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Research Article

# Improving LC-MS/MS measurements of steroids with differential mobility spectrometry

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ABSTRACT

**Introduction:** Steroid measurements are important for diagnosis and monitoring of many conditions and treatment regimens; however, due to structural and chemical similarities amongst steroids, these analyses are challenging, even for highly specific techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential mobility spectrometry (DMS) has the potential to improve these analyses by providing an orthogonal and complementary separation technique.

**Methods:** Initially, the potential for DMS to improve signal-to-noise ratio (S/N) and reduce interference was tested by comparing chromatograms acquired with and without DMS when performing measurements of six different steroids. Subsequently, a full clinical validation of cortisol and cortisone in urine was performed with the LC-DMS-MS/MS method.

**Results and Discussion:** DMS significantly reduced interferences observed in the chromatograms and boosted S/N by between 1.6 and 13.8 times. Additionally, DMS improved the agreement between quantifier/qualifier fragment ion results for cortisol and cortisone as indicated by the increase in  $R^2$  from approximately 0.81 to 0.98. All validation studies met acceptance criteria and we observed exceptional analytical performance in terms of precision, with % CVs less than 8%.

**Conclusions:** DMS improved the specificity of the steroid measurements by reducing interferences and improving S/N. The validation studies prove that these benefits did not come at the expense of other aspects of analytical performance. This study indicates that DMS has the potential to benefit not just clinical measurements of challenging analytes, but many clinical LC-MS/MS analyses.

## 1. Introduction

Steroids are a class of biomolecules derived from cholesterol that are hydrophobic and exhibit similar chemical properties as lipids. Steroids have diverse and critical biological functions, serving as hormones to regulate metabolism, sexual development, fertility, blood pressure, salt retention, gastrointestinal processes, and growth; therefore, measurements of steroids are clinically useful for diagnosing and monitoring of a plethora of conditions [1,2]. However, due to the shared cholesterol backbone structure, there is a large amount of structural and chemical

similarity among steroids, leading to a tendency for interference and cross-reactivity [2–4]. Consequently, achieving highly specific measurements of steroids is challenging.

Historically, steroids measurements have been largely performed by immunoassays, but these tests suffered from inadequate specificity, with cross-reactivity and interference from other steroids too common [2–4]. Today, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely recognized as the preferred platform for steroid measurement due to its superior analytical specificity and sensitivity [3,5]. Yet, steroid analyses using LC-MS/MS are not without challenges. Circulating levels

**Abbreviations:** ACN, acetonitrile; AMR, analytical measuring range; ANST, androstenedione; BSA, bovine serum albumin; CCS, collision cross section; CLSI, Clinical & Laboratory Standards Institute; CV, coefficient of variation; DMS, differential mobility ion spectrometry; FAIMS, high field asymmetric ion mobility spectrometry; HCl, hydrochloric acid; IPA, isopropyl alcohol; IS, internal standard; LC, liquid chromatography; LLOQ, lower limit of quantitation; LOB, limit of blank; LOD, limit of detection; MeOH, methanol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; OHPG, 17-hydroxyprogesterone; QC, quality control; S/N, signal-to-noise ratio.

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of steroids are often low, and steroids are difficult to ionize, making sensitivity a challenge [6]. The aforementioned structural and chemical similarities make steroids difficult to isolate using standard sample preparation and chromatographic techniques [7,8]. Additionally, there is a high prevalence of isomers and many steroids produce shared product ions when fragmented in the mass spectrometer [8,9]. Hence, LC-MS/MS still struggles with completely eliminating steroid measurement interferences.

Ion mobility is an emerging technique in clinical laboratories that separates gas phase ions based on their mobility in the presence of a buffer gas and an electric field; therefore, the mechanism of separation is charge, size, and shape [10–13]. As a result, ion mobility has the potential to complement established LC-MS/MS techniques and further enhance the specificity of measurements for technically challenging analytes, such as steroids. Numerous studies have demonstrated successful separations of steroid isomers that are indistinguishable by mass spectrometry alone and difficult to separate chromatographically [10]. In these studies, cation adducts [14–21] or derivatization [22–24] have been utilized to enhance the separation of steroid isomers. Additionally, collision cross sections (CCS) have shown great potential for adding an additional level of specificity and confidence in steroid measurements [10,15,20,23–26].

However, despite the potential benefits outlined in these studies, ion mobility is not widely adopted for the routine analysis of low levels of steroids in biological matrices, such as those performed in clinical laboratories. One notable exception is the work of Guddat et al, which demonstrated improved signal-to-noise ratio (S/N) and specificity when using LC- high field asymmetric ion mobility spectrometry (FAIMS)-MS/MS compared to analyses without FAIMS [27]. Another study by Arthur and coworkers developed a high-throughput method for measuring sodium adducts of steroid metabolites in urine using LC-FAIMS-MS [28]. Expanding on this previous work, we present the results of our analyses using a commercially available differential mobility spectrometry (DMS) device for LC-MS/MS measurements of cortisol, cortisone, 24,25-dihydroxyvitamin D, androstenedione (ANST), and 17-hydroxyprogesterone (OHPG) in clinical samples. The chromatograms presented here clearly demonstrate the sensitivity and specificity benefits of using DMS when performing high-throughput analysis of clinical specimens. Additionally, a full validation of cortisol and cortisone measurements in urine was performed to demonstrate the robustness, reproducibility, and suitability of the technology for routine clinical testing.

## 2. Materials and methods

The Mayo Clinic Institutional Review Board (IRB)/Ethics Committee ruled that approval was not required for this study. Patient samples were deidentified prior to use in this study.

