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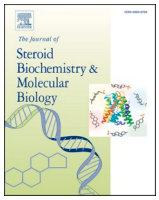

Fast and reliable quantification of aldosterone, cortisol and cortisone via LC-MS/MS to study 11β-hydroxysteroid dehydrogenase activities in primary cell cultures

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ABSTRACT

Cell culture experiments can support characterization of enzymatic activities in healthy and tumorous human tissues. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) enables simultaneous measurement of several steroids from a single sample, facilitating analysis of molecular pathways involved in steroid biosynthesis. We developed a reliable but fast method for quantification of cortisol, cortisone and aldosterone in cell culture supernatant. Validation, including investigation of matrix-matched calibration, was performed for two different cell types. Utility of the method was demonstrated in the study of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) activity under conditions of glucocorticoid and mineralocorticoid excess in different cell types. Aldosterone, cortisol and cortisone were extracted by liquid-liquid extraction (LLE) with methyl *tert*-butyl ether from 1 mL of cell culture supernatant. Steroids were separated on a Kinetex biphenyl column (50 ×2.1 mm, 2.6 μm) with gradient elution of water and methanol containing 2 mM ammonium format and analysed in multiple reaction monitoring mode after positive electrospray ionization. Application of the method included cell culture experiments with two different primary cell types, human coronary artery smooth muscle cells (HCSMC) and human coronary artery endothelial cells (EC). Cells were treated with different concentrations of cortisol, aldosterone and mifepristone, a glucocorticoid receptor antagonist and quantitative PCR was performed. The method exhibits high precision (CV ≤ 6 %) and accuracy (deviation from nominal concentration ≤ 6 %) for concentrations above the limit of quantification (LoQ) which is 0.11, 0.56 and 0.69 nmol/L for aldosterone, cortisone and cortisol, respectively. Calibration curves did not differ when prepared in media or solvent. The method enabled us to confirm activity of HSD11B2 and concentration dependent conversion of cortisol to cortisone in HCSMC (median conversion ratio at 140 nM cortisol = 1.46 %). In contrast we did not observe any HSD11B2 activity in EC. Neither addition of high aldosterone, nor addition of 1 μM mifepristone had impact on glucocorticoid concentrations. Quantitative PCR revealed expression of *HSD11B1* and *HSD11B2* in HCSMC but not in EC. We present a fast and reliable method for quantification of cortisol, cortisone and aldosterone in cell culture supernatants. The method enabled us to study HSD11B2 activity in two different cell types and will

Abbreviations: HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; HSD11B2, 11β-hydroxysteroid dehydrogenase type 2; C, concentration; HCSMC, human coronary artery smooth muscle cells; CV, coefficient of variation; DF, dilution factor; EC, human coronary artery endothelial cells; EMA, European Medicines Agency; ESI, electrospray ionization; GR, glucocorticoid receptor; IS, internal standard; LC, liquid chromatography; LLE, liquid-liquid extraction; LoQ, limit of quantification; ME, matrix effect; MR, mineralocorticoid receptor; MRM, multiple reaction monitoring; MS, mass spectrometry; MTBE, methyl *tert*-butyl ether; N, Number; PE, process efficiency; R^2, coefficient of determination; RE, recovery; SPE, solid phase extraction; QC, quality control; UHPLC, ultra-high performance liquid chromatography; V, volume.

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support future experiments investigating mechanisms of target organ damage in conditions of glucocorticoid and mineralocorticoid excess.

1. Introduction

Chronically elevated concentrations of cortisol are associated with unfavourable effects on human wellbeing and health, and contribute to increased mortality in affected patients [1–3]. Pathophysiological mechanisms include insulin resistance [1,4,5], hyperglycaemia [6,7] and hypertension [1,5,8] which subsequently leads to arteriosclerosis [9,10] and other cardiovascular diseases [1–3,11,12]. Understanding the mechanisms underlying cortisol-induced diseases is crucial to develop new treatment options. An effective approach to investigate such mechanisms are experiments with suitable cell lines. Those provide insights to cellular responses to glucocorticoid excess and could help to investigate causes of specific target organ damage.

Earlier studies using mammalian cells demonstrated a protective function of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) towards the mineralocorticoid receptor (MR) in mineralocorticoid target tissues [13–15]. At physiological cortisol concentrations (e.g., morning serum cortisol concentrations between 124 – 662 nmol/L, whereof only around 5 % are bioavailable [23]), MR is primarily activated by the mineralocorticoid aldosterone in mineralocorticoid target tissues [16–19], since HSD11B2 converts cortisol to inactive cortisone and prohibits MR activation by the glucocorticoid [8,19–21]. 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1), on the other hand, catalyses the reverse reaction, i.e., converts cortisone back to cortisol [22]. In contrast, at excessively high cortisol concentrations MR is also activated by cortisol which results in mineralocorticoid hypertension [8,19–21]. Determining cell-type specific vulnerability to glucocorticoid-mediated MR activation by the use of primary cell lines and modern measurement techniques may refine our understanding of the pathophysiology of steroid-induced organ damage. Ultimately, cell-specific protective interventions may ensue.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) provides a powerful measuring technique with high sensitivity and selectivity [24,25]. It is especially suited to measure small molecules like steroids and enables simultaneous measurement of several analytes within one sample. This makes it an ideal tool to monitor changes in steroid metabolism in cell culture experiments [26–28].

