

APPLICATION NOTE

# EVALUATION OF A STERIOD PANEL LC-MS KIT IN A ROUTINE CLINICAL LABORATORY.

Measurement of 17 steroid  
hormones and dexamethasone.



## ABSTRACT.

The presented study was conducted at the children's hospital in Erlangen (Kinder- und Jugendklinik, Universitätsklinikum Erlangen, Germany) and focuses on the evaluation of the clinical performance of the in-house method in comparison to a commercially available CE-IVDD kit solution, the Steroid Panel LC-MS from Tecan.

For clinical performance, the established in-house method was compared to the Tecan kit using serum samples with different clinical backgrounds. 17 steroids and dexamethasone were quantified in clinically relevant serum samples and in ring trial samples from SKML. An excellent correlation ( $r > 0.96$ ) between the commercial method and the in-house method has been observed for eleven different steroids (testosterone, 17-OH-progesterone, progesterone, 11-deoxycortisol, androstenedione, corticosterone, cortisone, cortisol, DHEAS, aldosterone and 11-deoxycorticosterone). The EQAS samples from SKML (testosterone, 17-OH-progesterone, progesterone, cortisol, 11-deoxycortisol, androstenedione, DHEAS, aldosterone, estradiol, DHEA and estrone) were measured to evaluate the clinical bias of the mentioned steroids. In this scope, the correlation between SKML target values and the results gained by Steroid Panel LC-MS kit was found with  $r > 0.94$ , demonstrating the reliable quantification of all measured analytes.

The results obtained with the commercial Steroid Panel LC-MS kit were in concordance with the established in-house method. Additionally, this assay allows the clinicians to gain a broader clinical picture of the patients, as it includes 17 steroids and dexamethasone which is helpful for the diagnosis and the understanding of endocrine disorders.

## INTRODUCTION.

Liquid Chromatography-Mass Spectrometry (LC-MS) allows for precise identification and quantification of a broad spectrum of analytes. Especially in the steroid analysis, LC-MS became the gold standard over the last years providing valuable insights into hormonal profiles and potential health implications. The benefits of measuring steroids via LC-MS include high sensitivity, and specificity enabling accurate identification and quantification of multiple steroids using only one extraction and/or measurement. Multi-steroid analysis offers a comprehensive view of hormonal status, aiding in research, clinical diagnostics, and monitoring conditions such as hormonal imbalances or endocrine

disorders. LC-MS also allows for simultaneous detection of various steroid classes with improved precision compared to traditional methods.

LC-MS is recommended for measuring circulating steroids, however, there is nearly no commercial solution available nor an external quality assessment scheme (EQAS) available for all clinically relevant steroids.<sup>[1]-[3]</sup>

This study aims to compare the results of the laboratory-specific procedure performed in the Erlangen Children's Hospital to the commercially available kit from Tecan for the measurement of 17 circulating steroids and dexamethasone. Additionally, an evaluation of the clinical bias of the mentioned kit by using external quality control samples from Stichting Kwaliteitsbewaking Medische Laboratorium diagnostiek (SKML) as a reference was performed. Since no reference measurements and/or ring trial samples are available for all steroids included in the commercial kit, the results of the serum samples were set in a clinical context afterwards.

## MATERIALS AND METHODS.

For method comparison, the in-house method from the Department of Pediatrics and Adolescent Medicine, University Hospital, Erlangen, Germany (in the following: Universitätsklinikum Erlangen), as well as the Steroid Panel LC-MS method described in the Instruction for use (IFU) of the product (Steroid Panel LC-MS, Cat# 30191875, IFU Version 2022-06, CE-IVDD, Tecan, IBL International GmbH) were used.

The in-house method established in the Universitätsklinikum Erlangen has been published already.<sup>[1]-[3],[5]</sup> The lab implemented its own sample preparation protocol as well as an acquisition and quantitation method incorporating commercial calibrators for their multi-steroid LC-MS/MS assay and regularly releases results for 17-OH-progesterone, androstenedione, cortisol, testosterone, 11-deoxycortisol, 11-deoxycorticosterone, 21-deoxycortisol, corticosterone, cortisone, progesterone, and aldosterone.

