β-Blocker Dialyzability in Maintenance Hemodialysis Patients
A Randomized Clinical Trial

Alvin Tieu, Thomas J. Velenosi, Andrew S. Kucey, Matthew A. Weir, and Bradley L. Urquhart

Abstract
Background and objectives There is a paucity of data available to describe drug dialyzability. Of the available information, most was obtained before implementation of modern hemodialysis membranes. Our study characterized dialyzability of the most commonly prescribed β-blockers in patients undergoing high-flux hemodialysis.

Design, setting, participants, & measurements Patients on hemodialysis (n=8) were recruited to an open label, pharmacokinetic, four-way crossover trial. Single doses of atenolol, metoprolol, bisoprolol, and carvedilol were administered on separate days in random order to each patient. Plasma and dialysate drug concentrations were measured, and dialyzability was determined by the recovery clearance and arterial venous difference methods.

Results Using the recovery clearance method, the dialytic clearance values for atenolol, metoprolol, bisoprolol, and carvedilol were 72, 87, 44, and 0.2 ml/min, respectively (P<0.001). Applying the arterial venous difference method, the dialytic clearance values of atenolol, metoprolol, bisoprolol, and carvedilol were 167, 114, 96, and 24 ml/min, respectively (P<0.001).

Conclusions Atenolol and metoprolol are extensively cleared by hemodialysis compared with the negligible dialytic clearance of carvedilol. Contrary to estimates of dialyzability on the basis of previous literature, our data indicate that bisoprolol is also dialyzable. This finding highlights the importance of conducting dialyzability studies to definitively characterize drug dialytic clearance.


Introduction
The importance of cardiovascular disease among patients with CKD cannot be overstated. It accounts for nearly 45% of deaths among patients on hemodialysis, an incidence 10–20 times greater than in the general population (1–3). Over the past four decades, β-adrenergic receptor antagonists (β-blockers) have emerged as a fundamental component of treating cardiovascular disease. β-Blockers decrease BP, heart rate, myocardial oxygen demand, arrhythmia, and oxidative stress, and they improve left ventricular function (4). More importantly, their ability to reduce mortality has been shown in numerous randomized clinical trials (5–8). Although β-blockers are widely prescribed in patients on hemodialysis, this is on the basis of their proven efficacy in patients with normal kidney function. Extrapolating findings from the general population to patients with CKD has a number of caveats. The pharmacokinetics of many drugs are altered in CKD, including both kidney- and nonkidney-mediated elimination (9–11). Expectations that β-blockers will deliver similar therapeutic efficacy in patients on hemodialysis compared with the general population are based on very little evidence.

For drugs to be approved for use in patients, detailed pharmacokinetic analysis must be undertaken to characterize parameters, such as bioavailability, metabolic elimination, and kidney-mediated drug clearance. The majority of drugs are not tested in patients on dialysis during the drug development process, which results in a lack of appropriate dosage recommendations (12–15). Drug disposition is an especially important consideration in patients on hemodialysis because there is minimal to no residual kidney function for drug excretion. Despite requirements to characterize kidney-mediated elimination during drug development, there are very minimal data on drug clearance in patients on hemodialysis. Specifically, drug dialyzability—the efficiency of drug removal by dialysis—is likely to vary among β-blockers, which should be considered when they are prescribed to patients. The use of drugs that are highly dialyzed can result in subtherapeutic plasma concentrations during and after hemodialysis and increase the risk for adverse clinical outcomes (16). Currently, definitive data on drug dialyzability are only available for 10% of medications.

The dialyzability of β-blockers is presumed largely on the basis of physicochemical characteristics and not experimental evidence (17). Of the available information, most data were obtained before implementation of modern high-flux dialysis membranes, rendering many of the older studies irrelevant (18). Therefore, this study was conducted to define the modern dialyzability of the most commonly used β-blockers.
We hypothesized, on the basis of physicochemical characteristics and previous literature (Table 1), that atenolol and metoprolol would be highly dialyzed, whereas bisoprolol and carvedilol would be minimally removed during hemodialysis (19–22).

Materials and Methods

Study Design and Participant Eligibility

We conducted an open label, four-way crossover trial of the four most commonly prescribed β-blockers in Ontario, Canada—atenolol (50 mg), metoprolol (50 mg) bisoprolol (5 mg), and carvedilol (6.25 mg)—among eight patients receiving maintenance hemodialysis at the London Health Sciences Centre hemodialysis units. The sample size was determined by a power calculation using preliminary data and assuming an α of 0.05 and power of 0.8. Patients were at least 18 years of age and receiving thrice weekly hemodialysis for at least 90 days before the study. We excluded patients with significant gastrointestinal/liver disease, patients with body mass index >40 kg/m², and those who could not safely receive a study β-blocker (treatment with contraindicated medications, prior adverse drug reactions to β-blockers, severe reactive airway disease, or hemodynamic instability during dialysis). Patients who met the eligibility criteria were randomized to one of the following treatment sequences: (1) atenolol, metoprolol, and carvedilol followed by bisoprolol, (2) bisoprolol, metoprolol, and carvedilol followed by atenolol, (3) carvedilol, metoprolol, and bisoprolol followed by atenolol, or (4) atenolol, carvedilol, and bisoprolol followed by metoprolol. A research assistant generated the randomization sequence, and the patient’s nephrologist or nurse provided the randomization code. The randomization sequence was concealed from the research assistant, the investigator, and the patients, and the randomization process was repeated with each of the three remaining β-blockers.

