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Development and optimization of liquid chromatography-tandem mass spectrometry methods for steroid analysis in endocrine research and clinical diagnostics

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Affidavit

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is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

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Confirmation of congruency between printed and electronic version of the doctoral thesis

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Signature doctoral candidate

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List of abbreviations

BMI	Body-mass-index
CLIA	Chemiluminescence immunoassay
cps	Counts per second
Da	Dalton
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
HPLC	High performance liquid chromatography
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
IA	Immunoassay
kDa	Kilodalton
KORA	Cooperative Health Research in the Region of Augsburg, publicly funded study at Helmholtz Center Munich
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDT	Laboratory developed test
LLE	Liquid-liquid extraction
LMU	Ludwig-Maximilians-Universität
LoQ	Limit of quantification
MR	Mineralocorticoid receptor
MS	Mass spectrometry
MTBE	Methyl *tert*-butyl ether
m/z	Mass to charge ratio
Q1	First quadrupole
Q2	Second quadrupole and collision cell
Q3	Third quadrupole
RI	Reference intervals
RIA	Radioimmunoassay
SPE	Solid-phase extraction
V	Volt

List of publications

Scientific Publications summarized in this thesis:

I. Kunz S, Wang X, Ferrari U, Drey M, Theodoropoulou M, Schilbach K, Reincke M, Heier M, Peters A, Koenig W, Zeller T, Thorand B, Bidlingmaier M. **Age and sex-adjusted reference intervals for steroid hormones measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a widely available kit.** *Endocr Connect.,* 2024 13(1):e230225.

DOI: 10.1530/EC-23-0225. PMID: 37938144.

II. Kunz S, Meng Y, Schneider H, Brunnenkant L, Höhne M, Kühnle T, Reincke M, Theodoropoulou M, Bidlingmaier M. **Fast and reliable quantification of aldosterone, cortisol and cortisone via LC-MS/MS to study 11β-hydroxysteroid dehydrogenase activities in primary cell cultures.** *J Steroid Biochem Mol Biol.,* 2024 244, 106610.

DOI: 10.1016/j.jsbmb.2024.106610. PMID: 39214289.

Further publications resulting during my time as a PhD candidate.

- Wang X, Heinrich DA, Kunz SL, Heger N, Sturm L, Uhl O, Beuschlein F, Reincke M, Bidlingmaier M. **Characteristics of preoperative steroid profiles and glucose metabolism in patients with primary aldosteronism developing adrenal insufficiency after adrenalectomy.** *Sci Rep. 2021* ;11(1):11181.
  DOI: 10.1038/s41598-021-90901-4. PMID: 34045650; PMCID: PMC8160266.

- Eisenhofer G, Kurlbaum M, Peitzsch M, Constantinescu G, Remde H, Schulze M, Kaden D, Müller LM, Fuss CT, Kunz S, Kołodziejczyk-Kruk S, Gruber S, Prejbisz A, Beuschlein F, Williams TA, Reincke M, Lenders JWM, Bidlingmaier M. **The Saline Infusion Test for Primary Aldosteronism: Implications of Immunoassay Inaccuracy.** *J Clin Endocrinol Metab.* 2022 107(5):e2027-e2036.
  DOI: 10.1210/clinem/dgab924. PMID: 34963138.

- Zhang R, Rubinstein G, Vetrivel S, Kunz S, Vogel F, Bouys L, Bertherat J, Kroiss M, Deniz S, Osswald A, Knösel T, Bidlingmaier M, Sbiera S, Reincke M, Riester A. **Steroid profiling using liquid chromatography mass spectrometry during adrenal vein sampling in patients with primary bilateral macronodular adrenocortical hyperplasia**. *Front Endocrinol (Lausanne).* 2022 6;13:1079508.
  DOI: 10.3389/fendo.2022.1079508. PMID: 36561559.

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  DOI: 10.3389/fendo.2023.1244342. PMID: 37693351.

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DOI: 10.1007/s40520-023-02574-w. Epub 2023 Nov 9. PMID: 37943405.

- Bruedgam D, Adolf C, Schneider H, Schwarzlmueller P, Mueller L, Handgriff L, Bidlingmaier M, Kunz S, Zimmermann P, Deniz S, Williams TA, Beuschlein F, Reincke M, Heinrich DA. **Postoperative ACTH-stimulated aldosterone predicts biochemical outcome in primary aldosteronism.** *Eur J Endocrinol.* 2023 189(6):611-618.
  DOI: 10.1093/ejendo/lvad159. PMID: 38048424.

- Buffolo F, Pecori A, Reincke M, Outland M, Veglio F, Schwarzlmüller P, Bidlingmaier M, Kunz S, Stremmel C, Mengozzi G, Priolo G, Mulatero P, Adolf C, Monticone S. **Long-Term Follow-Up of Patients With Elevated Aldosterone-to-Renin Ratio but Negative Confirmatory Test: The Progression of Primary Aldosteronism Phenotypes.** *Hypertension.* 2023.
  DOI: 10.1161/HYPERTENSIONAHA.123.21983. Epub ahead of print. PMID: 38084587.

Confirmation of co-authors

Hereby I declare that all authors contributed to the publications I and II gave written informed consent to use these publications for my dissertation.

1. My contribution to the publications

1.1 Contribution to publication I

For this publication with the title

Age and sex-adjusted reference intervals for steroid hormones measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a widely available kit

published in Endocrine Connections 2024, I was involved in planning and conceptualization of the project. I wrote parts of the project agreement and established the variable list for acquisition of sample-related data from our cooperation partner (KORA, Helmholtz center Munich). I performed data acquisition, including sample pretreatment, LC-MS/MS measurements and interpretation of chromatograms. I implemented method verification, including determination of the limit of quantification (LoQ), precision, and accuracy of the method. I managed data evaluation and interpretation by excluding samples from participants with hormone intake, excessively high BMI and repetitive samples. I prepared the data set for statistical calculation of the reference intervals by adjusting for LoQ and grouping according to age and sex. I performed statistical calculations of BMI correlation and generated all figures and tables, except distribution figures which were prepared by the coauthor Xiao Wang. I performed literature search, wrote the draft and revised the manuscript during revision process.

1.2 Contribution to publication II

For this publication with the title

Fast and reliable quantification of aldosterone, cortisol and cortisone via LC-MS/MS to study 11β-hydroxysteroid dehydrogenase activities in primary cell cultures

published in the Journal of Steroid Biochemistry and Molecular Biology 2024, I was involved in the planning and conceptualization of the project. I developed and validated the LC-MS/MS method. Method development included optimization of the extraction procedure, chromatography and mass transitions. Validation included investigation of precision, accuracy, LoQ, dilution linearity, matrix effect, recovery, robustness, calibration material and stability. I performed data acquisition, including sample pretreatment, LC-MS/MS measurements, processing and evaluation of raw data for all samples in cell culture supernatant. I wrote the draft, prepared all figures, performed all the statistical analysis and literature search and revised the manuscript during revision

process. I share the first authorship with Yao Meng who performed the cell culture experiments. Gene expression experiments were performed by Laura Brunnenkant. Conceptualization of the cell culture experiments was performed by Holger Schneider.

During the whole time of my PhD project, I was also responsible for the maintenance of the LC-MS/MS instrument, and measurement of samples from external quality assessment schemes.

