Supplementary Data

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The chromatograph consisted of a Waters 510 pump (Waters Corp., Milford, MA), a Lo-Pulse pulse dampener (Scientific Systems, Inc., State College, PA), a Rheodyne 7126 injector (Idex Health and Science, Rhonert Park, CA), a Cortecs C18 column (3 mm × 100 mm, 2.7 μm superficially porous particle) protected by a Cortects C18 guard column (Waters Corp.), a Thermasphere column oven (Phenomenex, Torrance, CA), and a 40D ultraviolet absorbance detector with a 10 μL analytical flow cell (Knauer, Berlin, Germany). Data were recorded using Clarity Chromatography Data Station software (DataApex, Prague, Czech Republic).

The mobile phase was 12:88 acetonitrile:aqueous triethylamine (TEA) adjusted to pH = 2.0 with phosphoric acid. The total concentration of TEA was 0.5%, and the pH of the aqueous portion was adjusted before addition of acetonitrile. The mobile phase was degassed offline before use. The flow rate was 450 μL/min, column temperature was maintained at 35°C, and full loop injection was effected with a 200 μL sample loop. Column effluent was monitored at 284 nm absorbance.

Calibrators were prepared daily using class A volumetric flasks (Pyrex, Corning, NY) and pipettes (Gilson, Inc., Middleton, WI). Stock standards of timolol and metoprolol were prepared from powder in class A volumetric flasks, divided into aliquots, and stored at –80°C until use. All calibrators and stock standards were prepared in ultrapure water from a Barnstead GenPure water system (Thermo Fisher Scientific, Waltham, MA).

SOLID PHASE EXTRACTION

Solid phase extraction (SPE) was performed using Supelclean LC-WCX cartridges with 3 mL tube volume and containing 500 mg sorbent. The cartridges were placed onto a vacuum manifold (Agilent Technologies, Santa Clara, CA) and conditioned first with 10 mL acetone, followed by 6 mL ultrapure water. One thousand five hundred microliters plasma (either study sample, drug-free plasma, or quality control [QC] sample) was transferred into a polypropylene test tube and spiked with 50 μL of internal standard solution (10 μg/mL metoprolol in water). After mixing, this was loaded onto the cation-exchange cartridges. Interferences were removed by rinsing with 2 mL water, followed by 2 mL 50:50 acetone:water, and then with 4 mL neat acetone. Elution was achieved using 10% (v/v) formic acid in acetone. First, 800 μL of this formic acid solution was passed through the cartridges and allowed to flow to waste. Next, 1,400 μL was passed through the cartridges and collected. The SPE eluent was then evaporated to dryness under a stream of nitrogen gas at 50°C and the residue redissolved in 500 μL ultrapure water. This solution was injected into the high-performance liquid chromatography for analysis. Solvent was allowed to flow through the SPE cartridges by gravity with exception of the first step, where positive pressure was used to push acetone through the cartridges.

BASELINE SUBTRACTION AND CONTROL SAMPLES

Since this project required quantification of trace amounts of timolol in a complex biological sample matrix, we found that analysis of drug-free plasma resulted in a nonzero assay result, which was consistent across donors. The analytical baseline was taken as the mean assay result from triplicate analyses of drug-free plasma, which were extracted side by side with each set of 10 study samples. The limit of detection was calculated as three standard deviations of the assay results from the drug-free plasma and was, on average, 50 pg timolol free-base per mL plasma. The limit of quantification was 165 pg timolol free-base per mL plasma, corresponding to a signal-to-noise ratio of 2.5 after purification and enrichment of the sample. Positive results that fell below the limit of quantitation were estimated for the purpose of calculation of the group means, and are indicated as such.

Two QC samples were extracted side by side with each set of 10 patient samples and 3 drug-free plasma samples. The QC samples consisted of drug-free plasma spiked with timolol to a known concentration and were used to verify that method percent accuracy was within 95% to 105% during each day of analysis. An intermediate calibrator was reinjected after every eight injections of plasma extract and at the end of each sequence to ensure that instrument calibration did not drift over the course of the day.