# Development and Validation of a Stability Indicating HPLC-UV Assay for Leucovorin Calcium Oral Suspension

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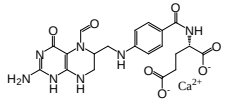
## INTRODUCTION

- Leucovorin, also known as Folinic acid, is used in combination with other chemotherapy agents (mainly methotrexate or 5-fluorouracil) in cancer treatment.
- The objective of this study is to develop and validate a stability indicating HPLC assay for Leucovorin calcium oral suspension.
- The stability of Leucovorin Calcium Oral Suspensions packaged in PTFE plastic multi-dose containers in controlled cold temperature and controlled ambient temperature conditions was evaluated.



## METHODS

- *Instrumentation:* Agilent (Santa Clara, CA) Series 1100 HPLC System equipped with an Agilient Poroshell EC-C8 4.6 x 150 mm 2.7 µm particle column was used for the stability indicating HPLC assay. Barnstead (Lake Balboa, CA) Nanopure Water Systems was used throughout the analysis. HPLC ChemStation Version A.10.02 (Windows XP) software was used for data analysis. Varian (Palo Alto, CA) VNMRS 500 MHz-NMR Spectrometer was used to characterize the Leucovorin standard.
- *Materials*: 1N Sodium Hydroxide (NaOH) and 1N Hydrochloric Acid from Ricca; Methanol from LabExpress; 30% Hydrogen Peroxide from Sigma-Aldrich; Acetonitrile from Fischer Scientific; Tetrabutylammonium Phosphate from Acros; Tetrabutylammonium Hydroxide from Spectrum, Leucovorin Calcium standard from USP; 5 mg/mL Leucovorin Calcium Oral Placebo and Leucovorin Calcium Oral Suspension were compounded by Flourish Integrative Pharmacy.
- *Chromatographic Conditions of stability indicating HPLC method:*

  Mobile Phase A: 5 mM tetrabutylammonium phosphate buffer solution 80:20 methanol, pH adjusted to 6.6 with tetrabutylammonium hydroxide

  Mobile phase B: Methanol.

  Gradient: 0-20 min (0-10% B), 20.1-30 min (0% B). Column Temp: 55 ºC; Flow Rate: 0.75 mL/min. UV detection at 290 nm.
- Forced degradation studies were performed by subjecting the Leucovorin Calcium oral suspension and placebo to conditions including acid (pH 1 overnight), base (pH 11 overnight), heat (4-hr at 80ºC), oxidation (3% $H_2O_2$) and UV light overnight. Each stressed sample was injected in duplicate. % Leucovorin recovered and retention time of any extra peaks were recorded. Peak purity analysis of the Leucovorin peak in the stressed samples was conducted using a photodiode array detector.
- Robustness: USP standard and samples with Leucovorin and all excipients at 100% of assay level (46.4 µg/mL) were analyzed with the following adjustment to the assay conditions: (+) 2 degree adjustment to the column temperature; (-) 2 degree adjustment to the column temperature; (+) 10% flow rate adjustment; (-) 10% flow rate adjustment; (+) 2% adjustment of methanol in mobile phase; (-) 2% adjustment of methanol in mobile phase; (+) 0.2 unit adjustment of pH in mobile phase; (-) 0.2 unit adjustment of pH in mobile phase.
- The stability of the samples were assessed based on appearance, pH, and potency assay. The samples were stored for 90 days under controlled cold temperature (2°C to 8°C) and controlled ambient temperature (20°C to 25°C) conditions. At time zero a homogeneity study was also conducted where Leucovorin content was determined in each sample bottle by sampling from the top, middle and bottom of the bottles.

## RESULTS

**Fig 1. Representative chromatograms of Leucovorin Calcium oral suspension stressed with (A) 0.1 N HCl, (B) 0.1 N NaOH and (C) 3% $H_2O_2$**

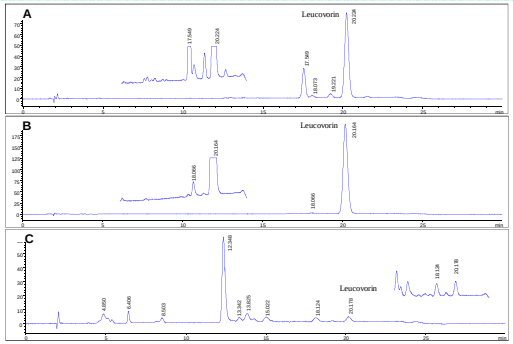



**Table 1. Examining the specificity of stability indicating HPLC assay through forced degradation**

| Sample type | Average Area of Leucovorin Peak (mAU's) | % Of Control | Additional Peaks (RRT) |
|---|---|---|---|
| Leucovorin Formulation Control | 2028.78 | N/A | 0.87 |
| Leucovorin Formulation + HCl | 705.73 | 35% | 0.11, 0.14, 0.24, 0.63, 0.67, 0.68, 0.73, 0.74, 0.87, 0.89, 0.95, 1.06 |
| Leucovorin Formulation + NaOH | 2007.1 | 99% | 0.26, 0.29, 0.83, 0.87, 0.95 |
| Leucovorin Formulation + $H_2O_2$ | 89.89 | 4% | 0.24, 0.25, 0.27, 0.32, 0.42, 0.61, 0.66, 0.69, 0.71, 0.74, 0.80, 0.84, 0.86, 0.95 |
| Leucovorin Formulation + UV | 2032 | 100% | 0.26, 0.29, 0.95 |
| Leucovorin Formulation + Heat/Humidity | 2316.32 | 114% | 0.26, 0.29, 0.87, 0.95 |

In all conditions, additional peaks were resolved from the API peak. Peak purity of Leucovorin peak was assessed to verify no co-eluting impurities.