2. Aim and relevance of this thesis

Steroid hormones are endogenous molecules that are involved in various regulation mechanisms in the human body (1). These include glucose metabolism (2–4), vascular function (5,6), blood pressure (7), reproduction (7,8), muscle formation (9–11), bone structure (12,13) and stress response (1). Hence, deficiencies in steroid production or metabolism result in severe disorders (14–16). Treatment and further research are helping to reduce mortality and improve quality of life of those affected (17–20). However, diagnosis of the disease and best possible treatment requires reliable and accurate measurement of steroid hormones.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is referred to as “gold standard” in steroid hormone analysis and exhibits high precision and accuracy but is still not available in every clinical laboratory (21,22). Objective of this thesis was to make steroid quantification by LC-MS/MS accessible for research and diagnosis of endocrine diseases at the endocrine laboratory of the Medizinische Klinik und Poliklinik IV of the LMU Klinikum. Method specific reference intervals (content of Publication I) and matrix specific method development for measurement of cell culture supernatants (content of Publication II) make a valuable contribution to this aim (23,24).

Reference intervals are essential for correct clinical interpretation of laboratory results (23,24). Since steroid concentrations are influenced by many different biological factors, such as age (25–29), sex (25,27–31), menstrual cycle (29,32), menopause status (25,28,30,32), time of the day (27,31) and stress level (33,34), establishing reference intervals is challenging (24,35). Additionally, reports about pronounced differences in measurement results between laboratories, even when the same devices or kits are used, suggest the use of method specific reference intervals (36–38).

Equally relevant in this context is the performance evaluation of the used method in the respective laboratory (24,39–41). Manufacturers usually provide values for limit of quantification (LoQ), accuracy and precision, but determination is not performed in a routine-like setting and there are differences in used laboratory equipment. Furthermore, commercial kits are not necessarily suitable for all clinical applications (39). Therefore, Publication I provides reference intervals for several clinically relevant steroids but also includes the evaluation of performance characteristics and a suitability check of the kit.

There are still limitations in patient care for endocrine diseases (42–44). Clinical research is one way to further improve treatment. It often starts with cell culture experiments. A

major advantage of LC-MS/MS in cell culture experiments is the possibility to monitor several analytes within one measurement. This applies in particular to experiments on enzyme activity, when educt and product can be measured simultaneously.

Reliable, high-quality research is based on reproducible results. However, uncommon sample materials such as saliva, sweat, dried blood spots, blood samples from animals or cell culture supernatants are usually not included in the validation procedure of a commercial kit. Therefore, revalidation or even redevelopment are required (45–47). Publication II describes a newly developed method for quantification of aldosterone, cortisol and cortisone in cell culture supernatants and its application in enzyme activity experiments.

Overall, this thesis provides valuable tools to enable the use of LC-MS/MS in the endocrine laboratory for research and routine applications.

3. Principles and methods

3.1 Technical principles of liquid chromatography-tandem mass spectrometry

Mass spectrometry (MS) is known to be a very sensitive detection method and adding a second MS unit increases specificity due to an additional separation function (48–51). The enhanced specificity of this method makes it especially suitable for substances with similar masses, as it is the case for steroids (49,50).

The use of triple quadrupole instruments is most common in clinical applications (51). A quadrupole consists of four conductive rods. Applying constantly changing alternating current and direct current to the opposite rod pairs forces charged molecules to pass the quadrupole on a predefined trajectory between the rods (24,45,52). By adjusting the currents, selected molecules pass the quadrupole on stable trajectories. All other molecules are following unstable trajectories and are not passing the quadrupole (24,45,52).

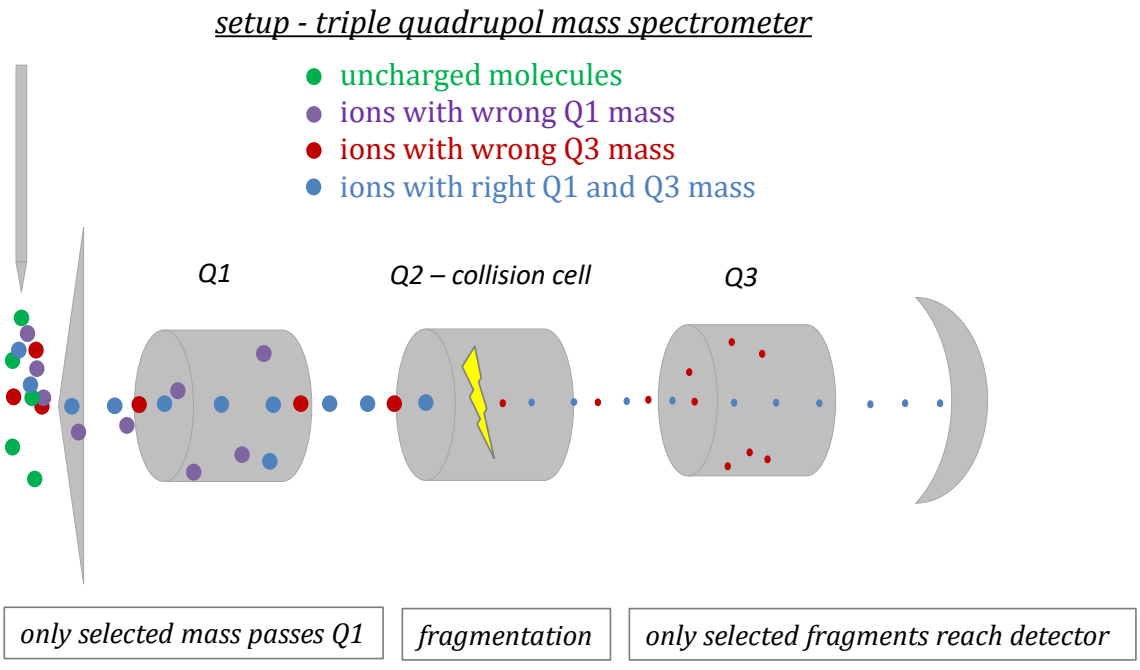

Figure 1: A triple quadrupole mass spectrometer exhibits high specificity. Schematic illustration of the separation mechanism includes selection of one mass in the first quadrupole (Q1), fragmentation of molecules with this preselected mass in the collision cell (Q2) and selection of a certain fragment in the third quadrupole (Q3).

Separation process includes the selection of the main ion in the first quadrupole (Q1), fragmentation of this ion in the second quadrupole (Q2) that works as a collision cell and

selection of molecule specific fragments in the third quadrupole (Q3) (49,52). A schematic setup of a triple quadrupole mass spectrometer is shown in Figure 1.

Ionization of the molecules is an important part of the procedure. Mass spectra usually show the mass to charge ratio (m/z) of the generated ions against the intensity measured in counts per second (cps). Only charged substances are attracted by the electrically field in the mass spectrometer, uncharged molecules are not entering the ion path (52) (Figure 1). This prevents the mass spectrometer from contaminants but can also lead to insufficient sensitivity when the amount of ionized analyte molecules is too low. Impact of ionization efficiency is further discussed in Chapter 3.5.

