Effect of Antioxidants on Vitamin A, E, and Carotenoid Stability in Dried Blood Spots

Francisco X. Arredondo and Neal E. Craft. Craft Technologies, Inc. Wilson, NC 27893.
www.crafttechnologies.com

Introduction

Dry blood spot (DBS) collection offers a more practical alternative to serum particularly in remote areas were cold storage is not possible. Unfortunately many analytes, such as retinol, retinyl palmitate, β-carotene and α-tocopherol, are sensitive to rapid oxidation as the DBS ages on the card, thereby generating unreliable measurements.
One report has indicated that, from day one post-collection, retinol values can drop up to 25 % and α-tocopherol up to 50% (1). Pretreatment of DBS cards with 0.5% sodium ascorbate as an antioxidant improved the recovery of α-tocopherol during a single determination in several individuals (2).

Objective

To investigate the influence of non-phenolic and phenolic antioxidants on the stability over time of carotenoids, retinol, retinyl palmitate and α-tocopherol in DBS cards.

Methods

We employed Whatman Protein saver 903 cards and known antioxidants; sodium ascorbate, (SIGMA-A7631); ascorbic acid-6-palmitate, (AA6P; SIGMA-A1968); butylated hydroanisole (BHA, SIGMA-B1253); 2,6,di-tertbutyl-4-methylphenol (BHT, SIGMA-B1378); tert-butylhydroquinone (TBHQ, SIGMA-B9161). We also used the Whatman/GE Healthcare FTA DMP-A, B and C cards (Whatman/DMPK starter pack Cat WB129248) without any antioxidant addition. Phenolic antioxidant solutions were made in methanol at concentrations ranging from 5 to 0.005% while sodium ascorbate was dissolved in distilled water.

Whole blood was collected from a healthy volunteer in sterile 10 mL vacutainer tubes containing sodium heparin as anticoagulant. Sixty five microliters of sodium heparin blood was used on each of the antioxidant treated cards. The DBS were allowed to dry at 20-25°C laboratory room temperature and in the dark for 8 hours, deposited in zip-lock bags with desiccant and stored at -20 °C. The DBS cards were kept at constant -20°C, and exposure to room temperature and humidity was minimized. Measurements were made at days 1, 3, 7, 14, 21, 28, and 63.

DBS extraction.
One 6 mm dry blood spot disc (DBS) was deposited in a 12x75 mm borosilicate disposable tube and extracted. Five hundred microliters of sterile distilled water with 0.05% Trolox (SIGMA-238813) was added, vortexed briefly, sonicated at room temperature for 10 minutes and then further incubated at room temperature for an additional 20 minutes. Then, 500 µL of acetonitrile with 0.036 µg/mL of Tocol, as internal standard, was added, mixed briefly and extracted two times with 1 mL of Hexane containing 0.5% BHT. The extracted organic phases were pooled and concentrated in a centrifugal evaporator. The concentrated extract was dissolved in 120 µL of acetonitrile/isopropyl alcohol (90/10 v/v) and deposited in an autosampler glass microvolume insert. Fifty microliters was injected.

HPLC system.
ThermoSeparation HPLC System (ThermoSeparation Products, CA) consisted of a SCM1000 solvent conditioning module (degasser), P4000 gradient pump, AS3000 autosampler with vial and column temperature control, and UV6000 LP photodiode-array UV/visible detector. The separation was on an ES Indsutries, Spherisor ODS2, 250 x 4 mm, 3 µm, 80 Å, with titanium frits at a flow of 1mL/min with detection at 450 nm, 290 nm, 325 nm. The run time was 25 minutes (3).

Results

No data is presented for any of the DMPK/FTA cards DBS recovery values for retinol, retinol palmitate β-carotene and α-tocopherol were lower than the values obtained with the protein saver plain, non-treated cards. The manufacturer states that upon eluting these cards with organic solvents, the precipitated proteins stay on the cards. Blood applied to the FTA-DMPK card B turned black, most likely indicating the formation of hematin.
Protein saver cards were soaked with the appropriate antioxidant solution and left to dry in the dark for 18 hours in a ventilated hood; the TBHQ and AA6P soaked cards acquired a light pink color.

Results – cont.

