Vitamin A In Dried Blood Spots: Update On HPLC Method Changes To Measure Retinol

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BACKGROUND

During the passed 10 years dried blood spots (DBS) have evolved as a sample matrix that can be collected in a field setting with certain advantages over liquid serum samples. Reported advantages include: easier collection, transport, and storage; accessibility to younger and more remote populations; and decreased risk of disease transmission. Craft Technologies, Inc. (CTI) first reported an HPLC method for the analysis of DBS retinol in 1995. The initial method was performed by reversed-phase HPLC and required a subsample of serum but has evolved over the years to normal-phase HPLC accompanied by measurement of sodium to estimate the serum volume in each sample. Recently a vendor discontinued production of a critical component. This resulted in the redevelopment and validation of a new methods with a different column and mobile phase.

OBJECTIVES

To develop and validate an improved extraction and HPLC separation of DBS retinol in the absence of a critical column component. To improve the ruggedness and quality control of the method.

METHODS

Blood samples were collected from volunteers with and without anticoagulants. Control DBS of known volume were prepared to determine if anticoagulants altered the extraction of retinol or contributed to errors in the estimation of sample volumes. DBS calibration materials were prepared from serum or plasma samples of established retinol concentrations and packed red blood cells. HPLC conditions were tested to first provide acceptable separation of standards and potential interferences. Then HPLC conditions were tested for interferences from serum extracts. DBS of known volumes (15 or 25 uL) were extracted using modified conditions to identify what aspects (extraction buffer, time, antioxidants, solvent volume, etc) were critical to accurate quantification.

HPLC Conditions

Column: Diol, 3µm,150 x 3.0 mm

Mobile phase: Hexane\Ethyl acetate\Isopropanol (930:54:16)

Flow rate: 1.0 mL/min.

Temperature: Room Temp

Detection: programmed wavelength UV (300 nm for Tocol and 325 nm for Retinol)

Figure 1

DBS Retinol Separation Using Old vs New Method

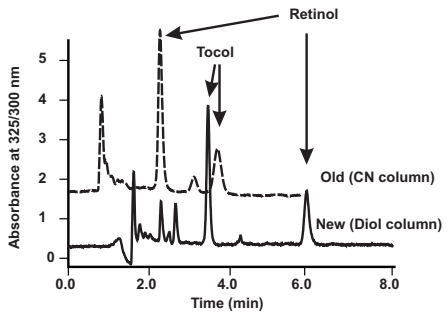

Figure 1. Since it was not possible to find an exact replacement for the NH2 guard column in the former method, we replaced the CN analytical column with a Diol column resulting in a reversal of the peak elution

Table 1

Effects of Treatments on DBS Retinol Recovery

Treatment	% Recovery	Indications
Anticoagulent		
Serum Retinol	100	Gold Standard
DBS w/o Anticoagulent	82	As Expected
DBS w Heparin	86	As Expected
DBS w EDTA	80	As Expected
Eluent		
Water	100	Control = 100%
+Pyrogallol 0.1%	111	Variable
+Pyrogallol 1%	117	Variable
+Ascorbic Acid 1%	105	Interferes w Na+
1% AA +Triton X100	90	Interferes w Tocol & Na+
0.1% AA +Triton X100	108	Interferes w Tocol & Na+
+Trolox	115	Protects Tocol & Retinol

Table 1. Effect of extraction variables on DBS retinol recovery. DBS made with serum or plasma resulted in 15-20% lower recovery than serum itself as reported previously.. Several components improved retinol recovery but interfered with the chromatography or Na+ measurement. Trolox improved recovery without compromising other aspects of the method.

Figure 2

DBS Calibration: Plasma Retinol vs DBS Retinol

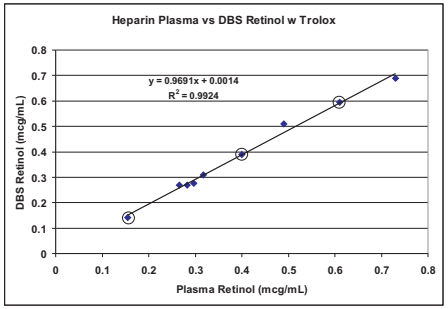

Figure 2. Multiple point calibration of DBS retinol using 12 serum samples and matching DBS. The points inside the circles were selected for use as routine QC samples and are included with each set of samples.

Figure 3

Honduran Serum Retinol vs DBS Retinol

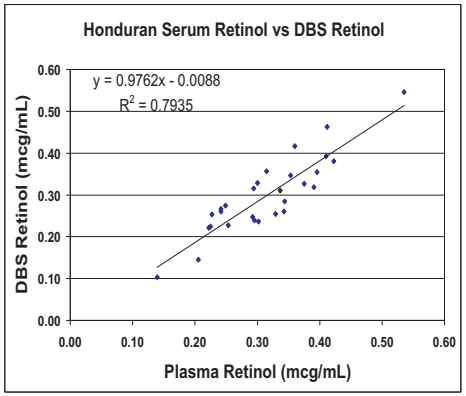

Figure 3. Comparison of Honduran plasma retinol to matching DBS retinol samples.

Results

Chromatography (Figure 1)

- Several HPLC columns and mobile phase conditions were tested.
- The new separation uses a Diol column, an NH2 guard column.
- Mobile phase is hexane\ethyl acetate\isopropyl alcohol (930:56:14) at 1.0 mL/min.
- Detection is monitored at 300nm and switched after Tocol elutes to 325nm in order to measure Retinol.
- Potentially interfering components, including the tocopherols, and carotenoids, did not coelute with the analytes.

Sample Treatment (Table 1)

- Elution time, extraction solvent and solvent volume remained the same as previously.
- Antioxidants (ascorbic acid, pyrogallol, Trolox) and detergent (Triton X100) were tested.
- Detergent did not improve the recovery of Retinol or Tocol
- Ascorbic acid and pyrogallol improved Retinol and Tocol recovery but interfered in other ways.
- Trolox prevented the loss of Tocol without interfering with the HPLC profile or altering the Na+ measurement.

Calibration and Quality Control (Figure 2)

- The DBS method was calibrated using several DBS (Figure 2) from 0.15 to 0.7 mcg.mL (0.5 to 2.5 umol/L).
- Based upon this multipoint calibration, three samples were selected to be used as QC/calibration materials.
- All three are included at the beginning and end of each batch of DBS samples measured.

Validation (Figure 3)

- Plasma retinol and DBS retinol values from Honduran samples were highly correlated (r^2 = 0.793, see Figure 3).

CONCLUSIONS

In summary, an improved HPLC method to measure DBS retinol was developed when the previous guard column was no longer available. The new method provides quantitative recovery of retinol and permits the use of Na+ to estimate sample volume. While ascorbic acid has been excluded due to its interference with the Na+ selective elctrode, Trolox has been added as an antioxidant. The chromatography is more robust and less dependent on a component from a single vendor. The QC now includes 3 DBS samples spanning 0.5 to 2 uM that are measured at the beginning and end of each batch. Results correlate well with matching plasma samples.