However, when applying a commercial kit, our method routinely used for quantification of steroids in human serum [29] to measurements in cell culture supernatants, we found insufficient extraction of steroids, evidenced by yellowish extracts and visible residues after evaporation (supplemental Figure ***A.1***). These residues severely impaired our measurements by mechanically blocking the LC system, but possibilities to optimize the method are limited since detailed information about the used materials are not available for the commercial kit. We therefore aimed to develop a method that allows more efficient extraction and simultaneously shorten chromatographic run time for analysis of 3 steroids in cell culture supernatant.

Our approach was to use liquid-liquid extraction (LLE) by a non-polar solvent and a newly developed LC-MS/MS method, optimized for application in cell culture media. Since composition of sample matrix can have an impact on measurement results [30–32] and composition of cell culture medium differs depending on cell type and purpose [33], we investigated the efficacy of the extraction method in terms of matrix effects with two different cell culture media.

Here we propose a rapid and robust method that allows selective simultaneous quantification of cortisol, cortisone and aldosterone in cell culture supernatants. We demonstrate applicability of the method in cell culture experiments by analysing the conversion of cortisol to cortisone in cultured human coronary artery smooth muscle (HCSMC) and endothelial cells (EC), providing evidence for HSD11B2 activity or inactivity in these cell types under ex vivo conditions.

2. Material and methods

2.1. Materials and chemicals

Standards of Aldosterone and Aldosterone d7 were purchased from Molekula (München, Germany) and Biomol (Hamburg, Germany), respectively. Cortisol and cortisone standard solutions, ammonium format (LC-MS grade) and water (LC-MS grade) were purchased from Sigma Aldrich (Schnelldorf, Germany). Standards of Cortisol d4 and Cortisone d8 were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and Biozol (Eching, Germany), respectively. Methyl *tert*-butyl ether (MTBE) and methanol (LC-MS grade) were obtained from Honeywell (Charlotte, USA). Human coronary artery smooth muscle cells, growth-medium (SmBM medium, CC-3181; EBM-2 Medium, CC-3156) and supplements (SingleQuots™ Supplement Pack, CC-4149, CC-4147) were purchased from Lonza (Basel, CH). Mifepristone (RU496) was purchased from Thermo Fisher (Karlsruhe, Germany).

2.2. Method development

2.2.1. Preparation of calibration samples, quality control samples and internal standards

Stock solutions of 1 μg/mL (aldosterone, aldosterone d7), 10 μg/mL (cortisol, cortisone) and 100 μg/mL (cortisol d4, cortisone d8) were prepared in methanol. Stock solution mixtures, including internal standard (IS) mix, were prepared in methanol/water (50:50, v/v). Concentrations in μg/mL and μmol/L are shown in supplemental Table ***A.1***. Calibration solutions were prepared in methanol/water (50:50, v/v) adding varying volumes of calibration stock solution mix to result in nine different concentration levels (supplementary Table ***A.2***). QC samples were prepared by adding varying volumes of the QC stock solution mix to the respective cell culture medium to result in three different concentrations (supplementary Table ***A.3***). All stock solutions, stock solution mixtures, IS mix, calibration and quality control samples were stored at ≤ -20°C until use.

2.2.2. Sample preparation

Sample preparation was performed using 1 mL of calibration solution, quality control sample, or cell culture supernatant in 10 mL glass tubes. Each was mixed with 20 μL of IS mix and incubated for 2 min. Steroids were extracted by adding 1.5 mL MTBE and mixing for 20 seconds. To facilitate phase separation, tubes were centrifuged at 1500 rpm for 1 min. One millilitre of upper phase was transferred to fresh glass tubes and evaporated to dryness under nitrogen stream at 37°C. Dried extracts were reconstituted with 200 μL of methanol/water (50:50, v/v). Concentrated extracts were transferred to a 96-well plate and placed into the autosampler of the LC-MS/MS system.

2.2.3. HPLC conditions

Chromatographic separation was performed on a Kinetex® Biphenyl LC Column (50 ×2.1 mm, 2.6 μm; Phenomenex, USA) equipped with a SecurityGuard™ ULTRA Cartridge (UHPLC Biphenyl, 2.1 mm ID; Phenomenex, USA) on a 1290 Infinity II ultra-high performance liquid chromatography (UHPLC) system (Agilent, USA). Flow rate was 0.3 mL/min, injection volume was 20 μL and column temperature was 50°C. Gradient elution with 2 mM ammonium format in water (mobile phase A) and 2 mM ammonium format in methanol (mobile phase B) resulted in three baseline separated steroid signals in 4 min run time. Gradient elution started with 55 % mobile phase B for 2 min. After 2.5 min.

mobile phase composition reached 100 % B to remain for 1 min. and returned to initial composition of 55 % B after 3.0 min. Retention times for cortisol, cortisone and aldosterone were 1.9 min., 2.2 min. and 2.9 min., respectively. A typical chromatogram is shown in Fig. 1.