**Table 2. The accuracy and precision of HPLC assay for Leucovorin Calcium oral suspension**

| Accuracy and Precision | | | | | | |
|---|---|---|---|---|---|---|
| Expected Amount (µg/mL) | Calculated Conc. (Triplicate Sample Preparation and Analysis) (µg/mL) | | | Average Conc. (µg/mL) | % of Expected | % RSD |
| | 1 | 2 | 3 | | | |
| 37.1 | 36.3 | 36.3 | 36.2 | 36.3 | 98% | 0.32% |
| | 36.5 | 36.3 | 36.2 | | | |
| | 36.5 | 36.3 | 36.2 | | | |
| 46.4 | 45.3 | 45.2 | 45.2 | 45.4 | 98% | 0.36% |
| | 45.4 | 45.5 | 45.5 | | | |
| | 45.6 | 45.6 | 45.6 | | | |
| 55.7 | 55.6 | 55.4 | 55.4 | 55.2 | 99% | 1.00% |
| | 56.1 | 55.5 | 54.4 | | | |
| | 54.7 | 54.8 | 54.7 | | | |

**Figure 2. (A) Solution stability and (B) Filter/Centrifuge suitability of Leucovarin standard and assay samples**

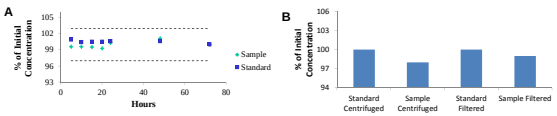



**Table 3. Robustness of Leucovorin Calcium method**

| Condition Variations | % RSD of Five Standard injections | Tailing Factor (First Std/Last Std/Spl) | Column Efficiency (First Std/Last Std/Spl) | Resolution (First Std/Last Std/Spl) |
|---|---|---|---|---|
| Flowrate + 10% | 0.1 | 0.972/0.979/0.979 | 26555/31020/30218 | NA/NA/5.3 |
| Flowrate -10% | 1 | 1.001/1.004/1.012 | 3910/4057/4054 | 1.6/1.5/1.6 |
| Column temp + 2°C | 0.2 | 1.001/1.006/1.011 | 36957/37261/37085 | NA/NA/8.0 |
| Column temp - 2 °C | 0.1 | 1.170/1.141/1.130 | 32788/33001/33483 | NA/NA/6.7 |
| pH + 0.2 | 0.2 | 0.884/0.897/0.901 | 20605/21170/22072 | NA/NA/3.9 |
| pH - 0.2 | 0.2 | 0.928/0.930/0.941 | 32084/34124/34002 | NA/NA/9.9 |
| Organic in MP + 2% | 0.2 | 0.878/0.874/0.878 | 17129/18124/18997 | NA/NA/6.0 |
| Organic in MP - 2% | 0.4 | 0.972/0.984/0.997 | 2485/2684/2667 | 1.9/2.1/2.1 |

**Figure 3. (A) Linearity of HPLC assay and (B) homogeneity of Leucovorin Calcium oral suspension in sample bottles**

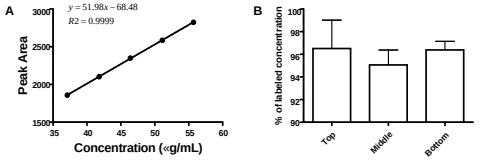



**Figure 4. Plot of average Leucovorin recovery relative to the label potency (% of Label) for samples stored at refrigerated and ambient conditions for 90 days. Dashed lines represent lower and upper limits for Leucovorin potency (90-110%).**

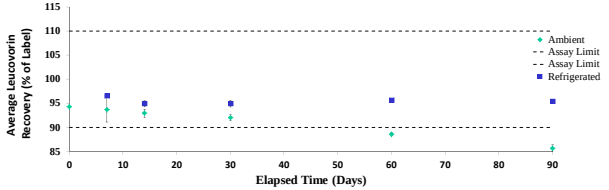
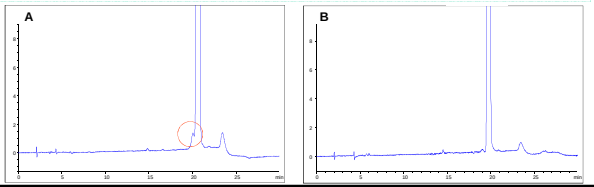



**Figure 5. Representative Leucovorin chromatograms with (A) and without (B) artificial peak caused by tetrabutylammonium phosphate during thorough investigation.**




## CONCLUSIONS

- The developed stability-indicating HPLC method enables a quantitative evaluation of the stability of Leucovorin Calcium oral suspension. The validation demonstrates that the method is specific, linear, precise, accurate, and robust.

**ACKNOWLEDGMENT** This study was supported by The United States Pharmacopeial Convention.