

Abstract

A rapid and inexpensive method to measure retinol would be welcome in developing countries where vitamin A deficiency is a life-threatening problem for infants and children. Analytical technologies to measure serum retinol are either simple to perform but nonspecific and inaccurate, or they are highly specific and accurate but require extensive sample preparation and long run times. A simplified HPLC method reported herein represents a compromise between an easy-but-inaccurate non-chromatographic method and a labor-intensive HPLC method. Sample preparation and run times are cut in half. The method uses an HPLC system fitted with a 3µm reversed-phase column eluted at 0.9 mL/min with 78% acetonitrile. Sample preparation involves one-step extraction using 25 µL serum, 50 µL retinyl acetate (prepared in ethanol) as an internal standard, and 125 µL acetonitrile. Mixing is followed by centrifugation and sampling of the supernatant. Retinol, measured at 325 nm, elutes within 4 minutes. Neither hemolysis nor lipemia have an impact upon the result.

This method offers a significantly more rapid sample preparation process with use of fewer steps, fewer supplies, less expensive equipment, and a commercially available internal standard. The correlation between this rapid method and the conventional HPLC method is excellent (average bias = + 0.5 µg/dL; $y (\mu\text{g/dL}) = 1.0949x - 3.1031$; $r^2 = 0.9729$). Method validation results show excellent recovery (94 ± 4%), accuracy (98 ± 7%), and precision (3.6 ± 3.5% intra- and 4.6 ± 2.5% inter-assay CV).

Simplified HPLC/UV detection method for measuring serum retinol

M Chaudhary-Webb, JG Erhardt, MB Haynes, RL Schleicher

CDC, Atlanta, Georgia 30341 USA; University of Indonesia, Jakarta 10038 Indonesia

Retinol – Come see what it's all about!

Method Validation



LOD Measurements

- LOD = 0.52 µg/dL ± 0.43
- 20 intra-run measurements of a low QC pool diluted to 1 µg/dL.

Spike Recovery

- Spiked a low QC pool with retinol to 25, 40, 50, and 100 µg/dL.
- Analyzed over 7 runs
- Overall Mean Recovery = 94.2 ± 3.6%

	25 µg/dL Spike	40 µg/dL Spike	50 µg/dL Spike	100 µg/dL Spike
% Recovery	96.8%	95.9%	88.9%	95.1%

Diagnostic Accuracy

- Measured NIST 968 I and II over several runs
- Overall Mean Accuracy = 98.3%, Overall Mean Precision (% RSD) = 7.2%

Temperature Degradation Study

Evaluated retinol recovery under adverse storage conditions to simulate potential international conditions. Ideal Storage Conditions = -70°C. Adverse Field Conditions = approximately 25°C

- Minimal degradation of retinol (≤ 4%) in serum stored at 25°C for 3 weeks

Sample ID	Ideal Conditions (µg/dL)	% Change 1 day	% Change 2 days	% Change 3 days	% Change 1 week	% Change 2 weeks	% Change 3 weeks	% Change 4 weeks
Patient 1	3.5	8	-4	6	-9	-7	-1	-17
Patient 2	19.8	22	19	5	32	17	-30	-44
Patient 3	19.8	3	4	-6	0	3	-4	-10
Patient 5	37.9	3	-3	-5	0	-7	0	-4
Patient 4	40.4	1	-1	-3	-4	-3	1	-5
Patient 6	56.1	2	-8	-4	-6	-6	-2	-16
Patient 7	64.9	4	-3	-4	1	-3	3	-5
Patient 8	73.2	0	-7	-9	-6	-4	0	-8
Patient 9	87.0	4	-2	-3	-3	-5	1	-5
Patient 10	99.2	-2	-8	-10	-4	-1	-3	-9
Patient 11	107.4	1	-3	-4	-3	-5	1	-4
Mean Change (SD %)		4% (0)	-2% (0)	-4% (5)	0% (1)	-2% (7)	-3% (9)	-11% (12)

Intra-Assay Variability

- Intra – Measured 3 levels of stabilized quality control pools 22 times in a single run
- Measured 10 patient samples 5 times within a single run
- Measured 12 patient samples 4 times within a single run

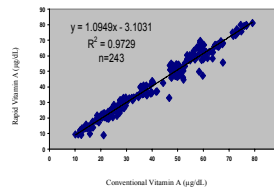
Samples	Number of Samples (Replicates per Sample)	Mean Vitamin A Concentration (µg/dL)	Intra-Assay % RSD
Quality Control - Low Pool	1 (22)	22	2.6
Quality Control - Medium Pool	1 (22)	45	2.3
Quality Control - High Pool	1 (22)	77	1.6
Severely Deficient	6 patients (4-5)	5-10	7.6
Deficient	4 patients (4-5)	11-20	3.4
Low to Sufficient	7 patients (4-5)	21-50	1.8
Sufficient to High	5 patients (4-5)	51-100	1.4

- Intra-Assay variability < 4% for specimens >10 µg/dL and < 8% for specimens ≤ 10µg/dL.