### 2.1. Chemicals and reagents

LC-MS grade Methanol (MeOH), acetonitrile (ACN), isopropyl alcohol (IPA), formic acid, and ammonium formate, ammonium acetate, hexane, methylene chloride, were purchased from MilliporeSigma (Burlington, MA). Hydrochloric acid (HCl) and acetone were purchased from Fisher Scientific (Hampton, NH).

### 2.2. Measurements of androstenedione and 17-Hydroxyprogesterone in serum

#### 2.2.1. Preparation of androstenedione and 17-Hydroxyprogesterone Calibrators, QC, and samples

This method has been described previously; thus, it is described here briefly with method changes [29]. OHPG (catalog#-H-085) and ANST (catalog#-A-075) calibration materials were purchased from Cerilliant (Round Rock, TX). Calibration material was diluted and spiked into 1 % BSA to yield calibration levels of 16, 40, 200, 1000, 2500, and 4000 ng/

dL. Four levels of quality controls samples (QC) are produced by spiking calibration material into charcoal stripped human serum (SeraCare Life Sciences, Milford, MA, catalog#-HS-230) to produce final concentrations of 60, 100, 700, and 2,000 ng/dL. Isotopically labeled ANST was purchased from IsoSciences (catalog#- S14216) and isotopically labeled OHPG was purchased from Cerilliant (catalog#-H-096). These solutions were used to create a working internal standard (IS) solution of 30 ng/mL by diluting in 70 % methanol. Calibrators, QC, and samples were prepared for analysis by placing 100  $\mu$ L into a 96 well plate followed by the addition of 25  $\mu$ L of IS solution and 370  $\mu$ L of acetonitrile. Samples were then vortexed for 30 sec and centrifuged for 15 min at 3000  $\times$  g.

#### 2.2.2. LC-MS/MS analysis of androstenedione and 17-Hydroxyprogesterone

The supernatant (50  $\mu$ L) of the sample preparation process outlined above was injected for LC-MS/MS analysis using a Thermo Scientific (Waltham, MA) TLX-2 system. The LC method is shown in Table S1 and the mobile phases are shown in Table S2. We purified samples by using a 4x3 mm C<sub>18</sub> Security Guard Cartridge (Phenomenex catalog#-AJO-4287, Torrance, CA) followed by separation on a 50  $\times$  3.0 mm Accucore RP-MS column (ThermoFisher Scientific catalog #-17626-053030). The LC eluates underwent mass spectrometry analysis using a Sciex 6500 + mass spectrometer equipped with a SelexION DMS system. All compounds were optimized by infusion of target compounds and using a tee to simulate LC elution conditions. First, separation voltage (SV) was set to 3875 V and the software ramping function was used to determine the optimum compensation voltage and DMS offset parameters. The SelexION has five options for resolution that range in specification of full width at half max of the compensation voltage transmission window from 0.6  $\pm$  0.2 (high resolution) to 3.7  $\pm$  0.4 (open resolution). Higher resolution comes at the expense of transmission, and it was determined that open or low resolution was appropriate for the applications shown here. The MS/MS parameters with and without SelexION are shown in Table S3. Source parameters for SelexION versus no SelexION can be seen in Tables S4 and S5, respectively.

### 2.3. Measurement of 24, 25 dihydroxyvitamin D in serum

#### 2.3.1. Preparation of 24, 25 dihydroxyvitamin D Calibrators, QC, and samples

This method was previously published [30] and is described here only briefly. Calibration material for 24, 25 dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> was purchased from Medical Isotopes, Inc. (Pelham, NH, catalog#-18821 and 18866). Calibrators were prepared by spiking both D<sub>2</sub> and D<sub>3</sub> into vitamin D depleted serum purchased from SeraCare Life Sciences (catalog#-502079-1L) to yield final concentrations of 0.1, 0.5, 1, 5, 10, and 25 ng/mL. Quality control samples were prepared by spiking materials in the same manner as calibrators at concentrations of 0.75, 7.5, and 20 ng/mL. 24,25 dihydroxyvitamin D<sub>2</sub>-d<sub>3</sub> was purchased from IsoSciences (Ambler, PA, catalog#-13043) and 24,25 dihydroxyvitamin D<sub>3</sub>-d<sub>6</sub> was purchased from Toronto Research Chemicals (North York, ON, Canada, catalog#-D455087). The labeled materials were used to make a working internal standard with a concentration of 25 ng/mL for both D<sub>2</sub> and D<sub>3</sub> in 70 % MeOH.

When preparing samples, 500  $\mu$ L was placed into a glass tube followed by the addition of 50  $\mu$ L of the working IS solution. The solution was then vortexed and incubated for 15 min. Next, 0.5 mL of 0.2 M HCl was added. 24,25 dihydroxyvitamin D was then enriched using solid phase extraction (SPE) with cartridges purchased from Agilent (Santa Clara, CA, catalog#-1210C18OHT) and positive pressure displacement. Cartridges were preconditioned with 2.5 mL of MeOH and then 2.5 mL of water followed by the addition of the sample. Cartridges were washed with 5 mL of 70 % MeOH and then 2 mL of 90:10 hexane:methylene chloride, and then sample was eluted with 5 mL of 90:10 hexane:isopropanol. This solution was then dried at 45 °C and extracts were reconstituted in 0.002 % 4-Phenyl-1,2,4-triazoline-3,5-dione

