LC/MS Application Note

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# Quantitation of a Dinitro Flavonoid (DNF) in Rat Plasma Using the Varian 1200L LC/MS/MS System

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## Introduction

Flavonoids are compounds that make up the pigments in fruits, vegetables, and herbs. There have been reports of flavonoids having multiple beneficial therapeutic effects for a large number of serious illnesses, such as rheumatoid arthritis, cancer, cataracts, anxiety, and depression. The objective of this work was to carry out a pharmacokinetic evaluation to determine a profile with the formulation being tested at increasing doses where there is pharmacological effect.

A Varian 1200L LC/MS/MS system was used for the quantitation for plasma levels of a novel dinitro-substituted flavone (DNF) in samples from a pharmacokinetic study following oral administration of the drug. Flavone was used as the internal standard. Structures are shown in Figure 1.

## Instrumentation

- Varian 1200L LC/MS/MS equipped with ESI source

## Materials and Reagents

- DNF provided by Faber-Kramer Pharmaceuticals, Houston, Texas.
- Flavone (Catalog Number F602) from Sigma-Aldrich.
- All other chemicals are reagent grade or HPLC grade.

## Sample Preparation

A 200 µL aliquot of rat plasma containing DNF is spiked with 10 µL of flavone (0.2 ng) as the internal standard (ISTD). The rat plasma is made basic and extracted with hexane: chloroform (2:1, v/v).

After separation of the phases by centrifugation, the organic layer is transferred by pouring into a silanized conical tube and evaporated to dryness. The dry residue is reconstituted in 50 µL of methanol:water (60:40, v/v).

A 10 µL aliquot is injected into an ESI-LC/MS/MS for analysis using Selected Reaction Monitoring (SRM).

## HPLC Conditions

| | | | | |
|---|---|---|---|---|
| Column | 5.0 cm by 2.0 mm, 5 µm C-18 | | | |
| Solvent A | acetonitrile:water (50:50) | | | |
| Solvent B | acetonitrile:water (95:5) in 0.1% formic acid | | | |
| LC Program | Time (min) | %A | %B | Flow (µL/min) |
| | 0:00 | 99 | 1 | 200 |
| | 3:50 | 1 | 99 | 200 |
| | 5:00 | 1 | 99 | 200 |
| | 5:10 | 99 | 1 | 200 |

## MS Parameters

| | |
|---|---|
| Ionization Mode | ESI positive |
| Collision Gas | 2.2 mTorr Argon |
| API Drying Gas | 25 psi at 260 °C |
| API Nebulizing Gas | 60 psi |
| Scan Time | 0.9 sec |
| SIM Width | 0.7 amu |
| Needle | 4850V |
| Shield | 300V |
| Capillary | 76V |
| Detector | 1850V |

**Compound Structures**

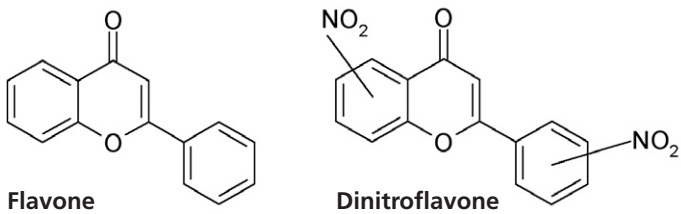


*Figure 1. Structure of flavone and dinitroflavone.*

## Scan Parameters

| Analyte | Precursor Ion (m/z) | Product Ion (m/z) | Collision Energy (V) |
|---|---|---|---|
| DNF | 313 | 221 | 30.5 |
| Flavone | 223 | 129 | 27.5 |

## Results and Discussion

The ratio of the area intensities of the product ion for DNF to the product ion of the internal standard [(m/z 221)/(m/z 129)] is used to calculate the DNF concentration in unknown rat plasma samples. The calibration curve is generated from the analysis of a drug-free rat plasma matrix fortified with various amounts of DNF and a fixed amount of the internal standard. The calibration curve range for DNF is from 0.10 ng/mL to 5.00 ng/mL of rat plasma.

Figure 2 shows the excellent response and peak shape obtained at the Lower Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ). Visual examination of the calibration curve in Figure 3 shows that good linearity was obtained with the linear-log, curve-fitting model used. Additional sensitivity can easily be obtained by increasing the sample volume used for the assay.

