Quantification of Steroids and 25-OH Vitamin D_3 in Dried Blood Spots and Serum using LC/MS/MS

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Steroids are important biomarkers for a number of clinical conditions. Most clinical assays for steroid quantification are based on immunoassays, which suffer from selectivity problems due to cross-reactivity, or GC/MS separations, which are time consuming and often involve complex sample preparation procedures. Analysis of a comprehensive steroid panel is challenging due to the need to rapidly separate structurally related compounds in order to quantify low concentrations present in complex biological specimens. LC/MS/MS has been introduced as an alternative for steroid analysis in the past few years due to its superior sensitivity and selectivity.

Most clinical assays are based on serum or plasma obtained by venous sampling in the clinic. Good alternatives are dried blood spot (DBS) samples, conveniently obtained from a prick of the finger or heel, and spotted onto filter paper. DBS analysis is desirable because it is simple, samples are easy to obtain, and the analysis uses only a small amount of blood from the patient.

This technical note will highlight a simple extraction method, requiring no derivatization of target compounds, which is capable of measuring a variety of clinically important steroids from DBS and serum using the AB SCIEX QTRAP® 5500 System coupled to a Shimadzu Prominence LC system. Concentrations of steroids in serum and DBS from 20 apparently healthy volunteers are compared.

Materials and Methods

Sample Preparation: Cortisol, 17-alphahydroxyprogesterone (17-HP), progesterone, testosterone and 25-OH vitamin D_3 are extracted from DBS using liquid-liquid extraction prior to analysis on an LC/MS/MS system. Dried blood spot calibration curves (7 points) were constructed in specially treated steroid-free whole blood, prepared in-house and spotted onto fresh filter paper (Whatman 903 Specimen Collection Paper) in 60 µL aliquots, left to dry at room temperature for 24 hours, and stored at -20°C until needed.

Three different DBS sample preparation procedures were evaluated including direct extraction using acetone/acetonitrile (50:50), methanol (100%), and liquid-liquid extraction.

The optimal DBS sample preparation procedure consisted of the following steps:

1. The dried blood spot was cut out and soaked in aqueous solvent for 60 min, after which an internal standard mixture was added, and then liquid-liquid extraction was performed.
2. The organic supernatants were transferred to glass tubes and dried under nitrogen gas.
3. Extracts were reconstituted with a solution of methanol and water (50:50) before loading onto the LC/MS/MS system.

Serum calibration curves were constructed in double-stripped human serum. The serum sample preparation procedure consisted of the following steps:

1. A 100 µL aliquot of serum sample or standard was transferred to a 1 mL Eppendorf vial.
2. Protein precipitation was carried out by adding to the serum sample 100 µL of methanol and excipient mixture containing the appropriate internal standard concentration for each analyte.
3. The mixture was vortexed for 5 minutes, centrifuged for 10 minutes, and the supernatant was transferred to glass injection vials for LC/MS/MS analysis.

Liquid Chromatography: Separation was performed using a 2.7 µm, 3x100mm C18 reverse phase column on a Shimadzu Prominence LC system.

Mass Spectrometer: Analysis was performed using the APCI ion source interfaced to the QTRAP® 5500 system. Steroids were identified using the Multiple Reaction Monitoring (MRM) mode and quantified versus an isotope-labeled internal standard. Two MRM transitions were used for each analyte, one quantifier and one qualifier. The integrated Valco valve was also used to keep the instrument clean.

Results

Method development included the evaluation of major challenges associated with analyzing endogenous level steroids: standard curve construction, different dried blood spot sample preparation procedures, interferences, separation and reproducibility of the assay. As a starting point for these analyses, we used a previously developed and validated LC/MS/MS method for serum, which removed interferences from 12 endogenous steroids[1].

Preliminary data yielded the following lower limits of quantitation for cortisol, 17-alphahydroxyprogesterone, progesterone, testosterone, and 25-OH vitamin D_3, respectively:
5 ng/mL, 100 pg/mL, 100 pg/mL, 50 pg/mL, and 10 ng/mL.
Figure 1 displays representative LC/MS/MS chromatograms for all analytes in a dried blood spot (DBS) sample at the low end of the standard curve. The chromatograms for all analytes in a DBS sample for a female patient are displayed in Figure 2. Good linearity and correlation coefficient (R>0.99) were observed for the calibration curves of all analytes in both serum and DBS samples (Table 1).

