

## LC–MS/MS Method for the Simultaneous Determination of Free Urinary Steroids

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**Abstract** Cortisol homeostasis is implicated in hypertension and metabolic syndrome. Two enzymes modulate cortisol availability; 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) preferentially converts inactive cortisone to cortisol, whereas 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) converts cortisol to cortisone. In contrast, 5 $\alpha$  and 5 $\beta$  reductases inactivate cortisol by conversion to its tetrahydrometabolites: tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone. A subtle local increase in cortisol can be detected by measuring 24-h urine metabolites, LC–MS/MS being the reference method. The 11 $\beta$ -HSD2 activity is assessed based on the cortisol/cortisone ratio, and the 11 $\beta$ -HSD1 activity on the (tetrahydrocortisol + allo-tetrahydrocortisol)/tetrahydrocortisone ratio. To better understand hypertension and/or metabolic syndrome pathogenesis a method for simultaneous determination

of cortisol, cortisone, tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone was developed and validated in an LC coupled with the new detector AB Sciex QTrap<sup>®</sup> 4500 tandem mass spectrometer. The steroids were extracted from 1 mL urine, using cortisol-D4 as internal standard. The quantification range was 0.1–120 ng/mL for cortisol and cortisone, and 1–120 ng/mL for tetrahydrometabolites, with >89 % recovery for all analytes. The coefficient of variation and accuracy was <10 %, and 85–105 %, respectively. Our LC–MS/MS method is accurate and reproducible in accordance with Food and Drug Administration guidelines, showing good sensitivity and recovery. This method allows the assessment of 11 $\beta$ -HSD2 and 11 $\beta$ -HSD1 activities in a single analytical run providing an innovative tool to explain etiology of misclassified essential hypertension and/or metabolic syndrome.

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### Introduction

Cortisol homeostasis is important in controlling blood pressure, and its deregulation could be implied in hypertension and metabolic syndrome (MetS). Several enzymes modulate cortisol availability. In the liver and adipose tissue, 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) preferentially converts the inactive cortisone to active cortisol. In the kidney and colon, 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) transforms cortisol to cortisone, protecting the mineralocorticoid receptor from erroneous activation by cortisol. Furthermore, in the liver, the 5 $\alpha$  and 5 $\beta$  reductases (in conjunction with 3 $\alpha$ -hydroxy steroid

dehydrogenase) inactivate cortisol and cortisone to the tetrahydrocortisol metabolites: 5 $\alpha$  tetrahydrocortisol, 5 $\beta$  tetrahydrocortisol (allo-tetrahydrocortisol), and tetrahydrocortisone [1]. Thus, the availability of cortisol to bind and activate the glucocorticoid and mineralocorticoid receptors may be influenced by both the local pre-receptor metabolism and the systemic cortisol level.

Based on the functions of the 11 $\beta$ -HSD isozymes, the impairment of their activity has been associated with the pathogenesis of arterial hypertension and MetS, which are common medical conditions. Assessing the enzymes' activities is critical to the study of these patients, where the urinary cortisol/cortisone ratio has been suggested as an index of 11 $\beta$ -HSD2 activity. In the presence of a normal cortisol/cortisone ratio, the ratio of the urinary metabolites tetrahydrocortisol and allo-tetrahydrocortisol to tetrahydrocortisone provides an estimate of 11 $\beta$ -HSD1 activity [1, 2].

The steroid hormones in body fluids are usually measured by immunoassay in the clinical laboratory due to the simplicity, speed, and analytical sensitivity. This is possible using automated analyzers that allow high-throughput testing [3]. Despite their many advantages, immunoassays have certain limitations, including the differences between compounds cross-reactivity that are structurally related to the target, suboptimal specificity, limited dynamic range, and matrix effects [4]. Alternative techniques, such as the combination of gas chromatography and mass spectrometry (GC-MS), previously the 'gold standard' for steroid profiling, are laborious and time consuming due to the low volatility of some compounds and the need of a derivatization step, limiting their application to routine diagnostics [5]. High-performance liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) fulfills the requirements of sufficient analytical sensitivity and specificity, as well as speed and robustness, for single-steroid analysis [6, 7].