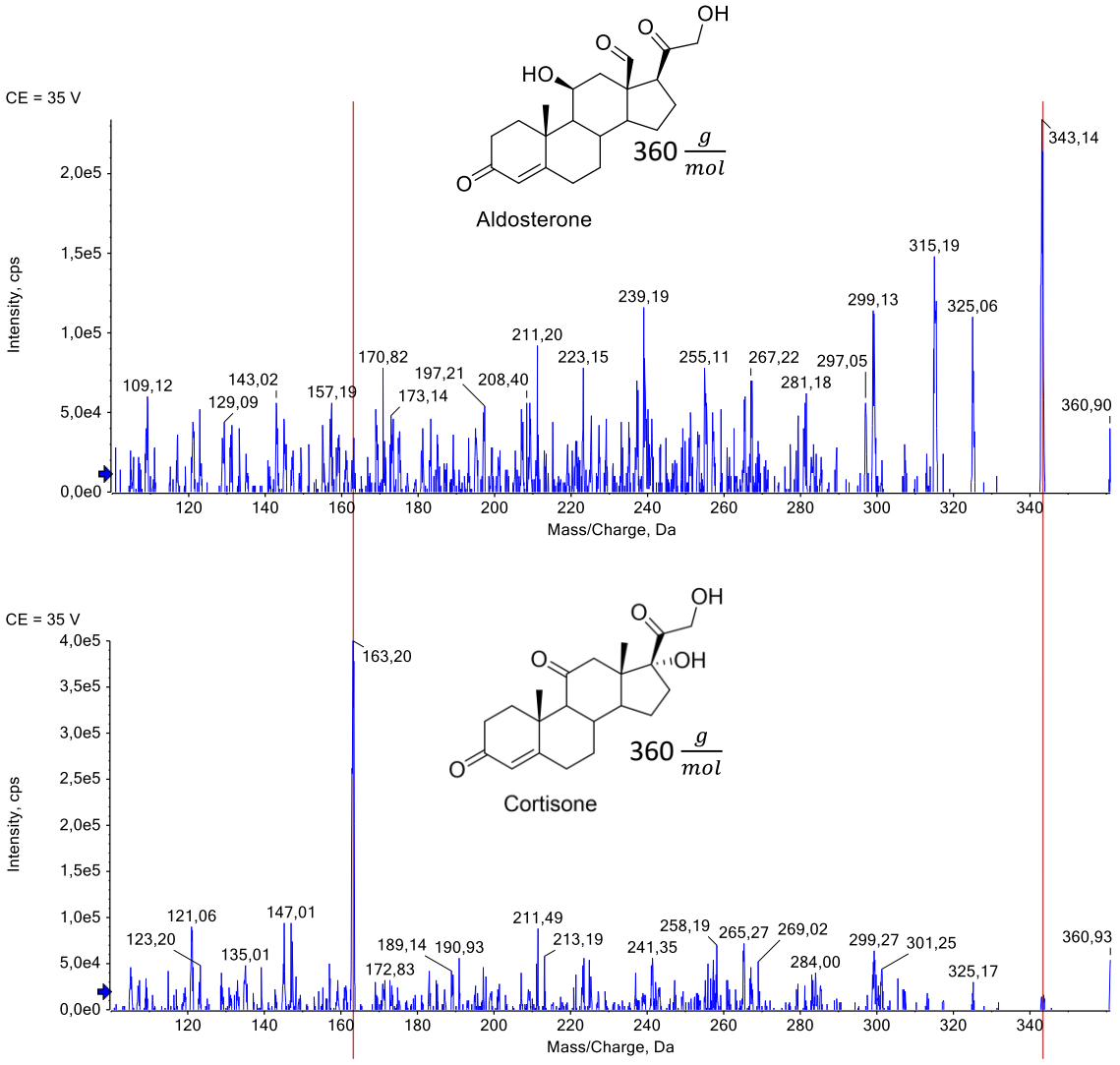

Figure 2: Most abundant fragment ions for aldosterone (m/z 343) and cortisone (m/z 163) occur in both mass spectra after fragmentation with optimized collision energy (35V). Spectra were generated with SciexOS Software Version 3.3.0.

Selection of fragment ions for aldosterone and cortisone was an essential part during method development for Publication II. These two steroids have the same mass and therefore cannot be distinguished according to their molecular ion in Q1. Differentiation

in the MS is only possible if both show individual fragment ions after fragmentation. Figure 2 shows that this is not the case. After fragmentation with an optimized collision energy of 35V, all fragments occurred in both spectra. Even though intensity difference between the most abundant fragment in one spectrum and the same fragment in the other spectrum was large, interferences could not be excluded. Therefore, it was decided to separate the signals chromatographically and use the most abundant fragments for quantification to maximize sensitivity for both steroids.

Liquid chromatography (LC) is used to separate molecules according to differences in their chemical or physical properties (45). Most common used type of analytical LC in clinical steroid analysis is reversed phase LC (45,48,53). It is based on a chromatographic column, packed with different materials and attached chemical groups, which is called the stationary phase (54,55). The solvent that is eluting the analytes from the column one after the other is called mobile phase (53,56).

Separation properties of a chromatographic column are depending on the type of chemical group used and its interaction with the analytes (49,57). Chromatographic separation occurs, when analytes have different affinities to stationary and mobile phase and bind to the column for different durations before they are again dissolved in the mobile phase (45,56). Careful selection of column and mobile phase adjusted to the intended use is therefore a critical step in method development (51,56).

Chromatographic performance and runtime are improved by column materials that withstand very high pressures and by gradient elution, which means that mobile phase composition is changed during the chromatographic run (53,57).

The chromatographic column that is used in Publication II, a Kinetex ® Biphenyl LC Column (50 x 3.1 mm, 2.6 µm; Phenomenex, USA), was selected to enable separation of the structurally very similar steroids. Reversible π-π interactions between biphenyl groups of the stationary phase and the steroids enable a strong binding of the steroids to the stationary phase (51,58) which were than successively released from the stationary phase with a gradient elution.

3.2 Relevance of liquid chromatography-tandem mass spectrometry in the clinical context

Clinical laboratories can choose from a variety of different methods for steroid quantification, including Immunoassay (IA), electrophoresis and chromatography with different detectors (45,50,54,59,60). In steroid analysis, nowadays, chemiluminescence

immunoassay (CLIA) and LC-MS/MS are the dominating analytical measuring methods with decreasing use of IA and an increasing application of LC-MS/MS methods (23,45,49,59) (Figure 3).

Reasons for the preferential use of LC-MS/MS in steroid analysis are various. However the most prominent reason are specificity issues of IA. Interferences of other endogenous substances like steroids and antibodies are known as well as cross-reactions of exogenous substances like medication and supplements (61–64). In a worst-case scenario, the lack of specificity leads to biased diagnosis and incorrect treatment of patients. Studies, describing such cases were published recently (65–68).