2.2.4. Mass spectrometry conditions

Ion source and MRM parameters of the QTrap 6500+ mass spectrometer (Sciex, USA) have been optimized for maximum signal intensity. Parameter, that are invariable during the run, such as source temperature and electrospray ionization (ESI) probe position were optimized to the favour of aldosterone that showed the poorest ionization efficiency. The optimized values for ion source temperature, ion spray voltage, curtain gas, nebulizer gas, drying gas and declustering potential were 650°C, 4500 V, 30 psi, 70 psi, 50 psi and 40 V, respectively. Constant values were used for entrance potential (10 V), cell exit potential (14 V) and collision gas (medium). Optimized MRM parameters for each compound are shown in Table 1. Positive ESI mode was applied throughout. Quantification was performed in multiple reaction monitoring (MRM) acquisition mode. Analyst 1.7 software was used for acquisition and processing (Sciex, USA). Peak integration and

Table 1
Multiple reaction monitoring parameter for aldosterone, cortisol, cortisone and their related internal standards aldosterone d7, cortisol d4 and corticosterone d8.

substance	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (V)
cortisol d4	367.2	121.0	30
cortisol	363.2	121.0	30
cortisol qual	363.1	109.0	34
cortisol qual2	363.1	97.0	29
cortisone d8	369.2	168.1	32
cortisone	361.1	163.1	31
cortisone qual1	361.1	121.0	36
cortisone qual2	361.1	105.0	39
aldosterone d7	369.2	323.2	29
aldosterone	361.2	343.2	23
aldosterone qual1	361.2	315.2	27
aldosterone qual2	361.2	325.2	27
aldosterone qual3	361.2	299.2	32

m/z – mass to charge ratio; Q1 – quadrupole 1; Q3 – quadrupole 3; V – volt.

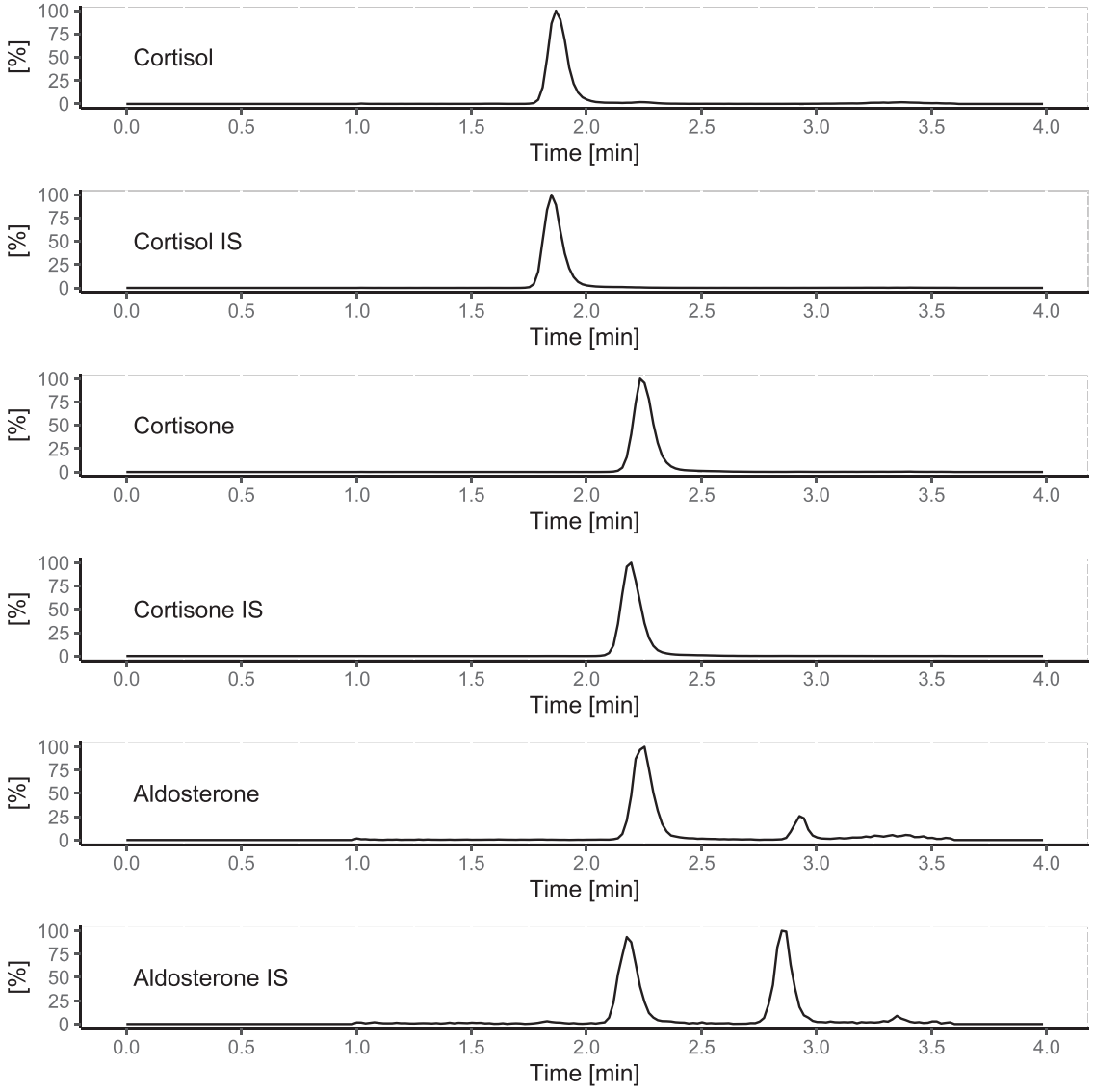

Fig. 1. Chromatographic method allows separation of steroids. Multiple reaction monitoring chromatograms for the 3 steroids and the respective stable isotope labelled internal standards (IS) are shown. Isobaric steroids cortisone and aldosterone are baseline separated (retention times cortisone 2.2 min, cortisone IS 2.2 min, aldosterone 2.9 min and aldosterone IS 2.8 min.

quantification were performed using SciexOS 1.6 software (Sciex, USA).