The aim of this study was to develop and validate an LC-MS/MS method, using US Food and Drug Administration (FDA) parameters for the simultaneous determination of free cortisol, cortisone, tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone in a clinical laboratory to gain a better understanding of hypertension and/or MetS pathogenesis.

## Materials and Methods

### Solvents and reagents

All solvents (methanol, acetone, hexane, and water) were high-performance liquid chromatography (HPLC) grade from Merck (Santiago, Chile). Formic acid (Merck) was 98–100 % pure. Cortisol (4-pregnen-11 $\beta$ , 17, 21-triol-3,

20-dione), cortisol-D4 (4-pregnen-11 $\beta$ , 17, 21-triol-3, 20-dione-9, 11, 12, 12-d4), cortisone (4-pregnen-17, 21-diol-3, 11, 20-trione), tetrahydrocortisol (5 $\beta$ -pregnan-3 $\alpha$ , 11 $\beta$ , 17, 21-tetrol-20-one), allo-tetrahydrocortisol (5 $\alpha$ -pregnan-3 $\alpha$ , 11 $\beta$ , 17, 21-tetrol-20-one), and tetrahydrocortisone (5 $\beta$ -pregnan-3 $\alpha$ , 17, 21-triol-11-20-dione) were purchased from Steraloids, Inc., (Andover, MA, USA). Stock solutions of all standards (1 mg/mL) and of cortisol-D4 as the internal standard (IS; 2  $\mu$ g/mL) were prepared in methanol and stored at  $-20^{\circ}\text{C}$ .

### Liquid Chromatography

The liquid chromatography separations were carried out using an Agilent Technologies Series 1200 high-pressure mixing pump equipped with a Series 1200 autosampler and microwell plate autosampler (Agilent, Boston, MA, USA). An Inertsil ODS-3 C18 (2.1 mm inner diameter  $\times$  150 mm) 3- $\mu$ m particle size column (GL Sciences Inc., Tokyo, Japan) was used with a mobile phase gradient of solvent A (water containing 0.1 % formic acid) and solvent B (methanol containing 0.1 % formic acid). The gradient conditions were 0–5 min with 40 % solvent A and 60 % solvent B, from 6 to 8 min 50 % solvent A and 50 % solvent B, from 9 to 10 min 60 % solvent A and 40 % solvent B, from 11 to 13 min 10 % solvent A and 90 % solvent B, and from 14 to 18 min 40 % solvent A and 60 % solvent B.

### Mass Spectrometry

An AB Sciex QTrap<sup>®</sup> 4500 tandem mass spectrometer (Foster City, CA, USA) operated with a Turbo-V IonSpray source was used to obtain the mass spectra and the selected reaction monitoring (SRM) determinations. The optimized conditions were as follows: ion spray voltage, 5.5 kV; gas source 1, 60; gas source 2, 40; turbo temperature, 600  $^{\circ}\text{C}$ ; entrance potential, 10 V; and declustering potential (DP), 60 V. The collision gas pressure was set at 12 mPa and the curtain gas pressure at 20 mPa. The SRM transitions and the related optimized DP, collision energy, and collision cell exit potential for the different analytes are shown in Table 1.

### Standards and Quality Control Samples

Working solutions of eight standards (0.1, 1, 5, 10, 30, 60, 90 and 120 ng/mL) and three quality control samples (QCs; 20, 40 and 80 ng/mL) were prepared in 1 mL steroid-free urine by dilution of the stock solution of each analyte. The standards were prepared on the day of analysis, and the QCs were prepared in bulk monthly and stored at  $-20^{\circ}\text{C}$ .