Clinical Data Collection, Interventions, and Follow-Up

We randomly administered a single oral dose of one of the study β-blockers 4 hours before hemodialysis initiation. To achieve randomization, each β-blocker was assigned a number between one and four, and a random number generator was used to determine treatment order. A research assistant generated the randomization sequence, and the patient’s nephrologist or nurse provided the β-blocker on the study day. Because this was an open label study, there was no concealment of treatment. This process was repeated with each of the three remaining β-blockers during separate dialysis sessions, which were a minimum of 48 hours apart. During each hemodialysis treatment, blood samples were collected from the arterial and venous ports at six time points. During 4-hour dialysis treatments, we collected blood at 0, 0.5, 1, 2, 3, and 4 hours. During 3.5-hour dialysis treatments, we collected blood at hours 0, 0.5, 1, 1.5, 2.5, and 3.5. For 3-hour dialysis treatments, we collected blood at hours 0, 0.5, 1.5, 2, 2.5, and 3. Arterial blood samples were used to determine hematocrit. For all treatments, we also collected the total spent dialysate into a 200-L food-grade plastic barrel. We collected demographic information, health status, and dialysis prescription details at the time of study enrolment. The study period was from February 2015 to March 2016.

β-Blocker Extraction Analyses with Mass Spectrometry

β-Blockers were extracted from plasma and dialysate samples using solid-phase extraction cartridges (Phenomenex, Torrance, CA) conditioned according to the manufacturer’s instructions. β-Blocker concentrations in plasma (free plus protein bound) and dialysate were determined by ultraperformance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (Waters, Milford, MA). Methodologic details can be found in Supplemental Material.

Determining Dialytic Clearance

The two main methods to evaluate the clearance of medications during hemodialysis are (1) the arterial venous (A-V) difference method and (2) the recovery clearance method, and they are described by the following equations (18,23–25):

\[ CL_{A-V} = Q_P \left( \frac{A_{conc} - V_{conc}}{A_{conc}} \right) + Q_{UF} \left( \frac{V_{conc}}{A_{conc}} \right), \]

\[ Q_P = Q_{df} (1 - Hct), \]

where \( CL_{A-V} \) is A-V difference clearance, \( Q_P \) is mean plasma flow rate, \( Q_{df} \) is mean dialysis flow rate, \( Q_{UF} \) is mean ultrafiltration rate, \( Hct \) is hematocrit, \( A_{conc} \) is arterial plasma drug concentration, and \( V_{conc} \) is venous plasma drug concentration. Also,

\[ CLR = R_{drug}/AUC_{0-T}, \]

where \( CLR \) is dialyzer clearance, \( R_{drug} \) is total amount of drug recovered in dialysate, and \( AUC_{0-T} \) is the area under the plasma concentration-time curve during dialysis. The \( AUC_{0-T} \) was calculated by the trapezoidal method using GraphPad Prism (version 6.01 for Windows; GraphPad Software, San Diego, CA).

Statistical Analyses

We performed all statistical analyses using GraphPad Prism (version 6.01 for Windows; GraphPad Software). We conducted hypothesis testing of atenolol, metoprolol, bisoprolol, and carvedilol clearance using one-way ANOVA. After rejection of the null hypothesis, multiple comparisons were evaluated by Tukey post hoc test. Results are presented as mean ± SD. We considered a \( P \) value <0.05 statistically significant.

Results

Assay Validation and Performance

The recovery percentages of atenolol, metoprolol, bisoprolol, and carvedilol using solid-phase extraction were 101%, 112%, 93%, and 112%, respectively. The intraday accuracy and precision were assessed using five analytical replicates of the lowest concentration on the dialysate calibration curve. Accuracy, expressed as a bias percentage, was determined by comparing the mean measured concentration with the nominal concentration. Precision was determined by calculating the coefficient of variation percentage of the five replicates. Using a signal-to-noise ratio of at least 10:1, the lower limit of quantification of the calibration curve displayed acceptable accuracy (<10%) and precision (<10%) for all β-blockers, except for the precision of carvedilol, which was slightly above this limit. Specifically, the biases and coefficients of variation were 4.2% and 1.1% for atenolol, 1.9% and 1.5% for metoprolol, 2.0% and 2.7% for bisoprolol, and −6.0% and 12.5% for carvedilol, respectively.

Clinical Characteristics of Subjects

All eight subjects completed the pharmacokinetic four-way crossover trial, and baseline clinical information is
presented in Table 2. The mean age was 58 years old (ranging from 28 to 80 years old), mean height was 1.7 m, and mean weight was 95 kg. The mean body mass index was 32.6 kg/m². There were four different dialyzers used in this study: FX600, FX800, FX1000, and Revaclear Max 400. The higher-value FX hemodialysis filters have minor increases in their $K_t$ and clearance of metabolites (e.g., urea and creatinine) (26). The Revaclear Max 400 dialyzer has a similar $K_t$ but an elevated clearance of metabolites compared with the FX1000 (27).

