Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität München

Novel approaches to corticosteroid profiling by stable isotope dilution tandem mass spectrometry

Johanna Magdalena Lindner

München

2018

Aus dem Institut für Laboratoriumsmedizin
der Ludwig-Maximilians-Universität München

Direktor: Univ.-Prof. Dr. med. Daniel Teupser

Novel approaches to corticosteroid profiling by stable isotope dilution tandem mass spectrometry

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Johanna Magdalena Lindner
aus
München
2018

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Berichterstatter:	Prof. Dr. med. Michael Vogeser
Mitberichterstatter:	PD Dr. Peter Düwell
	Prof. Dr. Axel Imhof
	Prof. Dr. Philip Tinnefeld
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung:	07.12.2018

Eidesstattliche Versicherung

Johanna Lindner

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

“Novel approaches to corticosteroid profiling by stable isotope dilution tandem mass spectrometry"

selbstständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Johanna Lindner

München, den 16.04.2018

Kumulative Dissertation

Table of contents

1. Introduction

1.1. Background

1.1.1. Corticosteroids

Corticosteroids are steroid hormones which are synthesized in the adrenal cortex and are involved in many physiologic processes, e.g. inflammatory reactions or the biochemical stress response. The synthesis of the corticosteroids is regulated by the hypothalamic-pituitary-axis: Different influences like stress, low blood levels of cortisol or illness lead to the secretion of the peptide corticotropin releasing hormone (CRH) from the hypothalamus. CRH triggers the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH, in turn, stimulates the biosynthesis of corticosteroids in the adrenal gland. In a negative feedback mechanism, the synthesized corticosteroids affect the hypothalamus and anterior pituitary and inhibit the secretion of CRH and ACTH and thus, the synthesis of corticosteroids [1].

All corticosteroids are derived from cholesterol via various precursors through reactions of several enzymes [2, 3]. Depending on the involved enzymes and their localization in the adrenal cortex, there are three major pathways which lead to the three groups of corticosteroids: Glucocorticoids are synthesized in the zona fasciculata, mineralocorticoids in the zona glomerulosa and the synthesis of adrenal androgens takes place in the zona reticularis. The biosynthetic pathway of corticosteroids is schematically illustrated in Figure 1.

Corticosteroids have various physiological functions: While mineralocorticoids, e.g. aldosterone, influence the electrolyte balance, androgens have an effect as sex hormones. Glucocorticoids, with the main representative cortisol, are important for the carbohydrate, lipid and protein metabolisms [1]. Latter also play an important role in stress situations, like severe infections, mental stress or surgeries. In such situations, the synthesis of CRH and ACTH is increased while simultaneously the cortisol plasma clearance is reduced. This leads to elevated cortisol concentrations, which are necessary to cope with the stress situation [4]. However, the significant effects of glucocorticoids during the stress response are up to now not yet completely understood [1]. However, their importance for the biochemical stress reaction becomes obvious in critical illness, where low cortisol levels are associated with poorer outcomes [5].

Malfunction of the biosynthetic corticosteroid pathway leads to different endocrinological disorders whereby both overproduction and lack of corticosteroids have serious consequences. Some of these disorders can be traced back to a defect enzyme of the biosynthetic pathway.

The lack of the product or accumulation of the substrate of the respective enzyme can then be used for diagnosing purpose, e.g. elevated 17-OH-progesterone levels indicate congenital adrenal hyperplasia [6]. Thus, corticosteroids are important biomarkers for the functionality of the adrenal gland and reliable quantification methods are essential. However, corticosteroids are structurally closely related and therefore, accurate and selective quantification of these substances is challenging.

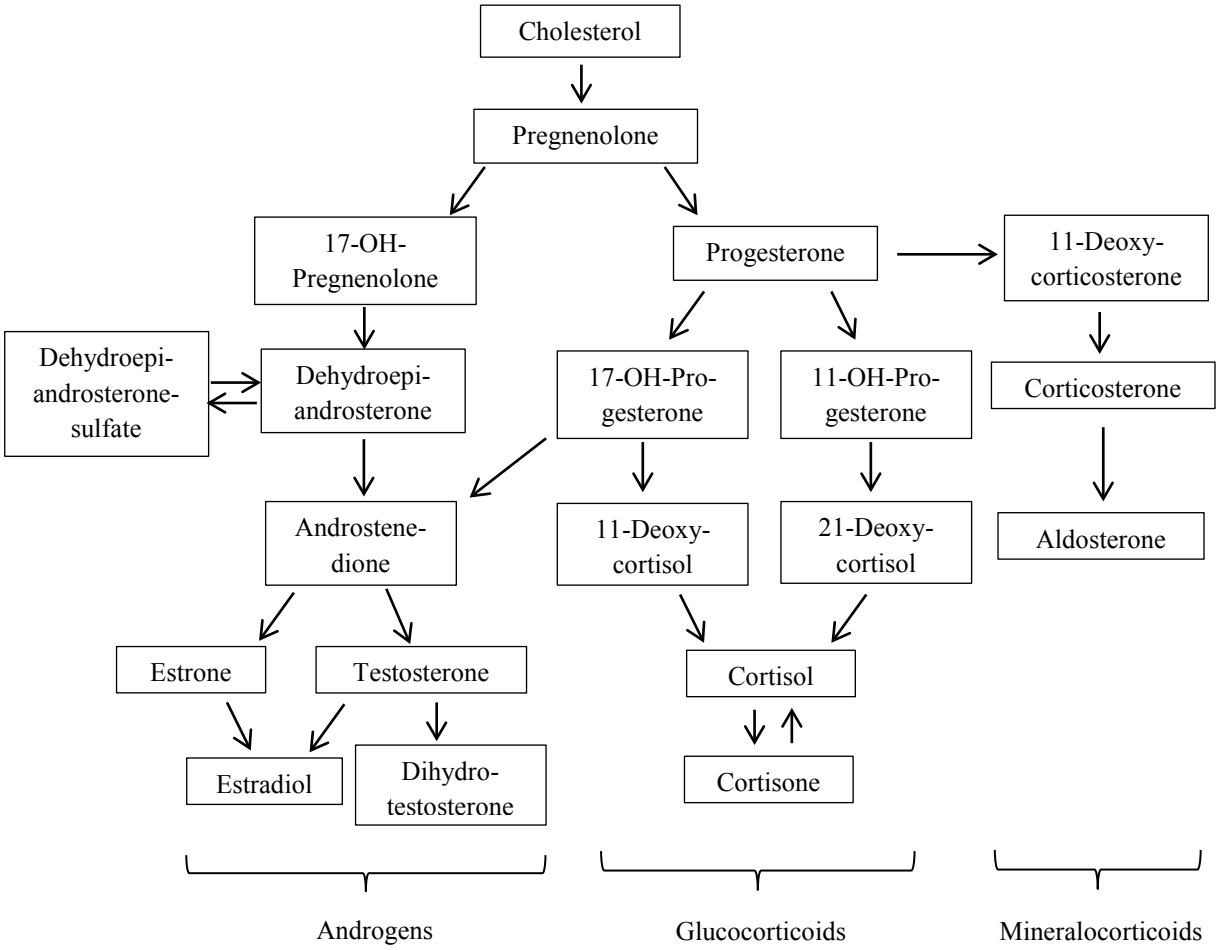

Figure 1: *Schematic biosynthetic pathway of corticosteroids.*

1.1.2. Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS)

There are different analytical measuring principles available for the quantification of corticosteroids and their precursors in human serum. In clinical routine laboratories they are usually quantified by immunoassays [7]. However, there are significant drawbacks of this technique such as cross reactivity with structurally related analytes, which can lead to ambiguous results as well as standardization issues between different laboratories, which can lead to substantial between-method bias [7, 8]. To overcome these problems, isotope dilution

liquid chromatography mass spectrometry is increasingly applied for quantification of steroid hormones [7, 9].

Liquid chromatography is an analytical technique used to separate different compounds of a mixture of substances such as diagnostic serum samples. By pumping the mixture of analytes in a mobile phase through an analytical column, the compounds interact differently with the stationary phase of the column according to their chemical properties, mainly their polarity. Thus, the retention time of the analytes on the stationary column varies, which leads to separation of the compounds [10].

To detect the separated analytes, liquid chromatography is often coupled to mass spectrometry, which differentiates the analytes due to different mass-to-charge-ratios and determines their molecular mass and their fragmentation patterns. To this end, the analytes are transferred into the gas phase, ionized in the ion source, e.g. by electrospray ionization, and accelerated through an electrostatic field into the analyzer. There are different analyzers; the most widely used in clinical laboratories are triple quadrupoles. A quadrupole consists of four parallel metal rods, to which an AC and DC voltage is applied, forming an electrical quadrupole field when diagonally opposing rods are on the same neighboring rods on inverted electrical potential. Only ions with a selected mass-to-charge ratio can pass this quadrupole field along the metal rods, other ions are emitted laterally and discharged by collision with the rods (see Figure 2).

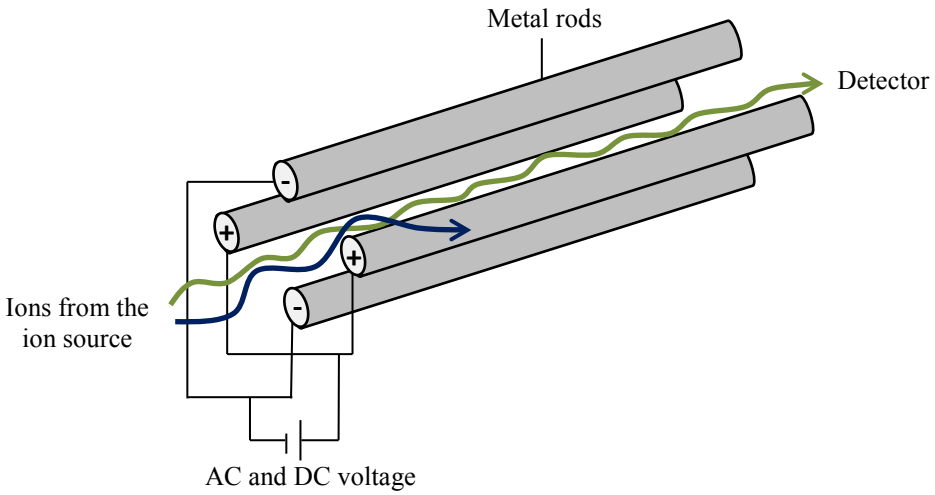

Figure 2: *Schematic structure of a quadrupole. Only ions with a determined mass-to-charge ratio have a stable flight pass and reach the detector (green line), other ions (blue line) are discharged.*

A triple quadrupole system consists of two quadrupoles which are connected via a collision cell (which is also built as a quadrupole). This structure is often referred to as tandem mass spectrometry due to the multiple steps of mass spectrometric selection. For quantification, the multi-reaction monitoring is widely used as mode of action: In the first quadrupole, ions with a defined mass-to-charge-ratio are selected. In the following collision cell these ions, the so-called precursors, are fragmented by collision with a collision gas (e.g. Nitrogen). The formed fragment ions, the so-called product ions, are highly specific for the analyte and they are transferred to the following quadrupole. Here, a determined ion fragment is selected and can pass to the detector, e.g. a photomultiplier [11] (see Figure 3).

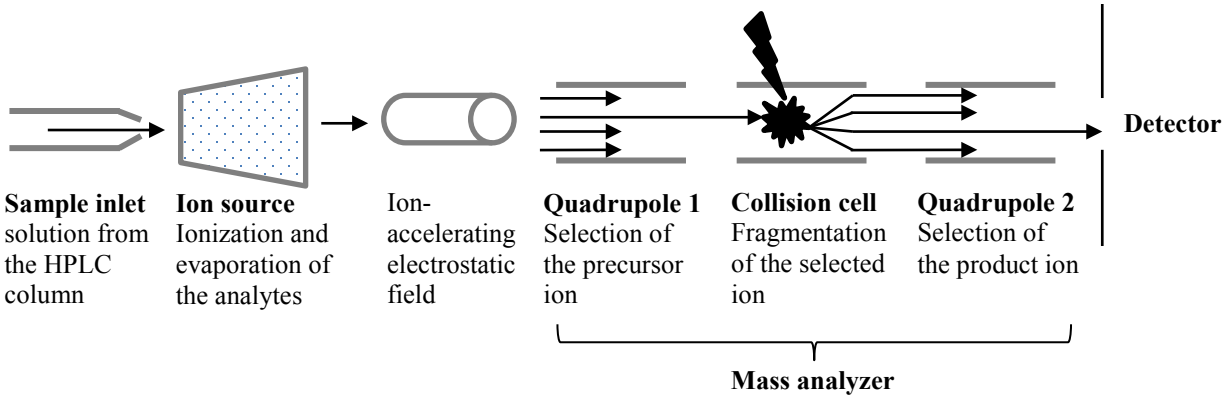

Figure 3: *Schematic diagram of a triple quadrupole mass spectrometer operating in multi-reaction monitoring.*

This technique enables an improved selectivity compared to immunoassays. Therefore, LC-MS/MS is becoming the method of choice for steroid measurement in clinical laboratories [7, 9, 12]. A further advantage of this technique is the possibility of simultaneous quantification of several analytes within one run [12] which is especially useful with regard to metabolic profiling, e.g. steroid profiling (see section 1.1.4.). Furthermore, LC-MS/MS methods can be developed independently, and thus, there is no dependence on industrial suppliers. Another essential aspect is the use of stable isotope-labelled internal standards in LC-MS/MS methods which is known as "isotope dilution" [13]. These internal standards have almost the same physical and chemical properties as the analytes but include an isotope labelling, e.g. the exchange of ^{12}C atoms with ^{13}C atoms or the replacement of hydrogen with deuterium. Due to the exchanged atoms the complete molecule has a different mass and can be discriminated in the mass spectrometer. At the beginning of the sample preparation, a known amount of those isotope-labelled substances is added to a determined sample volume which contains the respective analyte. For the quantification of the analyte, the area ratio of analyte area to internal standard area is used. Because the analyte and its isotope-labelled counterpart

undergo the same processing steps, the use of stable isotope-labelled internal standards corrects analyte losses due to the sample preparation. Furthermore, it compensates possible effects during ionization, e.g. ion suppression or ion enhancement, due to co-eluting components of the sample matrix [13-15], which is known as matrix effect. Because of different challenging matrices used in clinical laboratories, like serum, whole blood or urine, the isotope dilution approach is an important advantage of LC-MS/MS compared to other analytical approaches ensuring reliable and robust quantification.

1.1.3. ACTH stimulation test

Quantification of corticosteroids is an important task of clinical laboratories and there are diagnostic tests, e.g. the ACTH stimulation test, which rely on the measurement of at least one corticosteroid as read-out. The ACTH stimulation test assesses the functional capacity of the adrenal gland [16]. Therefore, 250 µg of synthetically produced peptide, which consists of 24 amino acids (Tetracosactidhexaacetat), with a comparable biological efficacy to ACTH, is intravenously administered. Blood sampling is performed before and 30 or 60 minutes after the intravenous injection; as standard read-out, serum cortisol is determined. According to the physiological function of ACTH described in section 1.1.1, the concentration of cortisol in healthy individuals increases after injection of the ACTH analogue. A missing increase is an indication of malfunction of the adrenal gland and therefore, the test is used as diagnostic tool for adrenal insufficiency [16, 17].