The standards and QCs were spiked with 10  $\mu$ L IS (cortisol-D4; 20  $\mu$ g/mL in methanol; final concentration,

**Table 1** LC/MS–MS data for the corticosteroids studied

Steroid	Molecular weight (Da)	Precursor ion	Product ions (ESI+) <sup>a</sup>	Declustering potential	Collision energy	Collision cell exit potential
Cortisol	362.21	363.25	327/309/ <u>121</u>	80/85/82	21/24/36	16/14/10
Cortisone	360.19	361.24	343/301/ <u>163</u>	110/100/75	23/29/33	08/12/12
Tetrahydrocortisol	366.24	331.27	313/ <u>301</u> /295	95/100/100	17/17/17	13/08/16
Allo-tetrahydrocortisol	366.24	331.27	313/295/ <u>271</u>	95/75/80	17/18/18	13/16/15
Tetrahydrocortisone	364.22	347.28	329/311/ <u>243</u>	105/107/110	19/21/30	16/18/13
Cortisol-D4 <sup>b</sup>	366.23	367.27	<u>331</u> /273/121	85/85/85	24/24/36	14/14/10

<sup>a</sup> Quantitative ions are underlined

<sup>b</sup> Internal standard

20 ng/mL). Before analysis, steroids were extracted on SPE columns (Oasis<sup>®</sup> HLB 3 mL, 60 mg) preconditioned sequentially with 3 mL methanol and 3 mL water, followed by the addition of 1 mL urine to the columns. The columns were washed sequentially with 3 mL water, 3 mL acetone/water (200 mL/L), and 1 mL hexane. After the columns were dried by suction for 2 min, the steroids were eluted with 2 mL methanol. The extracts were dried under nitrogen and dissolved in 100  $\mu$ L methanol/water (1:1) containing 0.1 % formic acid, and 10  $\mu$ L samples were injected in the LC–MS/MS system.

#### Method Validation

The method was validated according to the FDA parameters [8]. The linearity was evaluated by the regression analysis of standards over the concentration range of the calibration curve. The lower limit of detection (LLOD) was defined as the lowest concentration of each analyte that could be reliably differentiated from background noise assessed with a blank sample; the lower limit of quantification (LLOQ) was defined as the lowest concentration that could be measured with an interday coefficient of variation (CV) of <20 % and accuracy between 80 and 120 %.

The precision and accuracy were assessed using the three QCs. The QCs were assayed five times within the same day for the determination of intraday precision and on five consecutive days in duplicate to determine interday precision, and the CV was calculated. The CV determined at each concentration level should not exceed 15 %. The accuracy was defined as the percentage of the nominal concentration and was estimated based on the percent deviation of the mean value from the target following five measurements of each of the three QCs. The mean value should be within 15 % of the actual value. Calibration curves were prepared on each day of analysis.

The matrix effect (ME), recovery (RE), and process efficiency (PE) for each analyte were assessed using post-extraction addition, established by Matuszewski et al. [9]

and others. Briefly, three sets of standards (20, 40 and 80 ng/mL) were prepared. Set A contained the standard and IS in methanol; set B contained extracts spiked with the standards and IS after extraction; and set C contained regular samples (i.e., urine spiked with the standards and IS before extraction). The peak area for the standards in these three sets was used to calculate ME, RE, and PE for each analyte according to the following equations:

$$\text{Matrix effect (\%)} = B/A \times 100$$

$$\text{Recovery (\%)} = C/B \times 100$$

$$\text{Process efficiency (\%)} = C/A \times 100$$

where *A* = peak area of each analyte from set A; *B* = peak area of each analyte from set B; and *C* = peak area of each analyte from set C.

