# Challenges in the Development of Certified Vitamin Reference Solutions

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## Abstract

Vitamins are essential for general health and well-being. From patient samples in clinical laboratories to manufacturers of dietary supplements and fortified foods, use of Certified Reference Materials (CRM) for vitamin testing applications is critical to ensure accurate results. Effective reference materials must be quantitative, accurate, consistent, and stable. Certified solution reference standards with suitable diluents at appropriate concentrations and in sealed ampoules under inert conditions can offer significant advantages over neat reference materials in terms of accuracy, consistency, and stability.

Factors such as chromatographic purity, hygroscopicity, solubility, and stability of the neat material must be determined and assessed prior to preparation of the reference standard solution. Handling and preparation of vitamin reference solutions present significant challenges due to their sensitivity to air, light, and solution pH. This poster will discuss the unique challenges in development of certified solution standards of certain vitamins, including vitamins A , B, D and E.

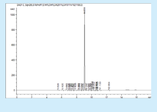

## Ampouled Certified Solutions – A Better Alternative

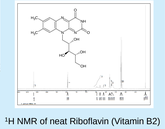
| | Ampouled Certified Solutions | Laboratory Prepared (Solutions from Neat Materials) |
|---|---|---|
| **Stability over time** | Years | Weeks-months |
| **Lot to lot consistency and reproducibility** | Larger batches - larger weighings - greater accuracy. | Frequent smaller weighings may not be sufficiently accurate due to the sample size and relative error of the analytical balance – minimum 25mg weighing necessary on typical 5-place balance. |
| **Homogeneity / Concentration** | Ampoule to ampoule and across the lot. | Cannot be ensured – precipitation/evaporation.<br><br>Hygroscopicity of the neat can affect concentration from weighing to weighing. |
| **Convenience of use** | “Snap and Shoot” | Weigh, dilute, qualify. |
| **Efficiency** | Reduced labor – eliminates bench preparation & certification . | Repeated weighing, handling, qualification . |
| **Material usage/cost** | Eliminates waste – stable single use format. | More frequent preparation – more disposal. |
| **Contamination/ Degradation risk** | Single use format – very low risk.<br><br>Protects against air, light and moisture. | Multiple use format – higher risk for bulk contamination.<br><br>Open container - risk of degradation & changes in concentration due to evaporation. |

## Neat Material Certification

- Source of material may impact results – research grade vs. CRM
- Full characterization is necessary to ensure:
  - Proper weighing adjustment is assigned
  - Lot-to-lot consistency
  - No degradation or absorption of water has occurred during transit or over time
- CRM certification includes:
  - Chromatographic Purity determination by HPLC, GC/FID and/or LC/MS
  - Determination of residual water, solvent, and inorganics
  - Identity verification by NMR, FTIR and/or MS

HPLC of neat Riboflavin (Vitamin B2)





$^1$H NMR of neat Riboflavin (Vitamin B2)

| Compound | Chrom. Purity (%) | Residual Solvent Content (%) | Trace Inorganic Content (%) | Residual Water Content (%) | Purity Factor (%) |
|---|---|---|---|---|---|
| Thiamine HCl (Vitamin B1) | 99.7 | None detected | 0.43 | <0.2 | 99.2 |
| Nicotinamide (Vitamin B3) | 99.9 | None detected | 0.03 | <0.2 | 99.9 |
| Nicotinic acid (Vitamin B3) | 99.9 | None detected | None detected | <0.2 | 99.9 |
| Pyridoxine (Vitamin B6) | 99.9 | 0.09% | 0.01 | <0.2 | 99.8 |
| (±)-α-Tocopherol (Vitamin E) | 98.2 | 0.05% | None detected | None detected | 98.2 |
| (±)-γ-Tocopherol (Vitamin E) | 98.1 | None detected | 0.08 | None detected | 98.0 |
| Vitamin D2 | 99.2 | None detected | None detected | <0.2 | 99.2 |
| Vitamin D3 | 99.5 | 0.04% | 0.08 | <0.2 | 99.4 |

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## Pre-Formulation Studies

A pre-formulation study was performed for determination of the diluent as well as solubility and stability of the analyte in the diluent selected.