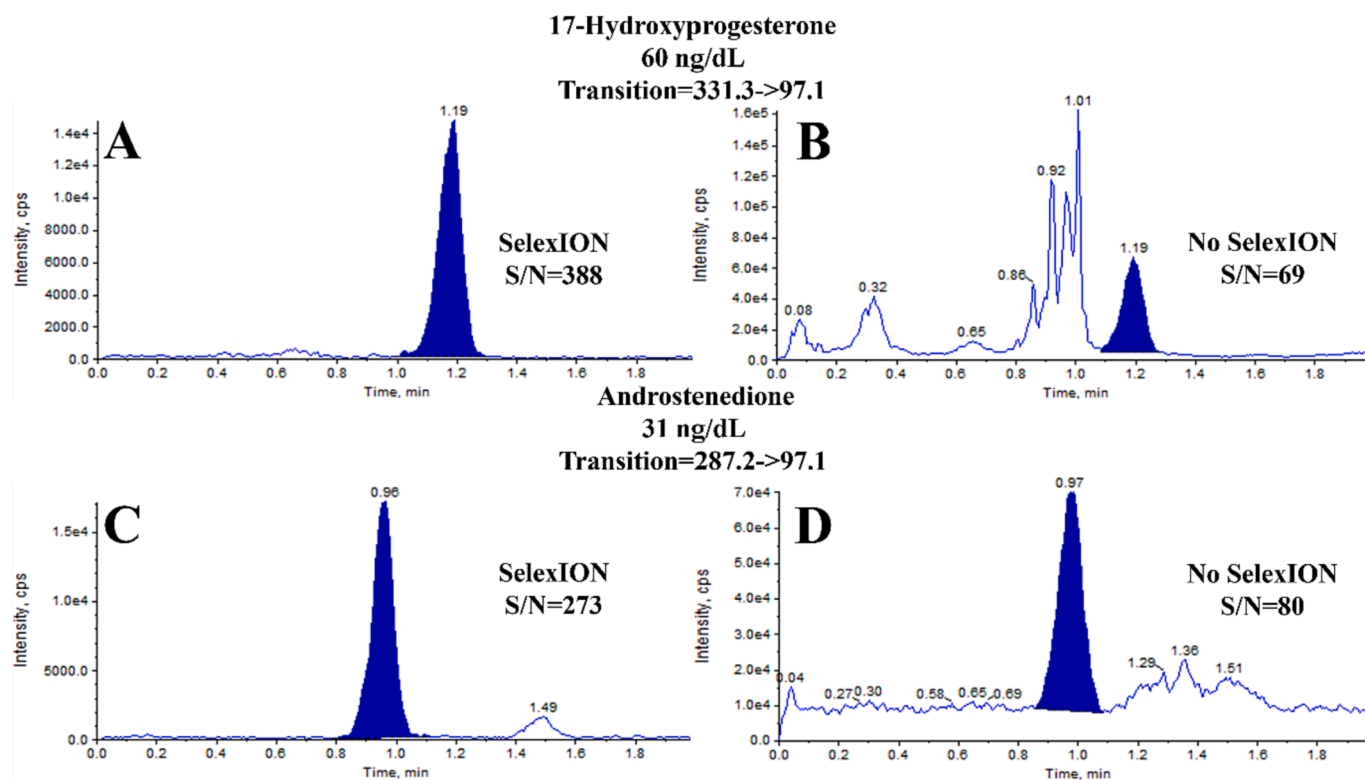


Fig. 1. Comparison of chromatograms from measurements of OHPG and ANST performed with and without the use of SelexION DMS. A) OHPG with SelexION, B) OHPG without SelexION, C) ANST with SelexION, D) ANST without SelexION.

(MilliporeSigma) in ACN to be derivatized while drying at 60 °C. The final derivatized product was then reconstituted in 150  $\mu$ L of 70 % MeOH.

### 2.3.2. LC-MS/MS analysis of 24, 25 dihydroxyvitamin D

Instrumentation used was as described in section 2.2.2. This method did not use TurboFlow online purification. The injection volume was 50  $\mu$ L. Mobile phase A was 1 mM Ammonium Acetate in water and mobile phase B was 100 % methanol. The analytical column was a 2.1x50 mm Agilent (Santa Clara, CA) XDB-C8 (catalog#- 971700–906). The LC method can be seen in Table S6. The mass spectrometry parameters are shown in Table S7 and source conditions with the SelexION are in Table S8 and without are in Table S9.

## 2.4. Measurement of cortisol and cortisone in urine

### 2.4.1. Preparation of cortisol and cortisone calibrators, QC, and samples

This method was originally published by Taylor et al. in 2002 [31] and changes to the method are outlined here. Cortisol (catalog#-C-106) and cortisone (catalog#-C-130) calibration materials were purchased from Cerilliant. Calibration levels were 0.08, 0.25, 1, 4, 10, and 20  $\mu$ g/dL in stripped urine purchased from BioChemed Services (Winchester, VA). QC samples were also prepared by spiking calibration material into stripped urine at concentrations of 0.16, 1.5, 4, and 10  $\mu$ g/dL. Deuterated ISs were purchased from IsoSciences and used at a working concentration of 0.03  $\mu$ g/mL cortisol and 0.06  $\mu$ g/mL cortisone. Samples were prepared for analysis by first putting 100  $\mu$ L urine into a 96 well plate followed by the addition of 50  $\mu$ L of working IS solution and vortex mixing.

### 2.4.2. LC-MS/MS analysis of cortisol and cortisone

The solution described in the previous section was then injected directly (30  $\mu$ L) for online purification and LC-MS/MS analysis. Instrumentation used was as described in section 2.2.2. The LC method is

shown in Table S10 and mobile phases can be seen in Table S11. The parameters for mass spectrometry analysis are shown in Table S12 and source conditions with the SelexION are in Table S13 and without are in Table S14.