The Tecan Steroid Panel LC-MS kit was used according to the IFU. The kit solution provides all necessary standards, reagents, and consumables, apart from methanol and ultra-pure water to perform the assay. Additionally, the calibration standards as well as the controls are prepared in human serum and are shipped lyophilized. For sample preparation, only 250  $\mu\text{L}$  of each serum sample, calibrator, and control was used followed by the addition of 50  $\mu\text{L}$  of internal

standard. The sample preparation was performed utilizing a Solid Phase Extraction (SPE) allowing the removal of potential interferences. The overview of the steroids included in the kit with their respective calibration ranges is listed in Table 1.

For both methods, an Agilent 1260 Infinity II UPLC System (Agilent, Santa Clara, CA, US) coupled to an API 6500 QTRAP LC-MS/MS System (SCIEX, Danaher Corporation, Washington D. C., US) was used for analysis. The injection volume was 20  $\mu$ L for both methods. The acquired data were evaluated using MultiQuant Software (SCIEX, Danaher Corporation, Washington D. C., US). The Regression type was chosen as linear with weighting type 1/x. The statistical analysis, namely the Passing-Bablok fit, was performed using Analyse-it (Version 6.15, Analyse-it Software, Ltd.; Leeds, UK).

For method comparison, 13-19 human clinical samples collected for routine measurement at

the endocrinological laboratory of the Erlangen Children's Ambulance Hospital were used. These samples are considered to be from affected children suffering from endocrine disorders. Some of the patients underwent ACTH stimulation as part of their clinical diagnosis. All the samples were anonymous and measured in single determination in one batch.

For evaluation of clinical bias, SKML ring trial samples (proficiency samples containing defined concentrations of steroid hormones in serum, panel "Hormones in Serum", including testosterone, 17-OH-progesterone, progesterone, cortisol, 11-deoxycortisol, androstenedione, DHEAS, aldosterone, estradiol, DHEA, estrone and 21-deoxycortisol) were collected from 2022 and measured in single determination in one batch. The overview of the analytes measured in the current study is listed in Table 1.

**Table 1:** Overview of the analytes with their corresponding calibration ranges with the commercial kit provided by Tecan compared to the in-house method of Universitätsklinikum Erlangen, and information of the expected ranges from the measured SKML ring trial samples. N/A: Non Applicable.

Analyte	Calibration ranges Steroid Panel LC-MS [ng/mL] (nmol/L)	Calibration ranges in-house method by Erlangen [ng/mL] (nmol/L)	Ring trial samples from SKML with expected ranges [ng/mL] (nmol/L)
<b>Testosterone</b>	0.04-13.4 (0.138-46.5)	0.053-12.00 (0.183-41.6)	0.196-11.18 (0.68-38.8)
<b>17-OH-Progesterone</b>	0.1-12.8 (0.303-38.7)	0.087-14.5 (0.26-43.9)	0.23-6.01 (0.70-18.2)
<b>Progesterone</b>	0.1-12.8 (0.318-40.7)	0.134-14.5 (0.426-46.1)	0.11-12.73 (0.35-40.5)
<b>Cortisol</b>	2-256 (5.52-706)	10-279 (27.6-770)	85.18-349.08 (235-963)
<b>11-Deoxycortisol</b>	0.1-12.8 (0.20-36.9)	0.091-13.7 (0.26-39.5)	0.20-88.76 (0.60-256)
<b>Androstenedione</b>	0.1-12.2 (0.34-42.5)	0.184-13.50 (0.64-47.1)	0.40-4.58 (1.4-16.0)
<b>DHEAS</b>	50-6,412 (135-17,376)	109-5,664 (295-15,349)	585-6.548 (1,590-17,700)
<b>Cortisone</b>	0.5-64.1 (1.38-177)	1.03-41.0 (2.85- 113)	N/A
<b>Corticosterone</b>	0.3-38.5 (0.86-111)	0.531-45.9 (1.53-132)	N/A
<b>11-Deoxycorticosterone</b>	0.04-5.13 (0.12-15.5)	0.044-2.91 (0.13-8.81)	N/A
<b>21-Deoxycortisol</b>	0.1-12.8 (0.289-36.9)	0.069-5.01 (0.199-14.4)	N/A
<b>Aldosterone</b>	0.1-4.59 (0.27-12.7)	0.023-2.74 (0.063-6.84)	0.064-0.39 (0.18-1.09)
<b>Estradiol</b>	0.03-3.85 (0.11-14.1)	N/A	0.22-0.43 (0.82-1.60)
<b>Estrone</b>	0.01-1.28 (0.037-4.73)	N/A	0.021-0.048 (0.08-0.18)
<b>DHT</b>	0.15-1.57 (0.51-5.40)	N/A	N/A
<b>Dexamethasone</b>	0.50-64.1 (1.379-176)	N/A	N/A
<b>17-OH-Pregnenolone</b>	0.3-38.47 (0.909-116)	N/A	N/A
<b>DHEA</b>	1-45.9 (3.47-159)	N/A	0.92-6.83 (3.2-23.7)