The interday variability of the assay with different sample preparation procedures, and for analyses of both serum and DBS patient specimens, ranged from 0.2 to 16.7%. In general, better reproducibility (<4%) was observed in the serum samples (Table 2). Progesterone and 17-alphahydroxyprogesterone are less reproducible than the other analytes, most likely related to individual patient specimen matrices.

Consistent quantifier-to-qualifier ratios were observed for all analytes across standard and patient samples, in both serum and DBS matrices. The consistent retention time and internal standard response over all the injections indicated that the assay is rugged in real sample matrices (Figure 3).

Figure 1. Representative LC/MS/MS chromatograms for analytes in a dried blood spot (DBS) sample at the low end of the standard curve.

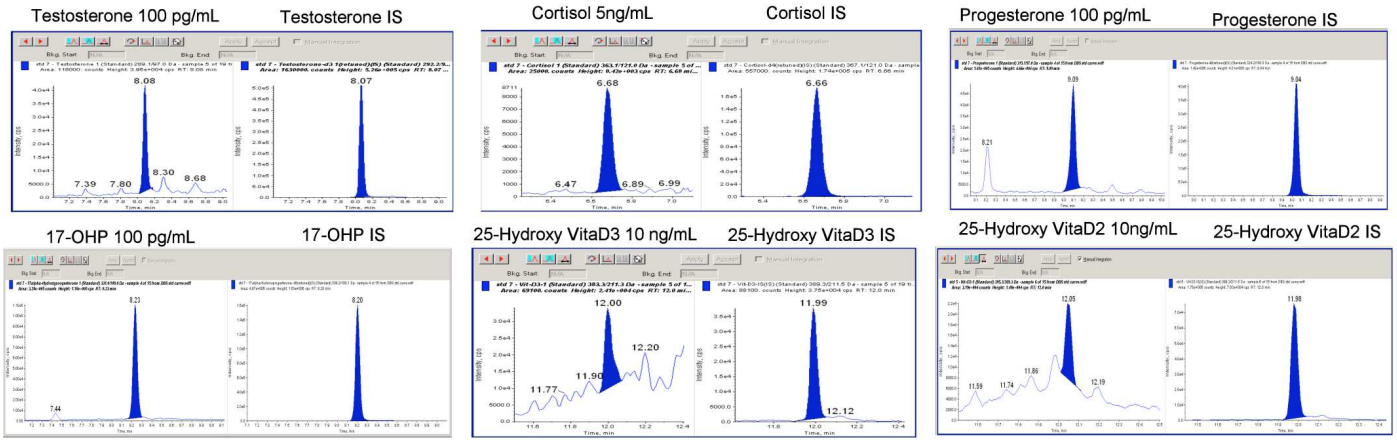


Figure 2. LC/MS/MS chromatograms for all analytes in a dried blood spot (DBS) sample for a female patient

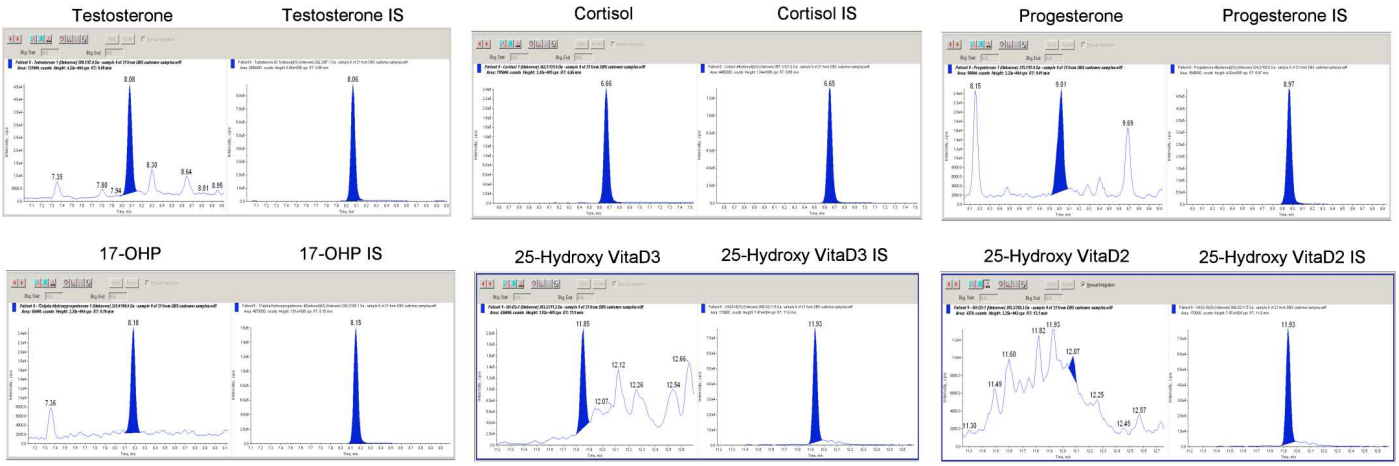


Figure 3. Evaluation of assay ruggedness for testosterone in dried blood spot.