Furthermore, ACTH stimulation can also be used as a model for stress situations because it simulates maximum physiological stress. Thus it can be used for studying the biochemical stress response of the human body. By broadening the read-out to a set of steroids, a deeper insight to the physiological reactions to stress is possible. In this context, a profile of different steroids may be advantageous compared to solely measuring cortisol.

1.1.4. Steroid profiling

The quantification of several endogenous steroids at once is referred to as steroid profiling and allows for a deeper insight into the underlying biochemical processes of different disorders compared to measuring cortisol as single marker. Analogous to the more general metabolic profiling - the simultaneous assessment of various analytes related to a metabolic pathway [18] - steroid profiling is an increasingly widespread tool in clinical diagnostics

because of the gain of additional information. There are several clinical conditions in which measuring and monitoring steroid hormones and their corticoid precursors are meaningful. Steroid profiling is for example used to confirm congenital adrenal hyperplasia [19], in the diagnostic workup of adrenal dysfunction [20], and for differential diagnosis of adrenal tumors [21].

Besides its application in the diagnostic workup of different diseases, steroid profiling is also important in the field of clinical research. As mentioned in section 1.1.1., cortisol plays a crucial role in the biochemical stress response of the human body. However, the underlying processes of the stress reaction are up to now not yet completely understood. By measuring a panel of steroids instead of solely cortisol, a better understanding may be achieved.

1.1.5. Aim and scope

In this research project we aimed at utilizing the advantages of the LC-MS/MS technique for the measurement and quantification of corticosteroids. Because this technique overcomes the above-mentioned drawbacks of immunoassays, which are at present commonly used in routine laboratories for measuring corticosteroids, this can contribute to an improvement of diagnostic methods in clinical laboratories and thus to a better patient care.

In the first part of the project the objective was to develop and evaluate a LC-MS/MS method for the reliable quantification of 12 corticosteroids.

In the second part the clinical application of such a LC-MS/MS method was addressed. We obtained a corticosteroid profile of healthy individuals involved in an ACTH stimulation study by using a LC-MS/MS method. These data contribute to the establishment of normal ranges for corticosteroid concentrations in humans before and after ACTH stimulation.

1.2. Development of a multi-analyte isotope dilution LC-MS/MS method for corticosteroid profiling

As mentioned in section 1.1.1., diagnosing and monitoring of several endocrine disorders depend on the quantification of corticosteroids. Thus, the development of reliable and specific quantification methods for corticosteroids is important to provide meaningful information for the clinicians. Due to the above-mentioned advantages, LC-MS/MS is well suited for steroid analysis. Nevertheless, the method must be thoroughly established. Thereby, the major challenge of the method development is the sufficient chromatographic separation of isobars

which are substances with the same molecular mass and fragmentation pattern. These analytes cannot be differentiated in the mass spectrometer and chromatographic separation is therefore crucial for a reliable quantification of corticosteroids.

Most of the existing LC-MS/MS methods for steroid quantification use stationary phases having C18 groups as surface modification. To improve the chromatographic separation, we investigated the suitability of a reversed phase column with biphenyl groups on the surface. An improved resolution of our targeted analytes was achieved using such a stationary phase compared to the standard modification of C18-groups. This is expected as a consequence of possible π-π interactions between the biphenyl rings of the stationary phase and the steroid molecules [9], additional to the common van-der-Waals interactions between lipophilic groups, which represent the main interaction with a C18 modification. Another important aspect in method development is the sample preparation, which should be preferably short und not elaborate. Nevertheless, it is essential to remove matrix components like phospholipids and proteins, which could interfere with the measurement and contaminate the instruments. Therefore, we performed a protein precipitation with zinc trifluoroacetate (TFA) in methanol as a novel precipitation agent. TFA is volatile and therefore contamination of the mass spectrometer can be minimized. A comparison between this precipitation agent and the long-established methanol zinc sulfate precipitation showed a comparable precipitation efficacy regarding protein content and phospholipid profile. Additionally, we applied ammoniumfluoride as a rarely used additive for the mobile phase to improve the sensitivity of the method. This aspect is important due to the low concentrations of some endogenous steroids.

After the development, a method should be comprehensively validated to ensure that it provides reproducible, reliable results and to characterize the method performance. Unfortunately, widely accepted guidelines for method validations for methods assessing endogenous compounds are lacking. Thus, these assays are often validated according to the *Guideline of bioanalytical method validation* by the European Medicines Agency, which was actually established for methods addressing drug concentrations in biological matrices [22].

There are different method characteristics which should be addressed during validation. Very important is the assurance of specificity, in particular in the context of isobaric compounds. Accuracy and precision are also essential method characteristics which should be investigated during method validation. While precision is an indication of the consistency of repeated measurements and shows the spread of measuring points, accuracy describes the closeness of

the value measured by the method to the nominal value. The matrix effect is a further aspect which should be addressed during validation and reflects the potential influence of sample matrices on the measurement. Furthermore, the validation should describe carry-over, lower limit of quantification and stability [22].

These aspects were addressed in the first publication in which we could present a thoroughly developed and validated LC-MS/MS method for the quantification of 12 steroids which is suitable for steroid profiling.

The author of this doctoral thesis was involved in all parts of the described publication: Planning of the project, experimental laboratory work for the LC and MS method development, preparation of the samples series for the method validation, performing the measurements, data analysis and writing the manuscript.

1.3. Application of a multi-analyte isotope dilution LC-MS/MS method for corticosteroid profiling after ACTH administration in individuals without endocrine disorders

Since steroid profiling is becoming a frequently used tool in endocrinology, normal corticosteroid concentration ranges for individuals without endocrine disorders are necessary for the correct assessment of values measured in patients' samples. At the time of our investigation, there was already data available in literature for basal corticosteroid concentrations in healthy individuals, but there was only sparse comparative data available for corticosteroid concentrations after ACTH stimulation. Thus, the biochemical stress response to ACTH in healthy individuals regarding the respective corticosteroid concentrations has not been addressed sufficiently so far.

Therefore, we performed an ACTH stimulation study with individuals who are not suspected to have endocrinological disorders. The concentrations of 6 corticosteroids (cortisol, cortisone, corticosterone, 11-deoxycorticosl, 17-OH-progesterone, and 11-deoxycortico-sterone) were measured in the blood samples via a validated LC-MS/MS method before and after ACTH stimulation.

With this work we contributed to the establishment of normal ranges of corticosteroids after intravenous injection of ACTH. These data are necessary to study differences in the human stress response depending on the physical condition. A characteristic pattern of changes in the concentrations of the measured corticosteroids was obtained. Our results particularly suggest further investigation of corticosterone as a sensitive new stress marker for stress research but

maybe also for diagnostic testing, because it showed a much more pronounced increase after ACTH administration than cortisol, the standard read-out of the test. A further aspect revealed in our study is the missing standardization in steroid measurement among different laboratories and methods. Thus, inter-laboratory method comparisons seem to be necessary to standardize results of corticosteroid measurements.

To this publication, the doctoral candidate contributed by performing sample preparations and measurements of the study samples. Data analysis and writing of the manuscript are further contributions to this work.

1.4. Summary / Zusammenfassung

Corticosteroid profiling is becoming increasingly significant for diagnosing and differentiating various endocrinological disorders and for stress research. Therefore, reliable quantification methods for corticosteroids are mandatory. Due to the advantages of LC-MS/MS methods like improved selectivity compared to immunoassays and the possibility of using isotope-labelled internal standards, this technique is very appropriate to use it for the diagnostic workup of patients' samples.

Therefore, a LC-MS/MS method for quantification of 12 corticosteroids was developed in the first part of this doctoral thesis. By using a biphenyl column as stationary phase, very good separation of the isobaric compounds was achieved. The validation of the developed method proofs the suitability for the targeted steroid profiling.

In the second part, a profile of 6 corticosteroids of individuals not suspected to be suffering from endocrinological disorders was assessed before and after ACTH stimulation by using a LC-MS/MS method. The collected data can support the establishment of normal ranges of corticosteroid concentrations after ACTH stimulation and therefore contribute to the investigation of the biochemical stress response. Additionally, corticosterone was identified as potential new stress marker.

Corticosteroid Profiling gewinnt zunehmend an Bedeutung in der Diagnose und Differenzierung verschiedener endokrinologischer Erkrankungen sowie in der Stressforschung. Voraussetzung dafür sind verlässliche Methoden zur Quantifizierung der einzelnen Corticosteroide. Aufgrund der Vorteile der LC-MS/MS Methoden, z.B. der verbesserten Selektivität gegenüber Immunoassays und der möglichen Verwendung von

isotop-markierten internen Standards, sind sie für die diagnostische Bewertung von Patientenproben gut geeignet.

Im Rahmen dieser Doktorarbeit wurde im ersten Teilprojekt daher eine LC-MS/MS Methode zur Quantifizierung von 12 Corticosteroiden entwickelt. Durch die Verwendung einer Biphenylsäule als stationäre Phase konnte eine sehr gute Isobarentrennung erzielt werden. Die Validierung der entwickelten Methode zeigte, dass diese für die Erfassung von Steroidprofilen gut geeignet ist.

Im zweiten Teilprojekt der Arbeit wurde mittels einer LC-MS/MS Methode ein 6 Corticosteroide umfassendes Steroidprofil von Probanden ohne Verdacht auf endokrinologische Erkrankungen vor und nach ACTH Stimulation erfasst. Die erhobenen Daten können zur Erstellung von Normbereichen für Corticosteroidkonzentrationen nach ACTH Stimulation beitragen und stellen dadurch einen Beitrag zur Untersuchung der biochemischen Stressantwort dar. Zudem konnte Corticosteron als möglicher neuer, sensitiver Stressmarker identifiziert werden.

2. Original Articles

2.1. J.M. Lindner, M. Vogeser, S.H. Grimm, Biphenyl based stationary phases for improved selectivity in complex steroid assays, *J Pharm Biomed Anal* 142 (2017) 66-73

Journal of Pharmaceutical and Biomedical Analysis 142 (2017) 66–73

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Biphenyl based stationary phases for improved selectivity in complex steroid assays

Johanna M. Lindner*, Michael Vogeser, Stefanie H. Grimm

Institute of Laboratory Medicine, Hospital of the Ludwig-Maximilians-University Munich, Marchioninistraße 15, 81377 Munich, Germany

ARTICLE INFO

Article history:
Received 31 January 2017
Received in revised form 13 April 2017
Accepted 15 April 2017
Available online 19 April 2017

Keywords:
LC–MS/MS
Biphenyl column
Steroid profiling
Validation
Precipitation agent
Isobaric compounds

ABSTRACT

The measurement of steroid hormones and their corticoid precursors is an important aspect in endocrinology since these analytes are biomarkers for several endocrine disorders. Over the last few years, HPLC–MS/MS has become the method of choice to analyze these compounds. There are already several methods using stationary phases modified with C18 groups. However, since these columns sometimes do not enable sufficient separation of some isobaric steroids, we investigated the potential of a different RP modification using biphenyl groups for the separation of challenging isobars such as corticosterone, 11- and 21-deoxycortisol.

The aim of our work was the development of an isotope dilution UHPLC–MS/MS assay for clinical research that combines simple and effective sample preparation with a powerful MS method quantifying a broad steroid panel (aldosterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, 17-OH-progesterone, progesterone, and testosterone) in human serum.

After a manual protein precipitation step using zinc trifluoroacetate (ZnTFA) in methanol, the supernatants were directly injected into the UHPLC–MS system. Chromatographic baseline separation of all isobaric compounds (corticosterone ↔ 11-deoxycortisol ↔ 21-deoxycortisol, 17-OH-progesterone ↔ 11-deoxycorticosterone, and aldosterone ↔ cortisone) was achieved using a Kinetex Biphenyl column (150 × 2.1 mm, 1.7 μm) with a mobile phase consisting of 0.2 mM ammonium fluoride in water and methanol. The total run time was 10 min. For detection we used a Xevo TQ-S mass spectrometer operating in the ESI positive and negative modes. The method was validated according to the EMA guideline for bioanalytical method validation.

The results for accuracy (within-run: 92.3%–115%, between-run: 92.4 %–113%) and imprecision (within-run: 0.80%–9.05%, between-run: 1.98 %–15.2%) were satisfying. The recovery ranged from 95% to 111%. The matrix effect was between 93% and 112% and an excellent linearity with $R^2 > 0.99$ for all analytes was achieved.

It was demonstrated that biphenyl based columns are a powerful tool for comprehensive, MS based steroid assays including various isobaric substances. Additionally, we could evince that ZnTFA is a convenient precipitation agent suitable for steroid analysis.

1. Introduction

Steroid hormones and their corticoid precursors are important biomarkers to diagnose and monitor a broad spectrum of endocrine disorders such as Cushing's disease, adrenal failure, primary hyperaldosteronism, or congenital adrenal hyperplasia [1–3]. The assessment of a broad steroid panel could contribute to a better differentiation and understanding of these disorders [4–6]. Additionally, cortisol and its precursors are involved in the human stress response [7], therefore steroid profiling can contribute to a better understanding of the underlying biochemical processes under stress conditions. As a consequence, it may allow insight into the stress situation of the human body in critical situations such as surgery and critical illness, especially since it was found that the total serum cortisol level correlates with the severity of the illness of critical ill patients [8].

In clinical routine laboratories, steroid hormones are commonly measured by immunoassays. Although this analytical technique is well established and has been used for a long time, there are some important drawbacks that can compromise the results. Cross

* Corresponding author.
E-mail address: johanna.lindner@med.uni-muenchen.de (J.M. Lindner).

http://dx.doi.org/10.1016/j.jpba.2017.04.020
0731-7085/

reactivity with structurally related components or metabolites is an issue as well as a poor agreement between commercially available immunoassays and a lack of sensitivity [9–12]. Recently, HPLC–MS/MS assays addressing steroid hormones started to replace conventional immunoassays [13]. Due to its sensitivity and, more importantly its specificity, the HPLC–MS/MS technique is a highly attractive analytical method for differentiating between structurally very similar steroids that cannot be achieved by immunoassays. Furthermore, it allows for the possibility of analyzing a broad panel of target analytes simultaneously. As a consequence, this technique is becoming the gold standard in the field of clinical research and routine diagnostics [11,13].

Even if mass spectrometry allows discrimination between structurally similar compounds, it cannot differentiate between so-called isobaric substances, which have the same mass of the intact molecule but also of the respective fragments. Thus, the main challenge of steroid HPLC–MS/MS assays is the chromatographic separation of these isobaric compounds. So far, stationary phases modified with C18 groups are the standard for steroid assays. However, separating isobaric compounds with these stationary phases requires complex solvent gradients combined with long run times. Reverse phase columns based on biphenylic groups seem to be a promising tool for corticosteroid separation because of the formation of π-π interactions with the steroid molecules [13].