## RESULTS AND DISCUSSION.

### Method comparison based on clinical samples.

For both methods, clinical samples were measured for the determination of the concentration of testosterone, 17-OH-progesterone, progesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, corticosterone, cortisone, cortisol, and 11-deoxycorticosterone. Additionally, up to 12 ring trial samples have been included in order to cover DHEAS and aldosterone as well. After quantitative analysis, the Passing-Bablok fits were plotted to evaluate the comparison of the used methods (see Figure 2). The Passing-Bablok fit takes into consideration at least nine samples for each analyte. Values under LoQ were excluded in the method comparison. As only one sample was measured above the LoQ for 21-deoxycortisol for the in-house method, this parameter is not represented graphically. The presented results show a good correlation for all the other measured analytes: The correlation  $r$  for each analyte is given in Table 2 and exceeds 0.96 for all the 11 steroids measured. Overall, it can be stated that the in-house method and the Steroid Panel LC-MS achieved comparable results for the mentioned analytes, leading to the same clinical statement. A deeper look at the clinical interpretation is made in the corresponding chapter.

**Table 2:** Method Comparison – Correlation of in-house method and commercial kit quantified by  $r$ . Data generated via Analyse-it. \*Data solely based on SKML samples.

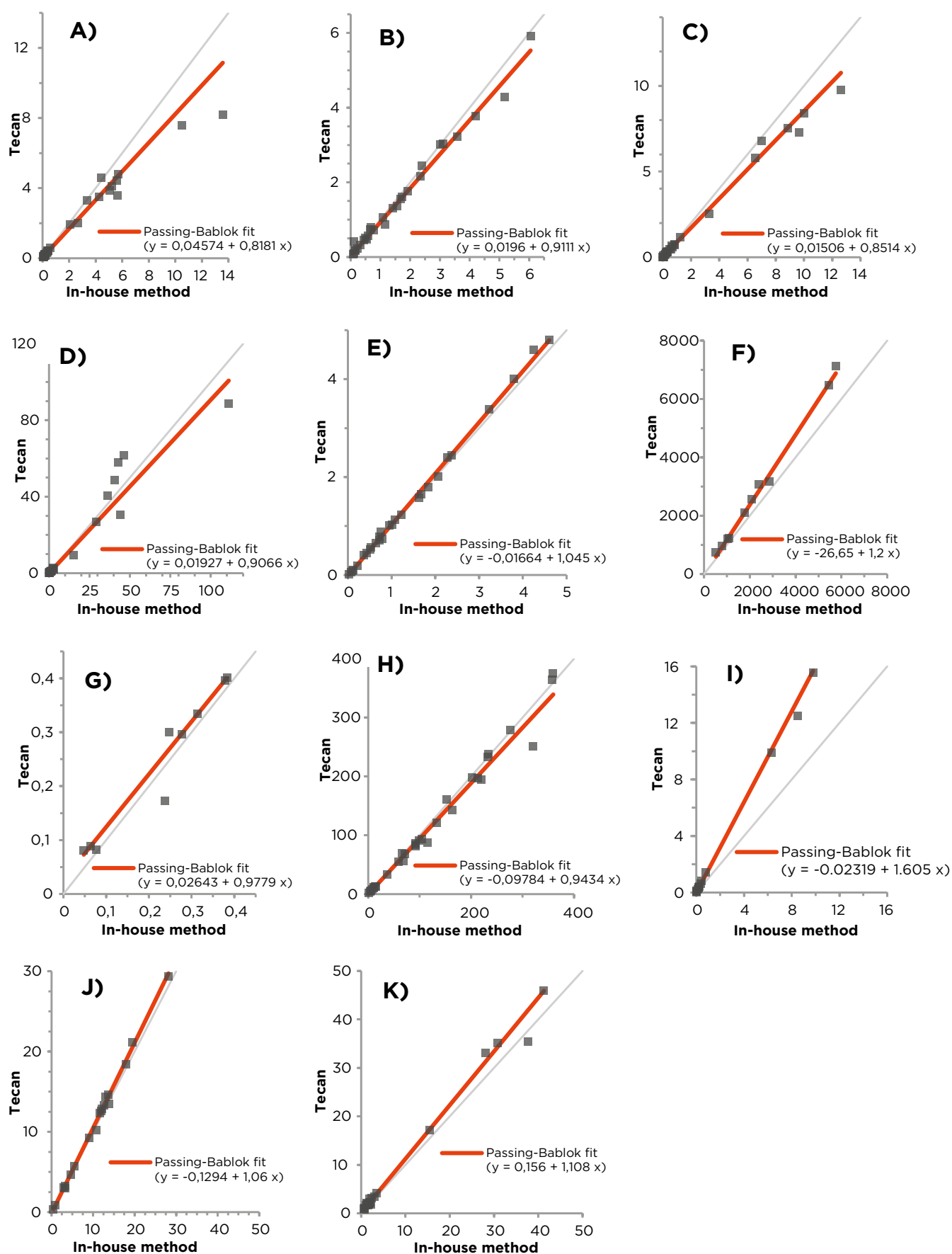
Analyte	n	r
Testosterone	31	0.982
17-OH-Progesterone	29	0.994
Progesterone	27	0.996
11-Deoxycortisol	29	0.962
Androstenedione	28	0.999
DHEAS*	10	0.998
Aldosterone*	9	0.971
Cortisol	31	0.990
11-Deoxycorticosterone	13	0.999
Cortisone	19	0.997
Corticosterone	24	0.995