2.3. Method validation

Method validation was performed following the guideline on bioanalytical method validation of European Medicines Agency (EMA) [34] with special focus on precision, accuracy, limit of quantification, selectivity, matrix effect and dilution linearity.

2.3.1. Precision, accuracy and limit of quantification

To determine precision and accuracy of the method, SmBM medium was spiked with three different steroid concentrations. Six aliquots of each concentration level were measured on three different days. Intra- and interday coefficient of variation (CV) and deviation from the nominal concentrations were calculated. To investigate sensitivity limits of the method, SmBM cell culture medium was spiked with concentrations at and below the lowest calibration level. CV and deviations from the nominal concentration were calculated for five aliquots of each sample. Lowest concentration with signal-to noise ratio (S/N) $>$ 10, CV $<$ 20 % and deviation from the nominal concentration $<$ 20 % was defined as limit of quantification (LoQ), illustrated in supplemental Figure ***A.3***, ***Figure A.4*** and ***Figure A.5***. Acceptance criteria for concentrations above LoQ were CV $<$ 15 % and deviation from nominal concentration $<$ 15 %, respectively.

2.3.2. Matrix effect and extraction efficiency

Separation of analytes from interfering sample components is often crucial to obtain robust LC-MS/MS methods. Unfortunately, composition of cell culture supernatants varies depending on the application. Insufficient extraction from both cell culture media could be observed when applying our routine extraction method (supplemental Figure ***A.1***). To investigate extraction efficiency of LLE with MTBE for the three steroids, we calculated matrix effect (ME) and recovery (RE) according to Matuszewski et al. [32]. Therefore, three aliquots of each cell culture medium were spiked with a defined steroid concentration. Set A was spiked before extraction, Set B was spiked after extraction and set C was 50 % methanol spiked with the respective steroid concentrations for measurement without extraction. ME and RE were calculated according to the following equations.

$$ME\ [\%] = \text{mean(peak area set B)/mean(peak area set C)} \times 100$$

$$RE\ [\%] = \text{mean(peak area set A)/mean(peak area set B)} \times 100$$

2.3.3. Robustness of calibration and dilution linearity

Peak area, linear regression and 1/x weighting were used for calibration. Calibration solutions were prepared freshly every day during validation. To investigate robustness of calibration, we prepared calibration in 50 % methanol and two different cell culture media (SmBM, EBM-2). We compared slope, coefficient of determination (R^2), visual linearity and deviation from nominal value.

Since cell culture experiments might cover a wide concentration range, as seen in previously published reports [35–37], we also investigated whether high concentrated samples can be diluted to meet the calibration range. For this purpose, SmBM cell culture medium was spiked with a cortisol and cortisone concentration of 270 nmol/L and an aldosterone concentration of 10 nmol/L and diluted with various amounts of LC-MS grade water. Dilution factors (DF) were 2, 3 and 6, calculated according to following equation.

$$DF = (V(\text{sample}) + V(H_2O))/V(\text{sample})$$

To investigate dilution linearity, samples were measured in duplicates. Back calculation to original concentration was performed with SciexOS software (Sciex, USA) using the respective dilution factor.

2.3.4. Selectivity and carry-over

Gradient elution was optimized to avoid overlapping steroid signals in the chromatogram and prohibit interferences amongst the target steroids. Furthermore, a standard mix, containing estradiol, testosterone, androstenedione, dehydroepiandrosterone, corticosterone 11-deoxycortsiol, dehydroepiandrosterone-sulfate, 17-hydroxyprogesterone, progesterone, 18-oxocortisol, 18-hydroxycortisol, dihydrotestosterone and 11-deoxycorticosterone was measured and visually checked for interfering signals.

To investigate carryover of steroids from one measurement run to the next, which can result in falsely elevated values, two double blank samples were measured before calibration and after the highest concentrated calibration solution. Acceptance criteria was $<$ 20 % signal area compared to LoQ.

2.3.5. Stability of calibration and quality control samples

Stability of calibration and quality control samples was investigated since production and storage of aliquots is practical and time saving. Aliquots of calibration and QC samples were stored at −20°C for up to two years. Stability of extracted calibration and QC samples was investigated for one day at autosampler conditions (12°C) and one month at −20°C. Deviation from nominal concentrations was calculated using freshly prepared calibration solutions.

2.4. Application - cell culture experiments

Our validated method was applied to measure aldosterone, cortisol and cortisone concentrations in cell culture supernatants of HCSMC and EC. Both cell types were cultured in specific growth-medium (SMBM medium, CC-3181; EBM-2 Medium, CC-3156; Lonza, Basel, CH) with supplements (SingleQuots™ Supplement Pack, CC-4149 for HCSMC and CC-4147 for EC, both from Lonza, Basel, CH) according to the manufacturer's recommendations. All experiments were conducted between passages 5–9 and both cell lines were maintained at 37°C, 5 % CO2 and 95 % O2 in a humidified incubator. Cells were serum starved over night before treatment in the respective medium as the confluence reached 85–90 %. Pharmacological treatment was performed in serum-free medium for an additional 48 hours.

