Pharmacokinetic Analysis

Bexarotene Sampling in Plasma and Tumor Tissue Preparation

Venous Blood

Eight to 10 ml blood samples were collected in heparinized tubes at the time of tumor tissue collection from all patients and the time was recorded. Blood samples were centrifuged at 1500 g for 10 minutes and the plasma separated and stored at -70^{o}C until analysis.

Tumor Tissue Preparation

An approximate 50 mg piece of tumor tissue obtained at operation was cut, blotted dry, weighed and placed in 500 μL of 4g/L collagenase Type 1A suspension in Cellgro Dulbecco's Phosphate-buffered saline (DPBS 1X) with calcium and magnesium. The tissue was then ground for 30-60 seconds in a glass pestle and matching tube. The suspension was transferred to a polypropylene centrifuge tube and the glass tube and pestle were washed twice with 250 μL of the collagenase suspension and these washings were added to the tissue suspension (1 ml total volume). The suspension was then shaken at 200 rpm at 37^{o}C for 2 hrs. After digestion, internal standard solution (100 μL of 500 μg/ml LG 100268) was added and mixed. After this point, the tumor tissue sample was treated as if it were plasma, with the addition of five separate 250 μL aliquots of acetonitrile (see below).

Plasma Sample Preparation

To 1 ml of plasma or digested tissue slurry 100 µL of a 500 µg /ml solution (9:1 ethanol: DMSO) of LG 100268 (internal standard) was added. Five separate 250 µL aliquots of acetonitrile and 1000 µL 0.5N HCl were then added, and the solution well mixed. Then 5 ml of the extraction solvent, hexane:amyl alcohol (98:2, v/v), was added and gently mixed for 20 minutes using a nutator apparatus (48 inversions per minute). Centrifugation of this mixture at 3200 x g for 10 minutes yielded a two phase separation. To facilitate removal of the upper organic layer, the aqueous layer was frozen using a dry ice/alcohol bath. The organic layer was then evaporated to dryness under a stream of nitrogen. The residue was resuspended in 400 µL of acetonitrile using a vortex genie (30sec) followed by 2 minutes sonication in an E/MC Ultrasonic cleaner, model 250. Then 100 µL of 10 mM ammonium acetate was added and the mixture centrifuged at 13,000x g for 5 minutes in a Fisher MicroCentrifuge, model 235B. A 200 µL aliquot of this sample was transferred to sample vials in the HPLC autosampler. Samples for plasma and tissue standard curves and QCs were derived from expired blood bank plasma and stored normal human renal tissue.

HPLC System and Analysis for Bexarotene Concentrations

A modified HPLC method was used to determine bexarotene concentrations in plasma and tumor tissue. In the modified assay, an analog of bexarotene, LG 100268 (provided by Ligand Pharmaceuticals) was used as the internal standard. The HPLC System consisted of a Varian Prostar 410 autosampler which injected 50 µL of the extracted sample onto a Phenomenex reverse phase Prodigy C-8, 5 µm , 150 x 4.6 mm column. A

pre-column, C-8 Brownlee cartridge (30 x 4.6 mm) was used to remove non-eluting plasma or tissue components. The column was conditioned at 35^{o}C using an Eppendorf CH-30/TC-50 column heater retrofitted to accommodate a 150 mm length column. A Varian Prostar 210 Delivery Module (pump) delivered the mobile phase, which consisted of 10mM ammonium acetate:acetonitrile:acetic acid (1:4:0.04, v/v/v), at 1ml/min at 23^{0}C. Detection was via a Rainin Dynamax Fluorescence Detector FL-1, set at 260nm excitation and 430nm emission wavelengths. Using this system the retention time for LG 100268 was 8.0 minutes and bexarotene 9.7 minutes with good baseline separation. The HPLC operation and data collection were performed using a Star LC Workstation running version 5.5 software installed on a Dell Optiplex G110.

Assay Validation: Plasma and Tumor Tissue Bexarotene

Plasma

The assay recovery of bexarotene from was 88%. The assay was linear over the range of the standard curve of 3-1200 ng/ml. Accuracy was between –9.8% and 3.8% bias over this concentration range. The r^2 for these data was always =/> 0.9999. Intra-day precision at six validation concentrations (3, 10, 60, 120, 400, and 1200 ng/ml) had a coefficient of variation (CV) of between 0.5%-3.9%; the inter-day precision at the same validation concentrations had a CV of between 0.5%-12.7%. The lower limit of quantitation (LLOQ) of the assay was 3ng/ml.

Tumor Tissue

The assay recovery of bexarotene from tissue was 95%. The assay was linear over the range of the standard curve of 3-1200 ng/ml. Accuracy over this concentration range was between –2.0% and 9.1%. The r^2 for these data was always =/> 0.998. Intra-day precision at six validation concentrations (3, 10, 60, 120, 400, and 1200 ng/ml) had a CV of 0.2%-10.3%; the inter-day precision at the same validation concentrations had a CV of 14.3 % or less except for the 3 ng/ml which had a CV of 20.2%. The LLOQ of the assay was 3ng/ml.