Supplementary material

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A Phase 1, Open-Label Study to Evaluate the Effects of Food and Evening Dosing on the Pharmacokinetics of Oral Trofinetide in Healthy Adult Subjects

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Online Resource 1

Bioanalysis and Pharmacokinetic Assessment

Human urine and lithium heparinized blood samples were analyzed for concentrations of trofinetide by Inotiv (West Lafayette, Indiana, United States) using validated liquid chromatographic–tandem mass spectrometric bioanalytical methods.

Sample processing for blood was performed by solid phase extraction of a 50-µL volume at alkaline pH using an Oasis® MAX solid phase extraction plate (Waters Corp., Milford, Massachusetts, United States). High-performance liquid chromatography (HPLC) was performed using a Restek™ Ultra PFPP column (100 × 3.0 mm, 3 µm) (Bellefonte, Pennsylvania, United States) and analyzed using a Sciex API 4000 mass spectrometer with Turbolonspray ionization source in positive ion mode and Analyst™ control software versions 1.5.2 and 1.7.1. Mass spectrometric detection was done using multiple reaction monitoring (MRM) with transitions (m/z) 316.2→169.1 for trofinetide and 322.2→169.0 for the internal standard [$^{13}C_5$,^{15}N]-trofinetide. The assay range was 0.100–100 µg/mL for trofinetide.

Sample processing for urine was performed by solid phase extraction of a 20-µL volume at alkaline pH using an Oasis® MAX solid phase extraction plate (Waters Corp., Milford, Massachusetts, United States). High-performance liquid chromatography (HPLC) was performed

using a Restek™ Ultra PFP propyl column (100 × 3.0 mm, 5 µm) (Bellefonte, Pennsylvania, United States) and analyzed using a Sciex API 4000 mass spectrometer with Turbolonspray ionization source in positive ion mode and Analyst™ control software version 1.7.1. Mass spectrometric detection was done using MRM with transitions (m/z) 316→169 for trofinetide and 322→169 for the internal standard [$^{13}C_5$,^{15}N]-trofinetide. The assay range was 0.0500–50.0 mg/mL for trofinetide.

The precision (coefficient of variation [%CV]) and accuracy (relative error [RE%]/mean % difference [Bias%]) of both of the HPLC methods were acceptable for trofinetide (≤ 15% [≤ 20% at the lower limit of quantification]). Mean recoveries of trofinetide and its $^{13}C_5$,^{15}N-labeled internal standard were 61.8% and 64.0%, respectively, from human whole blood and were 71.4% and 68.1%, respectively, from human urine. Validation and acceptance criteria were based on the FDA's Guidance for Industry: Bioanalytical Method Validation [1].

Multiple reaction monitoring conditions

Matrix	Declustering potential	Collision energy	Cell exit potential
Whole Blood	41.00	19.00	4.00
Urine	56.00	19.00	10.00

Reference

1. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for industry: bioanalytical method validation. May 2001. https://www.moh.gov.bw/Publications/drug_regulation/Bioanalytical%20Method%20Validation%20FDA%202001.pdf. Accessed October 2021.