### Bias evaluation based on SKML ring trial samples.

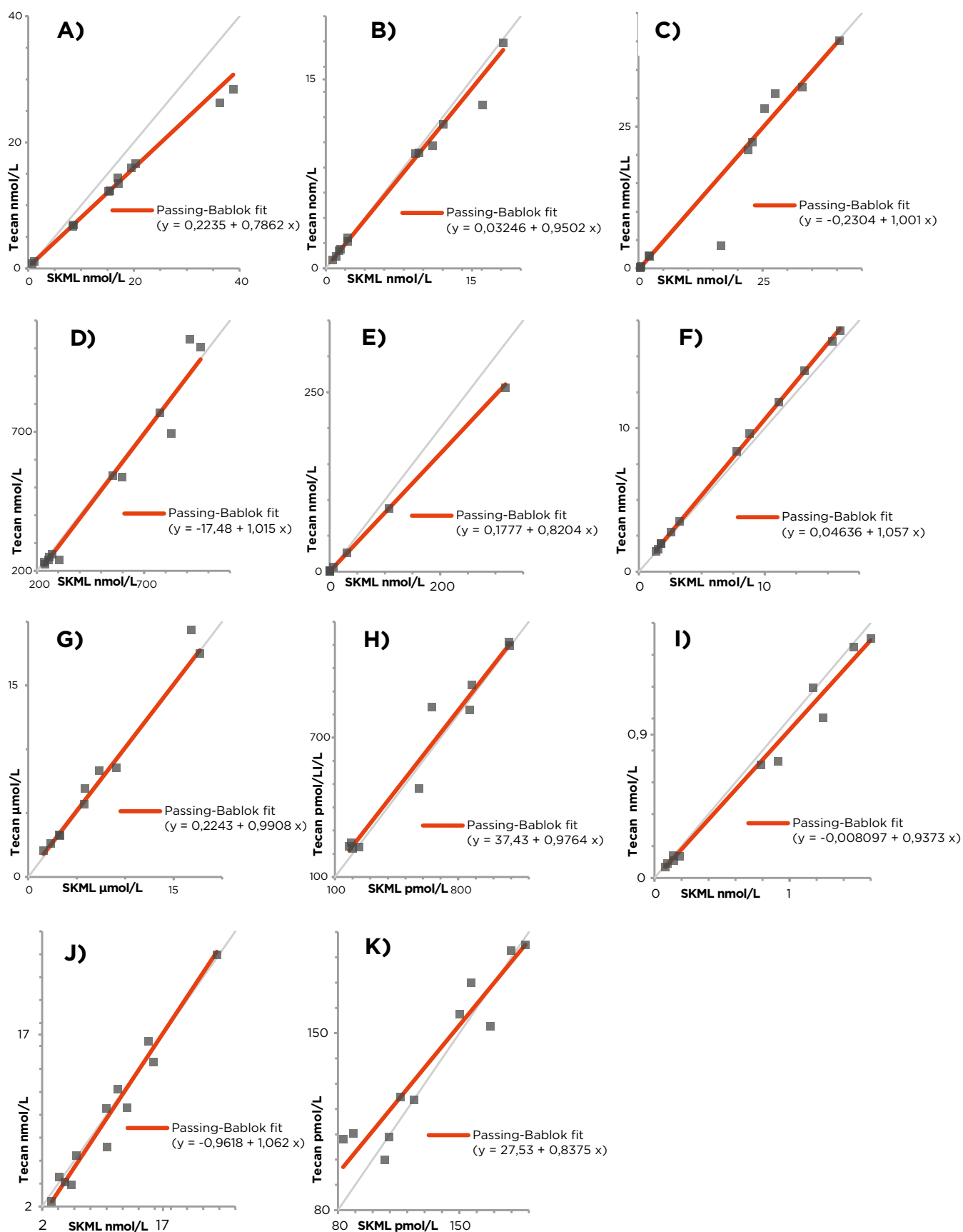
In order to evaluate the clinical bias, ring trial samples from SKML were analyzed with Steroid Panel LC-MS at Universitätsklinikum Erlangen. Figure 3 shows the Passing-Bablok fit for testosterone, 17-OH-progesterone, progesterone, cortisol, 11-deoxycortisol, androstenedione, DHEAS, aldosterone, estradiol, DHEA and estrone comparing the concentration gained with Tecan kit to the target value given from SKML. All the results have shown a good correlation between measured and target values, with  $r$  greater than 0.94 (see Table 3).

