Utilization of AMS and HRMS for Metabolite Profiling and Identification of [^{14}C]Revexepride (SSP-002358) in a Human Microtracer Phase I Study

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Introduction

Revexepride (a 5-HT4 receptor agonist) is among the first of a new class of selective prokinetic agents shown to accelerate gastric emptying and stimulate gastrointestinal motility. Although preliminary results of animal/preclinical absorption, metabolism and excretion (AME) data are available, the elimination pathways of revexepride in humans are largely unknown. Thus, one of the main purposes of this open-label, non-randomized Phase I study was to explore the AME profile of [^{14}C]revexepride in healthy male study participants.

Based on the quantitative whole-body autoradiography (QWBA) data after oral administration of [^{14}C]revexepride to rats, the estimated radiation to human tissues after oral administration of 100µCi radioactivity of [^{14}C]revexepride would be approximately 1.8 x 10^{-9} Sv/Bq (with 80% of this going to the eye), which would exceed the maximum exposure limits allowed by the FDA (21 CFR 361.1) by about 7 times. As the radiochemical analysis using the traditional measurement method of liquid scintillation counting would be compromised at doses lower than 100µCi, an alternative, more sensitive method needed to be employed in this study, i.e., accelerator mass spectrometry (AMS).

The sensitivity of AMS permits the use of a much lower level of radioactivity such that the dose of radioactivity given in this study was approximately 200nCi. In this study, AMS was used for radioanalysis and metabolite profiling to obtain detailed quantitative information on the circulating metabolites, the extent of metabolism, and routes and rates of excretion for revexepride and its metabolites, while high-resolution accurate mass (HRAM) mass spectrometry was used for metabolite identification.

Methods

The clinical study (ClinicalTrials.gov identifier: NCT01786876) was conducted at Covance Clinical Research Unit, 3402 Kinsman Boulevard, Madison, WI according to the clinical protocol. Six study participants received a single oral dose of 200 nCi/2 mg (free base equivalents) of [^{14}C]revexepride. Feces samples were freeze-dried in a clean room free of radioactivity at the Covance DMPK laboratory, 3301 Kinsman Boulevard, Madison, WI. All collected samples were sent to Xceleron Inc. for analysis by liquid scintillation counting (LSC) and accelerator mass spectrometry (AMS) to determine the total ^{14}C-content, in plasma, whole blood, urine and freeze-dried feces. Based on these results, pooling schemes were devised and pooled plasma and feces samples underwent solvent extraction prior to HPLC analysis. Urine was injected directly onto the HPLC. The HPLC eluate was fractionated and the fractions were analyzed by AMS to explore the extent of metabolism of revexepride. In the meantime, aliquots of the pooled urine and processed plasma/feces samples were sent back to Covance DMPK for metabolite identification by an HPLC coupled to an HRAM mass spectrometer.

Chemicals and Materials

Revexepride and six metabolite standards were supplied by Cambridge Major Laboratories (Weert, Netherlands). Copper (II) oxide powder (ACS), Cobalt powder (100 Mesh, 99.9%), Zinc powder (100 Mesh, 99%), Titanium (II) Hydride (325 Mesh, 98%), HPLC-grade Acetonitrile (ACN) and Methanol (MeOH), Ammonium Acetate and Ammonium Hydroxide was from Sigma-Aldrich (St. Louis, MO).
HPLC- and AR- grades MeOH, Borosilicate glass tubes, Chromosorb, glass scintillation vials were purchased from VWR (Radnor, PA). Sodium Benzoate and synthetic graphite (325 Mesh, 99.9999%) was obtained form Alfa Aesar (Ward Hill, MA). Aluminum cathode was from National Electrostatics Corp (Middleton, WI). Oxalic Acid was acquired from NIST Office of Reference Materials (Gaithersburg, MD). Urea was from Elemental Microanalysis Limited (Devon, UK). Graphitization tubes, sample tubes and Combustion tubes were from Lifehouse Glass (Sunrise, FL). Tin capsules, Ultima Gold scintillant, Carbosorb and Permafluor scintillant was from Perkin-Elmer (Akron, OH).

Analytical Instrumentation

- Liquid Scintillation Counter
  - PerkinElmer Tri-Carb TR/SL 3180
- HPLC System
  Agilent 1200 modular system, equipped with:
  - Autosampler, Binary pump with high-pressure mixing, UV detector, and Fraction collector (4 x 96-well plate capacity)
  - Shimadzu Nexera HPLC system, equipped with: Autosampler, Binary pump with high pressure mixing
- Accelerator Mass Spectrometer
  NEC SSAMS-250 AMS system
- Mass Spectrometer
  Thermo Q Exactive

LC-MS Conditions

Column: Waters Xbridge C18, 4.6 x 250 mm, 5 µm
Flow Rate: 0.8 mL/min
Column Temperature: 25°C
MP A: 0.1M ammonium acetate pH 7.0
MP B: 1M ammonium acetate pH 7.0:MeOH:ACN (10:45:45, v:v:v)
Ramp from 0 to 20% B in1 min; ramp from 20 to 35% B in 9 min; ramp to 37% B in 6 min; ramp to 100% B in 14 min; hold 5 min, return to initial conditions in 0.1 min and re-equilibrate for 4.9 min.
Q Exative was operated in either positive or positive/negative switching electrospray ionization (ESI) mode at resolution 70,000 FWHM and a *m/z* range of 100 to 1,000. Product ion spectra were acquired at resolution 35,000 FWHM or 17,500 FWHM. Spray voltage was ±3 kV, capillary temperature was 300°C, sheath gas flow rate was 75, auxiliary gas flow rate was 15, sweep gas flow rate was 3, S-lens level was 50, heater temperature was 500°C.