In both cell types, native activity ratios of HSD11B1 and HSD11B2 were studied by calculating the relative cortisol to cortisone conversion after treatment with 140 nM cortisol (n = 7 for HCSMC, n = 9 for EC). Relative conversion ratios in both cell lines were contrasted to gene expression data for *HSD11B1* and *HSD11B2*.

In HCSMC, saturation kinetics of the conversion of cortisol to cortisone were studied over a wide range of cortisol concentrations (1, 10, 140, 276, 414, 552, 690 nM) to investigate cell response under ex vivo conditions. Additional experiments were performed in the absence and presence of the glucocorticoid receptor (GR) antagonist mifepristone (1 μM) to determine GR-dependent regulation.

In EC, the impact of aldosterone excess on cortisol conversion was studied using a range of pathological aldosterone concentrations (0 nM, 1 nM, 10 nM and 100 nM).

2.5. Gene expression

Total RNA was extracted using the Maxwell 16 LEV simply RNA cells kit (Promega, Walldorf, Germany) and 100 ng were reversed transcribed using the GoScript™ Reverse Transcriptase Kit (Promega). Quantitative PCR was performed on a QuantStudio 5 machine (Thermo Fisher Scientific). Primers were purchased as Taqman probes from Thermo Fisher scientific. Each cDNA sample was assessed in triplicate. At least three independent repeats of each qPCR experiment were conducted. We used the $2^{-\Delta CT}$ method to normalize the expression levels of each gene of interest to the geometric mean of two housekeeping genes (EIF2B1 and HPRT1 for EC and PPIA and GAPD for HCSMC). Used assay IDs are shown in supplementary Table **A.4**.

2.6. Statistics and calculations

Unless specifically stated differently, concentrations below LoQ were arbitrarily set to the respective LoQ value for statistical calculations. Wilcoxon rank sum test was applied to compare group medians, with $p < 0.05$ considered significant. Statistics and figures were generated with R studio (R version 4.1.2). Relative conversion of cortisol to cortisone by HSD11B2 was calculated according to the equation

relative conversion [%] = concentration(cortisone)/(concentration (cortisol)+concentration(cortisone))

3. Results

3.1. Precision, accuracy and limit of quantification

LoQ for cortisol, cortisone, and aldosterone are 0.69 nM, 0.56 nM, and 0.11 nM, respectively. Precision and deviation from the nominal value are shown in supplemental Table ***A.5***. Results for intra- and interday precision and accuracy at three concentration levels are shown in Table 2. CV and deviation from the nominal concentration were ≤ 6 % in all cases, except the lowest aldosterone concentration, at LoQ level, where CV and deviation from the nominal value were ≤ 20 %.

3.2. Matrix effect and recovery

The extraction method resulted in colourless extracts and no visible residues remained after evaporation for both cell culture media (supplemental Figure ***A.2)***. Calculation of matrix factor according to Matuszewski et al. [32] reached values from 102 – 104 %. Recovery resulted in 51 – 54 % for cortisol and cortisone and 28 – 31 % for aldosterone. Data are shown in Table 3.

3.3. Robustness of calibration and dilution linearity

Relative differences between slope of calibration curve in 50 % MeOH compared to two different cell culture media was at or below the expected inter day variation of calibration curves in 50 % MeOH (< 2 %). A direct comparison of calibration in solvent and two different cell culture media is illustrated in supplemental Figure ***A.6***. Robustness of calibration was confirmed by CVs and deviation from nominal values < 5 % for all concentrations on eight different days. Linearity of the calibration curves was confirmed visually and by a correlation coefficient (R^2) > 0.995. Calibration parameters are shown in supplemental Table ***A.6***. All dilutions resulted in CV and deviation from nominal value below 5 %.

3.4. Selectivity and carry-over

Cortisone, isobaric aldosterone and cortisol signals were baseline separated in the chromatogram (Fig. 1). No signals were observed at the respective retention times after injection of a standard steroid mixture, confirming selective quantification of the target steroids in presence of the tested steroids. For double blank samples that were measured directly after the highest calibration level, area was below 2 % of LoQ area in all quantifier- and IS transitions.

Table 3
Matrix-effect (ME) and recovery (RE) for extraction of cortisol, cortisone and aldosterone from SmBM and EBM-2 cell culture medium using LLE with MTBE.

	ME [%]	RE [%]
cortisol (SmBM)	103	51
cortisol (EBM−2)	102	51
cortisone (SmBM)	102	53
cortisone (EBM−2)	102	52
aldosterone (SmBM)	102	30
aldosterone (EBM−2)	104	28

LLE – liquid-liquid-extraction; MTBE – methyl-tert-buthyl-ether.

3.5. Stability of calibration and quality control samples

Aliquots of calibration and QC samples stored at −20°C were stable for two years (deviation from nominal concentration < 15 %). Extracts of calibration in 50 % methanol and QC samples in both cell culture media were stable for at least 24 h at autosampler conditions (deviation from nominal concentration < 13 %) and at least one month at −20°C (deviation from nominal concentration < 10 %).