Therefore, we decided to develop and evaluate a clinical research LC–MS/MS assay for a broad steroid panel that includes several isobaric compounds using a biphenyl column as the stationary phase. Simultaneous, reliable and sensitive quantification of 12 endogenous steroids in human serum should be combined with a simple and effective sample preparation method employing a small sample volume.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and water were of UHPLC grade and purchased from Biosolve (Valkenswaard, The Netherlands). Standards for aldosterone, corticosterone, cortisone, cortisol, 11-deoxycorticosterone, 11-deoxycortisol, 17-OH-progesterone, progesterone, and testosterone; labelled internal standards for aldosterone-d_7, DHEA-d_6, DHEAS-d_5, progesterone-d_9, and testosterone-$^{13}C_3$; ammonium fluoride as mobile phase additive, and zinc trifluoroacetate (ZnTFA) as precipitating agent were purchased from Sigma-Aldrich (Steinheim, Germany). 21-Deoxycortisol, DHEA, DHEAS, 21-deoxycortisol-d_8, and cortisol-d_3, were obtained from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA) and cortisone-d_8, 11-deoxycorticosterone-d_8, and 11-deoxycortisol-d_7, were from Toronto Research Chemicals (Toronto, Canada). Corticosterone-d_8 and 17-OH-progesterone-d_8 were purchased from CDN Isotopes (Pointe-Claire, Canada).

Steroid stripped serum used for calibration and QC samples was obtained by unspecific depletion with charcoal.

2.2. Calibrators, quality control samples (QC) and internal standard solution

Stock solutions of the steroids and internal deuterated standards were prepared in methanol containing a nominal concentration of 100 μg/mL. Aldosterone-d_7 was dissolved in acetonitrile. Solutions were stored at −80 °C. The respective steroids were combined to obtain a methanolic master calibration solution. The calibrators were prepared by serial dilution of this methanolic master calibration solution with human steroid stripped serum. Three concentration levels of QCs based on human steroid stripped serum were spiked at low, medium, and high concentrations within the calibration range. Additionally, certified controls [GC–MS and LC–MS reference measurement procedure specified proficiency testing materials purchased by the *reference institute for bioanalysis* (RfB Bonn, Germany)] [14–18] were used for aldosterone, cortisol, DHEAS, 17-OH-progesterone, progesterone, and testosterone. The exact concentrations of the master calibration solution, calibrators and QCs are shown in Table 1 and Table 3.

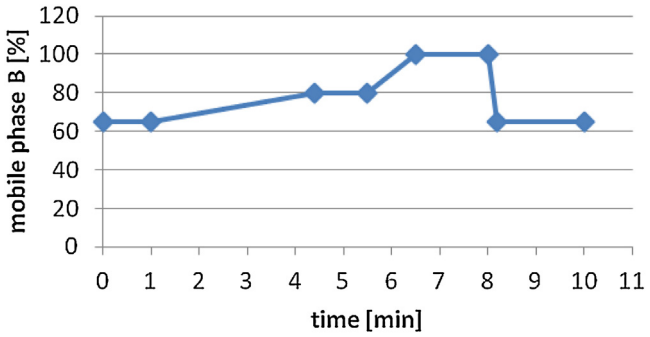

Fig. 1. LC-gradient of the chromatographic method. Mobile phase A: 0.2 mM ammonium fluoride in water; mobile phase B: methanol.

The internal standard solution was prepared in methanol by mixing the stock solutions of the deuterated standards to the following concentrations: cortisol-d_3 50 ng/mL; cortison-d_8, corticosterone-d_8, 11-deoxycortisol-d_7, and 21-deoxycortisol-d_7 10 ng/mL; 17-OH-progesterone-d_8, 11-deoxycorticosterone-d_8, aldosterone-d_7, progesterone-d_9, and testosterone-d_3 5 ng/mL; DHEA-d_6 70 ng/mL, and DHEAS-d_5 200 ng/mL.

2.3. Sample preparation

The precipitation agent was prepared freshly every day by diluting the internal standard solution 1:100 with a methanolic 124 mM ZnTFA solution. An aliquot of 50 μL of the sample (or calibrator, QC, blank) was mixed with 25 μL precipitation agent. The samples were vigorously mixed for 10 s and shaken for 10 min. A centrifugation step followed (10 min, 15 °C, 15 000 rpm). The clear supernatants were transferred to glass vials with micro inserts (Chromatographie Handel Müller, Fridolfing, Germany) and placed into an autosampler.

2.4. UHPLC and MS/MS conditions

For the analysis of the samples an Acquity UHPLC system, consisting of an autosampler, a switching valve, a column oven, and two pairs of pumps, coupled to a Xevo TQ-S was used (Waters, Milford, Massachusetts, USA). For controlling the instruments, data acquisition, and processing Mass Lynx V 4.1 software (Waters) was used. Chromatographic separation was performed on a Kinetex Biphenyl column (150 × 2.1 mm, 1.7 μm; Phenomenex, Torrance, California, USA) equipped with a Security Guard ULTRA cartridge (UHPLC Biphenyl, for 2.1 mm ID columns, Phenomenex). The column oven was kept at 50 °C, the injection volume was set to 5 μL. Mobile phase A was composed of 0.2 mM ammonium fluoride in H_2O (pH ~5.6); mobile phase B consisted of methanol. The gradient is shown in detail in Fig. 1. The flow rate was set to 0.3 mL/min. The total run time amounted to 10 min. In order to reduce contamination of the mass spectrometer the first 0.5 min of the eluents were sent to waste.

The parameters of the mass spectrometer were optimized as follows: source temperature 150 °C, source gas flow 150 L/h, desolvation temperature 500 °C, desolvation gas flow 500 L/h, a capillary voltage of 1.3 kV and a cone voltage of 30 V for positive electro-

Table 1
Concentrations [ng/ml] of the master calibration mix (MCM) and the calibrators (Cal). ALDO: aldosterone, 17-OHP: 17-OH-progesterone, 21-DF: 21-deoxycortisol, B: corticosterone, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone sulfate, DOC: 11-deoxycorticosterone, E: cortisone, F: cortisol, PG: progesterone, S: 11-deoxycortisol, T: testosterone.

	ALDO	17-OHP	21-DF	B	DHEA	DHEAS	DOC	E	F	PG	S	T
Range	0.25-48.7	0.08-36.9	0.19-93.0	0.23-115	4.76-476	20.1-5025	0.02-25.1	1-250	1.08-538	0.05-24.6	0.12-59.2	0.02-25.1
MCM	243	923	465	575	4755	50250	251	2498	2692	246	592	251
Cal 1	0.02	0.08	0.04	0.04	0.38	4.02	0.02	0.20	0.22	0.02	0.04	0.02
Cal 2	0.05	0.18	0.09	0.11	0.95	10.05	0.05	0.50	0.54	0.05	0.12	0.05
Cal 3	0.10	0.37	0.19	0.23	1.90	20.1	0.10	1.00	1.08	0.10	0.24	0.10
Cal 4	0.25	0.93	0.47	0.58	4.76	50.3	0.25	2.50	2.69	0.25	0.59	0.25
Cal 5	0.97	3.69	1.86	2.30	19.0	201	1.00	9.99	10.8	0.98	2.37	1.00
Cal 6	4.87	18.5	9.30	11.5	95.1	1005	5.02	50.0	53.8	4.92	11.8	5.02
Cal 7	9.73	36.9	18.6	23.0	190	2010	10.1	99.9	108	9.83	23.7	10.03
Cal 8	24.3	92.3	46.5	57.5	476	5025	25.1	250	269	24.6	59.2	25.1
Cal 9	48.7	185	93.0	115	951	10050	50.3	500	538	49.2	118	50.2

Concentrations printed in grey are outside the calibration range.

Table 2
MS/MS parameters for the targeted steroids and their internal standards.

Analyte	Molecular weight [g/mol]	Corresponding internal standard	Retention time [min]	Precursor ion [m/z]	Quantifier product ion [m/z]	Quantifier CE [eV]	Qualifier product ion [m/z]	Qualifier CE [eV]	Dwell time [s]
Aldosterone[a]	360.19	Aldosterone-d_7	3.30	361.0	343.0	16			0.012
Corticosterone[b]	346.21	Corticosterone-d_8	4.21	347.0	120.9	24	96.9	24	0.012
Cortisol	362.21	Cortisol-d_3	2.47	363.1	120.9	20	309.2	18	0.020
Cortisone[a]	360.19	Cortisone-d_8	2.74	361.0	163.1	24	121.0	28	0.012
11-Deoxycorticosterone[c]	330.21	11-Deoxycorticosterone-d_8	5.85	331.1	96.9	22	109.0	26	0.049
11-Deoxycortisol[b]	346.21	11-Deoxycortisol-d_7	3.86	347.1	96.9	26	109.0	26	0.012
21-Deoxycortisol[b]	346.21	21-Deoxycortisol-d_8	2.91	346.9	120.9	26	311.1	14	0.012
DHEA	288.21	DHEA-d_6	4.53	271.1	252.8	16	196.7	20	0.016
DHEAS	368.17	DHEAS-d_5	1.68	367.2	96.9	−20			0.024
17-OH-Progesterone[c]	330.21	17-OH-Progesterone-d_8	4.85	331.1	96.9	24	108.9	26	0.016
Progesterone	314.22	Progesterone-d_9	7.33	315.1	108.9	24	96.9	24	0.055
Testosterone	288.21	Testosterone-d_3	4.80	289.1	96.9	24	109.0	28	0.016
Aldosterone-d_7	367.19		3.23	369.2	351.0	16			0.012
Corticosterone-d_8	354.21		4.13	355.0	337.2	14			0.012
Cortisol-d_3	365.21		2.46	366.0	120.9	22			0.020
Cortisone-d_8	368.19		2.71	369.1	168.0	24			0.012
11-Deoxycorticosterone-d_8	338.21		5.77	339.0	99.9	26			0.049
11-Deoxycortisol-d_7	353.21		3.81	354.0	99.9	24			0.012
21-Deoxycortisol-d_8	354.21		2.87	354.9	319.2	14			0.012
DHEA-d_6	294.21		4.49	277.0	259.1	14			0.016
DHEAS-d_5	373.17		1.68	372.1	97.8	−28			0.024
17-OH-Progesterone-d_8	338.21		4.80	339.0	99.9	22			0.016
Progesterone-d_9	323.22		7.26	324.0	99.9	20			0.055
Testosterone-$^{13}C_3$	291.21		4.78	292.1	97.0	24			0.016

[a,b,c]Respective isobaric compounds.

spray ionization (ESI +), which was used for all analytes except for DHEAS. For this analyte, we used negative electrospray ionization (ESI-): source temperature 150 °C, source gas flow 150 L/h, desolvation temperature 500 °C, desolvation gas flow 700 L/h, a capillary voltage of 2.5 kV and a cone voltage of 40 V. For all analytes and internal standards, multiple reaction monitoring was performed. The acquired mass transitions are shown in Table 2.

Post-column infusion of neat solutions was used to optimize MS parameters, mass transitions, and collision energy of the respective analytes.

The software TargetLynx (Waters) was used for quantification. The ratio of the peak area of the quantifier (first mass transition of an analyte) to the peak area of the internal standard was calculated. The qualifier (second mass transition) was used to verify the retention time of the analytes. In order to improve selectivity and detect potential interferences, especially in native samples, we monitored the ratio of the quantifier and the qualifier. For linear regression, a $1/x^2$ weighting was used and the origin was excluded. For smoothing of the peaks, the mean smoothing algorithm with two iterations and a smoothing width of three was employed.

2.5. Method validation

The method was validated according to the *Guideline of bioanalytical method validation* by the European Medicines Agency (EMA) [19].

Five independent sample series were analyzed on different days. Each series was composed of a blank sample, a zero sample (containing internal standards but no analytes), calibrators, and QC samples. The calibrators were prepared and analyzed in duplicate in each series; the QC samples and the LLOQ sample (lower limit of quantification = lowest calibration standard) were prepared and analyzed 5 times.

The linearity of the method was investigated by measuring the calibration standards for at least 5 different concentration levels. The correlation coefficient and the slope were calculated.

For evaluation of within- and between-run imprecision and accuracy, three levels of QC samples and the LLOQ samples were examined (n = 5). The variation coefficient (CV), which demonstrates the imprecision, was calculated for each concentration level and analyte and should not exceed 15% according to the EMA guide-

line. For the accuracy, the deviation from the nominal value should not exceed 15%.

The LLOQ of corticosterone, cortisone, cortisol, 11-deoxycortisol, DHEAS, 17-OH-progesterone, progesterone, and testosterone was defined as a reasonable concentration regarding its physiological range or for aldosterone, 11-deoxycorticosterone, 21-deoxycortisol, and DHEA as a concentration that has a signal-to-noise ratio of at least 5:1 combined with an inaccuracy and imprecision $\leq$20 %.

To investigate potential carry-over, methanol was injected after the measurement of the highest calibrator. The methanol sample was examined for analyte peaks.

Dilution integrity was evaluated by spiking a sample with a concentration above the calibration range and dilution of this sample in two different ways (1:5 and 1:10). The measured concentration was compared to the expected concentration.

The freeze and thaw stability of the QC samples was investigated in 5 freeze and thaw cycles, in which the samples were completely thawed and refrozen.

To evaluate the stability of the processed samples, they remained in the autosampler at 8 °C for 24 h and were measured again.

We evaluated the matrix effect and the recovery by using a pre- and post-spiking experiment according to the procedure given by Matuszewski et al. [20]. In this way, 6 different lots of serum were assessed. The serum was collected in Sarstedt tubes (S-Monovette REF 01.1601). Three aliquots of a serum sample were spiked before extraction and 3 aliquots were spiked after extraction. The recovery was calculated as follows:

$$\text{Recovery} = 100\% \times c_{\text{serumpre}}/c_{\text{serumpost}}$$

To examine the matrix effect, the baseline value of steroids in the serum lots had to be determined by measuring unspiked aliquots of each lot (n = 3). These values were subtracted from the measured values in the samples spiked after extraction. The peak areas of the samples spiked after extraction were then compared to the peak areas of the pure analyte solution in absence of matrix, which was spiked with the same amount of analytes.

$$\text{Matrixeffect} = 100\% \times (c_{\text{serumpost}} - c_{\text{serumbaseline}})/c_{\text{solvent}}$$

Additionally, the *Guideline of bioanalytical method validation* from the EMA suggests calculating the *IS normalized matrix factor* and the variation coefficient of this factor, which should not be greater than 15%:

$$\text{Matrixfactor(MF)} = \text{Area}_{\text{matrix}}/\text{Area}_{\text{solvent}}$$

$$\text{MF}_{\text{normalized}} = \text{MF}_{\text{analyte}}/\text{MF}_{\text{IS}}$$

A post-column infusion experiment was used to further evaluate the matrix effect. Unspiked matrix was processed and injected on the column according to the procedure outlined by Bonfiglio et al. [21]. A solution of all analytes was simultaneously infused into the mass spectrometer using T-tubing and a syringe pump. The infusion chromatogram of the injected processed matrix was compared to an infusion chromatogram of injected methanol. Observation of differences would indicate an influence of the matrix.