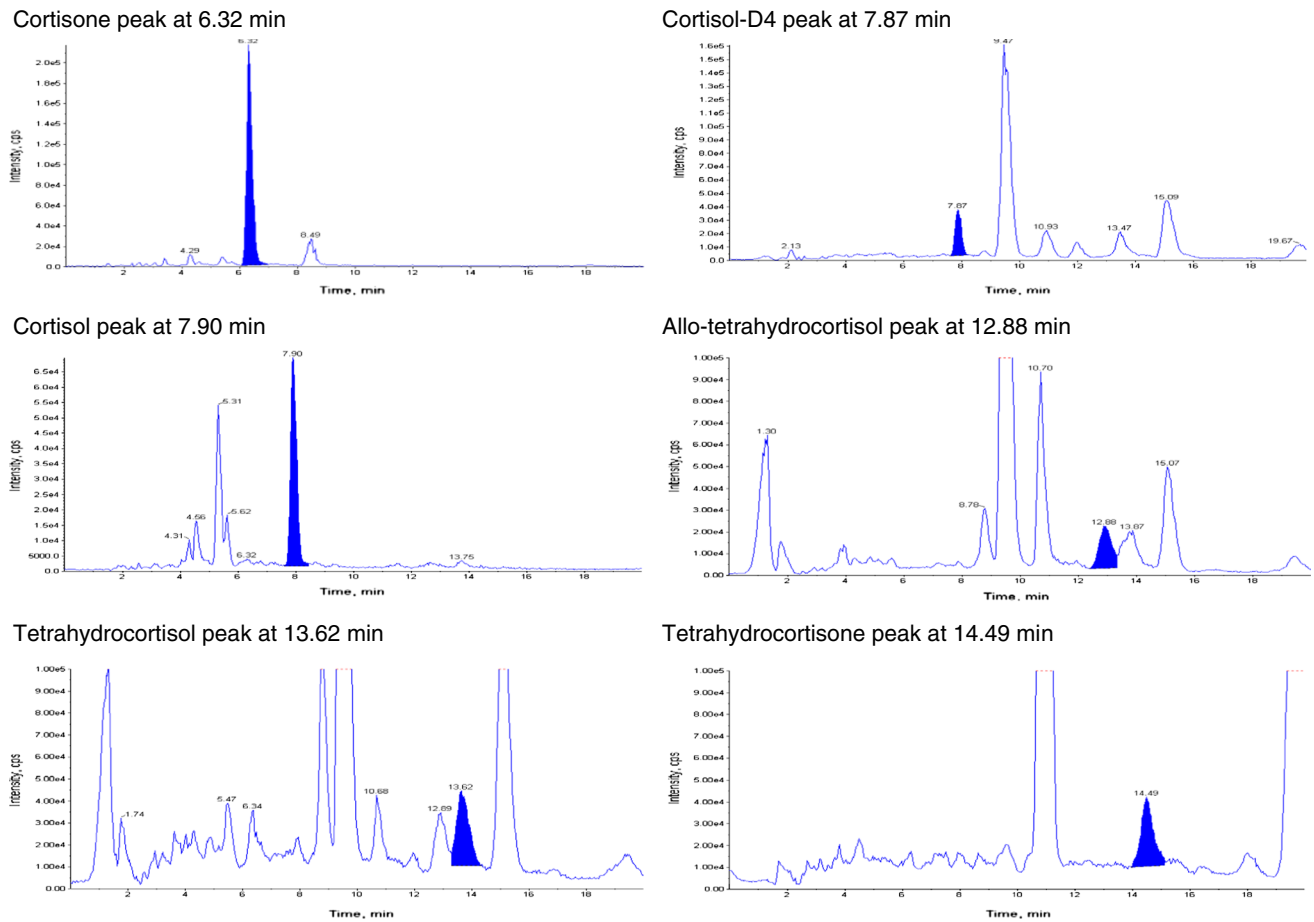
## Results

### LC–MS/MS Characteristics

The method described here allows the detection and quantification of the five free steroids in one analytical run of 18 min, with good resolution of each analyte. The retention times for cortisone, cortisol-D4, cortisol, allo-tetrahydrocortisol, tetrahydrocortisol, and tetrahydrocortisone were 6.53, 8.00, 8.02, 13.02, 13.80, and 14.61 min, respectively. A representative mass chromatogram of a physiological human urine sample is shown in Fig. 1.

### Linearity, Lower Limit of Detection, Lower Limit of Quantification, Precision, and Accuracy

The assay was linear from 0.1 to 120 ng/mL for cortisol and cortisone, and from 1 to 120 ng/mL for tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone. The regression coefficient ( $r^2$ ) was >0.999 for each of the five analytes. LLOD was 0.05 ng/mL for cortisol and cortisone,



**Fig. 1** Physiological human sample urine with five steroids and internal standard. Cortisone peak at 6.32 min, cortisol-D4 peak at 7.87 min, cortisol peak at 7.90 min, allo-tetrahydrocortisol peak at 12.88 min, tetrahydrocortisol peak at 13.62 min, tetrahydrocortisone peak at 14.49 min