3.6. Application – cell culture experiments

In HCSMC treated with 140 nM cortisol, cortisol and cortisone concentrations were easily detectable. Median percentage of cortisone to total cortisone and cortisol in cells, treated with 140 nM cortisol, was 1.46 % in HCSMC (Fig. 2 ***A***). Absolute cortisone concentrations increased with increasing cortisol concentration (Fig. 3 ***A***), but relative conversion of cortisol to cortisone decreased with increasing cortisol concentration reaching a constant value of 1 % at 276 nM cortisol (Fig. 3 ***B***). At 1 nM cortisol, measured cortisone values were below LoQ. To enable calculation of the cortisone ratio we arbitrarily set the cortisone concentration to the LoQ (0.56 nmol/L). Treatment with steroid receptor antagonist mifepristone did not affect absolute cortisone concentrations, or relative cortisol conversion in HCSMC (Fig. 3). Furthermore, concentrations of cortisol and cortisone did not change after addition of 1 nM aldosterone (supplemental Figure ***A.7 C, D***).

In contrast, no conversion ratio could be calculated in EC. While cortisol concentrations were detectable, cortisone concentrations were below LoQ (supplemental Figure ***A.7***). Only for the purpose of illustrating the difference to the situation in HCSMC, we calculated a conversion ratio in EC by replacing cortisone concentrations with LoQ (0.56 nmol/L) (Fig. 2***A)***. In EC, cortisol concentrations did not change in the presence of 0 nM, 1 nM, 10 nM or 100 nM aldosterone (supplemental Figure ***A.7 A***).

Table 2
Mean calculated concentration ($\bar{x}$ in nmol/L), imprecision (CV in %) and deviation from the nominal value (Δ in %) for intra-day (n = 6, mean of three measurements) and inter-day evaluation (n = 18) in spiked SmBM cell culture medium.

		QC 1			QC 2			QC 3		
		$\bar{x}$	CV	Δ	$\bar{x}$	CV	Δ	$\bar{x}$	CV	Δ
aldosterone	Intra-day batch	0.13	5 %	19 %	1.05	5 %	5 %	3.49	3 %	5 %
	Inter-day	0.13	6 %	20 %	1.04	5 %	5 %	3.53	6 %	6 %
cortisol	Intra-day batch	2.74	3 %	1 %	24.49	1 %	1 %	81.70	2 %	2 %
	Inter-day	2.71	4 %	2 %	24.48	2 %	1 %	82.25	3 %	1 %
cortisone	Intra-day batch	2.62	2 %	5 %	24.75	1 %	2 %	81.25	1 %	2 %
	Inter-day	2.60	2 %	6 %	24.56	2 %	1 %	80.96	2 %	3 %

CV – coefficient of variation; QC – quality control.

