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Short Communications

# Improving the long-term storage stability of all-*trans*-retinol in biological matrix

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ABSTRACT

All-*trans*-retinol or Vitamin A1 is an unstable lipid present in serum and formulations such as creams for acne, rhytids, and dyschromia. The retinoid is used as a biomarker for subclinical Vitamin A deficiency. Here, we report improved stability of retinol on long-term storage for over two years. Briefly, serum was used as a biological matrix. It was collected, stored, and processed in conditions simulating field conditions encountered in population-based studies in low/middle-income countries. Retinol was extracted in n-hexane and analyzed by HPLC. The analyte started deteriorating soon at room temperature but was stable for over 2 years when the matrix was stored under specified conditions. No additive was used. This study defines the collection, transportation, storage, handling, and processing conditions to ensure long-term storage stability of retinol in serum. The paper has implications in the analysis of Vitamin A1 in biological matrices and formulations stored over long periods.

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## INTRODUCTION

All-*trans*-retinol or Vitamin A1 is the predominant form of Vitamin A (VA) in human plasma and accounts for >95% of total VA (Aksnes, 1994; Zanotti and Berni, 2004). The retinoid is fat-soluble and is present in micrograms in serum bound to a protein called retinol-binding protein. A1 is also used as an adjunct in dermatological formulations such as creams for the treatment of acne, rhytids, and dyschromia (Akhavan and Levitt, 2008). In the blood/serum, it is used as a biomarker for subclinical VA deficiency (VAD), a common nutrition-related health problem in infants and children causing night-blindness and other health-related problems in high-risk populations, especially in South and South-East Asia, South Pacific, sub-Sahara, and parts of South and Central America, as well causing the risk of long-term respiratory disability in infants with a very low birth weight (Dowling and Wald, 1958; Spears et al., 2004; Bruins and Kraemer, 2013; Wiseman et al., 2017). VAD is also associated with increased severity and incidence of certain infections, immunity, and cancer risk (Semba et al., 1994; Castetbonet al., 2000; Huang et al., 2018). However, the compound is photosensitive and unstable (Akhavan and Levitt, 2008), a property which compromises its long-term storage stability in drug formulations and biological matrices. This paper describes the conditions that ensure the long-term storage stability of all-*trans*-retinol in serum. The paper has implications for the

analysis of retinol in biological matrices and formulations stored over long periods.

## MATERIALS AND METHODS

Retinol was measured by HPLC (Shimadzu, LC-10ATvp) equipped with a binary gradient and a multiple wavelength detector (SPD-10Avp) and C-18 column (Table 1). System was operated by SPINCHROM. Briefly, 100 µl serum was transferred to a 5-ml conical glass centrifuge tube and mixed with 100 µl internal standard (retinyl acetate in absolute ethanol) for 15 seconds (exact) and placed on ice for 5 minutes. Thereafter, 1 ml *n*-hexane was added and vortexed for exactly 1 minute. The mixture was centrifuged at 1,200-1,500 rpm for 5 minutes (25°C) and the upper clear hexane layer was drawn in another conical tube (5-ml). Hexane was evaporated under a gentle stream of Grade I $N_2$ gas. Finally, 100 µl methanol was added to the tube, contents were mixed (minimum 15 seconds) and a 20 µl aliquot was injected into the HPLC loop. All quality assurance exercises were completed as per the procedures. Purity of reagents, system suitability, selectivity, linearity, accuracy, and precision were checked as per the standard protocols. Conditions for the storage stability exercises are defined in Table 2. These conditions were employed in one of our population-based studies where the samples were collected and processed similarly. Freeze-thaw (FT) and bench-top stability exercises were also performed (Naime et al., 2011). Briefly low (10 µg/dl) and high (50 µg/dl) retinol concentrations were used (Table 3). For all practical purposes, retinol was analyzed in triplicate within 2-h after thawing.

## RESULTS

Retinol stored at room temperature started deteriorating a month after storage in dark. Deterioration was 50% by day 75 and the analyte was almost undetectable (3.78 µg/dl) after a year of storage when compared with the control (43.51 ± 0.17 µg/dl) preserved in a deep freezer at -20° C. Storage at 4 °C did not cause deterioration by day 75 and the sample was stable for a year (Fig. 1). At -20° C, values were identical when compared with the fresh control (43.35 ± 0.91 *vs* 43.51 ± 0.17 µg/dl). In conditions simulating field conditions, no significant variation was recorded *vs* control. Samples were stable for over 2 years under specified conditions (Fig. 1, Table 2). No additive was used in any of the samples. The analyte was stable after 2 FT cycles (Table 3). Stability in FT cycle 1 was b/w 97.17 and 103.6%, and in FT cycle 2, it was between 98.8 and 105.83%. Benchtop stability studies for 6 hours showed that the samples were stable on the bench during processing.

Table 1. High Performance Liquid Chromatography: Column specifications and mobile phase.

| | |
|---|---|
| Column | : α Bond™ C18 column, 125A 10µm, 300 x 3.9mm |
| Mobile phase | : Methanol/HPLC water (95/5) |
| Flow rate | : 1.2 ml/min |
| Pressure | : 120-145 Kgf/cm$^2$ |
| Detector | : UV-Visible |
| Wavelength | : 326 nm |

*C-18 column protected by a guard column was used. The column was washed using HPLC grade methanol. Analyte was extracted from the matrix/ serum in n-hexane and 20 µl of the aliquot was injected into HPLC loop using a Hamilton microliter syringe. Pure crystalline all-trans-retinol and all-trans-retinyl acetate (Sigma Chem. Co., St. Louis, MO, USA) were used as standard. Retention time for retinol was 5.78 minutes and for the internal standard retinyl acetate, it was 8.55 minutes. Pooled fresh serum from healthy human volunteers was obtained from Rotary Blood Bank.*