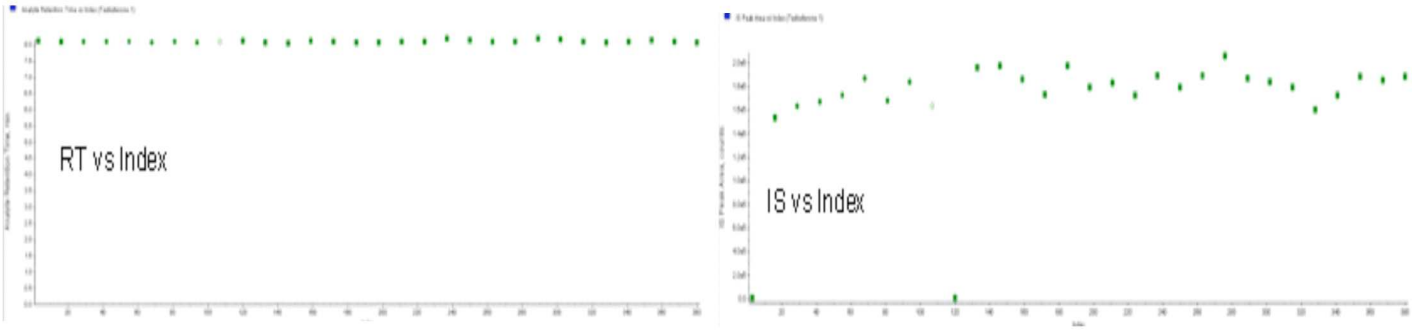
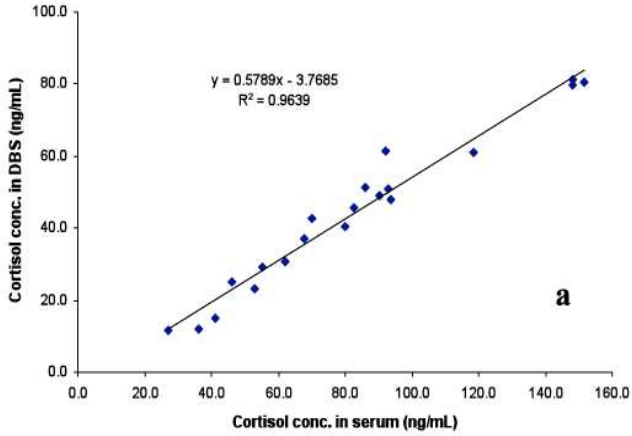
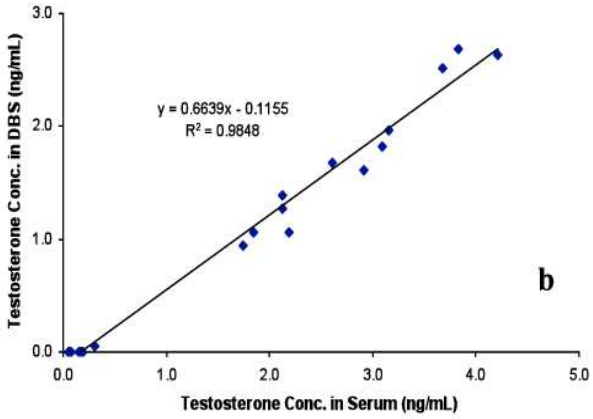
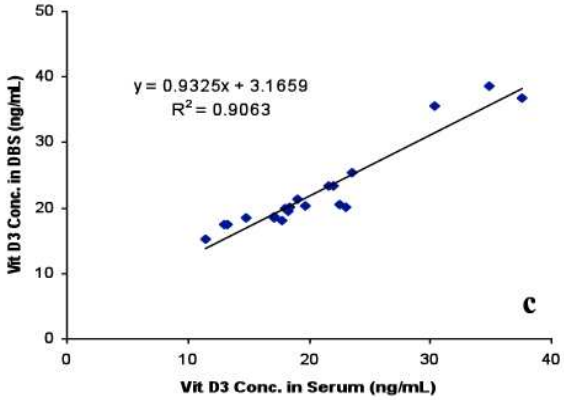


Figure 4. Correlation between analyte concentrations measured in dried blood spot (DBS) and serum samples.