### Riboflavin (Vitamin B2): Diluent Solubility Study

The Riboflavin (Vitamin B2) study indicated that the Certified Reference Standard should be prepared at a 50 or 100 µg/mL concentration in water with gentle heating to dissolve the solid.

| Diluent | Concentration | Dissolved | Did NOT Dissolve |
|---|---|---|---|
| 0.1% Formic Acid in Water | 100 µg/mL | | X |
| 0.1% Acetic Acid in Water | 100 µg/mL | | X |
| 0.1% Citric Acid in Water | 100 µg/mL | | X |
| 1% Urea in Water | 100 µg/mL | | X |
| 1% Urea in Water | 50 µg/mL | X | |
| Water with Sonication Only | 100 and 50 µg/mL | | X |
| Water with Sonication Only | 10 µg/mL | X | |
| Water with Gentle Heating | 100 µg/mL | X | |

### Retinol (Vitamin A): Diluent Stability Study

The Retinol (Vitamin A) study demonstrated that the Certified Reference Standard should be prepared at a concentration of 100 µg/mL in Ethanol containing 0.1% (w/v) BHT. Due to the known relative instability of Retinol in solution over time, the standard will be stored at sub-freezer conditions (-70˚C to -80˚C) to ensure its long-term stability.

| Retinol in Ethanol (No Stabilizer), 100 µg/mL | | | | |
|---|---|---|---|---|
| Time interval | Storage Temperature | | | |
| Time (weeks) | Freezer | Refrigerator | Room Temperature | 40˚C |
| 0 | 98.6% | 98.6% | 98.6% | 98.6% |
| 1 | 95.4% | 90.6% | 90.1% | 72.5% |
| 2 | 88.9% | 88.2% | 85.0% | 70.7% |

| Retinol in Ethanol with 0.1% (w/v) BHT, 100 µg/mL | | | | |
|---|---|---|---|---|
| Time interval | Storage Temperature | | | |
| Time (weeks) | Freezer | Refrigerator | Room Temperature | 40˚C |
| 0 | 98.7% | 98.7% | 98.7% | 98.7% |
| 1 | 98.4% | 98.4% | 98.4% | 98.2% |
| 2 | 98.7% | 98.8% | 98.7% | 98.3% |
| 4 | 98.4% | 98.4% | 98.2% | 97.6% |

**Temperature Ranges**
Freezer: -10˚C to -25˚C
Refrigerator: 2˚C to 8˚C
Room temperature: 15˚C to 30˚C

## Dispensing

Solution standards prepared in bulk can be dispensed into single use volumes and flame sealed under inert atmosphere in amber ampoules.

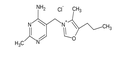
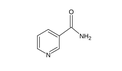
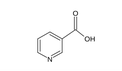
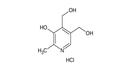
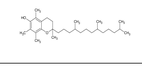
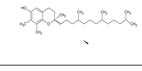
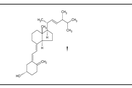
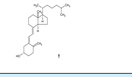
- Rigorous process controls ensure:
  - Consistency of volume dispensed
  - Homogeneity from vial to vial and across the lot
- Amber ampoules protect from light
- Single use format prevents cross-contamination
- Inert atmosphere promotes long-term stability and protects against oxidative & evaporative changes





## Release Testing of the Vitamin Reference Solution

Release testing of the standard determines accuracy of the solution concentration in comparison to an independently-prepared calibration solution. Lot-to-lot consistency is determined by comparison to the previous lot with homogeneity verified by analyzing random ampoules across the batch, purity by testing for contamination and degradation, and stability through comparison to older lots.