## Conclusion

The pharmacokinetic plot derived from DNF plasma levels obtained with six rats is shown in Figure 4. Visual observation of the plot reveals that at three hours post-dose there is a drop in DNF concentration, which "rebounds" an hour later, and from that point the concentration drops gradually in a linear fashion.

This fluctuation in concentration may be due to a phenomenon known as "entero-hepatic recirculation." Entero-hepatic recirculation is a process defined as a recycling of a drug through the liver by its excretion in bile, followed by re-absorption of the drug from the intestines into the portal circulation and passage back to the liver, where it is re-excreted in the bile.

Another possibility to explain the fluctuation in concentration is related to the limited solubility of DNF in aqueous media. DNF may be precipitating in the stomach and then later moving into the intestines where its absorption takes place. More studies are needed to determine if either of the processes described above are in fact taking place.

From the results of this study, it can be concluded that pharmacokinetic studies using the rat will be useful for comparison of different DNF formulations and different modes of administration.

**SRM at LLOQ and ULOQ**

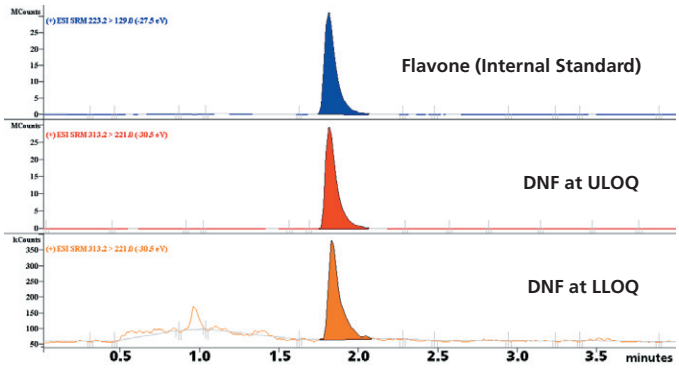


*Figure 2. Excellent detector response and peak shape for DNF at the LLOQ.*

**Standard Calibration Curve for DNF**

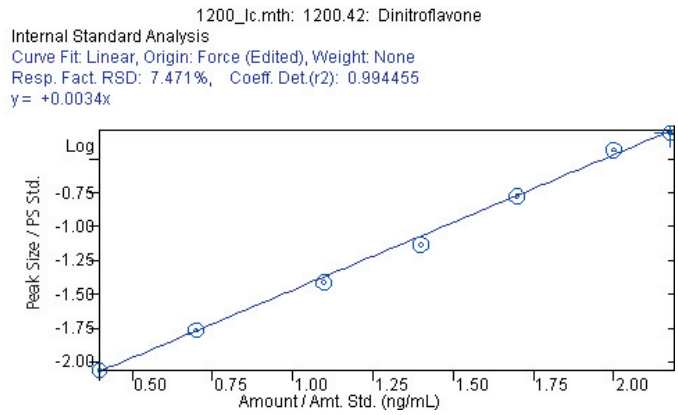


*Figure 3. Excellent linearity in the range of 0.10 ng/mL to 5.00 ng/mL for DNF in rat plasma.*

**Pharmacokinetic Plot of DNF Rat Plasma Levels**

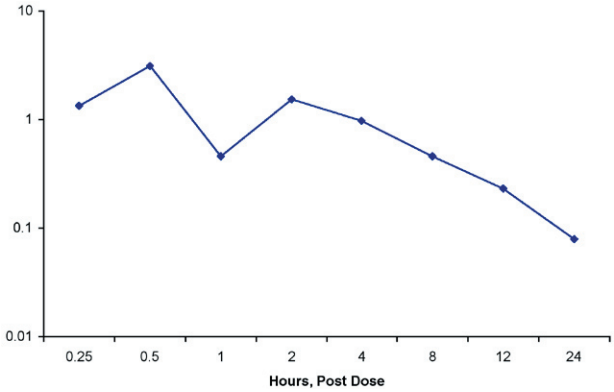


*Figure 4. DNF plasma levels drop dramatically between two and three hours post-dose, but "rebound" within the next hour.*

*These data represent typical results.*
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