### 2.4.3. Validation of cortisol and cortisone in urine using LC-DMS-MS/MS

This method was validated in accordance with Clinical & Laboratory Standards Institute (CLSI) guidelines. Intraassay and interassay imprecision were tested using 20 replicate measurements of five pools with concentrations starting at the lower limit of quantitation (LLOQ) and spanning the analytical measuring range (AMR). The acceptance criterion for precision was less than 20 % CV at the LLOQ and less than 10 % for the other levels. The limit of the blank (LOB) was established by 20 replicate measurements of extracted blank matrix across 10 days using the calculation:  $LOB = \text{Mean of blank} + \text{unidirectional Z-score for 95 \% probability} (=1.65) \times \text{SD of the blank}$ . The limit of detection (LOD) was established by spiking concentrations above the upper 95 % boundary of the LOB (again 20 replicates over 10 days) using the corresponding calculation:  $LOD = LOB + \text{unidirectional Zscore for 95 \% probability} (=1.65) \times \text{SD of the low-sample pool}$ . Only the primary fragment ion transition was used for LOD/LOB studies. To be considered acceptable, the LOD had to be less than the LLOQ.

Linearity across the analytical measuring range was confirmed by serial dilutions of five patient samples and subsequent linear regression analysis had to yield a slope of  $1 \pm 0.1$  and a  $R^2 \geq 0.98$  to be considered acceptable. Carryover was monitored by injecting a blank sample after highest calibrator during each analysis and the resulting calculated concentration had to be less than the LLOQ to be acceptable. Accuracy was confirmed by comparing results with an established LC-MS/MS test using the same extraction protocol, but without the use of DMS. Results were compared using Passing-Bablok regression analysis and the acceptance criteria were a slope of  $1 \pm 0.1$  and a  $r \geq 0.98$ .

The potential impact of ion suppression or enhancement was assessed using ten randomly selected patient samples with low cortisol