Table 2. Serum retinol concentration in specified matrix storage and handling conditions.

| Time of analysis | Retinol, µg/dl | |
|---|---|---|
| | Handling & storage conditions simulating the field conditions | Fresh pool serum analyzed before and after the storage in deep freezer |
| Fresh serum ↓ | 43.51 ± 0.17 | 43.51 ± 0.17 |
| 08 h in ice ↓ | 43.29 ± 0.17 | |
| 30 Days in liquid nitrogen↓ | 43.31 ± 0.35 | |
| 01 Day in gel packs ↓ | 43.80 ± 0.52 | |
| 05 Days in deep-freezer ↓ | 43.75 ± 0.14 | |
| 20 Days in deep-freezer ↓ | 43.52 ± 0.19 | |
| 47 Days in deep-freezer ↓ | 43.27 ± 0.53 | |
| 287 Days in deep-freezer ↓ | 43.18 ± 0.73 | |
| 329 Days in deep-freezer ↓ | 43.27 ± 0.52 | |
| 363 Days in deep-freezer ↓ | 43.57 ± 0.59 | |
| 750 Days in deep-freezer → | 43.40 ± 0.91 | 43.40 ± 0.91 |

*Each value represents average of measurements in triplicate (µg/dl, Mean ± SD). Samples were spiked with internal standard in the concentration range of 20-30 µg/dl. Fresh serum obtained from the pool of serum intended to be stored in different conditions was analyzed and used as control. Conditions simulating field conditions included preparation of the serum at room temperature, followed by 8 h on ice, 30 days in liquid nitrogen (-196 °C),*

*one day in gel pack (3-4 °C) and 24 months in deep-freezer.*

Table 3. Stability of retinol after two freeze-thaw (FT) cycles at 10 (low) and 50 (high) μg/dl dose levels under the specified conditions.

| Retinol | Fresh | FT cycle I | FT cycle II |
|---|---|---|---|
| 10 | 10.29 ± 0.51 | 10.66 ± 0.49 | 10.89 ± 0.12 |
| 50 | 44.95 ± 0.81 | 43.68 ± 2.94 | 44.41 ± 3.04 |

*Each value represents average of five measurements (μg/dl, Mean ± SD). The samples were frozen at 0 °C for 24 h followed by an unassisted thaw at room temperature. Retinol was analyzed within 2-h of thaw.*

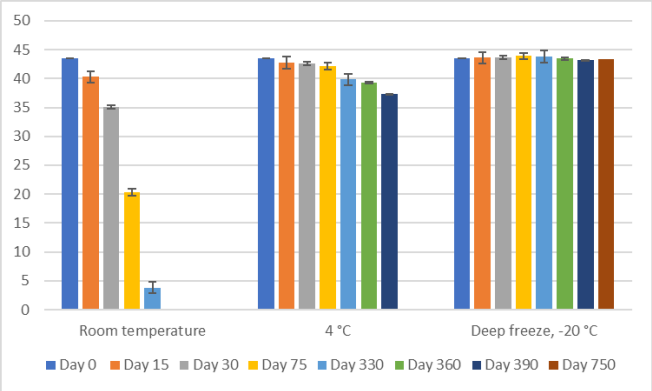


Figure 1. Serum retinol levels in samples stored at room temperature, 4 °C and -20 °C for different duration of time. Each value (μg/dl) is a representative estimation in triplicate. Samples were spiked with the internal standard, retinyl acetate in the concentration range of 20-30 μg/dl.

## DISCUSSION

This study defines the conditions to ensure the stability of all-*trans*-retinol in serum on long-term storage. The collection, storage, and handling conditions included the conditions defined in Table 2. These conditions were employed in one of our population-based studies where the samples were collected and processed similarly. Liquid nitrogen was used for transportation of serum collected from the field due to a lack of cold chain in low/middle-income countries. The samples were kept in ice/gel packs for at least one day and then transferred to a deep freezer before analysis on days 0, 15, 30, 75, 330, 360, 390, and 750 or 25 months. No change in serum retinol was recorded in samples stored under specified conditions *vs* control (Table 2). Earlier, long-term matrix/serum storage has been reported to cause a loss of retinol on storage at -20 °C (Driskell et al., 1985). Our results showed no loss of activity for over 2 years. This can be attributed to the specified handling conditions and sample preparation/extraction method. Contrary to this study, where ethanol was used for extraction (and retinol began to degrade in the extraction step soon after the addition), we used *n*-hexane. FT cycles (2 Cycles) and the bench-top stability exercises further suggested the analyte stability in our method. Bench-top stability demonstrates the stability of an analyte for the period the sample is usually exposed at room temperature during analysis. The bench-top studies for 6 h suggested that both retinol and retinyl acetate were intact. In a nutshell, handling and storage conditions defined in this study are crucial for maintaining the stability of all-*trans*-retinol over long periods. Other methods such as the Dried Blood Spot (DBS) method have been developed to facilitate collection and measurement from the field (Craft et al., 2000). However, the use of serum as the matrix for retinol estimation by HPLC is a foolproof method showing consistency in results. DBS method is suggested with caution until the conditions under which the sample is collected, handled, and stored are properly defined.

## CONCLUSIONS

Standardization of storage conditions for retinol in biological matrices, drugs, and cosmetic formulations is important to ensure long-term stability. This study defines the protocols for handling and storage of serum for the analysis of retinol in conditions simulating the field conditions, especially in low/middle-income countries where a cold chain is usually missing. In the conditions defined in this study, the sample collected for the estimation of retinol was stable under specified conditions for over 2 years. No additive was used in this study.

## CONFLICT OF INTERESTS

None declared.

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