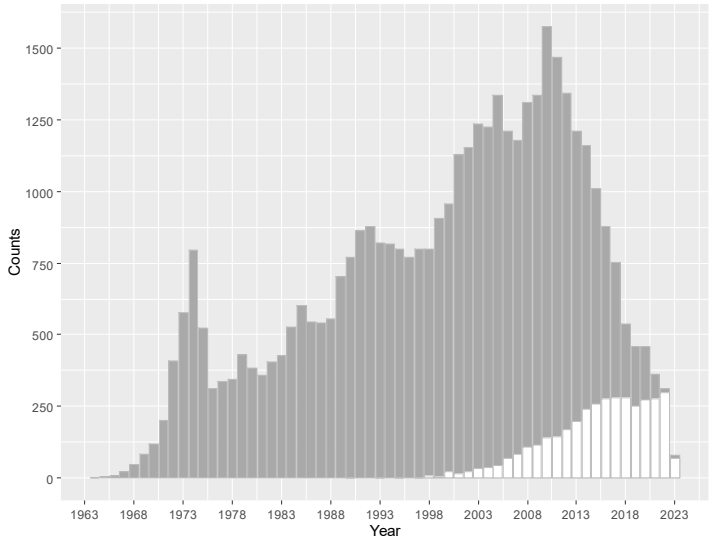

Figure 3: Citation report of the scientific data base Pubmed on the subjects “immunoassay-Steroids” (grey) and “LC-MS/MS-Steroids” (white) shows different trends for both analytical methods (https://pubmed.ncbi.nlm.nih.gov; 2023-03-17)

Reasons for the different specificity of the two methods are evident. IAs rely on biological material. In an organism, the binding of an antigen to the binding site of an antibody is usually co-regulated by several other mechanisms and does not need to be completely specific (69,70). However, when transferred to an isolated system without regulatory mechanisms, characteristics such as unfavorable size differences between antigen and antibody become problematic. Steroids have low molecular weight (250-400 Da) in comparison to their antibodies (> 40 kDa) and have very similar chemical structures, therefore errors are expectable. LC-MS/MS, on the other hand, is based on physical and chemical mechanisms. Steroids are differentiated according to their molecular mass, chemical structure and specific fragmentation pattern, which increases specificity (45,52,71–73).

3.3 Advantages and disadvantages of liquid chromatography-tandem mass spectrometry

The complex technology behind LC-MS/MS makes it precise and accurate but prone to cost-intensive down-times and other drawbacks (72,73) (Table 1). Hence, benefits and

drawbacks of each method should be considered when choosing the appropriate analytical method.

The most frequently reported benefit of LC-MS/MS over other quantification methods for steroids in literature is high specificity paired with high sensitivity and the possibility to measure several steroids in one measurement run (23,45,52,73,74). The latter is particularly advantageous for steroid analysis when activity of steroid converting enzymes is to be investigated (23).

Table 1: Advantages and disadvantages of LC-MS/MS quantification of steroid hormones.

Advantages	Disadvantages
- High specificity and sensitivity (23,52,73,74)	- technical complexity, requiring highly trained personal and expertise (39,72,73,75)
- Simultaneous measurement of several steroids in one run (23,45,52,74)	- High instrument and maintenance cost (39,72–75)
- Large dynamic range (23,52)	- Lack of standardization (39,74)
- Small required sample volume (23)	- Not fully automatically(75)
- Improved robustness and accuracy due to use of internal standards (45)	- Absence of Internal standards for uncommon steroids (45)
- Identification of interferences (23)	- Matrix effects, ion suppression/enhancement and interferences (39)
- Flexibility and fast development of methods for “new” steroids (73)	- Pretreatment required (72)

Equally advantageous is the large dynamic concentration range of LC-MS/MS methods (23,52,72). This is especially important in steroid analysis, as steroid concentration can cover a wide physiological range (23). Examples are the variation of female sex hormones during the menstrual cycle (28,29,32), sex hormones in general when comparing young and elderly or males and females (25,30), diurnal variation of glucocorticoids (27,31) also depending on stress level of the respective individuum (33,34) as well as suppressed or enhanced secretion of steroids in different diseases (76) also depending on the particular sample material (77,78).

Further on, LC-MS/MS is using small sample volumes. This particularly refers to the measurement of several steroids in one sample, where less sample volume is required than for a separate measurement of each individual steroid (23,48).

The use of stable isotope labeled internal standards makes LC-MS/MS robust and accurate as it corrects for matrix effects and analyte losses during sample preparation (73,79). The possibility to simultaneously measure several ion transitions of one steroid allows the identification of interferences (49,80,81).

Lastly LC-MS/MS offers flexibility regarding the addition of new substances, use of different matrices and method development for all kinds of different steroids. It only requires commercially available standards of the substances and adequate validation (45,73).

Despite all these advantages, LC-MS/MS requires high acquisition and maintenance costs, trained personal, specially equipped laboratory rooms and profound expertise (39,72–75). Therefore, down times of the instruments are expensive and maintenance contracts reasonable. Often, high trough put of samples is required for a cost-effective use.

The lack of standardization (39,74) and missing solutions for fully automated measurements (75) are still counterarguments for use of LC-MS/MS in clinical routine. Notably recently the application of instruments for full automation of LC-MS/MS measurements are reported but are still limited to simple sample preparation techniques, like protein precipitation and filtration and therefore not usable for all applications (82).

LC-MS/MS is not free of interferences and matrix effects that cause ion suppression or ion enhancement. Hence sample pretreatment and chromatographic separation is mandatory for most applications (73). Noteworthy, isotope labeled internal standards often correct for those matrix effects, but are not commercially available for all steroids (45).

In contrast there are fully automated IA methods (61,64,73), requiring short personal training times (31,45,73) and allowing short analysis times and high throughput (31,61) with good sensitivity (23,64,73). However, automation and speed, primarily achieved by omitting preparation techniques, often are at the cost of specificity (73). Steroid analysis via IA is limited to manufacturers portfolio or requires time-consuming development of new antibodies (52,73). Measurements are usually limited to one analyte at a time (52) and advanced automation also requires technicians in the case of instrument failures.

Considering the above-mentioned advantages and disadvantages of LC-MS/MS and IA, it appears, that – if both methods are available – all aspects should be considered to choose the most appropriate quantitation method for steroid analysis. Accuracy and urgency of the measurement are crucial factors. Exemplary, confirmatory testing for diagnosis of primary hyperaldosteronism requires accurate quantification of low

concentrated aldosterone, therefore quantification with LC-MS/MS is advisable (65). In case the correct position of a catheter during adrenal vein sampling needs to be checked during the procedure, LC-MS/MS cannot provide a sufficiently fast analysis result and immunoassay is the better option.

3.4 Challenges of determining reference intervals

Laboratory specific reference intervals from a good characterized and sufficiently large cohort are recommended to be established in each laboratory to allow for an appropriate clinical interpretation of the measured concentrations (39,83). The choice of an appropriate cohort to serve as a reference population might differ on the underlying clinical intentions or differences in health status or composition of the regional population. As already mentioned in Chapter 2 and Chapter 3.3, biological factors that are influencing steroid concentrations are various. Examples are variations due to age, sex (25,30), time of the day (27,31) stress level (33,34) or menstrual status in females (28,29,32). This makes it necessary to use reference intervals that take these factors into account. However, the availability of samples, capacity of the laboratory and applicability in clinical routine are also important factors that have to be considered. Furthermore, inter-laboratory variability and variability between different methods are still present and let recommend the use of laboratory specific and method specific reference intervals (36).

3.5 Difficulties with standardization of analytical methods

LC-MS/MS is commonly referred to as “gold standard” for steroid quantification. The method exhibits high precision and accuracy, however uniformity of results between laboratories is not guaranteed by using this technique. Evaluation of external assessment schemes shows apparent variability, even between LC-MS/MS laboratories that are using the same commercial kit (Figure 4).