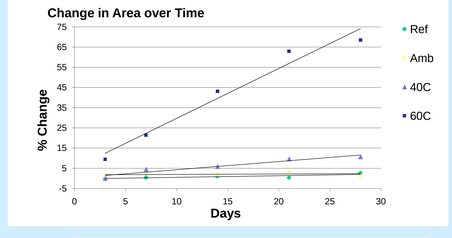
| Solution (1.0 mg/mL) | Structure | Solution Homogeneity: %RSD | % Conc Difference from Target | Solution Purity | Analytical Method |
|---|---|---|---|---|---|
| V-014 Thiamine HCl (Vitamin B1) |  | 0.93% | -0.07% | 99.8% | Analysis Method: HPLC/UV<br>Column: Discover HS F5 3µ, 4.6 x 100 mm<br>Mobile Phase: Acetonitrile::0.1% Ammonium acetate (10::90)<br>Flow Rate: 1.0 mL/min<br>Wavelength: 265 nm |
| V-016 Nicotinamide (Vitamin B3) |  | 0.67% | -0.06% | 99.8% | Analysis Method: HPLC/UV<br>Column: Discover HS F5 3µ, 4.6 x 100 mm<br>Mobile Phase: Acetonitrile::0.1% Ammonium acetate (3::97)<br>Flow Rate: 1.0 mL/min<br>Wavelength: 265 nm |
| V-017 Nicotinic acid (Vitamin B3) |  | 0.83% | 0.40% | 100.0% | Analysis Method: HPLC/UV<br>Column: Ascentis C18, 4.6 x 100 mm<br>Mobile Phase: 0.1% $H_3PO_4$<br>Flow Rate: 1.0 mL/min<br>Wavelength: 260 nm |
| V-018 Pyridoxine HCl (Vitamin B6) |  | 1.50% | -0.61% | 100.0% | Analysis Method: HPLC/UV<br>Column: Discover HS F5 3µ, 4.6 x 100 mm<br>Mobile Phase: Acetonitrile::0.1% Ammonium acetate (10::90)<br>Flow Rate: 1.0 mL/min<br>Wavelength: 325 nm |
| V-020 (±)-α-Tocopherol (Vitamin E) |  | 0.79% | 0.28% | 98.2% | Analysis Method: UV/VIS<br>Wavelength: 295 nm<br>Slit Width: 1.0 nm<br>Response: 0.5 s |
| V-021 (+)-γ-Tocopherol (Vitamin E) |  | 0.44% | 2.81% | 99.4% | Analysis Method: UV/VIS<br>Wavelength: 220 nm<br>Slit Width: 1.0 nm<br>Response: 0.5 s |
| V-024 Vitamin D2 |  | 0.30% | 1.70% | 99.2% | Analysis Method: UV/VIS<br>Wavelength: 290 nm<br>Slit Width: 1.0 nm<br>Response: 0.5 s |
| V-025 Vitamin D3 |  | 1.26% | 0.22% | 99.5% | Analysis Method: UV/VIS<br>Wavelength: 292 nm<br>Slit Width: 1.0 nm<br>Response: 0.5 s |

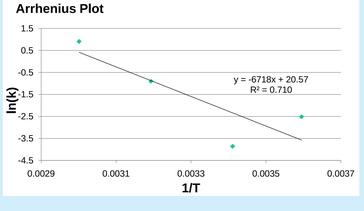
## Stability

The solutions were monitored at various storage temperatures over time to obtain real-time stability information including shipping stability. The stability of V-014 Thiamine HCl (Vitamin B1) was monitored by HPLC under refrigerated, room temperature, 40˚C, and 60˚C temperatures over the course of 1 month.

| Percent (%) Purity at Designated Storage Conditions | | | | |
|---|---|---|---|---|
| Time (Days) | Refrigerated | Ambient | 40˚C | 60˚C |
| 0 | 99.7 | 99.7 | 99.7 | 99.7 |
| 3 | 99.8 | 99.8 | 98.8 | 92.2 |
| 7 | 99.7 | 99.6 | 98.4 | 83.5 |
| 14 | 99.7 | 99.4 | 97.0 | 66.8 |
| 21 | 99.8 | 99.4 | 95.6 | 47.3 |
| 28 | 99.8 | 99.3 | 94.2 | 40.4 |
| Time 0 data is freezer storage conditions. | | | | |

**Using the Arrhenius Plot, V-014 can be estimated to be stable for 4 years.**




## Conclusions

Certified reference solutions offer a significant advantage over neat reference materials in terms of accuracy, consistency and stability. Long-term stability of solution-based Certified Reference Materials is achievable when appropriate parameters are chosen in the design, preparation, packaging, and storage of the solution standard. With proper parameter selection, stable and accurate certified reference solutions of notably sensitive compounds – Vitamins A, B, D, and E - have been developed.