**Table 2** Intraday and interday precision and accuracy in quality control pools

Steroid	QC <sup>a</sup> 20 ng/mL				QC <sup>a</sup> 40 ng/mL				QC <sup>a</sup> 80 ng/mL			
	Mean (ng/mL)	SD <sup>b</sup>	CV <sup>c</sup> (%)	Accuracy (%)	Mean (ng/mL)	SD <sup>b</sup>	CV <sup>c</sup> (%)	Accuracy (%)	Mean (ng/mL)	SD <sup>b</sup>	CV <sup>c</sup> (%)	Accuracy (%)
Cortisol intraday	20.58	0.58	2.81	102.9	39.80	1.00	2.52	99.5	83.47	1.09	1.31	104.34
Cortisol interday	20.14	1.47	7.30	100.71	40.04	0.82	2.04	100.10	79.73	6.69	8.39	99.67
Cortisone intraday	19.90	0.86	4.31	99.5	38.63	0.87	2.25	96.6	80.34	5.07	6.31	100.43
Cortisone interday	20.86	1.89	9.04	104.32	38.44	1.24	3.23	96.10	80.16	6.84	8.53	100.20
Tetrahydrocortisol intraday	20.18	1.04	5.14	100.9	38.15	1.86	4.87	95.4	76.99	5.42	7.05	96.23
Tetrahydrocortisol interday	17.12	0.34	2.00	85.60	39.94	1.61	4.03	99.85	75.85	5.62	7.41	94.81
Allo-tetrahydrocortisol intraday	18.22	1.38	7.55	91.1	35.60	1.44	4.04	89.0	73.12	2.68	3.67	91.40
Allo-tetrahydrocortisol interday	17.18	0.91	5.33	85.90	38.86	1.75	4.50	97.15	77.21	7.43	9.62	96.51
Tetrahydrocortisone intraday	19.20	0.65	3.39	96.0	37.03	1.72	4.64	92.6	77.75	5.32	6.84	97.19
Tetrahydrocortisone interday	17.94	0.99	5.53	89.70	39.46	1.56	3.95	98.65	79.69	6.56	8.23	99.62

<sup>a</sup> Quality control

<sup>b</sup> Standard deviation

<sup>c</sup> Coefficient of variation

**Table 3** Matrix effect, recovery and efficiency of the method

	Steroid	ME <sup>a</sup> (%)		RE <sup>b</sup> (%)		PE <sup>c</sup> (%)	
		Mean	SD <sup>d</sup>	Mean	SD <sup>d</sup>	Mean	SD <sup>d</sup>
	Cortisol	91.4	16.1	101.3	8.9	91.9	9.3
	Cortisone	94.5	19.6	104.5	9.4	98.9	17.9
<sup>a</sup> Matrix effect	Tetrahydrocortisol	76.9	14.4	99.6	9.4	75.5	14.3
<sup>b</sup> Recovery	Allo-tetrahydrocortisol	122.2	28.5	95.6	9.4	115.5	17.9
<sup>c</sup> Performance efficiency	Tetrahydrocortisone	90.0	16.7	105.2	6.0	94.5	13.3
<sup>d</sup> Standard deviation	Cortisol-D4	95.5	7.3	90.0	7.8	85.0	0.9

and 0.2 ng/mL for allo-tetrahydrocortisol, tetrahydrocortisol and tetrahydrocortisone, and the lowest concentration matching the LLOQ acceptance criteria was 0.1 ng/mL for cortisol and cortisone and 1.0 ng/mL for allo-tetrahydrocortisol, tetrahydrocortisol and tetrahydrocortisone.

The QCs for all five analytes had acceptable intraday and interday precision (<10 %) and accuracy (85–105 %); these results are shown in Table 2.

#### Matrix Effect, Recovery, and Process Efficiency

For all analytes, the mean analytical recovery was >89 %, with a matrix effect ranging from 90 to 122 % and a process efficiency >76 % (Table 3).