There is no external assessment scheme for all steroids that are included in the commercial kit that was used in publication I. However, we are regularly participating in the assessment scheme of the Referenz Institut für Bionalytik (Bonn, Germany), which includes aldosterone, cortisol, dehydroepiandrosteron-sulfate, estradiol, testosterone, 17α-hydroxyprogesterone and progesterone.

Figure 4 shows results for aldosterone and cortisol measured with LC-MS/MS and IA. These steroids are frequently measured in our laboratory as part of the diagnostic procedure of primary aldosteronism. Figure 4 illustrates that precision and accuracy of results are not necessarily improved with LC-MS/MS but are strongly variable depending on analyte, method and tested concentration range. Cortisol measurement with the DiaSorin assay (IA) for instance exhibits high precision but is less accurate than results measured with the Chromsystems kit (LC-MS/MS).

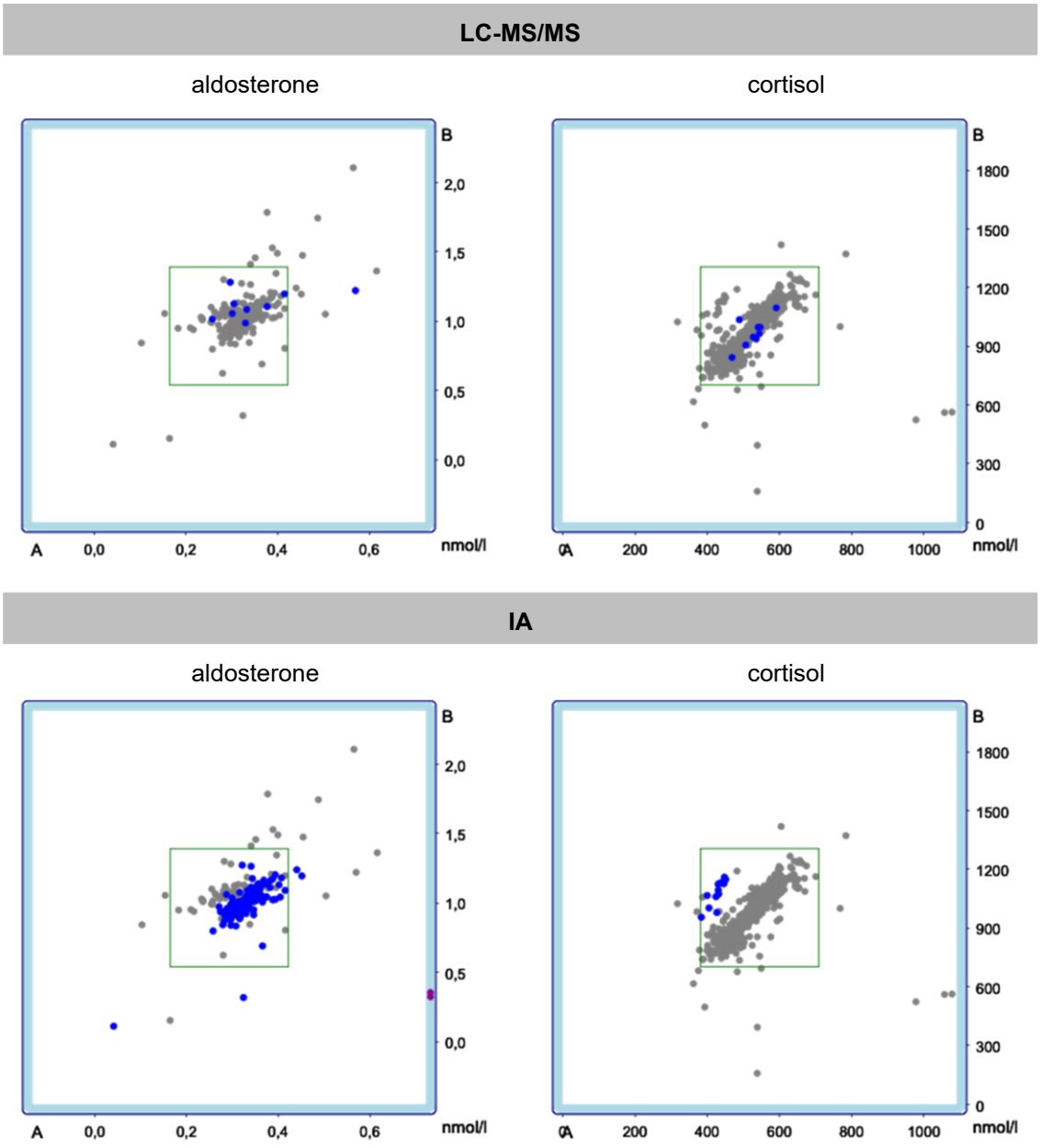

Figure 4: Youdenplots of an external assessment scheme (Referenzinstitut für Bioanalytik, HM1, Q1/24) show differences in precision and accuracy for aldosterone (left) and cortisol (right) measured with LC-MS/MS (top, blue: measured with Chromsystems kit) and IA (bottom, blue: measured with DiaSorin IA).
Image sources: https://www.rfb.bio/cgi/showPlot?statname=HM2411_011047&rvID=HM241 (2024-05-02); https://www.rfb.bio/cgi/showPlot?statname=HM2411_021047&rvID=HM241 (2024-05-02); https://www.rfb.bio/cgi/showPlot?statname=HM2411_010477&rvID=HM241 (2024-05-02); https://www.rfb.bio/cgi/showPlot?statname=HM2411_020477&rvID=HM241 (2024-05-02).

One critical parameter that has major influence for applicability and performance of LC-MS/MS for the respective analyte is the ionization efficiency (84–86), which can be seen as amount of analyte that is ionized in the ionization source of the MS. Performance of LC-MS/MS is the better the higher this amount is (86–88). Instrument settings such as flow rate, ionization voltage, and temperature in the ionization source must be optimized during method development but also before using a commercial kit. But besides this, chemical structure and physical properties are determining how efficient ionization for the respective substance is (86,88,89). Hence, for substances that have favorable chemical and physical properties, optimization of instrument settings is less critical than for substances that do not fulfill these specifications. Steroids in general have poor ionization efficiency due to a lack of basic or acidic groups in their chemical structure (78,85).

This is, besides different preanalytical and sample preparation procedures, a possible reason for variations between laboratories in steroid analysis. Therefore, use of a commercial kit alone may not achieve interlaboratory harmonization but seems to be a step in the right direction. However, it also underlines the need for individual reference intervals and the individual assessment of quality parameters even if commercial kits are used, as performed in Publication I.

3.6 Reasons to use a commercial kit

Admittedly, there are valid reasons to use laboratory developed tests (LDT) instead of commercial kits for steroid quantification. LDTs are usually designed to fit individual laboratory needs (51). For example, methods for multiple steroids can be optimized to the favor of one low concentrated one over other more abundant. A commercial kit, however, is usually designed to provide the best compromise for all included analytes. Composition of the kit components is usually not revealed, which prevents individual optimization. Therefore, clinical laboratories often use inhouse methods, partly with components from commercial kits (90).