In each run, the mean of the ion ratios of the calibration standards were calculated and compared to the mean of the ion ratios of the serum samples. Deviations in the ion ratios would indicate interferences in the sample. The ion ratio in the serum samples should not change by 20% from that of the mean ratio of the calibration standards [22]. The variation coefficient was calculated to monitor alterations of the ratios, which should be very little. The ratios of different runs were compared. Changes could be an indicative for ionization problems.

2.6. Evaluation of sample clean-up

To measure the content of proteins of the processed samples, and therefore the efficacy of the deproteination step, a NanoDrop C2000 (Thermo Scientific, Waltham, Massachusetts, USA) was used. To further investigate the purity of the processed samples, phospholipid profiles were assessed with the mass spectrometer according to the procedure described by Little et al. [23].

3. Results and discussion

3.1. Method development

3.1.1. HPLC–MS/MS method

The main challenge of the analytical development was the establishment of a chromatographic method that separates the isobaric compounds of the panel (corticosterone, 11-deoxycortisol and 21-deoxycortisol; 17-OH-progesterone and 11-deoxycorticosterone; and aldosterone and cortisone). Although there are published methods using C 18 reversed phase columns for similar panels [1], we could not achieve adequate separation of our targeted isobaric compounds, i.e., corticosterone and 21-deoxycortisol. Therefore, the suitability of a biphenyl column (100 mm of length) was investigated. In our case, this column improved the chromatographic resolution, but baseline separation of the isobaric compounds could only be achieved by using a rather long 150 mm biphenyl column (Fig. 2). In addition to the chromatographic separation, the sensitivity was also optimized. For this purpose, we employed two different additives of the mobile phase: ammonium formate and ammonium fluoride in different concentrations. Additionally, two different ionization techniques, ESI and APCI, were examined for several analytes. In the end, we decided to use ESI in combination with 0.2 mM ammonium fluoride as solvent additive.

3.1.2. Sample preparation

Methanolic zinc sulfate solutions are widely used as precipitation agents for steroids assays [24–26]. We compared this established reagent with a methanolic ZnTFA solution. In this regard, a mixture of 80% methanol and 20% aqueous zinc sulfate (89 g $ZnSO_4$ heptahydrate in 1 L water – equal to 62 mM) was compared to a mixture of 80% methanol and 20% aqueous ZnTFA solution (96 g ZnTFA in 1 L water – equal to 62 mM). According to the measured protein content (0.6% remaining proteins in the samples that were precipitated with ZnTFA and 0.7% remaining proteins in the samples that were precipitated with $ZnSO_4$) the deproteination efficiencies of the two reagents were comparable. The phospholipid profiles of respective processed serum samples were also similar, and neither a phospholipid carry over nor build up was observed for either precipitation reagent. Due to the volatility of TFA in contrast to sulfate, we decided to use ZnTFA as the precipitation agent to minimize the contamination of the mass spectrometer.

To further increase the sensitivity of the method, we reduced the volume of the precipitation agent while simultaneously increasing the concentration of ZnTFA. Thus, we could create an agent with the same efficacy, i.e., remaining protein content and phospholipid profile, but with less dilution of the sample. The final precipitation agent was composed of 124 mM ZnTFA in pure methanol.

3.2. Method validation

Linearity could be shown for all analytes over the whole calibration range with a correlation coefficient of $R^2 > 0.99$. The back calculated concentrations of all calibration standards of the 5 runs were within ± 15% of the nominal value. The responses of the LLOQs

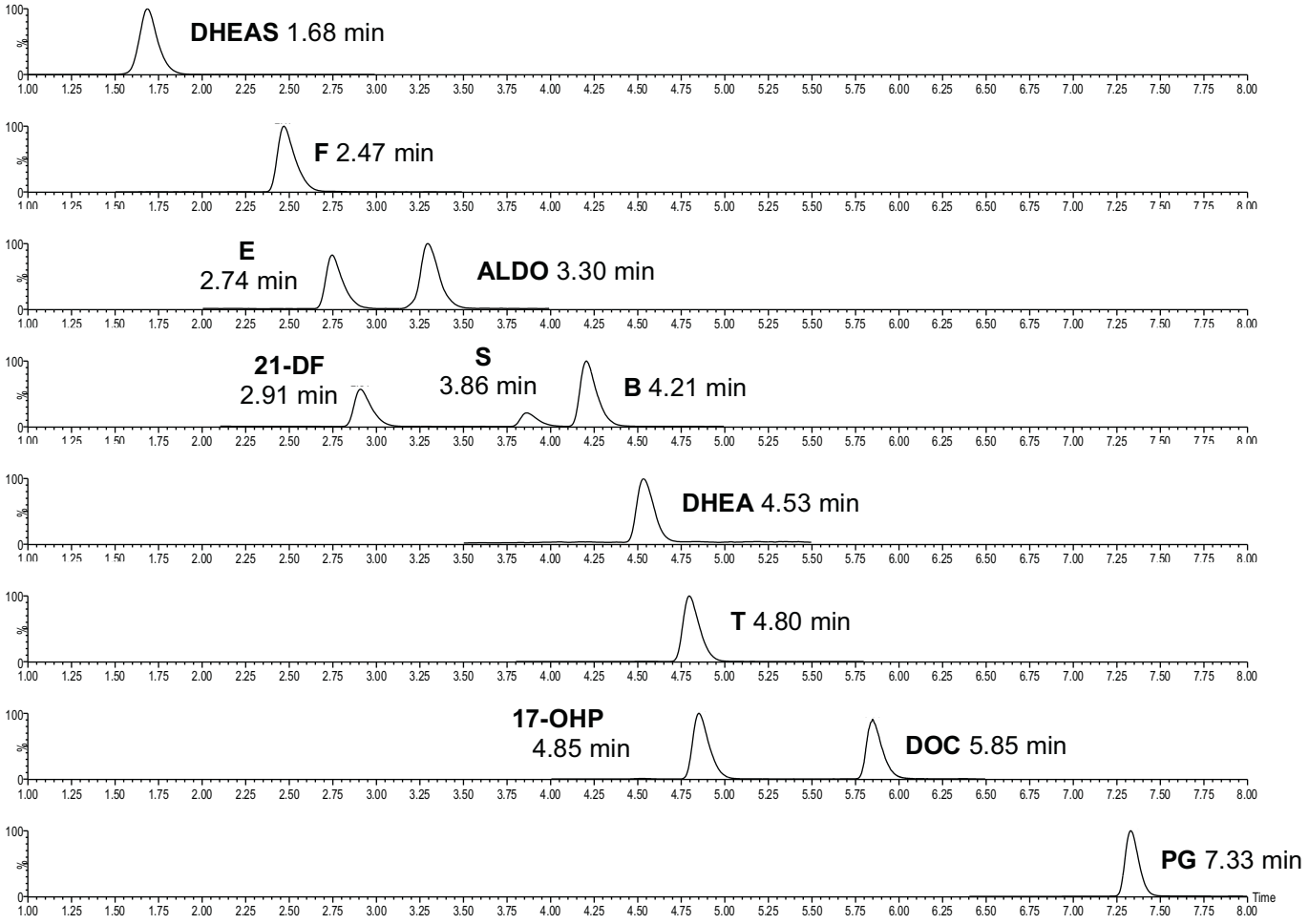

Fig. 2. Chromatograms of all analytes. ALDO: aldosterone, B: corticosterone, 21-DF: 21-deoxycortisol, DHEA: dehydroepiandosterone, DHEAS: dehydoepiandrosterone sulfate, DOC: 11-deoxycorticosterone, E: cortisone, F: cortisol, S: 11-deoxycortisol, 17-OHP: 17-OH-progesterone, PG: progesterone, T: testosterone.

were at least 5 times the response of the blank sample. Therefore, the criteria of the EMA were fulfilled.

Evaluation results for accuracy and imprecision (within- and between-run) were good for all analytes. All quality controls and the LLOQs were within the limits of the EMA guideline (±15% and ±20% for the LLOQs, respectively). Table 3 shows the accuracy and imprecision values of the QCs and LLOQs for all analytes.

Also the certified controls showed an accuracy and imprecision of less than 5% for cortisol, 17-OH-progesterone, progesterone and testosterone (see Table 3).

The carry-over was below the respective LLOQs and therefore negligible. The dilution integrity could be proven by deviations from the calculated values between 0.2% and 11.2% for all analytes and both dilution steps.

All analytes were stable for at least 5 freeze- and thaw cycles, the deviation ranged from 91.5% to 101%. The deviation of the samples stored for 24 h in the autosampler was also below the 15% suggested by the EMA (87.3%–107%). Therefore, the extracted samples were determined to be stable over the runtime of a large batch.

The results for the recovery were good: 103% cortisol, 94.9% cortisone, 104% corticosterone, 101% 11-deoxycortisol, 97.5% 17-OH-progesterone, 111% 11-deoxycorticosterone, 101% aldosterone, 94.6% progesterone, 96.0% 21-deoxycortisol, 96.2% DHEA, 107% DHEAS and 98.7% testosterone.

Additionally, the observed matrix effect has no relevant influence on the measurement (99.0% cortisol, 112% cortisone, 93.1% corticosterone, 100% 11-deoxycortisol, 101% 17-OH-progesterone, 110% 11-deoxycorticosterone, 104% aldosterone, 98.4% progesterone, 104% 21-deoxycortisol, 101% DHEA, 97.0% DHEAS and 100% testosterone). The variation coefficient of the *IS normalized matrix factor* ranged from 5.81% to 12.7%, only the value for DHEAS had a variation coefficient that was slightly higher than the recommended 15% (17.1%). The result of the post-column infusion experiment indicates ion suppression for all analytes of the matrix samples compared to a solvent injection (see Fig. 3, and Fig. S1 in the supplementary material in the online version at DOI: http://dx.doi.org/10.1016/j.jpba.2017.04.020). However, analyzing the results of the experiments according to Matuszewski et al., it could be assumed that the occurring matrix effect can be compensated by the internal standard, and no relevant matrix effect was observed.

It has to be noted that we could not achieve the required sensitivity for aldosterone and 21-deoxycortisol in healthy individuals. A modification of our LC systems that allowed a post-column infusion of 5% isopropanol to the eluent increased the signals but only up to 30%, which was still insufficient for our purpose. A promising alternative to overcome this obstacle is a more laborious sample preparation that enables an enrichment of the analyte, e.g., offline SPE or LLE [27,28]. However, we also present the chromatographic results for aldosterone and 21-deoxycortisol to demonstrate the separation performance of a biphenyl stationary phase.

The monitored ion ratios showed only little variation. The calculated within- and between- run CVs of the ratios ranged from 0.14%

Table 3
Results for accuracy and imprecision.

Analyt	LLOQ	QC 1	QC 2	QC 3	Certified Controls
Aldosterone [ng/mL]	0.25	0.30	0.97	29.2	0.44
Imprecision within-run [%]	8.32	4.04	2.47	4.50	2.44
Imprecision between-run [%]	8.91	5.28	11.1	6.43	7.66
Accuracy within-run [%]	102	110	106	110	104
Accuracy between-run [%]	101	110	108	107	104
Corticosterone [ng/mL]	0.23	0.33	11.5	68.9	
Imprecision within-run [%]	3.66	1.80	2.67	3.02	
Imprecision between-run [%]	8.39	2.12	4.30	4.04	
Accuracy within-run [%]	103	112	105	101	
Accuracy between-run [%]	102	112	105	101	
Cortisol [ng/mL]	1.08	3.24	80.8	323	101
Imprecision within-run [%]	3.13	2.08	2.64	4.04	1.70
Imprecision between-run [%]	9.79	3.53	5.56	5.09	2.59
Accuracy within-run [%]	97.5	106	106	107	100
Accuracy between-run [%]	95.6	106	106	107	100
Cortisone [ng/mL]	1.00	3.00	50.0	200	
Imprecision within-run [%]	3.25	1.99	2.96	5.26	
Imprecision between-run [%]	9.15	2.78	12.0	6.47	
Accuracy within-run [%]	96.9	108	113	111	
Accuracy between-run [%]	95.3	108	113	112	
11-Deoxycorticosterone [ng/mL]	0.02	0.03	1.01	15.1	
Imprecision within-run [%]	5.32	2.95	1.51	3.44	
Imprecision between-run [%]	5.36	5.49	2.34	4.60	
Accuracy within-run [%]	100	115	113	107	
Accuracy between-run [%]	100	109	113	108	
11-Deoxycortisol [ng/mL]	0.12	0.36	5.92	35.5	
Imprecision within-run [%]	3.36	1.75	2.84	3.15	
Imprecision between-run [%]	10.9	2.07	4.78	4.46	
Accuracy within-run [%]	104	113	111	109	
Accuracy between-run [%]	103	113	111	109	
21-Deoxycortisol [ng/mL]	0.19	0.57	9.30	55.8	
Imprecision within-run [%]	6.16	2.97	2.05	4.30	
Imprecision between-run [%]	15.2	5.51	5.63	6.99	
Accuracy within-run [%]	97.9	106	106	102	
Accuracy between-run [%]	96.1	107	106	101	
DHEA [ng/mL]	4.76	14.3	71.3	285	
Imprecision within-run [%]	9.05	2.55	3.01	2.79	
Imprecision between-run [%]	11.6	3.53	4.62	4.40	
Accuracy within-run [%]	103	109	110	109	
Accuracy between-run [%]	103	109	110	110	
DHEAS [ng/mL]	20.1	62.9	525	3147	4660
Imprecision within-run [%]	6.29	5.43	7.41	7.31	2.32
Imprecision between-run [%]	9.61	8.50	11.9	9.18	8.36
Accuracy within-run [%]	98.8	97.3	92.3	96.5	99.9
Accuracy between-run [%]	97.8	97.7	92.4	99.5	101
17-OH-Progesterone [ng/mL]	0.08	0.12	1.23	30.8	1.70
Imprecision within-run [%]	5.52	2.36	2.71	4.17	1.10
Imprecision between-run [%]	8.48	5.58	4.10	5.15	1.98
Accuracy within-run [%]	94.1	111	110	109	99.1
Accuracy between-run [%]	94.1	111	110	112	99.3
Progesterone [ng/mL]	0.05	0.15	0.98	14.8	3.14
Imprecision within-run [%]	6.45	2.15	3.16	3.80	0.95
Imprecision between-run [%]	11.5	3.24	5.54	6.02	2.05
Accuracy within-run [%]	103	111	112	108	101
Accuracy between-run [%]	102	112	112	108	101
Testosterone [ng/mL]	0.02	0.03	1.00	15.1	2.94
Imprecision within-run [%]	5.39	2.27	2.62	3.67	0.80
Imprecision between-run [%]	9.20	3.72	4.09	4.22	2.85
Accuracy within-run [%]	99.8	112	111	109	104
Accuracy between-run [%]	99.8	106	110	110	104

to 6.7%. The deviation between the ratios of the calibration standards and the ratios of the patient samples was <1.3%. Therefore, no indication of an unknown interference in the patient samples was observed. A detailed presentation of the ion ratios can be found in Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.jpba.2017.04.020, in the supplementary material.