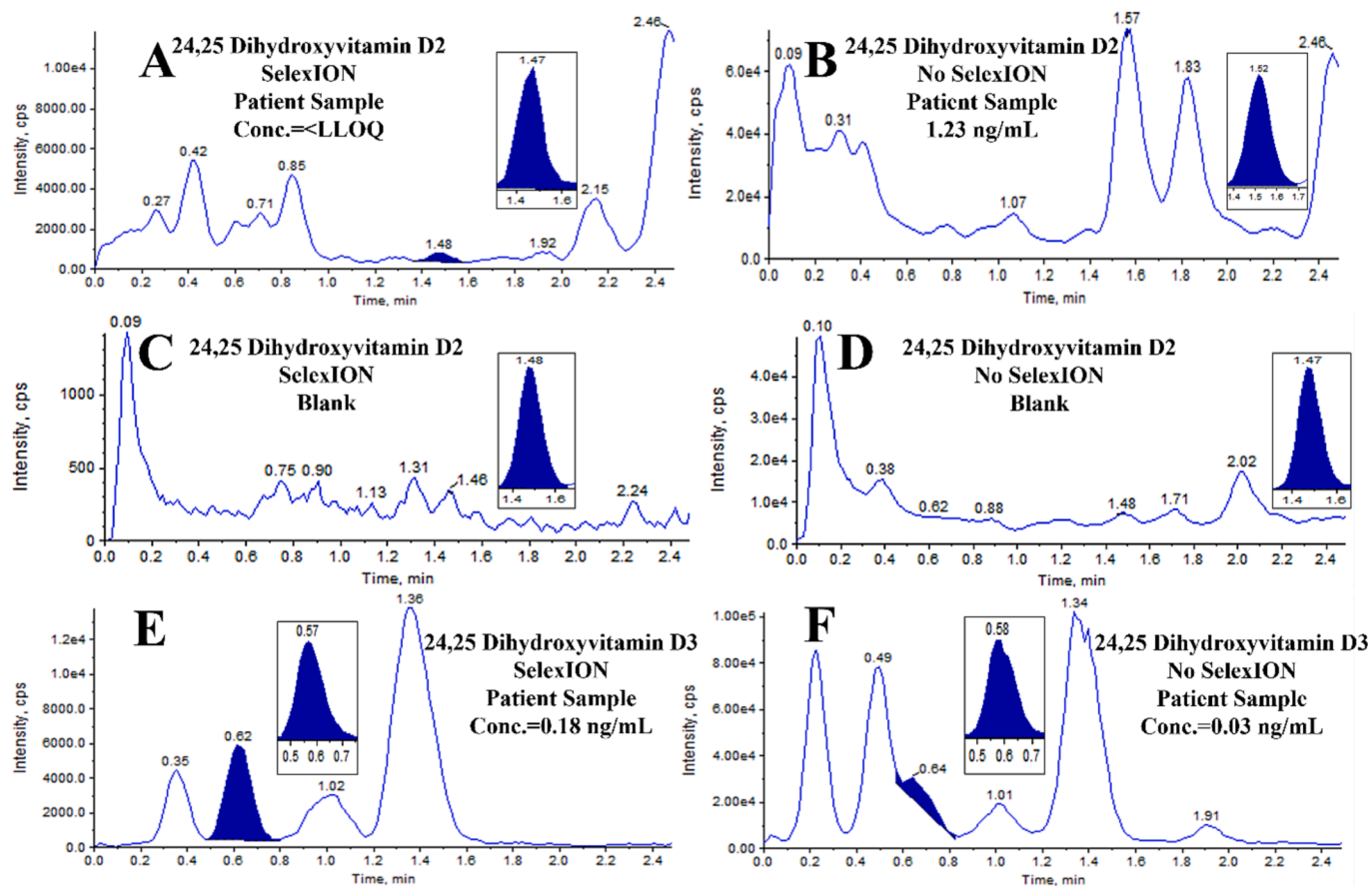


Fig. 2. Comparison of chromatograms from measurements of 24,25 dihydroxyvitamin D2 (transition 586.35->298.2) and D3 (transition 574.35->298.2) performed with and without the use of SelexION DMS. For retention time reference, the internal standard signal is inset. A) D2 in patient sample with SelexION, B) D2 in patient sample without SelexION, C) D2 extracted blank with SelexION D) D2 Extracted blank without SelexION E) D3 in patient sample with SelexION, F) D3 in patient sample without SelexION.

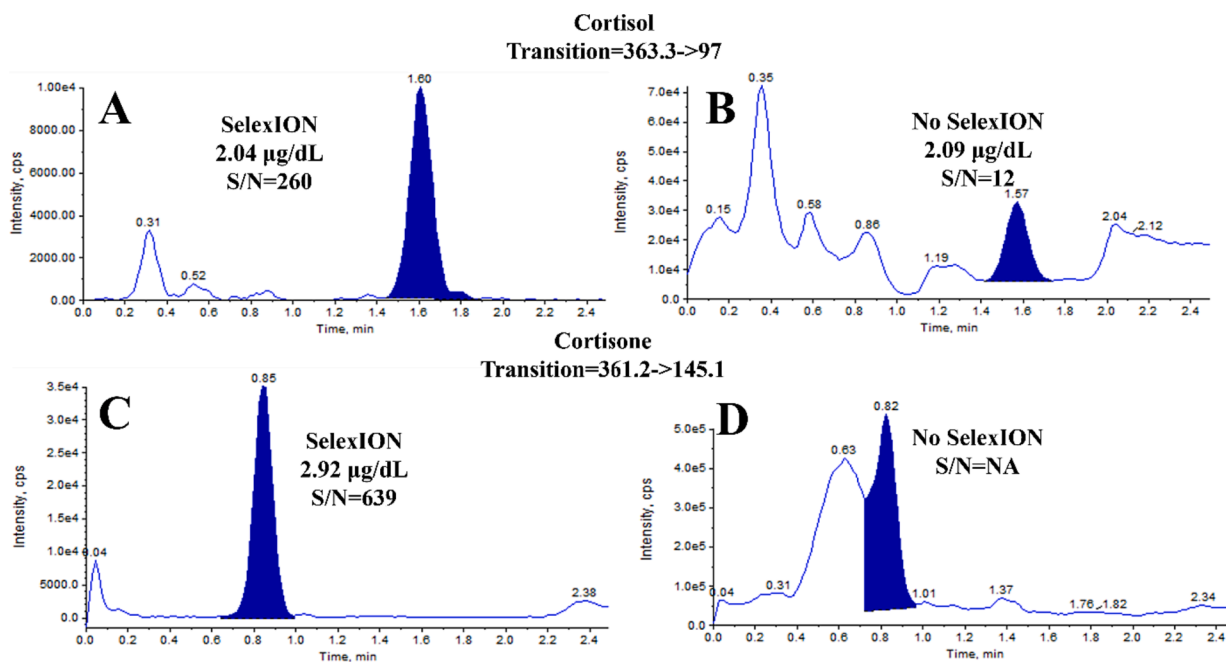
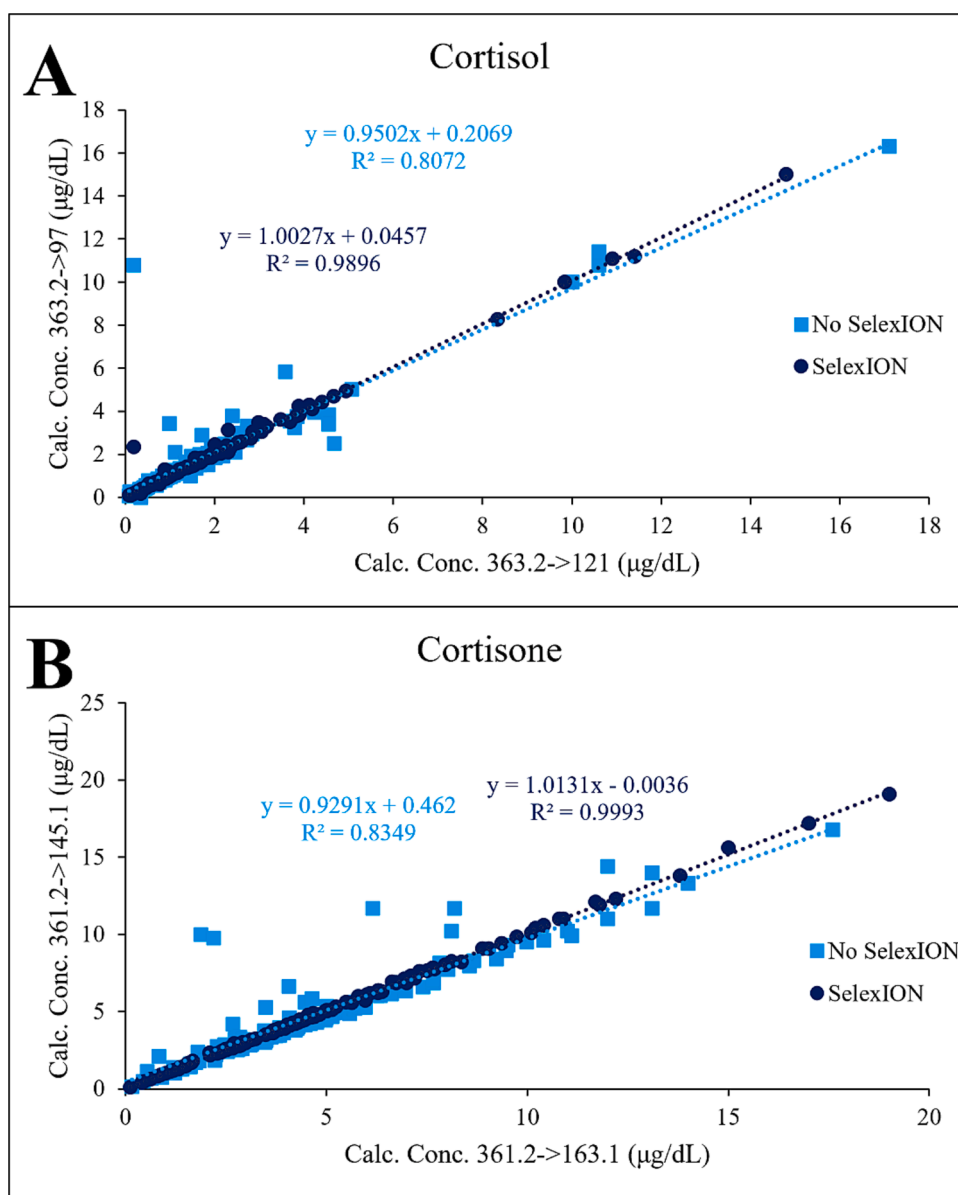