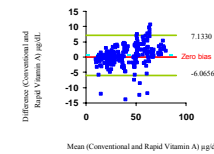
Bias Assessment

- Non-significant bias between two methods

Rapid Method vs Conventional Method Bias Assessment



Bland-Altman Comparison Bias → 0.5337 µg/dL

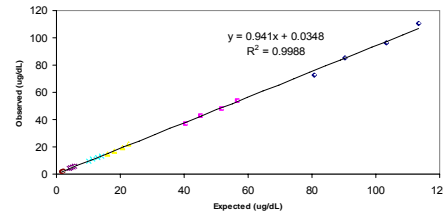


Linearity Assessments

- Patient samples analyzed at multiple dilutions. All dilution analysis per sample conducted within a single run
- Dilutions [None, 1:2, 1:5, 1:8, 1:20, 1:50]

- Mean difference comparing all diluted to undiluted rapid retinol method values = +4.6%
- Mean difference for all rapid retinol diluted to undiluted conventional retinol values = -2.9%

Serum Specimens diluted up to 50 times



Inter-Assay Variability

- Inter – Measured 3 levels of stabilized Quality Control pools in duplicate over 37 days
- Measured 8 patient samples repeatedly over 5 – 6 runs

Samples	Number of Samples (Assays per Sample)	Mean Vitamin A Concentration (µg/dL)	Inter-Assay % RSD
Quality Control - Low Pool	1 (74)	22	4.8
Quality Control - Medium Pool	1 (74)	45	4.2
Quality Control - High Pool	1 (74)	77	4.3
Severely Deficient	2 Patients (6)	5-10	8.0
Deficient	1 Patient (6)	11-20	4.5
Low to Sufficient	3 Patients (5)	21-50	3.6
Sufficient to High	2 Patients (5)	51-100	2.3

- Inter-Assay variability < 6% for specimens >10 µg/dL and ≤ 8% for specimens ≤ 10 µg/dL.

Materials and Supplies



- HPLC System with column heater/cooler, UV detector and Thermo Hypensil-Keystone Prism C18 column
- UV-VIS spectrophotometer for quantification of retinol and internal standard



Sample Preparation Supplies: adjustable pipette, pipette tips, gloves, centrifuge tubes, HPLC vials, inserts, and caps, biohazard disposal supplies, centrifuge



Calibrator Preparation Supplies: adjustable positive displacement pipette, positive displacement pipette tips, gloves, test tube, disposable transfer pipettes, HPLC vials, inserts, and caps

Rapid Method

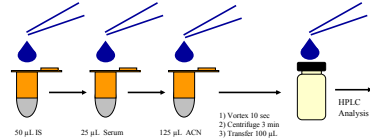
Obtain blood from patients via venipuncture or finger stick. Prepare serum.



Venipuncture method



Finger stick method



- 50 µL Internal Standard (IS) = Retinyl Acetate (Abs @ 325 nm in ethanol = 0.1321)

- 25 µL Serum
- 125 µL Extraction Solution = 100% filtered acetonitrile
- Shake using a vigorous up/down motion for 30 sec, or 10 sec via a mechanical vortexer
- Centrifuge the vials for 3 min at 25°C at 1,400 RPM
- Transfer 100 µL of supernatant into HPLC vials maintained at 10°C
- Inject 15 µL of the extract onto a Prism reverse phase HPLC column maintained at 35°C
- Isocratic Mobile Phase: 78% Acetonitrile with 0.1% Triethylamine / 22% Deionized Water
- Detect peaks at 325 nm

Cost/Time Considerations

Requirements for Method	Rapid Method	Conventional Method
Acetonitrile	✓	✓
Ethanol	✓	✓
Hexane	✓	✓
Triethylamine	✓	✓
Deionized Water	✓	✓
Ice Bath (with Dry Ice)	✓	✓
Syringes and Syringe Filters	✓	✓
HPLC Vials, Inserts and Caps	✓	✓
Pipettes and Pipette tips	✓	✓
HPLC System	✓	✓
SpeedVac Solvent Evaporator	✓	✓
Centrifuge	✓	✓
Cold Storage Facilities	✓	✓
Time per Test (Sample prep 20 samples)	15 min	90 min
Time per Test (HPLC analysis)	≤ 6 min	13 - 14 min
Cost per Test (supplies & small equipment)	\$6.19	\$17.49
Required Sample Volume	25 µL	100 µL

Typical Chromatogram

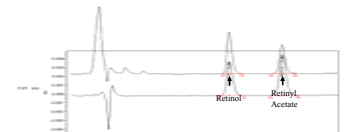


Figure 1. Chromatogram of a typical patient serum overlaid with a typical non-matrix matched standard. With a new column, full separation between the analyte (4.3 min) and internal standard (5.8 min) is observed with 1.5 min between the two. As the column ages, the retention times decrease and the separation diminishes between the analyte (3.4 min) and the internal standard (3.8 min).

Acknowledgments

- The entire staff of the CDC Nutrition Lab for all their collaborative inputs
- Michael Rybak for his technical assistance
- Christine Pfeiffer for superior leadership of the Lab