Piperacillin-tazobactam Chromatographic Analysis Detailed Methods

Collection

Blood is collected in PST Lithium Heparin Blood Collection tubes (#367963 - BD, Franklin Lakes, NJ) and dialysate is collected in blood collection tubes with no additives (#366431 - BD, Franklin Lakes, NJ). Samples are centrifuged, (10 min, 4ºC, 3,000 rcf), resultant plasma and dialysate are aliquoted into cryovials (1400 uL - 430488 – Corning, Corning, NY), and stored in liquid nitrogen within 1 hr of collection. Samples that are from centers outside the Cleveland clinic are shipped overnight on dry ice.

Calibration Standards

Five level standards are prepared (by mass) each day HPLC analysis is run. Each standard contains Piperacillin sodium salt (Sigma, St. Louis – P8396) and Tazobactam (LKT Laboratories, Inc., Montreal, Quebec – T0298). Calibration levels are prepared by diluting a fresh 1 mg/mL stock of each drug with PBS elution buffer at 0.5, 2, 25, 100, and 200 ug/mL for Piperacillin and 0.5, 2, 6, 25, 50 ug/mL for Tazobactam. Stocks were diluted on a balance to have precision to 0.01ug/mL when constructing a calibration curve. Penicillin-G (BP914 – Fisher, Fair Lawn, NJ) is prepared at 1 mg/mL and used as an internal standard (IS). One vial containing IS is placed in the autosampler. The autosampler is programmed to wash the needle, draw 1uL of IS from reservoir vial, then draw 9 uL from the sample or level standard.

Dialysate Preparation

Dialysate samples are thawed and pipetted directly into 96-well autosampler plate (#3363 - Costar, Corning, NY) and individually covered with a piercable lid (MP53001 - MicronicUSA, Aston, PA).

Free Drug Preparation

Plasma samples are thawed and aliquoted (300 uL) into centrifugal filter units (Centrifree YM-30 - Millipore, Ireland) and centrifuged with a fixed angle rotor (10 min., 4ºC, 2,000 rcf). All the filtrate is transferred to the 96-well autosampler plate.

Total Drug Preparation

HPLC grade acetonitrile (A998SK-4 – Fisher, Fair Lawn, NJ) is mixed 10:1 (v/v) with internal standard. This mixture is pipetted into micro-centrifuge tubes (200 uL). Thawed plasma samples are added drop-wise (163.6 uL) yielding a 1:1 mixture of acetonitrile to aqueous phase (IS+Sample). Each tube is vortex mixed (~5 seconds), allowed to stand (~1 min.), then centrifuged (10 min., 10,000 rcf). The resulting supernatant is pipetted directly into a 96-well autosampler plate and immediately capped with a piercable lid. Once in the temperature controlled autosampler, each sample is diluted prior to injection to mitigate poor peak shape from sample versus mobile phase differences. The autosampler needle is used to draw and eject sample (20 uL) into a remote 'mixing well' (14230238 – Fisherbrand, Pittsburgh, PA). Then an aliquot of mobile phase 'A' (40 uL - from a vial inside the autosampler) is added to the sample in the remote mixing well. In between each step the autosampler needle is washed to eliminate carry-over or contamination. The autosampler does a series of fast draws and ejects to mix sample and diluent, then injects 10 uL onto the column for analysis.

These added dilution steps increase the time to make an injection, but allow analysis without the need for solid phase extraction or liquid-liquid extraction. Level standards are processed identically to total drug patient samples yielding a unique calibration curve for total drug and eliminating the need for multipliers and/or dilution factors.

Chromatographic Conditions

The chromatographic system consists of a degasser (G13122A), binary pump (G1312B), a chilled autosampler (G1367D and G1330B), column heater (G1316A), and diode array detector (G1315C) all from Agilent (Santa Clara, CA). Separation is achieved on a Kinetex 2.6µ XB-C18 100Å, 100 x 3.00 mm column with a KrudKatcher inline filter (00D-4496-Y0 and AF0-8497 – Phenomenex, Torrance, CA) both kept in the column oven at 30ºC. The autosampler is kept at 4ºC. Mobile phase A: phosphate buffered saline (PBS - P4417 – Sigma, St. Louis, MO) with 5% HPLC grade methanol (A452-4 – Fisher, Fair Lawn, NJ). Mobile phase B: HPLC grade acetonitrile with 0.1% trifluoroacetic acid (HB9813-4 – Fisher, Fair Lawn, NJ). Gradient elution is performed at a flowrate of 0.8 mL/min run from 0% B to 30% B over 5 minutes. The mobile phase is returned to 0% B over 0.1 minutes and allowed to equilibrate for 1.9 minutes for a total run time of 7 min. Antibiotics and internal standards (IS) are quantified by UV absorbance at 214 nm.

Assay Characteristics

The chromatographic assay has a lower limit of quantitation for piperacillin or tazobactam of 1 µg/ml for the total drug assay and 0.5 µg/ml for free drug and dialysate. The within-day and between-day coefficients of variation for piperacillin at a concentration of 100 µg/ml were 0.07% and 2.9%, respectively. The within-day and between-day coefficients of variation for tazobactam at 25 µg/ml were 1.6% and 2.4%.