Sample Pooling and Processing

For plasma, "AUC$_{0\text{-}24}$" pools for each study participants were prepared, based on the blood sampling schedule, according to the method described by Hamilton *et al.*[1] Individual study participant urine and feces pools were prepared by taking aliquots of study participants 1001-1006 samples up to 72 hours and 96 hours, respectively, based on mass balance data and the amount of sample collected. Each individual study participants pool was combined into a single pool of urine and feces, respectively. The plasma and feces samples were extracted with 3 volumes of ACN twice, and were reconstituted at an appropriate volume of water:ACN (9:1, v:v) solution upon dryness under nitrogen. Total of 6 plasma samples, 1 urine sample, and 1 feces sample were subjected to LC-AMS and LC-HRAM Met-ID analysis.

Results and Discussion

Mass Balance

Mean total ^{14}C concentrations in plasma ranged 0.222-10.4 ng free base equivalents/mL and were <LLOQ after 72 hours postdose. Similar concentrations were seen in whole blood and were <LLOQ after 48 hours postdose. Blood:Plasma concentration ratios were consistent up to 24 hours postdose, with mean ratios ranging 0.72-0.92. Across the six study participants, excretion in urine was slightly lower than in feces ranging 27.8-50.6% (mean 38.2%); with excretion in feces ranging 45.3-69.6% (mean 57.3%). Drug elimination occurred rapidly, with a mean >90% of the administered radioactivity recovered in the first 96 hours postdose. The overall recovery of radioactivity in excreta over 0–240 hours postdose ranged 87.1-99.8% (mean 95.5%) across the six study participants and thus could be regarded as complete. Table 1 summarizes the radioactivity recovery data for each individual study participant.

Table 1. ^{14}C Radioactivity Recovery of revexepride and Metabolites in Urine and Feces Following 200 nCi Dose

Matrix	Subject 1001	Subject 1002	Subject 1003	Subject 1004	Subject 1005	Subject 1006	Mean	SD
	% of dose excreted							
Urine	50.6	30.4	27.8	29.8	49.5	40.8	38.2	10.3
Feces	47.6	69.4	69.6	57.2	45.3	54.5	57.3	10.4
Total	98.3	99.8	97.3	87.1	94.8	95.4	95.5	4.49

HPLC Method Development

Revexepride and the five authentic standards were used for HPLC method development with UV detection by Xceleron. The goal was to achieve baseline separation of the six analytes. Figure 1A illustrates the UV chromatogram of the separation. The HPLC conditions were then transferred to the Covance DMPK lab. Figure 1B shows the reconstructed ion chromatogram of the six analytes by LC-MS. A linear regression curve was generated to illustrate the retention time correlation between these two systems. The correlation equation was determined to be y=1.009x – 1.113, where x and y represent retention time from Xceleron's and Covance's HPLC systems, respectively.

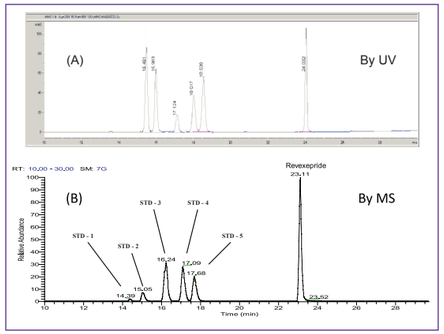

Figure 1. HPLC method development and retention time correlation.

LC-AMS Profiling and LC-HRAM Met-ID

Following oral administration of [^{14}C]revexepride (200 nCi / 2 mg free base equivalents) to humans, revexepride and up to 13 other radio peaks were detected and quantified by LC-AMS, 10 of which were identified by LC-HRAM and co-elution with reference standards where available. The proposed biotransformation pathway of [^{14}C]revexepride in humans is presented in Figure 2. In summary, unchanged revexepride (parent) was the largest circulating component in plasma, representing a mean of 37.8% of the total radioactivity AUC$_{0\text{-}24}$ and responsible for 16.0% of the administered dose in urine and 32.0% of the dose in feces. The quantitatively most important metabolites in plasma were hydroxy revexepride and revexepride -O-glucuronide, which together represented a mean of 26.6% of the total radioactivity AUC$_{0\text{-}24}$. N-desalkyl revexepride was another quantitatively important metabolite in plasma responsible for a mean of 10.5% of the total radioactivity AUC$_{0\text{-}24}$. Other metabolites detected and identified in plasma individually represented a mean of 0.2-6.6% of total radioactivity AUC$_{0\text{-}24}$. Metabolites detected and identified in excreta individually represented between 0.2 -2.9% and 0.4- 5.0% of the dose in urine and feces respectively and in total approximately 80% of the administered dose was identified in excreta.