Fig. 3. Comparison of chromatograms from measurements of cortisol and cortisone performed with and without the use of SelexION DMS. A) cortisol with SelexION, B) cortisol without SelexION, C) cortisone with SelexION, D) cortisone without SelexION.



**Fig. 4.** Linear regression comparison of the calculated concentration results from the qualifier/confirmatory fragment ion and the quantifier/primary fragment for (A) cortisol and (B) cortisone.

and cortisone concentration. Post-extraction, cortisol and cortisone were spiked into the reconstitution solvent at three levels across the AMR. The resulting signal was compared to spikes in reconstitution solvent in the absence of the matrix to yield percent suppression/enhancement. Interference from common drugs and steroids was also tested and is discussed further in the Results and Discussion section below. The resulting concentration from analysis had to be less than the LLOQ to not be considered an interference.

### 2.5. Data processing

Following acquisition, all data files were further processed and integrated in Analyst version 1.7.2. S/N was determined in Analyst by defining a 0.5 min noise region with minimal interfering signals. The same noise region was used for the data generated with and without the SelexION. The integrated signal was then automatically divided by calculated noise to determine the S/N. Data were exported to Microsoft Excel for further processing and plotting. Passing-Bablok regression analysis was performed using Analyse-it (Leeds, UK).

### 3. Results and discussion

The primary aim of this study was to assess the potential of using DMS to enhance the S/N and reduce interferences when performing quantitative analysis of steroids. To achieve this, samples were prepared and injected consecutively on the same LC-MS/MS system, with and without the SelexION DMS system. The only changes made between the two datasets were the optimized parameters for source conditions, depending on whether the SelexION was installed or removed.

The first comparison focused on the measurement of OHPG and ANST in serum. The chromatograms in Fig. 1 demonstrate the differences when using or not using the SelexION device. In the case of OHPG (Fig. 1A & B) and ANST (Fig. 1C & D), using the DMS system resulted in a reduction in both signal and noise, as well as potential interferences. Despite the drop in signal, there was a disproportionately larger decrease in noise and interferences, leading to an approximate 6-fold improvement in S/N. A similar comparison was made for 24,25-dihydroxyvitamin D2 (Fig. 2A–D) and 24,25-dihydroxyvitamin D3 (Fig. 2E & F). Without DMS, a chromatographic peak corresponding to the

**Table 1**

Average S/N increase when using SelexION DMS compared to not using SelexION DMS. The S/N of the chromatogram with the SelexION was divided by the S/N of the chromatogram without the SelexION to yield a S/N fold improvement for each patient sample. These results were then averaged. The number of patient samples with results within the AMR are also shown.