However, previous studies as well as the explanations in the previous Chapter 3.5 point out the need for better comparability and harmonization amongst clinical LC-MS/MS laboratories in steroid analysis (36,91,92). Generally valid clinical guidelines can only be established if all clinical laboratories provide uniform results (93,94). Using commercially produced, uniform sets of calibrators is an important step for this harmonization amongst clinical laboratories. One recent study demonstrates that imprecisions between laboratories can be improved when the same calibration solutions are used in all

laboratories (36). Further improvements by using same reagents and analytical columns are likely (91). Commercial kits provide standardized calibrators, reagents, preparation protocols and measurement methods.

Since there is no legally binding rule, that regulates the use of LC-MS/MS methods in clinical diagnostics (95), it is up to each laboratory to what extent validation, verification and quality control of analytical methods is performed. Certified manufacturers ensure consistent quality during manufacturing and between different production batches which enables a reduced inter-batch variability compared to individual production in the respective laboratory.

Since, development and validation of a LC-MS/MS method is complex, time-consuming, costly and requires a high level of expertise, using an already validated, commercial kit also saves time and resources.

Manufacturers provide validated materials, reagents and manuals. However, using a commercial kit does not prevent laboratories to investigate method specific parameter on their own. Variations between laboratories can occur due to different methodology, instrumental settings, preanalytical procedures, laboratory equipment or deviation from intended use (37,47). Verification of the method is therefore mandatory for all laboratories before using a commercial kit (47,83,96). The minimum of recommended quality parameter to check are the limit of quantification (LoQ), precision and accuracy of the method (47).

3.7 Reasons to use a laboratory developed test

Utilizing commercial kits can be inappropriate for certain applications (39). Sensitivity or measurement ranges might be insufficient for applications that deviate from the intended use of the kit. In an endocrine laboratory for instance, concentrations of pediatric samples, samples from patients after adrenalectomy or from suppression tests tend to fall below the measurement range (3,21). In contrast, concentrations in samples from adrenal vein sampling usually exceed the measurement range (60,97). Quantification of concentrations that are not within the validated measurement range can exhibit increased uncertainty. Therefore, many laboratories use in-house developed methods with adjusted measurement ranges (21,98). In our laboratory, we validated dilution for samples with a concentration above the calibration range of the commercial kit and introduced additional control parameters for concentrations at the lower end of the calibration range.

However, it is recommended to validate and, if necessary, adapt sample preparation and calibration with every new matrix to ensure accurate and robust results (46,47). In comparison to clinical routine analysis, where matrices such as serum, urine and saliva are common (23,95,99,100), clinical research applications may require less common matrices such as feces, breast milk, or cell culture supernatants (45,101–103).

Additionally, in cases where only few steroids are requested instead of a complete steroid profile, a commercial kit needs longer run times than necessary. Optimization of runtime enhances efficiency of the measurement (21).

4. Results

4.1 Clinical application – implementation of reference intervals

4.1.1 Using a commercial kit

In Publication I, a comprehensive evaluation of the performance of the commercial kit in our laboratory and comparison to the data provided by the manufacturer was performed. This includes precision, accuracy and LoQ. It was pointed out that generally recommended specifications for precision and accuracy could be met with the used kit in our laboratory and sensitivity was sufficient for the majority of the included steroids. Additionally, the suitability of calibration and quality control samples was discussed, as they showed lower background and fewer interfering signals compared to serum or EDTA-plasma samples, raising the question of whether the matrix was adequately accounted for the calibration of this kit. Publication I provides helpful information for other laboratories that want to use the kit and for manufacturers to further improve their kits.

4.1.2 Determining reference intervals

Publication I provides reference intervals for 9 steroids including clinical highly relevant (cortisol, 11-deoxycortisol, DHEA-S, androstenedione, testosterone, 17-OHP) and rarely used steroids (cortisone, corticosterone, DHEA). Samples from the KORA cohort (Cooperative Health Research in the Region of Augsburg) (104) were used in cooperation with Helmholtz Munich. A cohort of 585 subjects was selected, that was stratified by sex and age and represents a cross section of the regional population in terms of health conditions.

Total number of samples was in accordance with the capacity of the laboratory in the planned time range. We performed a rather rough differentiation of age groups to provide a table that is better applicable in clinical routine than a detailed calculation of individual reference intervals for each age. The cohort was only subdivided when the variability was big enough to make a statistical and clinical difference, based on statistical tests and in consultation of the co-authors that include specialists in laboratory medicine.

An intriguing finding was the sex specific BMI dependency of certain steroids, providing evidence that this should be considered when determining more detailed reference intervals. However, since a further reduction of sample size of the previously defined groups in our study would have reduced statistical power of the results, we decided to not include BMI to our reference intervals. We concluded that further studies with larger

cohorts and application of multivariant analysis considering age, sex and BMI need to follow to investigate this. The results of Publication I now serve as reference to facilitate clinical decisions.

4.1.3 Application of the reference intervals

Establishment of reference intervals enabled clinical application of the commercial kit for steroid quantification via LC-MS/MS in the endocrine laboratory. Since then, the method was applied to provide data for several clinical research publications listed as “Further publications resulting during my time as a PhD candidate“ in section “List of publications”. To date, main application of LC-MS/MS in the endocrine laboratory is the quantification of aldosterone for diagnosis of primary hyperaldosteronism. Other applications include quantification of cortisol for the diagnosis of Cushing’s disease, quantification of 17-OHP and 11-deoxycortisol for the diagnosis and monitoring of congenital adrenal hyperplasia and quantification of DHEA-S, testosterone and androstenedione for diagnosis of hyperandrogenism.

4.2 Research application – development of an in-house method

4.2.1 Selection of the appropriate extraction method

During method development for the method described in Publication II, mechanical blocking of the LC by visible residues after extraction with the commercial kit was observed. Additionally yellow color of the extracts indicated presence of phenol red that is often added to cell culture medium to allow pH monitoring during the experiments (105), but is also assumed to potentially cause interferences in LC-MS/MS (106). This hypothesis could not be confirmed due to the blockage. However, to separate steroids from phenol red in a precautionary manner, the extraction procedure was changed from solid-phase

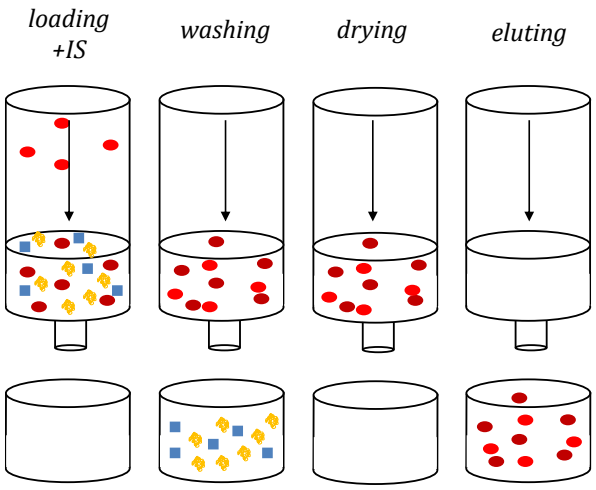

Figure 5: Schematic illustration of solid phase extraction procedure. Dark red: steroids, light red: IS, blue and yellow: interfering substances present in the sample material.

extraction (SPE) (Figure 5) used from the commercial kit to a laboratory intern developed liquid-liquid extraction (LLE) (Figure 6).