**Table 3:** Evaluation of clinical bias based on the measurement of SKML samples.

Analyte	n	SKML ranges		Correlation r
		Gained with Tecan kit [nmol/L]	Target values [nmol/L]	
Testosterone	12	0.76-28.38	0.68-38.8	0.997
17-OH-Progesterone	12	0.67-17.89	0.70-18.20	0.993
Progesterone	12	0.14-40.12	0.35-40.50	0.966
Cortisol	12	223-1,033	235-963	0.982
11-Deoxycortisol	12	0.60-318	0.60-256.17	1
Androstenedione	12	1.43-16.77	1.4-16.0	1
DHEAS	10	2,021-19,341	1,590-17,700	0.991
Aldosterone	10	0.22-1.112	0.18-1.09	0.981
Estradiol	11	0.06-1.49	0.82-1.60	0.992
Estrone	11	0.09-0.18	0.08-0.18	0.948
DHEA	12	2.43-23.95	3.2-23.7	0.980



**Figure 2:** Method comparison of the in-house method to Steroid Panel LC-MS by Tecan. Passing-Bablok fit based on at least 9 samples is shown for each analyte separately; all values are in ng/mL: **(A)** Testosterone, **(B)** 17-OH-Progesterone, **(C)** Progesterone, **(D)** 11-Deoxycortisol, **(E)** Androstenedione, **(F)** DHEAS, **(G)** Aldosterone, **(H)** Cortisol, **(I)** 11-Deoxycorticosterone, **(J)** Cortisone and **(K)** Corticosterone.