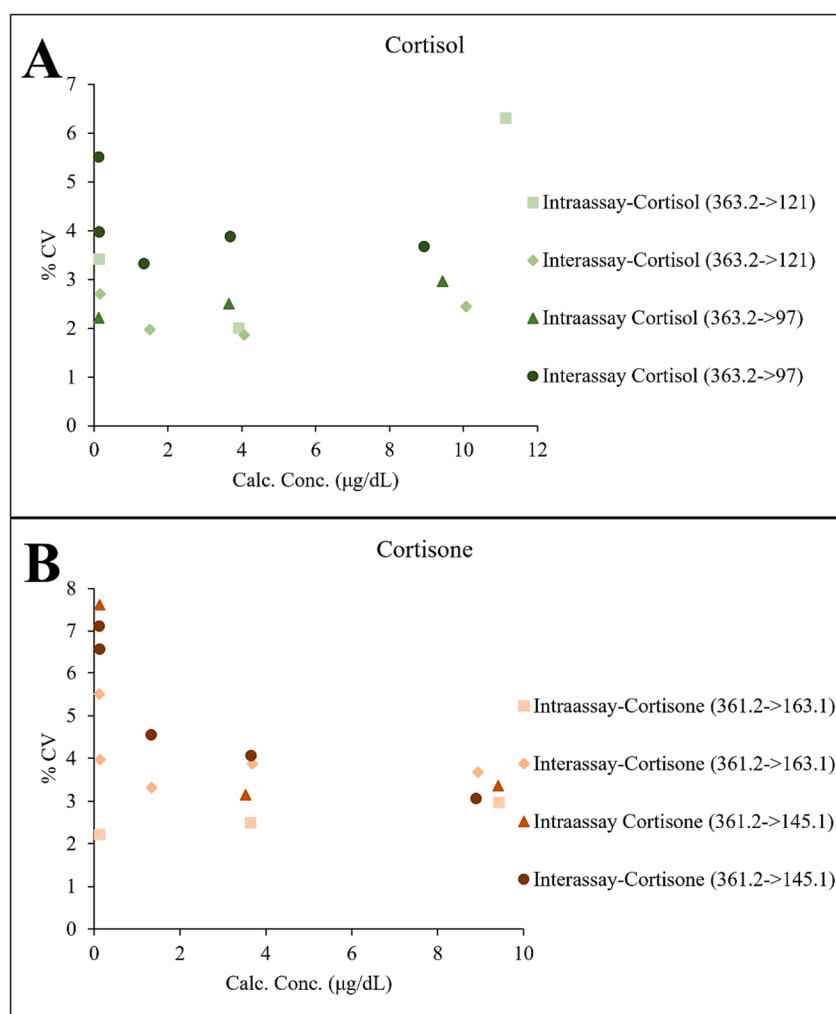
Analyte	ANST	OHPG	D2	D3	Cortisol	Cortisone
Number of Patient Samples	18	18	16	32	130	129
Ave. Fold S/N Increase with SelexION	2.4	6.7	2.2	1.6	13.8	7.7

retention time of the IS was observed for 24,25-dihydroxyvitamin D2 (Fig. 2B). However, when the DMS device was used, this signal was eliminated (Fig. 2A). For reference, extracted blanks are shown in Fig. 2C & D. Given the high prevalence of patient samples with undetectable levels of 24,25-dihydroxyvitamin D2 in the normal population and the inability of that signal to be detected when utilizing an additional level of separation, it is likely that this peak was an interference and would be falsely integrated as 24,25 dihydroxyvitamin D2 if DMS was not used, providing evidence of the specificity advantages offered by the DMS technology. Regarding 24,25-dihydroxyvitamin D3, it was not possible to integrate the chromatographic peak, which would have resulted in an inability to report a test result without DMS (Fig. 2F).

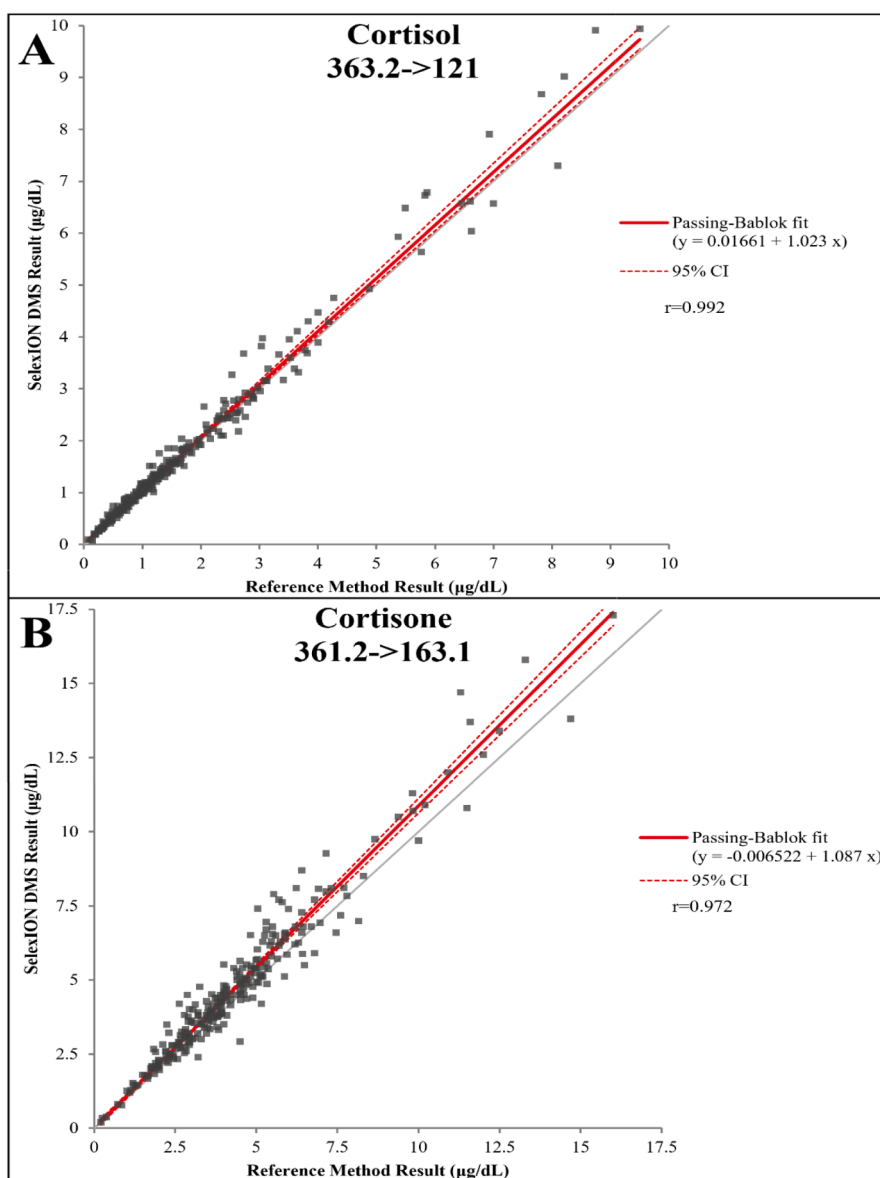
However, with the DMS system, a clear and detectable signal with sufficient S/N was achieved (Fig. 2E), enabling reporting of the test result.