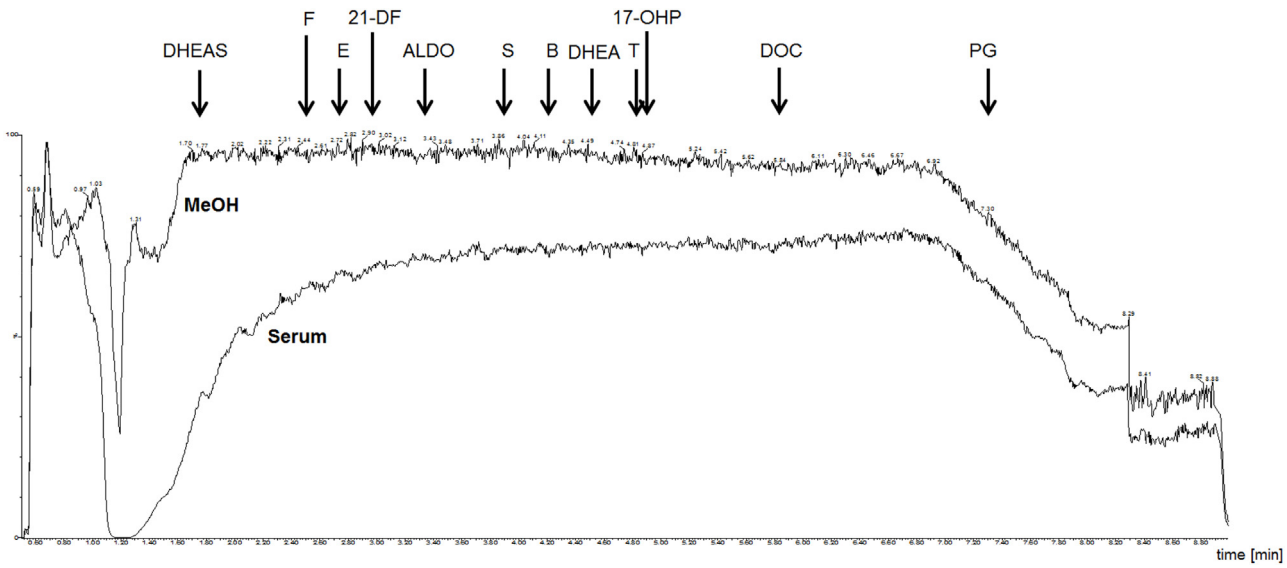

Fig. 3. Chromatogram of the post column infusion experiment. The infusion chromatograms of a methanol (MeOH) injection and a processed serum sample injection are shown. All analytes were post-column infused. ALDO: aldosterone, B: corticosterone, 21-DF: 21-deoxycortisol, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone sulfate, DOC: 11-deoxycorticosterone, E: cortisone, F: cortisol, S: 11-deoxycortisol, 17-OHP: 17-OH-progesterone, PG: progesterone, T: testosterone.

4. Conclusion

We have described a sensitive and selective UHPLC–MS/MS method for identification and quantification of a large steroid panel. This method for clinical research is innovative with respect to previous methods in two essential aspects:

1) The novel agent for protein precipitation, i.e., 124 mM zinc trifluoroacetate in methanol enables a good sample clean-up combined with a low dilution factor of the sample – a critical point for low abundant compounds such as 21-deoxycortisol and 11-deoxycorticosterone.
2) Concerning the selectivity of the assay, we could demonstrate that biphenyl based stationary phases are a powerful tool for steroid assays. Biphenyl groups enable a good separation of the isobaric compounds, which are widely occurring in steroid assays. Therefore, these stationary phases can be tested as an alternative in cases in which C 18 columns do not lead to the required separation. In combination with an elaborate sample preparation that enables an enrichment of analytes, the resulting selectivity may facilitate the assessment of new, expanded steroid profiles including highly related parameters such as corticosterone, 11- and 21-deoxycortisol.

Acknowledgement

This work was supported by the Hans Fischer Society Munich, Germany.

References

[1] M. Peitzsch, T. Dekkers, M. Haase, F.C. Sweep, I. Quack, G. Antoch, G. Siegert, J.W. Lenders, J. Deinum, H.S. Willenberg, G. Eisenhofer, An LC-MS/MS method for steroid profiling during adrenal venous sampling for investigation of primary aldosteronism, J. Steroid Biochem. Mol. Biol. 145 (2015) 75–84.
[2] C. Rossi, L. Calton, G. Hammond, H.A. Brown, A.M. Wallace, P. Sacchetta, M. Morris, Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography-tandem mass spectrometry, Clin. Chim. Acta 411 (2010) 222–228.
[3] L.K. Nieman, B.M. Biller, J.W. Findling, J. Newell-Price, M.O. Savage, P.M. Stewart, V.M. Montori, The diagnosis of Cushing's syndrome: an endocrine society clinical practice guideline, J. Clin. Endocrinol. Metab. 93 (2008) 1526–1540.
[4] G. Eisenhofer, T. Dekkers, M. Peitzsch, A.S. Dietz, M. Bidlingmaier, M. Treitl, T.A. Williams, S.R. Bornstein, M. Haase, L.C. Rump, H.S. Willenberg, F. Beuschlein, J. Deinum, J.W. Lenders, M. Reincke, Mass spectrometry-based adrenal and peripheral venous steroid profiling for subtyping primary aldosteronism, Clin. Chem. 62 (2016) 514–524.
[5] J.P. Holst, S.J. Soldin, R.E. Tractenberg, T. Guo, P. Kundra, J.G. Verbalis, J. Jonklaas, Use of steroid profiles in determining the cause of adrenal insufficiency, Steroids 72 (2007) 71–84.
[6] N. Janzen, F.G. Riepe, M. Peter, S. Sander, U. Steuerwald, E. Korsch, F. Krull, H.L. Muller, S. Heger, C. Brack, J. Sander, Neonatal screening: identification of children with 11beta-hydroxylase deficiency by second-tier testing, Horm. Res. Paediatr. 77 (2012) 195–199.
[7] E.R. de Kloet, H. Karst, M. Joels, Corticosteroid hormones in the central stress response: quick-and-slow, Front. Neuroendocrinol. 29 (2008) 268–272.
[8] I.E. Widmer, J.J. Puder, C. Konig, H. Pargger, H.R. Zerkowski, J. Girard, B. Muller, Cortisol response in relation to the severity of stress and illness, J. Clin. Endocrinol. Metab. 90 (2005) 4579–4586.
[9] A.N. Hoofnagle, M.H. Wener, The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry, J. Immunol. Methods 347 (2009) 3–11.
[10] A.E. Kulle, M. Welzel, P.M. Holterhus, F.G. Riepe, Principles and clinical applications of liquid chromatography – tandem mass spectrometry for the determination of adrenal and gonadal steroid hormones, J. Endocrinol. Invest. 34 (2011) 702–708.
[11] F.Z. Stanczyk, N.J. Clarke, Advantages and challenges of mass spectrometry assays for steroid hormones, J. Steroid Biochem. Mol. Biol. 121 (2010) 491–495.
[12] S.J. Soldin, O.P. Soldin, Steroid hormone analysis by tandem mass spectrometry, Clin. Chem. 55 (2009) 1061–1066.
[13] B.G. Keevil, LC-MS/MS analysis of steroids in the clinical laboratory, Clin. Biochem. 49 (2016) 989–997.
[14] L. Siekmann, H. Breuer, Determination of cortisol in human plasma by isotope dilution-mass spectrometry. Definitive methods in clinical chemistry, I, J. Clin. Chem. Clin. Biochem. Zeitschrift fur Klinische Chemie und Klinische Biochemie 20 (1982) 883–892.
[15] L. Siekmann, Determination of steroid hormones by the use of isotope dilution-mass spectrometry: a definitive method in clinical chemistry, J. Steroid Biochem. 11 (1979) 117–123.
[16] D.A. Machacek, R.J. Singh, Development and validation of serum 17 alpha-hydroxy progesterone LC-MS/MS assay for screening and monitoring of congenitaladrenal hyperplasia, in: Presented at American Society of Mass Spectrometry Meeting, 2001, Chicago, 2001.
[17] S.A. Wudy, M. Hartmann, M. Svoboda, Determination of 17-hydroxyprogesterone in plasma by stable isotope dilution/benchtop liquid chromatography-tandem mass spectrometry, Horm. Res. 53 (2000) 68–71.
[18] L. Siekmann, B. Spiegelhalder, H. Breuer, Bestimmung von Aldosteron im Plasma des Menschen unter Verwendung der Kombination Gaschromatographie/Massenspektrometrie, Zeitung der Analytischen Chemie 261 (1972) 377–381.
[19] European Medicine Agency, Guideline on Bioanalytical Method, Validation, 2011, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf, Accessed November 2015.

[20] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[21] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom.: RCM 13 (1999) 1175–1185.
[22] CLSI, Liquid Chromatography-Mass-Spectrometry Methods; Approved Guideline, CLSI Document C62-A, Clinical and Laboratory Standards Institute, Wayne, PA, 2014.
[23] J.L. Little, M.F. Wempe, C.M. Buchanan, Liquid chromatography-mass spectrometry/mass spectrometry method development for drug metabolism studies: examining lipid matrix ionization effects in plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833 (2006) 219–230.
[24] A.C. Suhr, M. Vogeser, S.H. Grimm, Isotope Inversion Experiment evaluating the suitability of calibration in surrogate matrix for quantification via LC-MS/MS-exemplary application for a steroid multi-method, J. Pharm. Biomed. Anal. 124 (2016) 309–318.
[25] M. Vogeser, J. Briegel, K. Jacob, Determination of serum cortisol by isotope-dilution liquid-chromatography electrospray ionization tandem mass spectrometry with on-line extraction, Clini. Chem. Lab. Med.: CCLM/FESCC 39 (2001) 944–947.
[26] U. Ceglarek, L. Kortz, A. Leichtle, G.M. Fiedler, J. Kratzsch, J. Thiery, Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography-triple quadrupole linear ion trap mass spectrometry, Clin. Chim. Acta 401 (2009) 114–118.
[27] J.G. Van Der Gugten, J. Dubland, H.F. Liu, A. Wang, C. Joseph, D.T. Holmes, Determination of serum aldosterone by liquid chromatography and tandem mass spectrometry: a liquid–liquid extraction method for the ABSCIEX API-5000 mass spectrometry system, J. Clin. Pathol. 65 (2012) 457–462.
[28] S. Travers, L. Martinerie, C. Bouvattier, P. Boileau, M. Lombes, E. Pussard, Multiplexed steroid profiling of gluco- and mineralocorticoids pathways using a liquid chromatography tandem mass spectrometry method, J. Steroid Biochem. Mol. Biol. 165 (2017) 202–211.

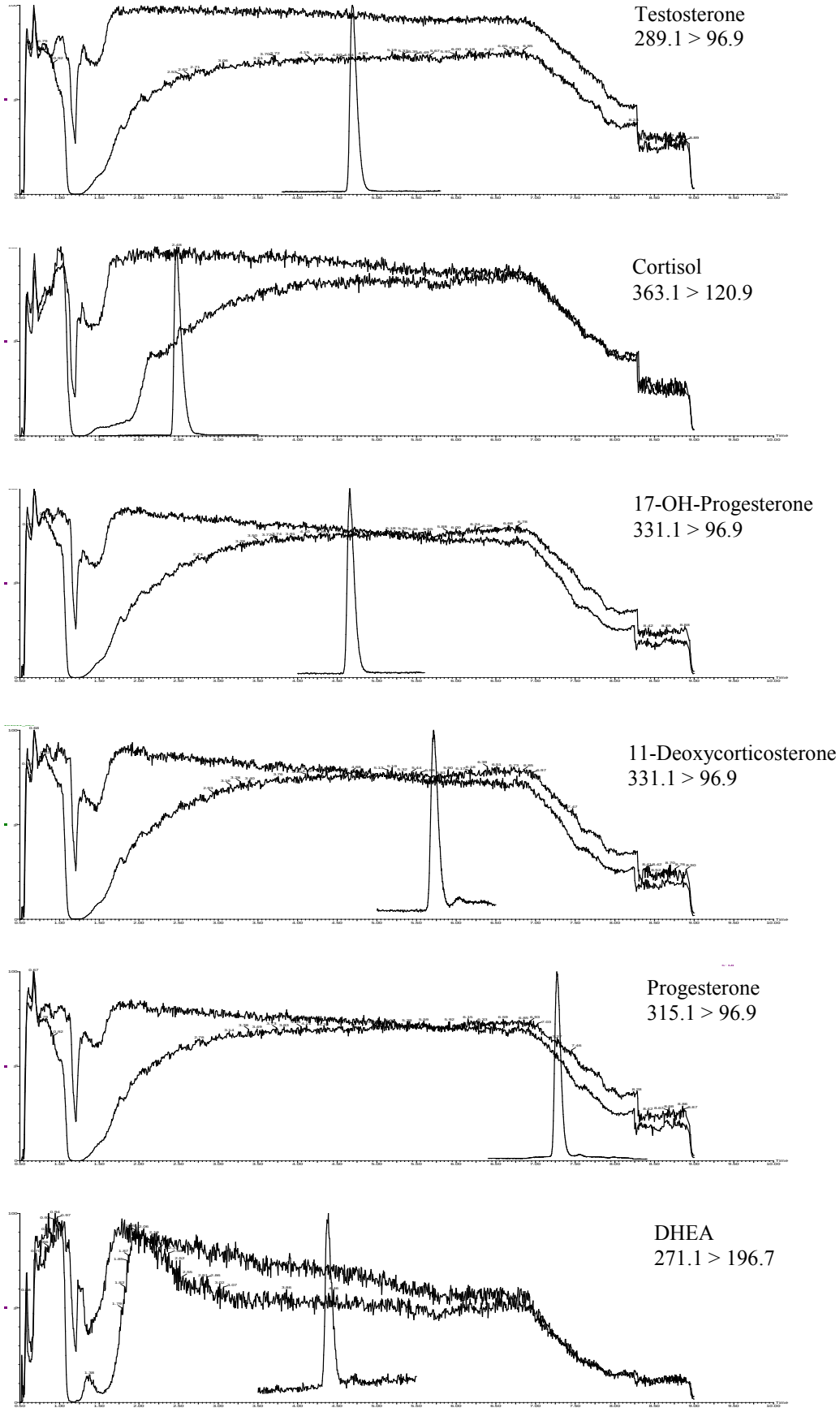

Figure S1 Exemplarily chromatograms of 6 analytes of the post column infusion experiment using specific transitions. The upper line displays the infusion chromatogram of injected methanol; the lower line displays the injected processed matrix. The peak represents the retention time of the respective analyte.

Table S1 Ion ratios of the calibration standards (CAL) and the serum samples.