#### Discussion

We developed an analytical LC–MS/MS method for the determination of five steroids in a single analytical run that is reliable, accurate, and precise. Based on FDA regulatory guidelines [8], this method is suitable to be implemented in a clinical laboratory for routine diagnostic purposes. The assay achieved good analytical separation of all five steroids with an acceptable intraday and interday precision and accuracy. The ability to measure the five analytes over a wide analytical range, from 0.1 to 120 ng/mL for cortisol and cortisone, and 1–120 ng/mL for tetrahydrometabolites, makes this method well suited for the evaluation of 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 activity. This dynamic range broadly covers the reference values reported previously by our group [1].

Recovery and performance efficiency evaluation using Matuszewski et al. [9] procedure showed that good results were achieved for all the five steroids. Assessment of ME is mandatory in the validation of a quantitative assay using LC–MS/MS in biological samples, since endogenous impurities can affect the ionization process and may reduce or increase the efficiency of formation of the desired analyte ions. In this way, ME affects assay reproducibility and accuracy. Our study documented the absence of a significant ME, being allo-tetrahydrocortisol the one that had the

largest one with a mean enhancement of 22 %. This could be caused by the fact that for tetrahydrometabolites the pattern of fragmentation is quite complicated, giving rise to spectra containing a large number of fragments which most of them are common to several steroid compounds [10]. For these reasons, it is necessary to separate the three tetrahydrometabolites in a successful chromatographic run prior to subsequent MS measurement. Because, in our method, allo-tetrahydrocortisol is the analyte with the least analytical sensitivity, it is the analyte that will be most affected by any loss of sensitivity and selectivity.

The use of positive or negative ionization mode was evaluated by Cuzzola et al. [11] in the only report in literature that describes the measurement of the same five steroids in human urine in a single analytical run. In this paper, the negative mode was selected for further fragmentation and quantification of the compounds. This approach was used by Turpeinen et al. [12], although this group measured only tetrahydrometabolites, and also by Pavlovic et al. [13] who were able to measure all five steroids but the method was validated in bovine urine. In our study, all the results were generated in positive ion mode and achieved a good LLOQ of 0.1 ng/mL for F and E, and 1 ng/mL for the tetrahydrometabolites, although it has been reported that the negative ion mode produces better signal-to-noise ratio and reduced fragmentation than the positive ion mode [11]. Other groups have used the positive ion mode to measure cortisol and cortisone and their tetrahydrometabolites but were unable to separate the isomers tetrahydrocortisol and allo-tetrahydrocortisol [10, 14]; only Yamashita et al. [15] accomplish this issue but their method needs picolinyl derivation of cortisol and cortisone A ring metabolites.

The LLOQ achieved in our work for free steroids measurement is relevant because in urine, only a small fraction of the steroids are in the free form, with the larger fraction being conjugated to glucuronides and sulfates. The advantage of measuring free steroids is that there is no need to deconjugate the urine samples, which is time consuming and can increase the chance of inaccurate results due to incomplete hydrolysis and variability in the enzyme preparations [10].

The simultaneous measurement of cortisol, cortisone, tetrahydrocortisol, allo-tetrahydrocortisol, and tetrahydrocortisone will be useful in clinics and, in particular, contribute to a better understanding of the pathophysiology of essential arterial hypertension and/or MetS. The measurement of clinical diagnostic biomarkers should be reliable, robust, and suitable for high-throughput analysis. Since its introduction as a highly specialized analytical tool, LC–MS/MS has evolved into an accepted, routine diagnostic technique in the clinical laboratory. LC–MS/MS is now considered the method of choice for the measurement of steroid hormones, particularly ones that circulate at low concentrations in plasma and/or urine.

## Conclusions

The LC–MS/MS method described here is accurate, precise and is in accordance with FDA guidelines. Good sensitivity, RE, and ME were obtained for the simultaneous measurement of the five analytes.

The analytical characteristics of this method make it suitable for implementation as a routine technique in the clinical laboratory, allowing the assessment of 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 activities in a single analytical run.

An adequate clinical endocrinological exam followed by the LC–MS/MS analysis of these steroids and accurate interpretation of data should aid physicians in identifying the etiology of misclassified essential hypertension and/or MetS.

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