The rationale behind this was, that SPE procedures usually use solutions that contain high amounts of organic solvents such as methanol or acetonitrile for the last elution step (Figure 5). Since phenol red and steroid hormones are equally soluble in organic solvents, both are eluted and remain in the extract.

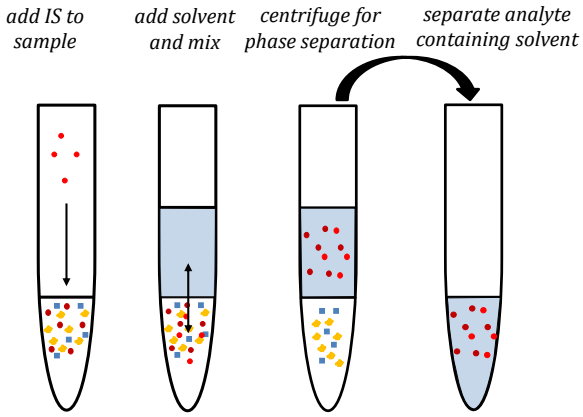

Figure 6: Schematic illustration of liquid-liquid extraction procedure. Dark red: steroids, light red: IS, blue and yellow: interfering substances present in the sample material. Blue background: nonpolar solvent.

In contrast, LLE uses nonpolar solvents such as methyl *tert*-butyl-ether (MTBE) for extraction. Since phenol red is soluble in organic solvents but not in nonpolar solvents, and steroids are soluble in both and LLE was the overall better choice in this case. This was demonstrated by colorless extracts and absence of mechanical blockage of the instrument.

4.2.2 Development, validation and application of an in-house method

Endothelial cells, smooth muscle cells and their interaction play an important role in the cardiovascular system (107). Investigation of the cellular responses to excess cortisol and aldosterone levels is relevant, since patients with chronically elevated concentrations of these steroid hormones exhibit increased cardiovascular morbidity (108,109). The mineralocorticoid receptor (MR) is a link between steroid hormone concentrations and the cardiovascular system (110). Interestingly, it has comparable binding affinity to the glucocorticoid cortisol and the mineralocorticoid aldosterone (111,112). Because cortisol is present at much higher concentrations, a specific protection mechanism is required to ensure the MR is primarily and specifically activated by the mineralocorticoid aldosterone (113). By converting cortisol to physiological inactive cortisone, the tissue specific expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) favors the aldosterone mediated activation of MR (111–113).

In Publication II, a method for the quantification of aldosterone, cortisol and cortisone in cell culture supernatants of primary human coronary artery smooth muscle cells (HCSMC) and endothelial cells (EC) was developed. It was shown that this method

allows calibration in solvent instead of matrix and chromatographic run time could be reduced by half to improve efficiency of the method. The method was validated according to a common guideline (47) and applied to indirectly study HSD11B2 activity by measuring the cortisol to cortisone conversion.

A first application of the method provided evidence of HSD11B2 activity in HCSMC but not in EC. Furthermore, the results indicate a good correlation of cortisol conversion to *HSD11B2* expression. In addition, concentration dependent conversion of cortisol to cortisone could be observed, independent of the presence of aldosterone or a glucocorticoid receptor antagonist.

5. Summary (in English)

The quantification of structurally similar steroid hormones, which are often present at low concentrations, is challenging for a clinical laboratory. Nevertheless, precise and accurate measurement of these hormones is essential to ensure high quality diagnostic outcomes and research findings. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers both high sensitivity and specificity. Therefore, the availability of this methodology in the endocrine laboratory is crucial.

This dissertation project addresses two essential aspects of establishing LC-MS/MS in the clinical laboratory. First, it focuses on the implementation of a routine method for clinical diagnostics. Second, it includes the development of a method that is suitable for the application in research projects.

A commercial kit was used to enable clinical diagnostics, covering the quantification of 15 steroids (aldosterone, cortisol, cortisone, corticosterone, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone-sulfate, estradiol, testosterone, androstene-dione, dihydrotestosterone, dehydroepiandrosterone, 11-deoxycorticosterone, 17-hydroxyprogesterone, progesterone) in human serum and plasma. Laboratory specific validation was implemented, and method- as well as laboratory specific reference intervals were established using a sufficiently large, good characterized and regional cohort.

Laboratory-specific validation resulted in adequate precision and accuracy (< 15%) of the method within the specified measurement range, in accordance with common guidelines. However, pronounced discrepancies were observed between the lower limit of quantification (LoQ) determined in our laboratory and the manufacturer specifications. Application of the laboratory derived LoQs to the cohort revealed insufficient sensitivity (with <10% of values falling below the LoQ) for six steroids (aldosterone, 21-deoxycortisol, estradiol, dihydrotestosterone, 11-deoxycorticosterone, progesterone). Consequently, no reference intervals were established for these steroids.

For the remaining steroids, reference intervals were established considering age-dependency (dehydroepiandrosterone-sulfate, dehydroepiandrosterone, androstene-dione), sex-dependency (testosterone, 17-hydroxyprogesterone), or independence from both age and sex (cortisol, cortisone, corticosterone, 11-deoxydortisol). A negative correlation between concentration and body-mass-index was observed for testosterone, 17-hydroxyprogesterone, dehydroepiandrosterone and dehydroepiandrosterone-sulfate in males, for androstenedione, estradiol and cortisone in females and for progesterone in both sexes.

In addition to establishing a routine method for clinical diagnostics, the project also focused on enabling the application of LC-MS/MS in clinical research. Given the wide range of potential applications, the determination of aldosterone, cortisol and cortisone in supernatants of primary human coronary artery smooth muscle cells (HCSMC) and endothelial cells (EC) was selected as a concrete application.

The optimization process included the selection of an adequate sample preparation procedure, fragment optimization of the isobaric steroids aldosterone and cortisone, chromatographic separation of all steroids as well as a reduction of chromatographic run time. Finally, samples from cell culture experiments with stimulation and inhibition of steroid biosynthesis were analyzed to demonstrate the applicability of the optimized method.

Optimized sample preparation by Liquid-liquid extraction resulted in clear extracts and minimal matrix effect (102-104%). The specifications of common guidelines were met for precision (CV ≤ 6%), accuracy (deviation from nominal concentration ≤ 6 %) and sensitivity with LoQs of 0.11, 0.56 and 0.69 nmol/L for aldosterone, cortisone and cortisol, respectively.

The applicability of the method was demonstrated in various cell culture experiments. Important findings from these experiments were that the relative conversion rate of cortisol to cortisone decreases with increasing cortisol addition and reaches a constant low value of 1% at a cortisol concentration of 276nM. The characteristic conversion of cortisol to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase type 2 was observed in HCSMC but not in EC. The addition of high concentrations of aldosterone or mifepristone did not affect the conversion of cortisol to cortisone in HCSMC.

In conclusion, LC-MS/MS was successfully implemented in the endocrine laboratory during this dissertation project. Validation results obtained with the commercial kit provide valuable information for laboratories planning to use this commercial kit in future. The establishment of reference intervals enabled the application of LC-MS/MS in clinical routine diagnostics. Furthermore, the development of a method for the reliable quantification of steroids in cell culture supernatants enabled the analysis of enzyme activities in cultured cells and represents a promising tool for future research applications.