The final comparison was conducted on cortisol (Fig. 3A & B) and cortisone (Fig. 3C & D) in urine testing. Similar to the previously discussed analytes, these chromatograms show a significant increase in S/N for cortisol (20x) and the elimination of a major interference for cortisone. Additionally, the use of DMS for cortisol and cortisone provides another advantage, as depicted in Fig. 4, which shows a linear regression comparison of the calculated concentration results from the qualifier/confirmatory fragment ion and the quantifier/primary fragment. The SelexION device improved the agreement, as indicated by both the slope and the increase in  $R^2$ . This improvement holds considerable significance since many tests require the fragment ion results to be within 20 %. Therefore, enhancing this agreement instills greater confidence in the analysis and enables a larger number of sample results to be provided to patients and clinicians. Table 1 presents an overall comparison of the average increase in S/N for all patient samples tested. With the use of the SelexION DMS device, the average S/N improvements ranged from 1.6 to 13.8 when compared to not using DMS.

Given the apparent benefits from the use of the SelexION DMS device in terms of reducing interferences and improving S/N, we elected to fully validate the technology for the measurement of cortisol and cortisone in urine. The precision from 20 replicate measurements was excellent, with the %CV being below 8 % at all levels (Fig. 5). The calculated LOB and LOD for cortisol were 0.009  $\mu\text{g}/\text{dL}$  and 0.047  $\mu\text{g}/\text{dL}$ ,



**Fig. 5.** Intra and interassay precision of replicate measurements of (A) cortisol and (B) cortisone in urine ( $n = 20$ ). Data from both transitions for each analyte are shown.



**Fig. 6.** Passing-Bablok regression analysis comparing reference method results to SelexION DMS Results for (A) cortisol and (B) cortisone. The primary fragment ion transition comparison is shown. N = 267 for cortisol and 261 for cortisone.

respectively. For cortisone, the LOB was 0.017  $\mu\text{g}/\text{dL}$  and the LOD was 0.026  $\mu\text{g}/\text{dL}$ . The LOD met acceptance criteria for both analytes. Linear regression analysis of the linearity study for cortisol is shown in Fig. S1, and for cortisone it is shown in Fig. S2. The acceptance criteria were met for all linearity studies. The observed carryover after the highest calibrator was less than 30 % of the LLOQ, meeting the acceptance criteria.

Passing-Bablok regression analysis for assessment of cortisol and cortisone accuracy can be seen in Fig. 6 for the primary/quantifier fragment ion transitions and Fig. S3 for the secondary/qualifier fragment ion transitions. For this analysis, samples with obvious interferences observed in the chromatograms, or a greater than 20 % difference between the ion transitions, were removed for the reference method to minimize the effects of the readily apparent limitations on the comparison. After the removal of samples with interference and those outside the AMR, 267 samples for cortisol and 261 samples for cortisone were included in the Passing-Bablok regression analysis. The results from all four Passing-Bablok comparisons exceeded the acceptance criteria for  $r$  and slope and indicated strong agreement. Based on the ionization suppression/enhancement study, cortisol exhibited 1.4 %

suppression, and cortisone exhibited 0.7 % suppression after IS correction, suggesting that matrix effects are minimal when performing these analyses. Additionally, the compounds shown in Table S15 were tested as possible interferences at the shown concentrations, totaling 78 interference studies. No interferences were observed.

This study shows that the use of DMS enhances the specificity of the analysis without sacrificing other aspects of analytical performance. However, a limitation of this study was that full validation was only conducted for two compounds, due to resource and time constraints. Moving forward, we intend to assess the potential of DMS to improve analyses of additional compounds through small studies and additional validations. A potential benefit that was not addressed in this study, but is a future goal, is the potential for DMS to improve throughput by eliminating the need for long, complicated chromatographic methods. Instead, it would enable shorter separation and run times. It is conceivable that using DMS will provide benefits for most analytes, not just interference-prone and challenging analytes, as was the focus of this study.

#### 4. Conclusions

This study provided further evidence that DMS is a powerful complement to established LC-MS/MS instrumentation, improving the specificity of steroid analyses, which are notoriously prone to interference and cross-reactivity. For the six steroids tested, DMS reduced the prevalence of interference and improved the S/N of the analysis. Validation studies performed for cortisol and cortisone in urine demonstrated exceptional analytical performance when DMS was utilized, as all validation studies met acceptance criteria. Of note, the %CVs observed were all less than 10 % and the calculated concentration results when using DMS showed excellent agreement with a reference method. Based on the results of this investigation, DMS has the potential to greatly benefit not just clinical measurements of challenging analytes, but also most clinical LC-MS/MS analyses.

#### CRedit authorship contribution statement

**Yubo Chai:** Validation, Formal analysis, Writing – review & editing. **Stefan K.G. Grebe:** Supervision, Writing – review & editing. **Anthony Maus:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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N/A

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmsacl.2023.10.001>.

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