a. Correlation between cortisol concentration measured in serum and DBS
b. Correlation between testosterone concentration measured in serum and DBS
c. Correlation between 25-OH vitamin D_3 concentration measured in serum and DBS

Table 1. Summary of Linearity and Correlation Coefficients for Standard Curves in DBS and Serum

Analyte	Linear Regression in DBS	Correlation Coefficient R in DBS	Linear Regression in Serum	Correlation Coefficient R in Serum
Cortisol	y=0.0031x+0.0277	0.9996	y=0.0193x+0.0007	0.9991
Testosterone	y=0.1740x+0.0535	0.9998	y=0.8890x+0.0393	0.9990
17-alphahydroxyprogesterone	y=0.0236x+0.0105	0.9996	y=0.1580x+0.0100	0.9990
Progesterone	y=0.1790x+0.0166	0.9976	y=0.9290x+0.0162	0.9992
25-OH vitamin D_3	y=0.0388x+0.3650	0.9949	y=0.1170x+0.4560	0.9963

Table 2. Analyte Concentrations and Interday Reproducibility Measured in DBS and Serum

	Concentration in Serum (ng/mL)				Concentration in DBS (ng/mL)			
Patient	Set 1	Set 2	Average	% Difference	Set 1	Set 2	Average	% Difference
21	26.6	27.8	27.2	-2.2	12.1	11.2	11.7	-3.9
22	37.8	34.9	36.4	4.0	10.9	12.7	11.8	7.6
23	42.1	40.2	41.2	2.3	14.0	16.1	15.1	7.0
24	46.5	45.9	46.2	0.6	22.1	28.1	25.1	12.0
25	51.9	53.9	52.9	-1.9	19.5	27.3	23.4	16.7
26	55.3	55.5	55.4	-0.2	24.4	33.8	29.1	16.2
27	63.3	60.8	62.1	2.0	29.6	31.5	30.6	3.1
28	67.0	68.8	67.9	-1.3	37.6	36.3	37.0	-1.8
29	68.6	71.4	70.0	-2.0	38.9	46.2	42.6	8.6
30	79.1	81.0	80.1	-1.2	39.3	41.8	40.6	3.1
31	85.8	79.3	82.6	3.9	45.1	46.5	45.8	1.5
32	87.4	84.5	86.0	1.7	49.0	53.4	51.2	4.3
33	90.6	93.4	92.0	-1.5	70.6	52.0	61.3	-15.2
34	92.2	93.7	93.0	-0.8	48.1	53.7	50.9	5.5
35	92.3	88.4	90.4	2.2	45.5	52.5	49.0	7.1
36	93.8	93.9	93.9	-0.1	41.2	54.7	48.0	14.1
37	117	120	118.5	-1.3	54.8	67.5	61.2	10.4
38	147	149	148.0	-0.7	77.0	82.8	79.9	3.6
39	151	152	151.5	-0.3	78.6	82.2	80.4	2.2
40	153	143	148.0	3.4	72.7	90.2	81.5	10.7

Good correlation was observed between the measured concentrations in dried blood spot and serum samples for cortisol (Figure 4a, R^2=0.96), testosterone (Figure 4b, R^2=0.98) and 25-OH vitamin D_3 (Figure 4c, R^2=0.91) in 20 healthy volunteer subjects. The DBS to serum concentration ratio appeared to be related to lipophilicity with cortisol (0.5) < testosterone (0.6) ~17OHP (0.66) < progesterone (0.76) < 25-OH vitamin D_3 (1). This indicates that the less lipophilic compounds, cortisol and testosterone, are mainly distributed in plasma. However, 25-OH vitamin D_3 is almost evenly distributed between plasma and red blood cells. Similar DBS to serum ratios have been reported previously for 25-OH vitamin D_2.

Conclusions

Initial validation experiments have demonstrated that it is possible to quantify steroids from DBS specimens and that there is a good relationship between steroid concentrations measured in DBS and those measured in serum.

References

1. Liu, HF, et al., 2010, LC-MS/MS Method Development for Steroids Analysis at Low Detection Limits; *MSACL*, San Diego.
2. Newman, M., Brandon, T., Groves, M., et al., January 2009, A Liquid Chromatography/Tandem Mass Spectrometry Method for Determination of 25-Hydroxy Vitamin D_2 and 25-Hydroxy Vitamin D_3 in Dried Blood Spots: A Potential Adjunct to Diabetes and Cardiometabolic Risk Screening, *Journal of Diabetes Science and Technologies* , Vol 3, Issue 1, p. 156-162.

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