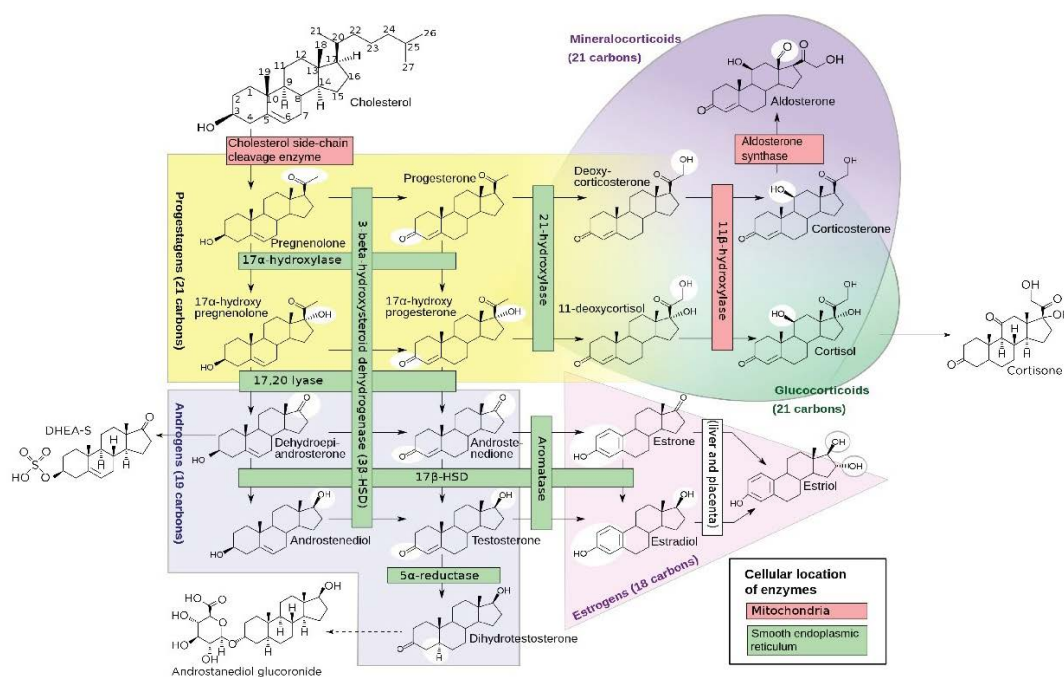
**Figure 3:** Passing-Bablok plot for the evaluation of bias of Steroid Panel LC-MS using SKML ring trial samples (at least 9 samples for each analyte), different plots refer to different steroids: **(A)** Testosterone, **(B)** 17-OH-Progesterone, **(C)** Progesterone, **(D)** Cortisol, **(E)** 11-Deoxycortisol, **(F)** Androstenedione, **(G)** DHEAS, **(H)** Aldosterone, **(I)** Estradiol, **(J)** DHEA and **(K)** Estrone.

### Analysis of clinically relevant samples.

The Tecan steroid kit allows the determination of 17 natural and circulating steroids as well as dexamethasone. In the following chapter, the results of the quantitative analysis obtained with the Steroid Panel LC-MS for clinically relevant serum samples were set in a clinical context. As these samples were collected in a clinical endocrine laboratory, they are considered to be from an affected population (not apparently healthy). Especially, five samples have shown particular patterns and values for specific steroids falling outside the expected normal ranges. For 11-deoxycorticosterone, the samples have shown values up to 27.86 ng/mL using the Tecan kit, which is a 100-fold increase from the normal range of 0.01-0.27 ng/mL<sup>[6]</sup>. The values gained with the in-house method also showed elevated values for 11-deoxycorticosterone. The values obtained for

11-deoxycortisol displayed a similar pattern for both methods being very high, while corticosterone and cortisol values were very low for both methods.

Such a pattern is expected to be observed after Metyrapone treatment. The latest, trade name Metopirone<sup>®</sup>, is a drug used in the diagnosis of adrenal insufficiency and occasionally in the treatment of Cushing's syndrome (hypercortisolism). Metyrapone blocks cortisol synthesis in healthy humans by inhibiting steroid-11 $\beta$ -hydroxylase in the cortex of the suprarenal gland. This stimulates adrenocorticotrophic hormone (ACTH) secretion by negative feedback, which results in an increase of 11-deoxycortisol and 11-deoxycorticosterone levels<sup>[5]</sup> The figure 4 describes the steroid pathway and shows the interdependency of the steroids and enzymes involved in their conversions.



**Figure 4:** Overview of the steroid pathway, reworked from the “Diagram of the pathways of human steroidogenesis”, WikiJournal of Medicine 1 (1). DOI:10.15347/wjm/2014.005. ISSN 20018762.

For 21-deoxycortisol, most of the samples were under the limit of quantitation of the Tecan kit as well as with the in-house method. Accordingly, the clinical interpretation of the values obtained with the Tecan kit is the same as the in-house method used in routine diagnostics.

The exemplary discussion for the mentioned steroids shows that the results obtained with both commercial and in-house methods lead to the same clinical interpretation. Table 4 lists the values obtained for all steroids included in the Tecan kit (split in Table 4-1 and 4-2 for better overview).