6. Zusammenfassung (deutsch)

Die Quantifizierung strukturell ähnlicher Steroidhormone, die oft in geringen Konzentrationen vorliegen, ist anspruchsvoll für ein klinisches Labor. Dennoch ist eine präzise und richtige Messung dieser Hormone unerlässlich, um qualitativ hochwertige Diagnose- und Forschungsergebnisse zu gewährleisten. Die Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS) bietet sowohl eine hohe Empfindlichkeit als auch Spezifität. Daher ist die Verfügbarkeit dieser Methodik im endokrinologischen Labor von entscheidender Bedeutung.

Dieses Promotionsvorhaben beschäftigte sich mit den beiden wesentlichen Aspekten der Etablierung von LC-MS/MS im endokrinologischen Labor. Zum einen mit der Implementierung einer Routinemethode für die klinische Diagnostik und zum anderen mit der Entwicklung und Validierung einer Methode, die für die Anwendung in speziellen Forschungsprojekten geeignet ist.

Die Anwendung in der klinischen Diagnostik wurde mit Hilfe eines kommerziell erhältlichen Kits für die Quantifizierung von 15 Steroiden (Aldosteron, Cortisol, Cortison, Corticosteron, 11-Deoxycortisol, 21-Deoxycortisol, Dehydroepiandrosteron-sulfat, Estradiol, Testosteron, Androstendion, Dihydrotestosteron, Dehydroepiandrosteron, 11-Deoxycorticosteron, 17-Hydroxyprogesteron, Progesteron) in humanem Serum und Plasma ermöglicht. Der Fokus lag dabei auf der laborspezifischen Validierung der Methode, sowie der Etablierung von methoden- und laborspezifischen Referenzintervallen in einer ausreichend großen, gut charakterisierten, regionalen Kohorte.

Die laborspezifische Validierung ergab eine ausreichend gute Präzision und Richtigkeit (<15%) der Methode innerhalb des festgelegten Messbereichs, gemäß gängiger Richtlinien. In Bezug auf die Quantifizierungsgrenzen ergaben sich deutliche Unterschiede zwischen den laborintern ermittelten Werten und den Herstellerangaben. Die Anwendung der laborinternen Quantifizierungsgrenzen in der Referenzkohorte ergab für sechs Steroide (Aldosteron, 21-Deoxycortisol, Estradiol, Dihydrotestosteron, 11-Deoxycorticosteron, Progesteron) eine unzureichende Sensitivität der Methode im Hinblick auf die Anwendung in der regionalen Kohorte (>10% der Werte unterhalb der Quantifizierungsgrenze). Für diese Steroide wurden daher keine Referenzintervalle festgelegt.

Für die restlichen Steroide wurden Referenzintervalle festgelegt. Dabei wurde berücksichtigt, ob eine Abhängigkeit der Konzentration von biologischen Variablen wie Alter (Dehydroepiandrosteron-sulfat, Dehydroepiandrosteron, Androstendion) und Geschlecht (Testosteron, 17-Hydroxyprogesteron) vorliegt oder nicht (Cortisol, Cortison, Corticosteron, 11-Deoxycortisol). Eine negative Korrelation der Konzentration mit dem Body-mass-Index wurde für Testosteron, 17-Hydroxyprogesteron, Dehydroepi-

androsteron und Dehydroepiandrosteron-sulfat bei männlichen Individuen, für Androsten-dion, Estradiol und Cortison bei weiblichen Individuen und für Progesteron bei beiden Geschlechtern festgestellt.

Zusätzlich zur Etablierung einer Routinemethode für die klinische Diagnostik sollte auch eine Anwendung von LC-MS/MS für spezielle Forschungsfragen ermöglicht werden. Als konkretes Anwendungsbeispiel wurde die Quantifizierung von Aldosteron, Cortisol und Cortison in Zellkulturüberständen von primären glatten Muskelzellen (HCSMC) und Endothelzellen (EC) aus menschlichen Koronararterien ausgewählt, da damit vielseitige Anwendungen vorstellbar sind.

Die Optimierung umfasste die Auswahl einer geeigneten Probenaufarbeitungstechnik, die Fragment-Optimierung der isobaren Steroide Aldosteron und Cortison, die chromatographische Trennung aller Steroide, sowie die Verkürzung der Analysenzeit. Zur Verdeutlichung der Anwendbarkeit der optimierten Methode wurden Proben aus Zellexperimenten mit Stimulation und Inhibition der Steroidbiosynthese analysiert.

Die Auswahl der Flüssig-flüssig-extraktion als Probenaufarbeitungstechnik führte zu klaren Extrakten und minimalem Matrixeinfluss (102-104%). Die Spezifikationen gängiger Richtlinien für Präzision (CV ≤ 6%), Richtigkeit (Abweichung von der Nominalkonzentration ≤ 6%) und Sensitivität (Quantifizierungsgrenzen von 0.11, 0.56 und 0.69 nmol/L für Aldosteron, Cortison und Cortisol) wurden erfüllt.

Die Anwendbarkeit der Methode konnte anhand verschiedener Zellexperimente gezeigt werden. Wichtige Erkenntnisse aus diesen Experimenten waren, dass die relative Umwandlungsrate von Cortisol zu Cortison mit steigender Cortisol-Zugabe abnimmt und ab einer Konzentration von 276nM einen konstant niedrigen Wert von 1% erreicht. Die für das Enzym 11β-Hydroxysteroid-Dehydrogenase Typ 2 charakteristische Umwandlung von Cortisol zu Cortison konnte nur in HCSMC beobachtet werden. Die Zugabe hoher Aldosteron- oder Mifepriston-Konzentrationen beeinflusste die Umwandlung von Cortisol zu Cortison in HCSMC nicht.

Zusammenfassend lässt sich sagen, dass die Etablierung der LC-MS/MS im endokrinologischen Labor durch dieses Promotionsvorhaben erfolgreich umgesetzt wurde. Die Validierungsergebnisse für das kommerzielle Kit liefern wertvolle Informationen für Labore, die dieses Kit in Zukunft nutzen wollen. Die Etablierung der Referenzintervalle ermöglicht die Anwendung der LC-MS/MS in der klinischen Diagnostik. Die neu entwickelte Methode zur quantitativen Bestimmung von Steroidhormonen in Zellkulturüberständen ermöglicht die Analyse von Enzym-aktivitäten in kultivierten Zellen und stellt ein wertvolles Instrument für zukünftige Forschungsanwendungen dar.

7. Publication I

Age and sex-adjusted reference intervals for steroid hormones measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a widely available kit

Authors: Sonja Kunz, Xiao Wang, Uta Ferrari, Michael Drey, Marily Theodoropoulou, Katharina Schilbach, Martin Reincke, Margit Heier, Annette Peters, Wolfgang Koenig, Tanja Zeller, Barbara Thorand, Martin Bidlingmaier

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8. Publication II

Fast and reliable quantification of aldosterone, cortisol and cortisone via LC-MS/MS to study 11β-hydroxysteroid dehydrogenase activities in primary cell cultures

Authors: Sonja Kunz, Yao Meng, Holger Schneider, Laura Brunnenkant, Michaela Höhne, Tim Kühnle, Martin Reincke, Marily Theodoropoulou, Martin Bidlingmaier

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