		Run 1		Run 2		Run 3		Run 4		Run 5	
		CAL	samples	CAL	samples	CAL	samples	CAL	samples	CAL	samples
Cortisol	**Mean**	2.534	2.513	2.524	2.510	2.529	2.541	2.549	2.536	2.583	2.581
	Min	2.51	2.475	2.481	2.464	2.335	2.485	2.444	2.5070	2.536	2.505
	Max	2.564	2.562	2.564	2.529	2.680	2.672	2.676	2.578	2.646	2.726
	CV [%]	0.8171	1.154	1.267	0.8843	3.102	2.317	2.172	0.9910	1.683	2.064
Cortisone	**Mean**	4.461	4.451	4.460	4.424	4.440	4.587	4.514	4.512	4.492	4.517
	Min	4.402	4.388	4.347	4.282	4.004	4.451	4.452	4.304	4.322	4.442
	Max	4.513	4.522	4.537	4.487	4.639	4.799	4.733	4.758	4.794	4.650
	CV [%]	0.9292	1.287	1.212	1.668	3.361	2.948	1.787	3.505	2.763	1.380
Corticosterone	**Mean**	2.594	2.582	2.567	2.575	2.594	2.622	2.650	2.632	2.608	2.622
	Min	2.511	2.505	2.490	2.445	2.479	2.556	2.490	2.612	2.558	2.503
	Max	2.668	2.638	2.643	2.628	2.650	2.689	2.890	2.644	2.659	2.750
	CV [%]	2.689	1.544	1.929	2.099	1.990	1.504	3.285	0.491	0.994	2.072
11-Deoxycortisol	**Mean**	2.077	2.053	2.092	2.057	2.088	2.058	2.098	2.087	2.088	2.077
	Min	2.007	1.971	1.826	1.975	2.018	2.007	2.051	2.073	2.04	2.031
	Max	2.109	2.074	2.169	2.084	2.124	2.140	2.257	2.138	2.159	2.102
	CV [%]	1.651	1.649	3.870	1.756	1.245	2.007	2.298	1.199	1.311	0.9003
17-OH-Progesterone	**Mean**	2.178	2.134	2.156	2.180	2.172	2.112	2.143	2.172	2.161	2.174
	Min	2.11	2.065	2.04	2.176	2.046	2.038	2.020	2.150	2.080	2.078
	Max	2.233	2.230	2.184	2.185	2.280	2.189	2.192	2.194	2.221	2.339
	CV [%]	1.623	2.514	1.671	0.1384	2.222	2.07	2.392	0.988	1.643	2.701
Deoxycorticosterone	**Mean**	2.234	2.228	2.218	2.213	2.227	2.205	2.234	2.218	2.251	2.239
	Min	2.195	2.204	2.110	2.179	2.172	2.172	2.186	2.167	2.128	2.187
	Max	2.274	2.249	2.266	2.248	2.257	2.274	2.294	2.241	2.381	2.258
	CV [%]	1.159	0.755	1.906	0.978	1.165	1.527	1.184	1.293	2.514	0.798
Progesterone	**Mean**	0.8578	0.8577	0.8603	0.8573	0.8691	0.8512	0.8622	0.8542	0.8577	0.8589
	Min	0.832	0.854	0.831	0.839	0.827	0.844	0.839	0.839	0.822	0.852
	Max	0.854	0.861	0.945	0.867	0.974	0.863	0.911	0.865	0.931	0.864
	CV [%]	2.095	0.3352	3.936	1.271	5.588	0.8052	2.636	1.300	3.449	0.5528
21-Deoxycortisol	**Mean**	1.511	1.524	1.538	1.530	1.557	1.533	1.564	1.533	1.554	1.511
	Min	1.491	1.504	1.506	1.517	1.526	1.506	1.531	1.519	1.533	1.467
	Max	1.529	1.534	1.620	1.540	1.581	1.555	1.608	1.567	1.573	1.530
	CV [%]	0.7794	0.7306	2.081	0.491	0.8996	1.540	1.288	1.208	0.8825	1.191
DHEA	**Mean**	0.3633	0.3575	0.3586	0.3578	0.3684	0.3628	0.3661	0.3695	0.3699	0.3719
	Min	0.356	0.349	0.345	0.352	0.354	0.354	0.347	0.363	0.361	0.361
	Max	0.387	0.362	0.395	0.364	0.429	0.370	0.379	0.375	0.375	0.38
	CV [%]	3.236	1.333	5.261	1.440	6.067	1.904	2.954	1.052	1.263	1.611
Testosterone	**Mean**	0.7564	0.7506	0.7566	0.7643	0.7585	0.7387	0.7541	0.7638	0.7618	0.7617
	Min	0.748	0.744	0.739	0.748	0.75	0.692	0.722	0.757	0.743	0.745
	Max	0.777	0.766	0.774	0.775	0.777	0.757	0.774	0.770	0.778	0.778
	CV [%]	1.166	0.9392	1.218	1.108	0.9160	2.957	2.020	0.5661	1.194	1.064

2.2. J.M. Lindner, A.C. Suhr, S.H. Grimm, P. Möhnle, M. Vogeser, J. Briegel, The dynamics of a serum steroid profile after stimulation with intravenous ACTH, *J Pharm Biomed Anal* 151 (2018) 159-163

Journal of Pharmaceutical and Biomedical Analysis 151 (2018) 159–163

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

The dynamics of a serum steroid profile after stimulation with intravenous ACTH

Johanna M. Lindner[a,*,1], Anna Catharina Suhr[a,1], Stefanie H. Grimm[a], Patrick Möhnle[b], Michael Vogeser[a], Josef Briegel[b]

[a] Institute of Laboratory Medicine, University Hospital, LMU Munich, Germany
[b] Department of Anesthesiology, University Hospital, LMU Munich, Germany

ARTICLE INFO

Article history:
Received 1 November 2017
Received in revised form
19 December 2017
Accepted 21 December 2017
Available online 26 December 2017

Keywords:
ACTH stimulation test
Biochemical stress response
Corticosteroid profiling
LC–MS/MS
Corticosterone

ABSTRACT

Background: Stimulation with intravenous adrenocorticotropic hormone (ACTH) is a widely used diagnostic procedure to characterize the adrenocortical function. Currently, the response of serum cortisol, mainly quantified by immunoassays, is the only established read-out of this test. By using liquid chromatography coupled with mass spectrometry (LC–MS/MS) simultaneous determination of several steroids that respond to ACTH stimulation is now possible. The aim of this study was to further characterize the typical effect of exogenous ACTH (250 mg) on a LC–MS/MS-serum steroid profile.
Methods: A set of 36 paired samples (pre-/post-IV-ACTH) was investigated (age range 22–58, 26 female and 10 male individuals). Serum steroid profiling was performed using a LC–MS/MS method covering cortisol, cortisone, corticosterone, 11-deoxycortisol, 17-OH-progesterone and 11-deoxycorticosterone.
Results: The concentrations of all measured steroids increased after stimulation with ACTH, except for cortisone. Serum corticosterone, 11-deoxycorticosterone and 11-deoxycortisol showed markedly more pronounced relative increases compared to cortisol. The strongest response was observed for corticosterone (15-fold median relative increase, compared to 1.4-fold median increase of cortisol).
Conclusion: Serum steroid profiling using LC–MS/MS after stimulation with IV ACTH demonstrates highly dynamic response patterns. Further studies should address in particular serum corticosterone as a potential novel marker of biochemical stress response.

1. Introduction

The assessment of panels of analytes which are related to a metabolic pathway, referred to as metabolic profiling [1], is promising for clinical diagnostics. The simultaneous quantification of various endogenous steroids has already become a valuable diagnostic tool in some areas of endocrinology [2] and beyond. The biosynthesis pathway of these steroids is shown in Fig. 1. So far, steroid profiling is used to confirm congenital adrenal hyperplasia [3], to investigate primary aldosteronism [4] and in the diagnostic work-up of other forms of adrenal cortical dysfunction [5,6]. Moreover, in endocrinological oncology, steroid profiling has become increasingly attractive as a tool for differential diagnosis, as demonstrated in recent work on adrenal tumours by Arlt et al. [7].

Stimulation testing with IV adrenocorticotropic hormone (ACTH) is a long-established approach to characterize the functional integrity of the adrenal cortex. Standard read-out of this test is the concentration of total cortisol in serum samples 60 min after ACTH administration. An increase of serum cortisol >18 μg/dL is considered normal [8]. ACTH stimulation testing is used in suspected adrenal failure due to different potential underlying conditions, including relative adrenal insufficiency in septicemia and septic shock [9].

Cortisol as the single read-out of the ACTH stimulation test is usually quantified by immunoassays. However, particularly in some clinical conditions such as sepsis [9], immunoassays can lead to inaccurate results due to cross reactivity with structurally similar analytes [10,11]. Serum steroid profiling using isotope dilution liquid chromatography mass spectrometry (ID-LC–MS/MS) enables the researcher to monitor several steroids simultaneously on a very high level of analytical specificity as well as accuracy – even for structurally closely related compounds. Therefore it is becoming the method of choice to quantify steroids in diagnostic samples [12,13].

* Corresponding author at: Johanna Lindner, Institute of Laboratory Medicine, Hospital of the University of Munich (LMU), Marchioninistrasse 15, 81377 Munich, Germany.
E-mail address: johanna.lindner@med.uni-muenchen.de (J.M. Lindner).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.jpba.2017.12.045
0731-7085/

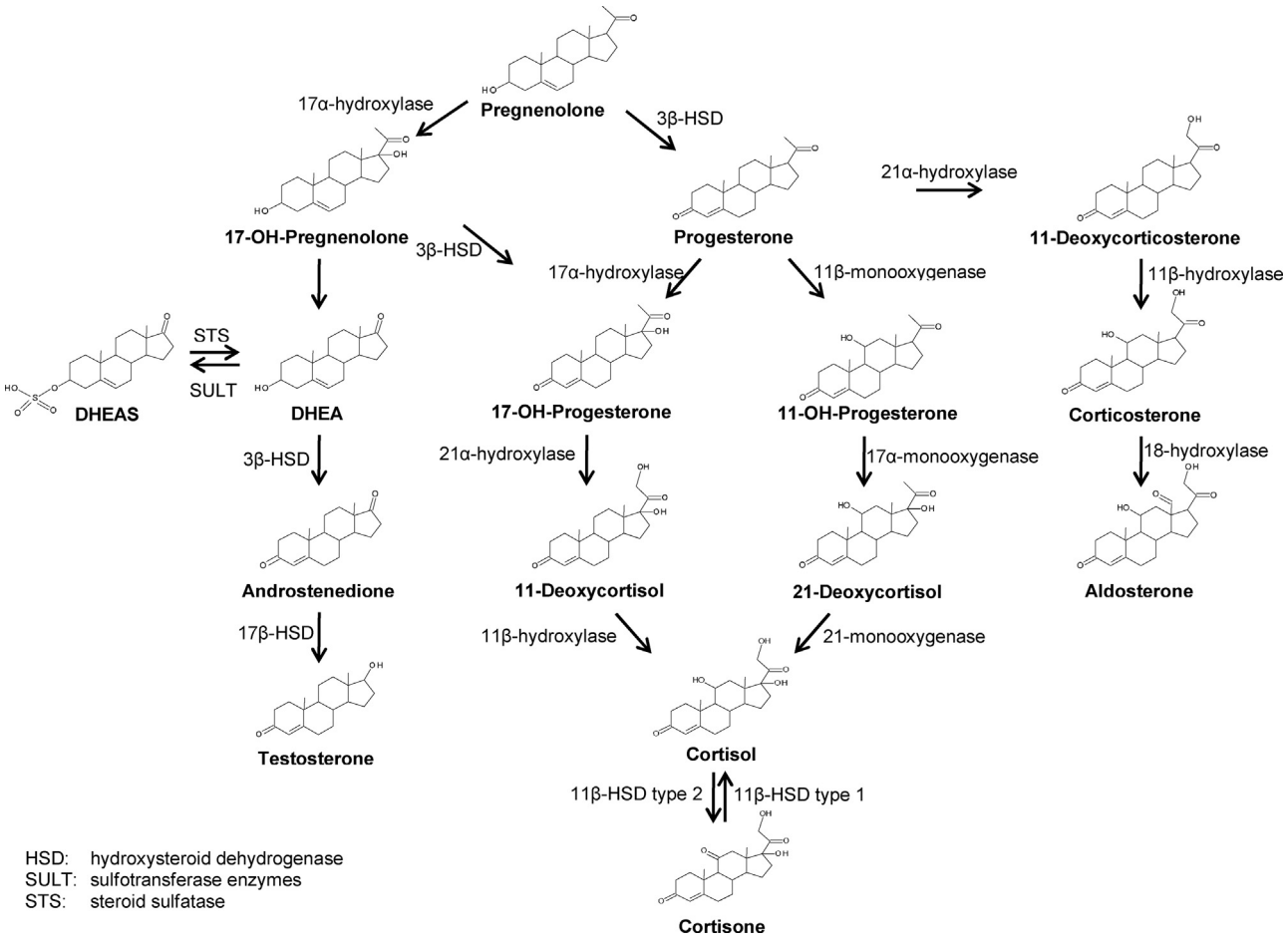

Fig. 1. Metabolic pathway of corticosteroids.

Additionally, IV ACTH stimulation simulates maximum physiological stress and allows studying biochemical stress responses on different biological levels. Steroid profiling of ACTH stimulated samples by LC–MS/MS represents a far more comprehensive approach to describe the biochemical response of the adrenal cortex to ACTH compared to mere immunometric cortisol measurement.

So far, there are solely data published on corticosteroid profiles after ACTH stimulation for cases of adrenocortical adenomas [14] and adrenal insufficiency [15], but there are only sparse data for control cohorts and healthy individuals [15].

Comparative and comprehensive data of steroid profiling in defined cohorts after ACTH administration are needed to study differences in the physiological reactions to stress. Therefore the aim of our investigation was to assess the characteristic pattern of changes in concentration of 6 steroids (cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone and 17-OH-progesterone) in human serum of individuals not suspected to be suffering from endocrinological diseases which can be reliably quantified using a recently developed ID-LC–MS/MS method for clinical research [16].

2. Materials and methods

2.1. Sample collection

Samples of 36 individuals were investigated. Of these, 24 paired samples (pre-/post-IV-ACTH) were residual, anonymized clinical samples from women attending a fertility outpatient service. Only samples were included if the increase in serum cortisol after ACTH administration was normal and above the cut-off (>18 μg/dL [8], measured by enzyme immunoassay), and if all requested routine laboratory tests did not show any abnormalities. Adrenal hyperplasia was excluded by the evaluation of serum 17-OH-progesterone values after ACTH stimulation measured by LC–MS/MS (<1 μg/dL, [17]). Testosterone was measured by an immunoassay and all values were ≤0.06 μg/dL for these females. The other 12 paired samples (10 male, 2 female, age 26–58 years) were obtained from volunteers after informed consent. These volunteers were free of health complaints (including sleep disorders) or evidence of diseases, were not overweight (BMI < 25) and didn't take steroid medication except contraceptives. The study protocol was approved by the institutional review board of the Ludwig-Maximilians-Universität Munich, Germany (No 84/15). ACTH stimulation was performed in all individuals by an intravenous injection of tetracosactidhexaacetat (250 micrograms = 25 I.E., Synacthen®, sigma-tau GmbH, Munich, Germany). Blood samples were taken in the non-fasting state in the morning (9–11 a.m.) prior to ACTH administration and 60 min after stimulation. In the sub-set of 12 volunteers serum sampling was also performed 30 min after stimulation. Blood samples were left to clot for 15 min and subsequently centrifuged. The obtained serum was anonymized and stored at −80 °C until analysis. Mean age of the entire cohort investigated in this study (n = 36) was 31 (range 22–58), 26 female (all pre-menopausal), 10 male.

