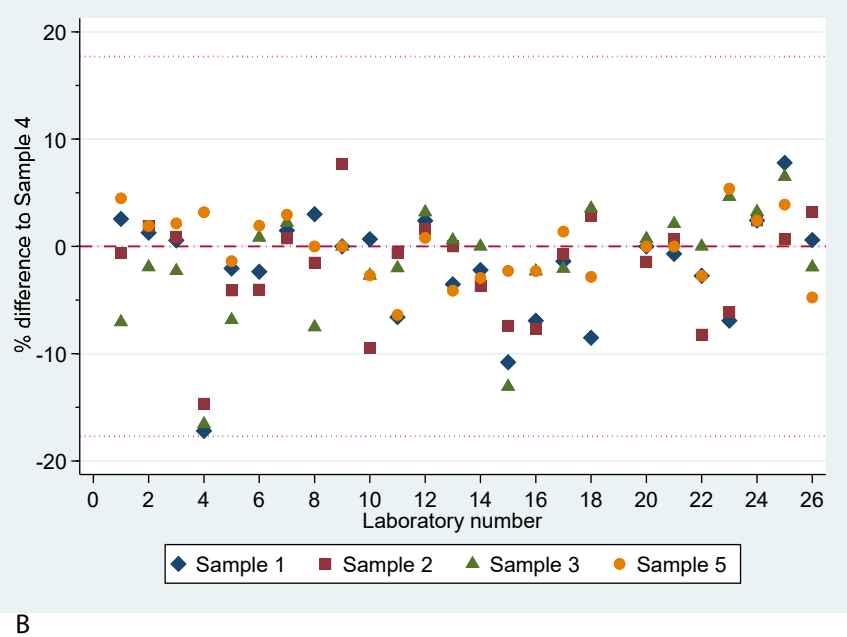

B

Figure 1: Comparison of 17OHP results returned. (A) Histogram of 17OHP results returned from the 26 laboratories. Red horizontal lines – dashed line is all group median of sample 4 – dot lines are the APS tolerance of ±20 %. Green horizontal lines – dashed line is the result returned by the orthogonal GC-MS/MS method of laboratory 26 – dot lines are the APS tolerance of ±20 %. (B) Comparison of percentage difference of samples 1, 2, 3, and 5 with reference to sample 4 within each laboratory. Laboratory 19 is excluded from this comparison because they did not return a result for sample 4. Red horizontal lines – dashed line is the zero percentage difference line compared to sample 4 – dot lines are calculated within laboratory allowable tolerance of ±17.7 %. Only laboratory 4 showed results that approached this limit of tolerance for sample 1 (–17.2 %), sample 2 (–14.6 %) and sample 3 (–16.6 %).

Whilst EQAP provides comparative performance for 17OHP, they do not always have the relevant interfering steroids present in their routine material to challenge laboratories on irregular errors related to isobaric/isomeric steroids [8]. To study whether isomeric interferences to 17OHP were present in EQAP samples, a retrospective chromatogram review on RCPAQAP Endocrine samples for serum 17OHP was conducted. One collaborating clinical mass spectrometry laboratory reviewed its chromatograms for RCPAQAP samples reported from 2015 to 2022. The LC-MS/MS method in this laboratory was previously described in Laboratory A, and the 17OHP service was accredited under ISO15189. The method remained unchanged over the study period [20]. No isomeric interference peak was found in any chromatogram reviewed from 2015 to 2022 material. Thus, the unacceptable variation in the RCPAQAP serum 17OHP results was not due to isomeric interference.