The blood deposited on the 5.0% and 0.5% AA6P cards did not saturate them so they were discarded. Upon depositing the blood on the 0.05% and 0.005% AA6P cards, the red blood cells (RBC) stayed in the center and the plasma quickly migrated to the periphery, creating an "halo" effect. The 5.0%, 0.5% BHA and TBHQ cards turned the blood to a dark brown color (alkaline hematin formation and precipitation).
Preliminary results are presented for four analytes: figure 2, retinol, figure 3, retinyl palmitate, figure 4, β-carotene, and figure 5, α-tocopherol. Plasma values for the analytes are considered to be 100% recovery. Plasma values remained unchanged during the course of the study. A representative chromatogram of the TBHQ treated DBS sample is provided in Figure 1. Stability data is presented in the figures for several of the antioxidants at the concentration that provided the best protection.
On day 63, the percent recovery for retinol, retinyl palmitate, β-carotene and α-tocopherol was 22.1, 5.1, 18.5 and 14.3% for the untreated DBS card compared to 68.2, 25. 5, 34. 3 and 69.2 % observed in the 0.05% TBHQ treated cards. For α-tocopherol this represents a substantial improvement compared to the 50% drop after 24 hours reported by Granado et.al (1).

Discussion

There is little information reported regarding the influence of antioxidants on DBS stability. Aging of the blood on the collection cards, exposure to high humidity and temperature can alter the measurement of any assayed molecule. The presence of hemoglobin in the DBS and oxygen in air can induce oxidation of the components in DBS. Under mildly acidic conditions, pH 6.0, hemoglobin oxidizes to deoxyhemoglobin and methemoglobin. In this study, we observed, in BHA and TBHQ treated cards, that the precipitation of hematin prevented the rapid oxidation of the reported analytes.
Some degree of protection was provided by all of the tested antioxidants.

Conclusions

The results indicate that the addition of antioxidants to the DBS collection cards improved the recovery of retinol, retinyl palmitate, β-carotene, and α-tocopherol.

References

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Fig 2. DBS- Retinol

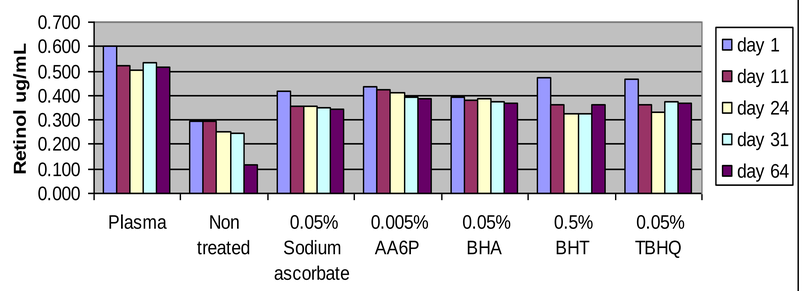

Fig 3. DBS-Retinyl palmitate

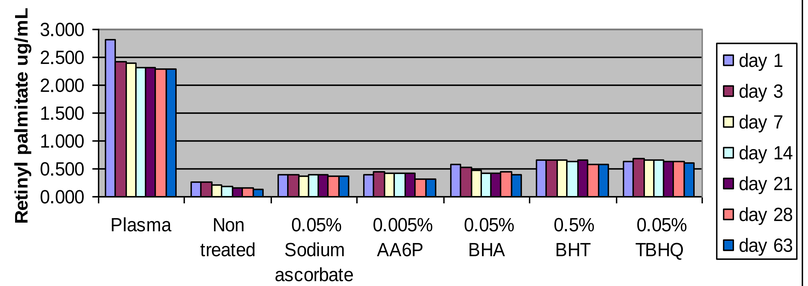

Fig 4. DBS-Total beta-Carotene

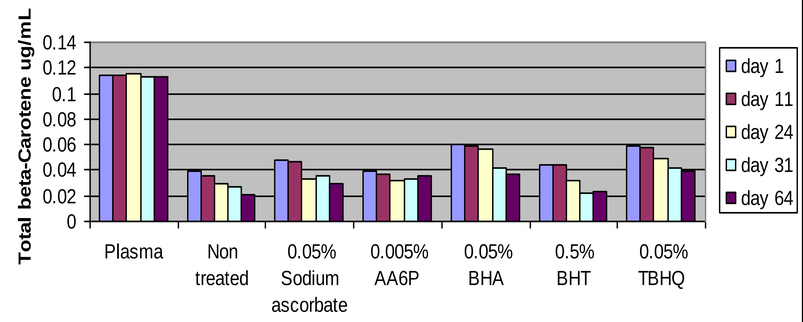

Fig 5. DBS-alpha-Tocopherol