**Table 4.1:** Concentration of steroids in five different serum samples after Metyrapone treatment. The grey underlined values were acquired using the Tecan kit whereas the other values were measured with the in-house method from Universitätsklinikum Erlangen. Bold values were elevated for the analyzed patient population.

No.	Method	Concentration [ng/mL]								
		P4	17-OHP4	DOC	11-DF	21-DF	B	F	ASD	E
<b>1</b>	In-house Erlangen	0.824	2.36	15.31	46.23	< LoQ	< LoQ	11.03	2.06	5.55
	Steroid Panel LC-MS	0.766	2.16	27.86	61.61	< LoQ	0.293	10.31	2.00	5.69
<b>2</b>	In-house Erlangen	0.316	1.15	6.32	29.09	< LoQ	< LoQ	5.82	0.726	3.20
	Steroid Panel LC-MS	0.295	0.87	9.91	26.77	< LoQ	0.315	5.72	0.708	3.02
<b>3</b>	In-house Erlangen	0.485	1.09	11.26	36.35	< LoQ	< LoQ	12.84	1.02	9.01
	Steroid Panel LC-MS	0.479	1.06	21.30	40.35	< LoQ	0.707	12.91	1.02	9.22
<b>4</b>	In-house Erlangen	0.294	1.41	8.55	40.61	< LoQ	< LoQ	3.92	1.83	2.94
	Steroid Panel LC-MS	0.299	1.30	12.50	48.60	< LoQ	0.383	4.32	1.80	3.10
<b>5</b>	In-house Erlangen	0.342	1.56	9.79	42.73	< LoQ	< LoQ	11.04	1.23	4.58
	Steroid Panel LC-MS	0.310	1.36	15.57	57.99	< LoQ	0.401	11.43	1.24	4.70

Abbreviations: P4 - progesterone, 17-OHP4 - 17-OH-progesterone, DOC - 11-deoxycorticosterone, 11DF - 11-deoxycortisol, 21DF - 21-deoxycortisol, B - corticosterone, F - cortisol, ASD - androstenedione, E - cortisone, LoQ - Limit of Quantitation.

**Table 4.2:** Concentration of steroids in five different serum samples after Metyrapone treatment. The grey underlined values were acquired using the Tecan kit whereas the other values were measured with the in-house method from Universitätsklinikum Erlangen. Bold values were elevated for the analyzed patient population. Values for the in-house method of Universitätsklinikum Erlangen marked with N/A are not available, as no in-house method was implemented for routine laboratory diagnostics.

No.	Method	Concentration [ng/mL]								
		DHT	DHEAS	DHEA	T	A	E1	E2	17-OHP5	DST
<b>1</b>	In-house Erlangen	N/A	N/A	N/A	0.293	N/A	N/A	N/A	N/A	N/A
	Steroid Panel LC-MS	< LoQ	869	4.05	0.323	< LoQ	0.031	< LoQ	7.60	< LoQ
<b>2</b>	In-house Erlangen	N/A	N/A	N/A	0.111	N/A	N/A	N/A	N/A	N/A
	Steroid Panel LC-MS	< LoQ	273	< LoQ	0.152	< LoQ	0.042	0.057	0.477	< LoQ
<b>3</b>	In-house Erlangen	N/A	N/A	N/A	0.165	N/A	N/A	N/A	N/A	N/A
	Steroid Panel LC-MS	< LoQ	6,231	6.77	0.181	< LoQ	0.034	0.014	12.2	< LoQ
<b>4</b>	In-house Erlangen	N/A	N/A	N/A	0.386	N/A	N/A	N/A	N/A	N/A
	Steroid Panel LC-MS	< LoQ	3,048	3.95	0.414	< LoQ	0.056	0.029	3.64	< LoQ
<b>5</b>	In-house Erlangen	N/A	N/A	N/A	0.166	N/A	N/A	N/A	N/A	N/A
	Steroid Panel LC-MS	< LoQ	769	< LoQ	0.148	0.045	0.034	< LoQ	1.60	< LoQ

Abbreviations: DHT - dihydrotestosterone, DHEAS - dehydroepiandrosterone-sulfate, DHEA - dehydroepiandrosterone, T - testosterone, A - aldosterone, E1 - estrone, E2 - estradiol, 17-OHP5 - 17-OH-pregnenolone, DST - dexamethasone, N/A - not available, LoQ - Limit of Quantitation.