Table 1
Serum steroid concentrations observed before and after ACTH administration in complete cohort [10 male, 26 female; μg/L].

	time	mean	median	range	relative increase [x-fold]	
					median	range
Cortisol	0 min	111	106	32.4–213	–	–
	60 min	267	261	181–407	1.40	0.300–4.58
Cortisone	0 min	19.9	19.9	11.2–29.4	–	–
	60 min	18.1	18.3	11.4–23.4	−0.084	−0.442 to 0.518
Corticosterone	0 min	2.68	1.81	0.684–9.20	–	–
	60 min	31.3	31.0	17.3–47.7	15.2	2.12–58.7
11-Deoxycortisol	0 min	0.243	0.229	<0.110–0.772	–	–
	60 min	0.897	0.822	0.241–4.37	2.75	0.181–10.8
11-Deoxy-corticosterone	0 min	0.048	0.027	<0.022–0.232	–	–
	60 min	0.235	0.190	0.037–1.15	5.72	1.45–15.4
17-OH-Progesterone	0 min	0.648	0.468	<0.070–2.42	–	–
	60 min	1.47	1.27	0.442–3.04	1.98	−0.257 to 9.06

2.2. LC–MS/MS assay

Steroid profiling of the serum samples was performed using a validated, semi-automated stable-isotope dilution-UHPLC–MS/MS assay as previously described in detail [16]. Isobaric compounds were chromatographically separated. For detection a Xevo TQ-S (Waters, Milford, MA, USA) was used and multiple reaction monitoring (MRM) was applied.

For quality assurance, a matrix-based quality control, "Lyphochek® Immunoassay Plus Control, Level 2" (Biorad, Munich, Germany) was used together with in-house prepared quality control samples. The exact concentrations of calibrators and quality controls are listed in the supplementary material (Table S1).

3. Results

The basal steroid levels, the concentrations after ACTH administration, and the x-fold relative increase $(c_{60min} - c_{0min})/c_{0min}$ of the steroids are given in Table 1. Marked increase in the concentration of all analytes except cortisone – the inactivated storage form of cortisol – was observed.

The most pronounced dynamics after ACTH administration were found for corticosterone, which showed a relative median 15.2-fold increase. Corticosterone also displayed the highest inter-individual variance, as illustrated in Fig. 2.

We differentiated these data between women and men to detect possible sex-specific differences in the adrenal secretion. Indeed, 17-OH-progesterone displays differing values between sexes: the median value for male individuals (1.72 μg/L) was higher compared to female individuals (1.12 μg/L), ACTH administration led to a clearly more pronounced increase in women (2.6-fold versus 0.9-fold) (see Table 2).

In the sub-set of volunteers (n = 12), serum sampling was performed after 60 min and additionally also after 30 min of ACTH administration. Thus, we were able to investigate the time course of the corticosteroid concentrations. We found that the main increase of the corticosteroids occurred within the first 30 min after ACTH stimulation. Sixty minutes after stimulation, the results were only slightly higher than or equal to the 30 min values (see Table 3).

4. Discussion

In this article, we report the application of a multi-analyte, stable isotope-dilution LC–MS/MS method for serum steroid profiling after stimulation with IV ACTH. Our main finding is that several steroids (11-deoxycorticosterone, 11-deoxycortisol, and corticosterone in particular) displayed a far more pronounced dynamic response to ACTH administration compared to cortisol, currently the only standard read-out of the ACTH stimulation testing. Most pronounced increase was observed for corticosterone (15-fold relative increase in relation to a 1.4-fold relative increase of cortisol, both expressed as median). This suggests this analyte as a novel and probably very sensitive marker in research addressing stress and the body's biochemical response to maintain haemostasis. Regarding the ACTH stimulation test in clinical diagnostic situations, abovementioned precursors as well as combinations of these might be a more powerful readout than cortisol alone; respective future studies to establish reference ranges and to test this hypothesis seem justified.

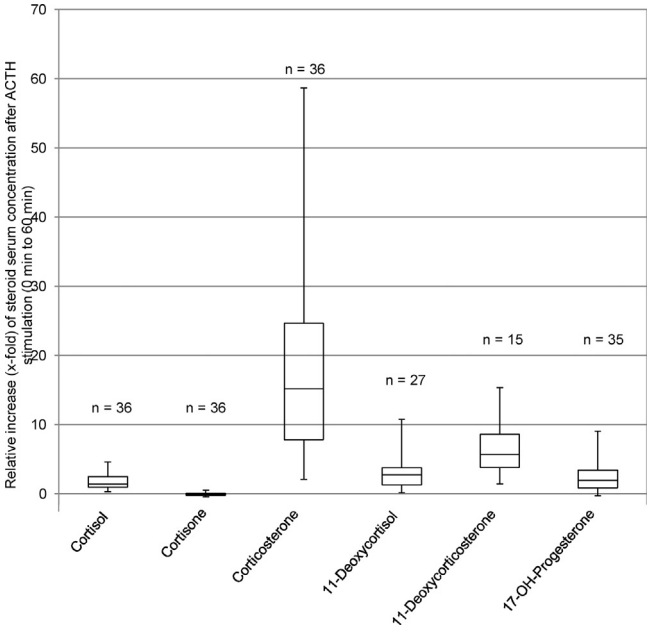

Fig. 2. Relative increase (x-fold) of steroids in serum after iv ACTH administration (250 μg) (0 min to 60 min). The bars display the minimum and the maximum of the respective increases. The different number of cases results from the lacking basal concentration of some samples due to their low concentrations (<LLOQ).

To the best of our knowledge, the steroid profile described in our work has not been investigated in the context of ACTH stimulation so far. In general there are very few data on steroid profiling in individuals without endocrine disorders after ACTH stimulation. However, we were able to compare our data with the results of Holst et al. who assessed steroid data of patients with adrenal insufficiency and also of healthy individuals after ACTH

Table 2
Serum steroid concentrations observed before and after ACTH administration, data separated in women (n = 26) and men (n = 10) [μg/L].

	time	mean		median		range		relative increase [x-fold] median		relative increase [x-fold] range	
		women	men	women	men	women	men	women	men	women	men
Cortisol	0 min	106	127	92.6	124	32.4–213	68.2–203	–	–	–	–
	60 min	272	261	267	260	181–407	236–302	1.73	1.24	0.668–4.58	0.300–2.70
Cortisone	0 min	20.0	19.5	17.7	18.3	11.2–29.4	15.4–25.6	–	–	–	–
	60 min	20.8	19.2	17.8	19.5	11.4–23.4	14.2–23.1	−0.155	0.016	−0.442 to 0.518	−0.374 to 0.247
Corticosterone	0 min	2.37	3.50	1.77	2.20	0.684–8.08	0.987–9.20	–	–	–	–
	60 min	31.4	30.9	32.4	29.3	17.3–46.8	21.5–47.7	17.0	13.7	3.26–58.7	2.12–28.1
11-Deoxycortisol	0 min	0.210	0.323	0.181	0.266	0.108–0.374	0.222–0.772	–	–	–	–
	60 min	0.894	0.906	0.758	0.924	0.241–4.37	0.323–1.70	3.39	2.65	0.181–10.8	1.07–3.78
11-Deoxy-corticosterone	0 min	0.041	0.059	0.034	0.025	0.022–0.088	0.021–0.232	–	–	–	–
	60 min	0.228	0.254	0.191	0.180	0.037–1.15	0.088–0.972	6.08	5.34	1.45–15.4	3.19–11.4
17-OH-Progesterone	0 min	0.481	1.08	0.305	1.08	0.058–2.42	0.485–1.968	–	–	–	–
	60 min	1.27	2.00	1.12	1.72	0.442–2.92	1.22–3.04	2.56	0.857	−0.257 to 9.06	0.152–2.61

Table 3
Serum steroid concentrations [μg/L] of a sub-cohort (n = 12). Sampling was performed before, 30 min and 60 min after ACTH administration.

	time	mean	median	range	relative increase [x-fold] median	relative increase [x-fold] range
Cortisol	0 min	134	124	68.2–213	–	–
	30 min	253	246	216–361	0.95	0.263–2.54
	60 min	272	260	236–407	1.12	0.300–2.70
Cortisone	0 min	19.2	18.1	15.4–25.6	–	–
	30 min	17.6	17.9	13.8–19.6	−0.04	−0.392 to 0.089
	60 min	19.3	19.5	14.2–23.1	0.035	−0.374 to 0.247
Corticosterone	0 min	3.24	2.10	0.987–9.20	–	–
	30 min	26.2	25.4	16.9–48.1	10.5	1.69–23.9
	60 min	30.3	28.9	21.5–47.7	13.3	2.12–28.1
11-Deoxycortisol	0 min	0.323	0.266	<0.110–0.772	–	–
	30 min	0.799	0.843	0.263–1.72	2.18	0.401–3.58
	60 min	0.874	0.920	0.323–1.70	2.65	1.07–3.78
11-Deoxy-corticosterone	0 min	0.066	0.025	<0.022–0.232	–	–
	30 min	0.205	0.146	0.062–0.911	6.60	1.30–8.09
	60 min	0.231	0.157	0.088–0.972	5.72	3.19–11.4
17-OH-Progesterone	0 min	0.916	0.963	0.058–1.97	–	–
	30 min	1.80	1.83	0.544–3.09	0.97	0.186–13.7
	60 min	1.80	1.67	0.592–3.04	0.94	0.152–16.1

administration [15]. The profile described by Holst et al. (cortisol, 11-deoxycortisol, 17-OH-progesterone, progesterone, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone-sulfate, estradiol, testosterone and aldosterone) overlaps partially our profile, i.e. regarding cortisol, 11-deoxycortisol and 17-OH-progesterone. While the values and relative increases for cortisol and 17-OH-progesterone agreed quite well with the results of our work (see Table 4), a striking difference was observed for 11-deoxycortisol: Holst et al. describe substantially higher concentrations of this analyte, both pre- and post ACTH administration. This might be explained by co-elution of 11-deoxycortisol together with its isomere corticosterone in one of the LC–MS/MS method applied by Holst et al.; notably the authors give no detailed information of specificity in the detection of these two compounds in one of their employed LC–MS/MS methods. This observation underlines the crucial importance of proper chromatographic separation of isobaric compounds in quantitative analysis using LC-MS/MS, in particular in steroid testing.

In further studies, only baseline values for healthy volunteers were available for comparison: Di Dalmazi et al. [14] assessed the steroid profile of patients with different disorders after ACTH administration by LC–MS/MS. In the respective healthy control

Table 4
Comparison with literature data [15] for corticosteroid profiling of healthy individuals [μg/L, expressed as mean].

	Holst et al., 2007	Own data	
		complete cohort	sub-cohort
Sex	38 female, 23 male	26 female, 10 male	2 female, 10 male
Age	24–61	22–58	26–58
n	61	36	12
		Cortisol	
basal	115	111	134
30 min	266	nd	253
60 min	311	267	272
		11-Deoxycortisol	
basal	0.79	0.243	0.323
30 min	5.71	nd	0.799
60 min	6.70	0.897	0.874
		17-OH-Progesterone	
basal	0.47	0.648	0.916
30 min	1.50	nd	1.80
60 min	1.62	1.47	1.80

nd, not determined.

group, only the basal levels were determined. Other studies addressing steroid profiling, e.g. those by Fanelli et al. [18] or Carvalho et al. [19] did not perform ACTH stimulation. A comparison of these published baseline values with our data can be found in the supplementary material (Table S2, S3 and S4). Whereas for some values good agreement was found, e.g. between the baseline ranges of serum steroids established by Carvalho et al. [19] and our results (see supplementary material Table S2) and the baseline cortisol values obtained by Di Dalmazi (see supplementary material Table S3), there are also several discrepancies: The baseline concentrations of cortisol, corticosterone, 11-deoxycortisol and 17-OH-progesterone of the pre-menopausal women measured by Fanelli et al. [18] are higher than the concentrations observed in the pre-menopausal women in our study (see supplementary material Table S4). Compared to the data of Di Dalmazi [14] the concentration of 17-OH-progesterone is lower than the concentration measured by us whilst the concentrations of corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol are higher than our values. These discrepancies raise the question if inter-individual biological ranges of steroids are indeed very wide or if the different results might originate from differences in quantification and standardization of methods. Thus, it seems to be important to perform method comparisons between different laboratories addressing steroid profiling by LC-MS/MS in order to achieve standardization of steroid profiling after ACTH challenge.

Our data presented herein demonstrate the feasibility of complex serum steroid profiling in the context of ACTH stimulation testing using a highly specific and carefully evaluated isotope dilution-LC–MS/MS assay. However, a limitation of our investigation is the incomplete characterization and the heterogeneity of the set of study samples. While the number of self-reporting healthy volunteers is rather small, little information was available concerning the left-over clinical samples. They originated from younger women addressing a fertility outpatient clinic; this fact and unremarkable results of standard laboratory tests make the presence of severe diseases in these individuals unlikely. Thus, our data display typical results but don't meet the criteria of reference ranges. The composition of the cohorts studied herein only allows qualitative interpretation – this is in particular the more dynamic response of several corticosteroids (and in particular of corticosterone) compared to cortisol. Definite sex and age-specific reference ranges for application in diagnostic studies remain to be established in well-characterized cohorts under highly standardized conditions (including fasting status, comprehensive data about general health and detailed endocrinological assessment).

In summary we conclude that isotope dilution-LC–MS/MS-based corticosteroid profiling after application of exogenous ACTH offers a promising and convenient tool for a far more detailed assessment of the adrenal function compared to the mere quantification of serum cortisol. This analytical approach may be relevant for diagnostic testing as well as for stress research and clinical research on suspected relative adrenal failure, including adrenal dysfunction associated with severe illness. In particular the evaluation of serum corticosterone concentrations as novel highly dynamic and potentially very sensitive biochemical stress marker seems worthwhile according to our data.

Conflict of interest

None.

Funding

This work was funded by the Hans Fischer Society Munich, Munich, Germany.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2017.12.045.