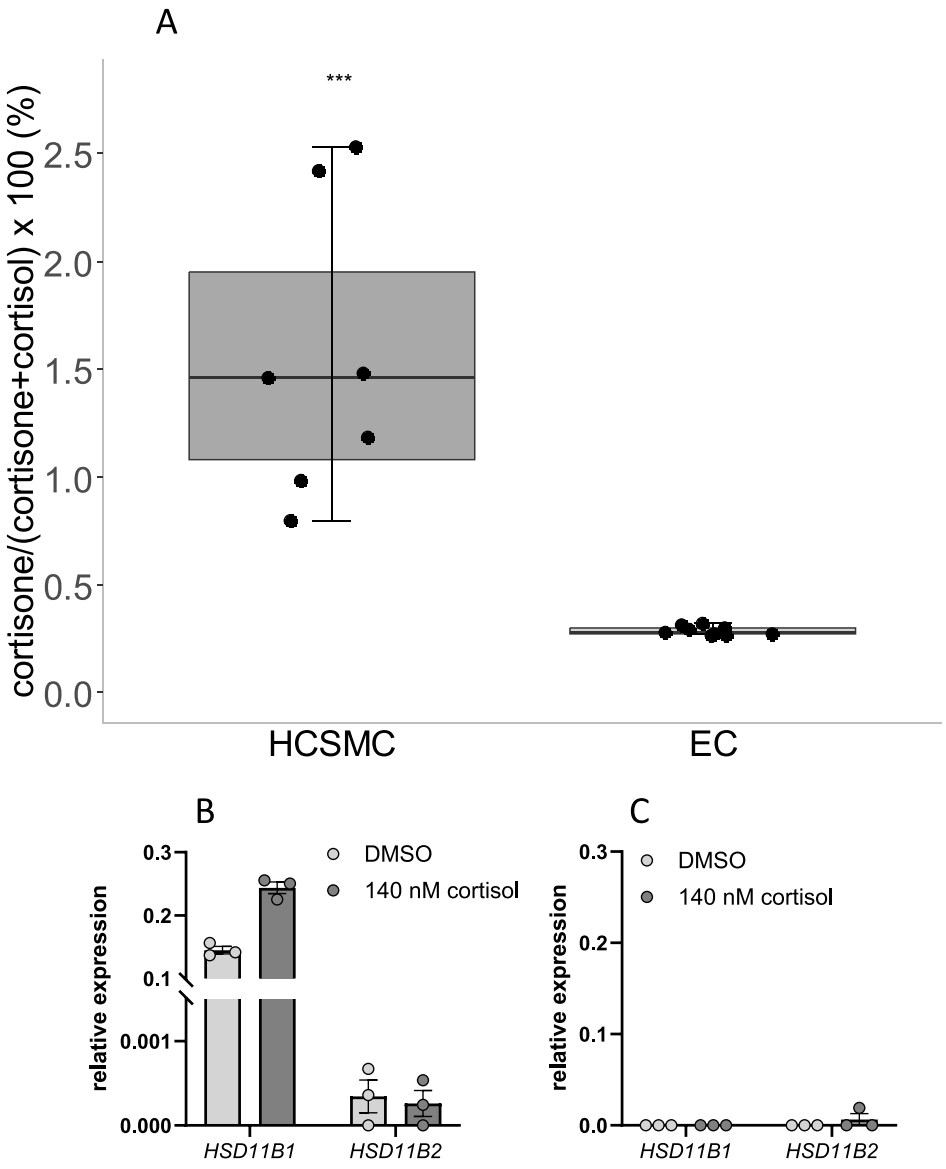

Fig. 2. Cortisol conversion corresponds to enzyme expression. Cortisol is converted to cortisone in HCSMC, but not in EC, where concentrations are below LoQ (A). The difference is significant (*** indicates $p < 0.001$). HSD11B1 and HSD11B2 are expressed in HCSMC (B) but not in EC (C).

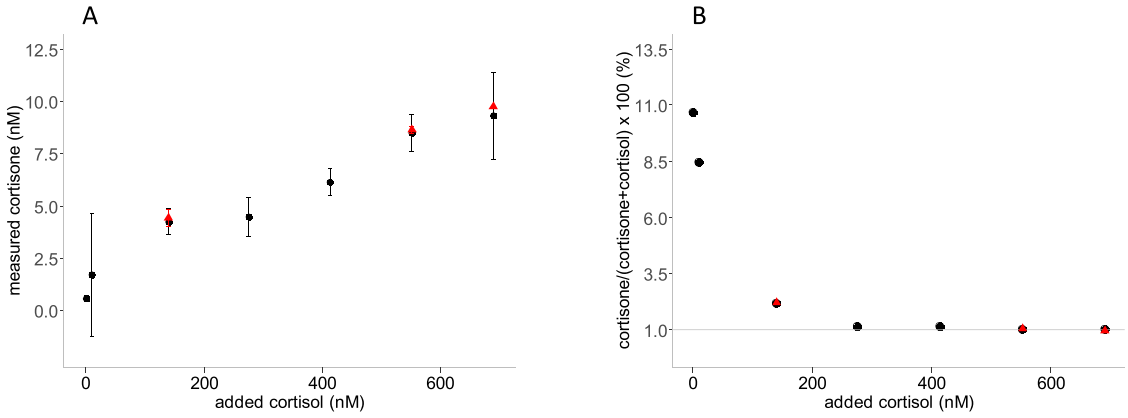

Fig. 3. Presence of glucocorticoid antagonist mifepristone does not alter cortisol conversion. Neither absolute (A) nor relative (B) cortisone concentrations are changed in presence of 1 μM mifepristone (red triangles).

3.7. Gene expression of HSD11B1 and HSD11B2

In line with the observed differences in cortisone concentrations, we found expression of *HSD11B2* and *HSD11B1* in HCSMC but not in EC (Fig. 2 ***B***, ***C***). In HCSMC, expression of *HSD11B1* was not significantly modified after addition of 140 nM cortisol ($p = 0.1$), and expression of *HSD11B2* also remained unchanged.

4. Discussion

We present a rapid, robust and fully validated LC-MS/MS method for the quantification of cortisol, cortisone and aldosterone in different cell culture media. We achieved selective quantification and complete chromatographic separation of three steroids with very similar molecular weight and structure in four minutes chromatographic runtime.

A modification of the extraction method used for routine serum samples was necessary not only to shorten runtime, but also because we had observed ineffective extraction of steroids from cell culture medium, accompanied by yellowish extracts and residues after evaporation (supplemental Figure ***A.1***). These residues accumulated when injected to the UHPLC and prevented further measurements. Regarding the yellowish colour, it is reasonable to assume that phenol red, a pH indicator commonly added to cell culture media and known to cause interferences in LC-MS/MS analysis [38,39], was still present in the extracts. We could, however, not test this hypothesis because injection of the extracts blocked the UHPLC almost immediately. Previously published LC-MS/MS methods for measurement of steroids in cell culture media all had used medium without phenol red [40–43]. Reasons included prevention of interferences in the LC-MS/MS, its known estrogen-like action [44] or were not specified. However, we wanted to keep the dye as a pH indicator during cell cultivation.

We chose LLE as alternative extraction method and confirmed its effectiveness by clear extracts (supplemental Figure ***A.2***) and matrix factors of 102–104 %. Similar results for matrix factors were published recently by Fanelli et al. [41] for a set of other steroids (94.9–104.7 %) and Abe et al. [42] for a set of steroids including aldosterone, cortisol and cortisone (85.3–112 %). However, both methods were using LLE for steroid extraction from cell culture media without phenol red. Our results suggest that our method can be used with different types of cell culture media, regardless whether they contain phenol red or not.