There were several considerations related to the matrix preparation and spike concentration. In earlier studies, the effect of lyophilisation and spiking on endocrine analytes had been evaluated, and the commutability of the material was presumed based on work conducted [9, 10]. The

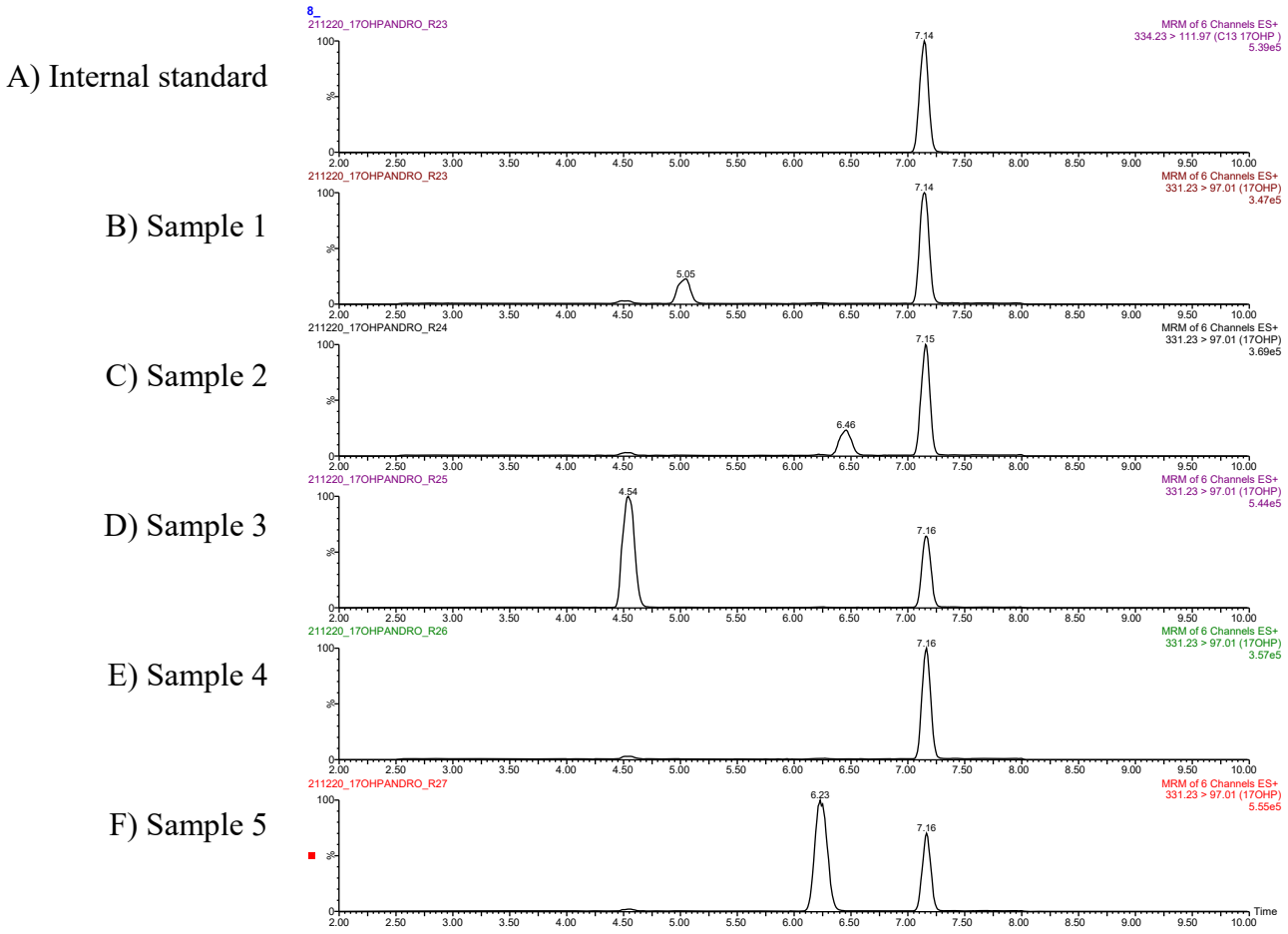

Figure 2: Chromatogram of the five distributed samples, peaks are: (A) 17OHP-C13 internal standard at retention time (RT) 7.14 min; (B) 11α-OHP and 17OHP at 5.05 and 7.14 min, respectively; (C) 11β-OHP and 17OHP at 6.46 and 7.15 min, respectively; (D) 16α-OHP and 17OHP at 4.54 and 7.16 min, respectively; (E) 17OHP at 7.16 min; and (F) 21OHP and 17OHP at 6.23 and 7.16 min, respectively. Sample 1 contained an equimolar concentration of 17OHP and 11α-OHP. Sample 2 contained an equimolar concentration of 17OHP and 11β-OHP. Sample 3 contained an equimolar concentration of 17OHP and 16α-OHP. Sample 4 only contained 17OHP. Sample 5 contained an equimolar concentration of 17OHP and 21OHP. The method associated with this chromatogram has previously been published as Laboratory C [20].

discarded venesection blood avoided using leftover endocrine material as this already had many analytes and could confound the interpretation. In preparing the challenge samples using this lyophilisation and spiking approach, it was considered that the base sample should have a low 17OHP. However, supplementation of 17OHP should be done to avoid issues with a lower measurement range, and the supplementation aimed to approximate 15.0 nmol/L (which demonstrated a 90 % recovery compared to the orthogonal reference method).

The choice of interfering steroids was based on the clinical relevance and availability of the compounds. So, potentially, not all clinically relevant steroids have been included in this study. In particular, additional endogenous interfering steroids might be of interest (6α-steroids and 6β-steroids). 15β-steroids can be found in the urine of children with CAH, and we query if this is a significant steroid group for serum measurement of babies in the neonatal period. Likewise, patients with rarer forms of CAH, such as cytochrome P450 oxidoreductase deficiency and 17α-hydroxylase deficiency, will have an altered steroid pathway and the potential for other novel clinically relevant isomeric steroid interferences. In some instances, using a second transition in the analysis will improve specificity. However, the most significant limitation of this study is that 32 % of laboratories failed to engage in this educational activity, and their ability to separate the isomeric steroids appropriately remains unanswered.