References

[1] D.I. Ellis, W.B. Dunn, J.L. Griffin, J.W. Allwood, R. Goodacre, Metabolic fingerprinting as a diagnostic tool, Pharmacogenomics 121 (2007) 1243–1266.
[2] M. Rauh, Steroid measurement with LC-MS/MS: Application examples in pediatrics, J. Steroid Biochem. Mol. Biol. 121 (2010) 520–527.
[3] C. Rossi, L. Calton, G. Hammond, H.A. Brown, A.M. Wallace, P. Sacchetta, M. Morris, Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography-tandem mass spectrometry, Clin. Chim. Acta 411 (2010) 222–228.
[4] M. Peitzsch, T. Dekkers, M. Haase, F.C. Sweep, I. Quack, G. Antoch, G. Siegert, J.W. Lenders, J. Deinum, H.S. Willenberg, G. Eisenhofer, An LC-MS/MS method for steroid profiling during adrenal venous sampling for investigation of primary aldosteronism, J. Steroid Biochem. Mol. Biol. 145 (2015) 75–84.
[5] P.C. Kao, D.A. Machacek, M.J. Magera, J.M. Lacey, P. Rinaldo, Diagnosis of adrenal cortical dysfunction by liquid chromatography-tandem mass spectrometry, Ann. Clin. Lab. Sci. 31 (2001) 199–204.
[6] L.K. Nieman, B.M. Biller, J.W. Findling, J. Newell-Price, M.O. Savage, P.M. Stewart, W.M. Montori, The diagnosis of cushing's syndrome: an endocrine society clinical practice guideline, J. Clin. Endocrinol. Metab. 93 (2008) 1526–1540.
[7] W. Arlt, M. Biehl, A.E. Taylor, S. Hahner, R. Libe, B.A. Hughes, P. Schneider, D.J. Smith, H. Stiekema, N. Krone, E. Porfiri, G. Opocher, J. Bertherat, F. Mantero, B. Allolio, M. Terzolo, P. Nightingale, C.H. Shackleton, X. Bertagna, M. Fassnacht, P.M. Stewart, Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors, J. Clin. Endocrinol. Metab. 96 (2011) 3775–3784.
[8] S.R. Bornstein, B. Allolio, W. Arlt, A. Barthel, A. Don-Wauchope, G.D. Hammer, E.S. Husebye, D.P. Merke, M.H. Murad, C.A. Stratakis, D.J. Torpy, Diagnosis and treatment of primary adrenal insufficiency: an endocrine society clinical practice guideline, J. Clin. Endocrinol. Metab. 101 (2016) 364–389.
[9] J. Briegel, C.L. Sprung, D. Annane, M. Singer, D. Keh, R. Moreno, P. Möhnle, Y. Weiss, A. Avidan, F.M. Brunkhorst, F. Fiedler, M. Vogeser, Multicenter comparison of cortisol as measured by different methods in samples of patients with septic shock, Intensive Care Med. 35 (2009) 2151–2156.
[10] A.N. Hoofnagle, M.H. Wener, The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry, J. Immunol. Methods 347 (2009) 3–11.
[11] U. Ceglarek, M. Werner, L. Kortz, A. Korner, W. Kiess, J. Thiery, J. Kratzsch, Preclinical challenges in steroid analysis of human samples, J. Steroid Biochem. Mol. Biol. 121 (2010) 505–512.
[12] B.G. Keevil, Novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for measuring steroids, Best Pract. Res. Clin. Endocrinol. Metab. 27 (2013) 663–674.
[13] B.G. Keevil, LC-MS/MS analysis of steroids in the clinical laboratory, Clin. Biochem. 49 (2016) 989–997.
[14] G. Di Dalmazi, F. Fanelli, M. Mezzullo, E. Casadio, E. Rinaldi, S. Garelli, V. Vicennati, U. Pagotto, R. Pasquali, Steroid profiling by LC-MS/MS in nonsecreting and subclinical cortisol-secreting adrenocortical adenomas, J. Clin. Endocrinol. Metab. 100 (2015) 3529–3538.
[15] J.P. Holst, S.J. Soldin, R.E. Tractenberg, T. Guo, P. Kundra, J.G. Verbalis, J. Jonklaas, Use of steroid profiles in determining the cause of adrenal insufficiency, Steroids 72 (2007) 71–84.
[16] A.C. Suhr, M. Vogeser, S.H. Grimm, Isotope Inversion Experiment evaluating the suitability of calibration in surrogate matrix for quantification via LC-MS/MS-Exemplary application for a steroid multi-method, J. Pharm. Biomed. Anal. 124 (2016) 309–318.
[17] P.C. White, P.W. Speiser, Congenital adrenal hyperplasia due to 21-hydroxylase deficiency, Endocr. Rev. 21 (2000) 245–291.
[18] F. Fanelli, I. Belluomo, V.D. Di Lallo, G. Cuomo, R. De Iasio, M. Baccini, E. Casadio, B. Casetta, V. Vicennati, A. Gambineri, G. Grossi, R. Pasquali, U. Pagotto, Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults, Steroids 76 (2011) 244–253.
[19] V.M. Carvalho, O.H. Nakamura, J.G. Vieira, Simultaneous quantitation of seven endogenous C-21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum. Journal of Chromatography B, Anal. Technol. Biomed. Life Sci. 872 (2008) 154–161.

Table S1 Concentrations (µg/L) of calibrators and controls

	cortisol	cortisone	cortico-sterone	11-deoxy-cortisol	11-deoxy-corticosterone	17-OH-progesterone
Cal 1	1.11	1.11	0.228	0.110	0.022	0.066
Cal 2	5.57	2.77	0.569	0.220	0.054	0.133
Cal 3	27.8	5.54	1.14	0.550	0.109	0.265
Cal 4	55.7	11.1	2.85	1.10	0.218	0.664
Cal 5	111	27.7	8.54	2.75	1.09	1.33
Cal 6	278	55.4	22.8	8.25	5.44	6.64
Cal 7	557	111	56.9	22.0	21.8	26.5
QC low	3.34	3.33	0.683	0.330	0.065	0.199
QC medium	77.9	22.2	5.69	1.65	0.544	0.929
QC high	334	66.5	22.8	11.0	10.9	13.3
Lyphocheck 2	198	8.50	1.54	0.29	-	1.83

Cal, calibrator
QC, quality control

Table S2 Comparison with literature data of baseline corticosteroid profiling of individuals without endocrine disorders (expressed as ranges in µg/L)

	Carvalho et al., 2008 [19]		**Own data**
Sex	female and male		26 female, 10 male
Age	18-67		22 - 58
n	*see below*		36
	[nmol/L]	**converted to [µg/L]**	**[µg/L]**
cortisol	176.6 - 714.6 (n = 58)	64.0 - 259	32.4 - 213
cortisone	22.1 - 97.1 (n = 58)	7.97 - 35.0	11.2 - 29.4
corticosterone	≤ 67.4 (n = 58)	≤ 23.4	0.684 - 9.20
11-deoxycortisol	≤ 2.3 (n = 138)	≤ 0.797	< 0.110 - 0.772
11-deoxycorticosterone	≤ 0.7 (n = 138)	≤ 0.231	< 0.022 - 0.232
17-OH-progesterone	≤ 5.1 (n = 53, only male)	≤ 1.69	< 0.485 - 1.97 (n = 10, only male)

Table S3 Comparison with literature data of baseline corticosteroid profiling of individuals without endocrine disorders (expressed as mean in µg/L)

	Di Dalmazi et al., 2015 [14]	**Own data**
Sex	128 female, 60 male	26 female, 10 male
Age	Mean = 61.1	22-58 (mean = 31)
n	188	36
cortisol	116	111
corticosterone	3.18	2.68
11-deoxycortisol	0.34	0.243
11-deoxycorticosterone	0.080	0.048
17-OH-progesterone	0.450	0.648

Table S4 Comparison with literature data of baseline corticosteroid profiling of individuals without endocrine disorders (expressed as median in µg/L)

	Fanelli et al., 2011 [18]	**Own data**
Sex	women pre-menopausal	women pre-menopausal
Age	18-54	22-34
n	134	26
cortisol	102	92.6
corticosterone	2.62	1.77
11-deoxycortisol	0.239	0.181
17-OH-progesterone	0.578	0.305

3. Appendix

3.1. List of abbreviations

ACTH	adrenocorticotropic hormone
CRH	corticotropin releasing hormone
LC	liquid chromatography
MS	mass spectrometry
TFA	trifluoroacetate

3.2. References

[1] K. Aktories (Hrsg.), U. Förstermann (Hrsg.), F. Hofmann (Hrsg.), K. Starke (Hrsg.) (2009). *Allgemeine und spezielle Pharmakologie und Toxikologie* (10. Auflage). München: Urban & Fischer.

[2] P.E. Marik, Critical illness-related corticosteroid insufficiency, *Chest* 135(1) (2009) 181-193.

[3] R.F. Greaves, G. Jevalikar, J.K. Hewitt, M.R. Zacharin, A guide to understanding the steroid pathway: new insights and diagnostic implications, *Clin Biochem* 47(15) (2014) 5-15.

[4] E. Boonen, G. Van den Berghe, Cortisol metabolism in critical illness: implications for clinical care, *Curr Opin Endocrinol Diabetes Obes* 21(3) (2014) 185-192.

[5] J. Briegel, C.L. Sprung, D. Annane, M. Singer, D. Keh, R. Moreno, P. Möhnle, Y. Weiss, A. Avidan, F.M. Brunkhorst, F. Fiedler, M. Vogeser, Multicenter comparison of cortisol as measured by different methods in samples of patients with septic shock, *Intensive Care Med* 35(12) (2009) 2151-2156.

[6] C. Dessinioti, A.D. Katsambas, Congenital adrenal hyperplasia, *Dermatoendocrinol* 1(2) (2009) 87-91.

[7] A.E. Taylor, B.G. Keevil, I.T. Huhtaniemi, Mass spectrometry and immunoassay: how to measure steroid hormones today and tomorrow, *Eur J Endocrinol* 173(2) (2015) D1-12.

[8] A.N. Hoofnagle, M.H. Wener, The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry, *J Immunol Methods* 347(1-2) (2009) 3-11.

[9] B.G. Keevil, LC-MS/MS analysis of steroids in the clinical laboratory, *Clin Biochem* 49(13-14) (2016) 989-997.

[10] V.R. Meyer (2009). *Praxis der Hochleistungsflüssigchromatographie* (10. Auflage). Weinheim: Wiley-VCH.

[11] H. Budzikiewicz, M. Schäfer (2012). *Massenspektrometrie - Eine Einführung* (6. Auflage). Weinheim: Wiley-VCH.

[12] C. Shackleton, Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis, *J Steroid Biochem Mol Biol* 121(3-5) (2010) 481-490.

[13] F.Z. Stanczyk, N.J. Clarke, Advantages and challenges of mass spectrometry assays for steroid hormones, *J Steroid Biochem Mol Biol* 121(3-5) (2010) 491-495.

[14] E. Ciccimaro, I.A. Blair, Stable-isotope dilution LC-MS for quantitative biomarker analysis, *Bioanalysis* 2(2) (2010) 311-341.

[15] E. Stokvis, H. Rosing, J.H. Beijnen, Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not?, *Rapid Commun Mass Spectrom* 19(3) (2005) 401-407.

[16] S.R. Bornstein, B. Allolio, W. Arlt, A. Barthel, A. Don-Wauchope, G.D. Hammer, E.S. Husebye, D.P. Merke, M.H. Murad, C.A. Stratakis, D.J. Torpy, Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline, *The Journal of Clinical Endocrinology & Metabolism* 101(2) (2016) 364-389.

[17] E. Charmandari, N.C. Nicolaides, G.P. Chrousos, Adrenal insufficiency, *Lancet* 383(9935) (2014) 2152-2167.

[18] D.I. Ellis, W.B. Dunn, J.L. Griffin, J.W. Allwood, R. Goodacre, Metabolic fingerprinting as a diagnostic tool, *Pharmacogenomics* 8(9) (2007) 1243-1266.

[19] C. Rossi, L. Calton, G. Hammond, H.A. Brown, A.M. Wallace, P. Sacchetta, M. Morris, Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography-tandem mass spectrometry, *Clin Chim Acta* 411(3-4) (2010) 222-228.

[20] P.C. Kao, D.A. Machacek, M.J. Magera, J.M. Lacey, P. Rinaldo, Diagnosis of adrenal cortical dysfunction by liquid chromatography-tandem mass spectrometry, *Ann Clin Lab Sci* 31(2) (2001) 199-204.

[21] W. Arlt, M. Biehl, A.E. Taylor, S. Hahner, R. Libé, B.A. Hughes, P. Schneider, D.J. Smith, H. Stiekema, N. Krone, E. Porfiri, G. Opocher, J. Bertherat, F. Mantero, B. Allolio, M. Terzolo, P. Nightingale, C. Shackleton, X. Bertagna, M. Fassnacht, P.M. Stewart, Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors, *J Clin Endocrinol Metab* 96(12) (2011) 3775-3784.

[22] European Medicine Agency, Guideline on bioanalytical method validation, 2011, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf, accessed November 2015.

3.3. Publications

Articles

- J.M. Lindner, M. Vogeser, S.H. Grimm, Biphenyl based stationary phases for improved selectivity in complex steroid assays, *J Pharm Biomed Anal* 142 (2017) 66-73.
- J.M. Lindner, A.C. Suhr, S.H. Grimm, P. Möhnle, M. Vogeser, J. Briegel, The dynamics of a serum steroid profile after stimulation with intravenous ACTH, *J Pharm Biomed Anal* 151 (2018) 159-163.

Posters

- J.M. Lindner, S.H. Grimm, M. Vogeser, Biphenyl based stationary phases for improved selectivity in complex steroid assays. MSACL 2016 EU, Salzburg, Sept 12th - 15th, 2016, Poster R04.
- J.M. Lindner, S.H. Grimm, M. Vogeser, Biphenyl based stationary phases for improved selectivity in complex steroid assays. 13th Annual Meeting of the German Society for Clinical Chemistry and Laboratory Medicine, Mannheim, Sept 28th - 30th, 2016, Poster P004.
- J.M. Lindner, S.H. Grimm, M. Vogeser, Quantification of 31 antidepressants in human serum by using a high resolution Orbitrap mass spectrometer. MSACL 2017 EU, Salzburg, Sept 10th - 14th, 2017, Poster H02.
- J.M. Lindner, S.H. Grimm, M. Vogeser, Quantification of 31 antidepressants in human serum by using a high resolution Orbitrap mass spectrometer. 14th Annual Meeting of the German Society for Clinical Chemistry and Laboratory Medicine, Oldenburg, Oct 11th - 14th, 2017, Poster P034.

3.4. Acknowledgements

At first, I want to thank Michael Vogeser for his proficient, reliable and kind mentoring during my thesis, for his moral support and for the opportunity to work on interesting projects.

Furthermore, I am very grateful for the professional support, the ideas and the scientific input of Stefanie Grimm.

I thank Daniel Teupser for giving me the opportunity to do research at the Institute of Laboratory Medicine.

My thanks also go to all colleagues of the Institute of Laboratory Medicine, especially to Carina Schuster, Natalie Moll, Katharina Sorg, and Martina Kneifel for their helpful advices and the pleasant working atmosphere.

Moreover, I thank Anna Suhr for the briefing at the beginning of my thesis and for the useful tips.

I am thankful for the financial funding of my work by the Hans Fischer Society Munich.

My special and sincere thanks go to my friends and my family. Their emotional support and their encouragements contributed a lot to this thesis. In particular, I am most grateful to my parents for always supporting me and believing in me.

Finally, I thank Johannes on whom I can always rely. Thank you for cheering me up when needed, for your confidence in my abilities, just for being you.