However, it has to be pointed out, that not all the steroids included in the Tecan kit are routinely measured at the Universitätsklinikum Erlangen, nor external quality assessment scheme is available for all parameters. Accordingly, the clinical performance for the missing steroids 17-OH-pregnenolone, DHT and dexamethasone is separately evaluated as follows:

The 17-OH-pregnenolone concentrations have been measured for all clinical samples between 0.3 and 16.90 ng/mL. These values are higher than the expected range between (0.41 to 4.80 ng/mL) for the children in healthy populations. Particularly, the 17-OH-pregnenolone values for the five highlighted samples in table 4-2 were elevated and above the normal ranges, showing concentrations up to 12.2 ng/mL. Such high concentrations can be expected as a consequence of the Metyrapone test, as 17-OH-pregnenolone is a precursor of 17-OH-pregnenolone and 11-deoxycortisol ultimately (Figure 4).

The concentration of dihydrotestosterone (DHT) in the analyzed serum samples is in a range of 0.13–0.38 ng/mL. These values met the normal range for individuals under 20 years (< 0.93 ng/mL).<sup>[8]</sup> DHT values in children are not expected to be elevated,<sup>[8]</sup> accordingly, the results obtained with the Tecan kit are coherent. Including this parameter in steroid profiles represents a new opportunity for the clinical laboratory to enhance the clinical picture of the patient.

For dexamethasone (DST), all samples measured with the Tecan kit show values below LoQ, which was expected as the patients coming to the clinic are not expected to be under treatment. Nevertheless, the steroid kit is able to measure the affected population since the calibration range is 0.50–64.1 ng/mL and dexamethasone concentration of patients subjected to 1 mg DST is expected to reach up to 20.2 ng/mL (median 4.8 ng/mL).<sup>[4]</sup> The clinical performance of dexamethasone in the Steroid Panel LC-MS is confirmed by F. Ponzetto et al. showing the ability to measure affected populations.<sup>[7]</sup>

Given the strong association of steroid hormones and endocrine disorders like adrenal insufficiency, Cushing's syndrome, and congenital adrenal hyperplasia e.g., steroid testing has become an integral part of diagnostic procedures and is recommended by many clinical practice guidelines.<sup>[5]</sup> The advantage of the current Tecan Steroid Panel LC-MS is the comprehensive analysis of 17 circulating steroids and dexamethasone. This allows the measurement of a complete steroid profile for the patient and as such is an aid to diagnosis and monitoring patients with suspected or confirmed endocrine disorders.

## CONCLUSION AND OUTLOOK.

This study performed a method comparison between the implemented in-house method for clinical routine measurements of Universitätsklinikum Erlangen and the Steroid Panel LC-MS by Tecan. A good correlation was found between both methods based on clinical samples. To verify these results, in the future, a method comparison study based on CLSI guidelines include at least 40 clinical samples should be performed. Even without this larger number of samples, both methods allow the reliable quantification of the aforementioned steroids due to the shown clinical performance. Clinical samples can be analyzed reliably and therefore both methods meet the laboratory needs. Furthermore, this can be confirmed by the performed accuracy experiments: The presented study showed an excellent agreement between measurements of 11 different steroids with SKML ring trial samples.

The results obtained with the commercial Steroid Panel LC-MS kit were in concordance with the established in-house method. Additionally, this assay allows the clinicians to gain a broader clinical picture of the patients, as it includes 17 steroids and dexamethasone which is helpful for the diagnosis and the understanding of the endocrine disorders. Particularly the addition of new parameters like DHT, dexamethasone and 17-OH-pregnenolone are beneficial for the investigation of the patient's hormone status.

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## ABOUT THE AUTHORS.

**Ayham Al Ahmad<sup>1</sup>, Maike Arndt<sup>1</sup>, Clarissa Müller<sup>2</sup>  
and Manfred Rauh<sup>2</sup>**

<sup>(1)</sup> Tecan, IBL-International GmbH, Flughafenstrasse  
52A, 22335 Hamburg, Germany.

<sup>(2)</sup> Kinder- und Jugendklinik, Universitätsklinikum  
Erlangen, Loschgestr. 15, 91054 Erlangen,  
Germany.

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**Italy** +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 17 4 **Nordic** +46 8 750 39 40 **Singapore** +65 644 41 886 **Spain** +34 93 595 25 31  
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