Figure 3 (Top panel) shows the reconstructed ion chromatogram of Revexepride and its identified metabolites from the initial LC-HRAM-analysis derived from pooled human urine (0–72 h) obtained from study participants 1001-1006. However, U2 (retention time of 9.6 min) showing in the radiochromatogram (Figure 3, bottom panel) was not identified initially. This early eluting peak has been observed but not identified in the previous animal/preclinical AME studies. Based on the retention time, this was also a peak representing 6.6% radioactivity in AUC$_{0\text{-}24}$ plasma.

According to the retention time correlation equation (y=1.009x – 1.113), U2 was predicted to be eluted out at ~8.57 min on Covance's LC-MS system. In the attempt to identify this peak, multiple injections of urine sample were fraction collected at 10-second intervals. Fractions from 8 to 9 minutes were dried and reconstituted. Enzymatic hydrolysis by sulfatase was performed on these 6 fractions. Each fraction was then analyzed on Q Exactive with positive/negative switching and data-dependent MS^2 for the entire LC run. By doing so, we confirmed that this metabolite was not a sulfate conjugate of Revexepride. With further comparison with a human control urine sample and an authentic standard solution, this early eluting metabolite was identified as the hydrolysis product of revexepride, from 8.33 to 8.67 min urine sample fractions. Figure 4 shows extracted ion chromatograms at *m/z* of (A) 242.0578 Da and (B) 240.0433 Da with a ±3ppm extraction window from analysis of the 8.33 to 8.50 min fraction.

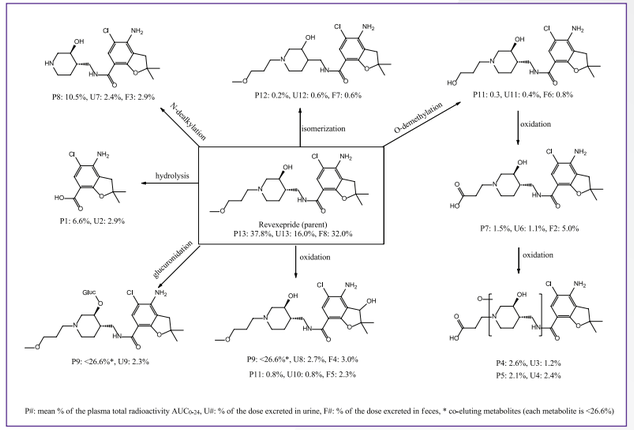

Figure 2. Overall quantitative metabolic pathway for [^{14}C]revexepride in humans.

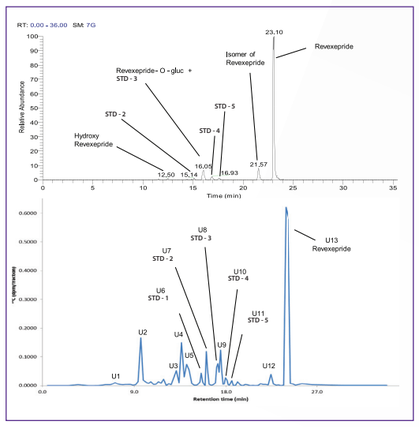

Figure 3. Reconstructed ion chromatogram (top panel) and radiochromatogram (bottom panel) derived from pooled human urine (0-72 h) obtained from study participants 1001-1006 administered 200 nCi/2 mg (free base equivalents) of [^{14}C] revexepride (HCl · H_2O salt).

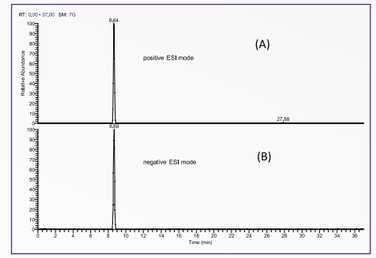

Figure 4. Extracted ion chromatograms from analysis of a fraction (8.33 to 8.50 min) from a pooled urine sample: (A) *m/z* 242.0578 Da, positive ESI mode; (B) *m/z* 240.0433 Da, negative ESI mode.

Conclusion

- The overall recovery of radioactivity was excellent, with nearly 100% of [^{14}C] recovered.
- AMS is a highly sensitive technique that enables the lowering of the [^{14}C] radioactive dose to levels considered insignificant in humans by regulators.
- Advanced LC-HRAM techniques allows successful identification of all major metabolites - despite their low concentrations.
- Modern LC-MS technique combined with AMS radioanalysis to identify and quantify drug metabolites allows AME data to be obtained far earlier in the development process.
- Good collaboration between AMS provider and Met-ID lab is essential for the success of the microtracer study.

References

1. Hamilton RA, Garnett WR, Kline BJ. Determination of mean valproic acid serum level by assay of a single pooled sample. Clinical Pharmacology and Therapeutics 29, 408–413 (1981).

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