Understanding and managing interferences in clinical laboratory assays is essential for evaluating the risk of analytical errors in all patient samples. Whilst many factors can contribute to irregular errors, including drugs [21], this

study specifically challenged the laboratory's appropriate chromatographic separation as this is an important factor for accurate quantitation in the presence of potential isomeric interferences to 17OHP [22]. Similar studies are suggested for 21 deoxycortisol with improvements in LC-MS/MS sensitivity as this is a recommended steroid marker for 21 hydroxylase deficiency and has isomeric steroids that require separation [23]. 21-Deoxycortisol is increasingly used for newborn and CAH screening, including programs in The Netherlands and Victoria, Australia [22, 24]. Overall, the literature emphasises the importance of separating isobars/isomers to ensure accurate measurements and not make erroneous decisions.

Conclusions

Most laboratories that returned results demonstrated their ability to reliably measure 17OHP in the presence of the four clinically relevant isomeric steroids. The performance of the 12 laboratories that did not engage in this educational activity remains unclear. We recommend all laboratories offering LC-MS/MS analysis of 17OHP in serum, plasma, or dried blood spots to determine that the isomeric steroids are appropriately separated.

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Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: All authors contributed to the conceptualisation of this project, interpretation of the results and writing of the manuscript. The initial interference study protocol was developed by RG for the RCPAQAP. The literature search of potential isobaric steroids was conducted by RZ and SB. Identification of clinically relevant interfering steroids was determined in the laboratories of CSH, CL, KH, BC, YdR, BZ, SB, MH and SW. The material was prepared by Australian Scientific Enterprise under the supervision of TA and distributed by SB and PG. The laboratory of KH and BC ran the material prior to distribution. The GC-MS/MS reference method was performed by MH with oversight from SW. Statistical expertise was provided by TPL. Clinical input was provided by SW.
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