We chose MTBE as extraction solvent because it has been reported to allow more comfortable handling compared to other common extraction solvents such as dichloromethane, hexane or chloroform: The extracts stay in the upper phase in LLE, and the solvent is less harmful to health [45,46]. In addition, MTBE has been presented as more suitable extraction solvent for steroids than hexane, dichloromethane, tetrachloromethane, diethyl ether and isopentane in previous reports [46–48].

We observed a recovery of 51–54 % for cortisol and cortisone, and 28–31 % for aldosterone. This suggests limited efficacy, particularly for aldosterone. While this did not represent a problem in our experimental setting, further optimization of the method might be required for applications requiring higher sensitivities. Recent reports describe the use of a mixture of 8:2 hexane:ethylacetat [41] and pure ethylacetat [42] for effective extraction of steroids from cell culture media.

We are aware about the recommendation in common guidelines [49,50] to use matrix based calibration for LC-MS/MS measurements to account for matrix effects. However, we obtained equal results in slope, linearity and deviation from nominal value for calibration solutions prepared in 50 % methanol and in two different cell culture media. This suggests that for our method, calibration curves can be prepared in solvent instead of the respective cell culture medium, which is more cost-effective, easier to handle and also facilitating standardization across experiments.

MS parameters were optimized to achieve accurate and sensitive measurement of high cortisol concentrations and low cortisone and aldosterone concentrations at the same time. The resulting sensitivities (LoQs of 0.69 nmol/L, 0.56 nmol/L, 0.11 nmol/L for cortisol, cortisone and aldosterone, respectively) are comparable or below the LoQs reported for previously published methods using SPE and other cell culture media [43,51,52]. Sensitivity could be further improved by performing multiple extraction steps instead of one, or reconstituting the extract after evaporation in a smaller volume than the 200 μL used in our protocol. However, since sensitivity was considered sufficient for the intended application, we decided to keep preparation time short and maintain a suitable volume for injection to allow repeated measurements.

Excessive cortisol and aldosterone concentrations are established causes of target organ damage [53–55]. Furthermore, it is known that MR and HSD11B2 are often co-expressed, but not in all mineralocorticoid target cells [56]. We therefore applied our method to study the cell-type specific protection by HSD11B2 as well as a potential interaction between aldosterone and cortisol metabolism in two different primary cell lines (HCSMC and EC). After treatment with cortisol, cortisone concentrations were detectable only in HCSMC, but below LoQ in EC (supplemental Figure ***A.7***). Accordingly, relative cortisol to cortisone conversion was significantly ($p < 0.001$) higher in HCSMC compared to EC, providing functional evidence of HSD11B2 activity in HCSMC, but not in EC. This was corroborated by gene expression data, indicating expression of *HSD11B2* in HCSMC but not in EC (Fig. 2***J***). In HCSMC, absolute cortisone concentrations increased with increasing cortisol concentrations added. However, the conversion rate seems to be higher at lower concentrations, and fell to about 1 % at cortisol concentrations above 276 nM, probably because enzyme capacity is saturated (Fig. 3 ***B***) [57]. Possible reconversion of cortisone to cortisol by HSD11B1 which is also expressed in HCSMC, might also play a role (Fig. 2). Off note, neither in ECs nor in HCSMC cortisol inactivation was affected under conditions of aldosterone excess (supplemental Figure ***A.7***).

Our data might suggest that in coronary arteries, SMC probably exhibit greater protection from MR activation by circulating cortisol than ECs. Since the protective effect shows saturation at 276 nM (corresponding to 10 μg/dL), which we consider to be a rather low concentration in our ex vivo experiment, it is tempting to speculate that MR signalling in both vascular cell types may be preferentially driven by cortisol in the early morning and by aldosterone around midnight, the natural peaks and troughs of systemic cortisol. Alternatively, the saturation kinetics of fractional conversion of cortisol in SMC may well reflect a system optimized for low, local concentrations (corresponding to 1–5 % of total circulating cortisol) [58]. However, it needs to be emphasized that during cultivation cells were isolated and exposed to concentrations of nutrients and substrates which are different from the respective in vivo conditions. The difficulty to exactly mimic physiological conditions are an obvious limitation of our and other experiments in cell culture systems. Further studies should aim to modify the presented method for tissue and cytosolic steroid concentrations to precisely determine bioavailability of steroids at tissue and cell-specific levels.

5. Conclusion

In conclusion, we developed a fast and reliable method that allows the accurate measurement of aldosterone, cortisol and cortisone in different cell culture supernatants, even if phenol red is present. We demonstrate that LLE with MTBE is a useful extraction method for steroids in different cell culture media and can prevent blockage and interference in LC-MS/MS analysis. Application of the method to characterize HSD11B2 activity in two different primary cell lines demonstrated significant differences in cortisol metabolism between them. The method can be expected to provide an interesting tool for future cell culture experiments.

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CRediT authorship contribution statement

Martin Reincke: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Tim Kühnle:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **Michaela Höhne:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Laura Brunnenkant:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Holger Schneider:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Yao Meng:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Sonja Kunz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martin Bidlingmaier:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marily Theodoropoulou:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Martin Bidlingmaier reports financial support was provided by German Research Foundation. Holger Schneider reports financial support was provided by German Research Foundation. Martin Reincke reports financial support was provided by German Research Foundation. Marily Theodoropoulou reports financial support was provided by German Research Foundation. Martin Reincke reports financial support was provided by European Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2024.106610.

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