Safety

$\beta$-Blocker treatments were well tolerated by all study participants, and no serious adverse drug reactions occurred. No abnormalities in heart rate and BP were observed during the study.

Dialyzability of $\beta$-Blockers in Patients on Maintenance Hemodialysis

Recovery Method. Collection and analysis of plasma samples over the duration of the hemodialysis session allowed us to create plasma concentration-time profiles (Figure 1) and determine $\beta$-blocker exposure for each subject (reported as AUC$_{0\rightarrow T}$). For atenolol, metoprolol, bisoprolol, and carvedilol, patient drug exposures were 827.9, 106.9, 180.7, and 150.5 ng*h/mL. The amounts of $\beta$-blocker recovered in total spent dialysate were 3.7, 0.6, 0.5, and 0.0 mg, respectively. Drug exposure values and the total amounts of drug recovered in dialysate were applied to the recovery clearance method (Equation 2) to calculate dialytic clearance. The null hypothesis that there is no difference in clearance between any of the $\beta$-blockers was rejected ($P<0.001$). Atenolol was readily dialyzable with a dialytic clearance of 72±21 ml/min, which was similar to metoprolol’s clearance of 87±28 ml/min (Figure 2). The clearances of atenolol and metoprolol were considerably higher than that bisoprolol at 44±9 ml/min ($P=0.02$ compared with atenolol) and $P<0.001$ compared with metoprolol). Carvedilol had a substantially lower clearance (0.2±0.6 ml/min; $P<0.001$) than all of the other study $\beta$-blockers.

A-V Difference Method. The blood flow and predialysis hematocrit for each subject are shown in Table 2. These two variables along with the difference in $\beta$-blocker concentrations between the arterial and venous ports were used to calculate dialyzability values by applying the A-V difference equation (Equation 1). The null hypothesis that there is no difference in clearance between any of the $\beta$ blockers
Table 2. Clinical characteristics of participants in a crossover trial assessing the dialyzability of four β-blockers

<table>
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<th>Subject Identification</th>
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<td>1.7</td>
<td>1.8</td>
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<td>35.6</td>
<td>19.2</td>
<td>37.7</td>
<td>38.7</td>
<td>33.8</td>
<td>30.3</td>
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<td>260</td>
<td>Anuric</td>
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<td>300</td>
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<td>DM and HTN</td>
<td>PCKD</td>
<td>Renux nephropath</td>
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<td>FX600</td>
<td>FX600</td>
<td>FX1000</td>
<td>FX800</td>
<td>FX600</td>
<td>Revaclear Max 400 313</td>
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<td>Mean blood flow rate, ml/min</td>
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<td>323</td>
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<td>1.4±0.11</td>
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<td>Central catheter</td>
<td>Fistula</td>
<td>Fistula</td>
<td>Central catheter</td>
<td>Central catheter</td>
<td>Fistula</td>
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<td>0.27±0.01</td>
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<td>0.33±0.01</td>
<td>0.29±0.01</td>
<td>0.27±0.01</td>
<td>0.28±0.01</td>
<td>0.29±0.02</td>
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<tr>
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<td>0.36±0.01</td>
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<td>0.30±0.01</td>
<td>0.32±0.03</td>
<td>0.37±0.01</td>
</tr>
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RPGN, rapidly progressive GN; DM, diabetes mellitus; HTN, hypertension; PCKD, polycystic kidney disease.
*Mean over the four dialysis sessions ± SD.
was rejected \((P<0.001)\). We found atenolol to be the most highly dialyzed \(\beta\)-blocker, with a mean±SD dialytic clearance of 167±23 ml/min. The dialytic clearances for metoprolol and bisoprolol were 114±19 and 96±17 ml/min, respectively (Figure 3), which were both significantly lower than atenolol \((P<0.001)\). The dialytic clearances of atenolol, metoprolol, and bisoprolol were all significantly higher than carvedilol’s clearance of 24±18 ml/min \((P<0.001)\).

**Discussion**

We determined the dialyzability of four commonly prescribed \(\beta\)-blockers in patients receiving maintenance hemodialysis. We found atenolol and metoprolol to be highly dialyzable \((72±21 \text{ and } 87±28 \text{ ml/min})\) compared with bisoprolol \((44±9 \text{ ml/min})\). All three of these \(\beta\)-blockers displayed dialytic clearance values markedly higher than that of carvedilol, which was found to have nearly negligible dialyzability \((0.2±0.6 \text{ ml/min})\). With only 10% of currently marketed medications having definitive dialyzability information on the basis of experimental data \((17)\), this is an important step in establishing how hemodialysis affects drug disposition.

Despite kidney excretion accounting for only 5% of metoprolol clearance \((28)\), both atenolol and metoprolol have physicochemical properties that enable them to be extensively dialyzed \((28–30)\). To our knowledge, no previous studies have determined the dialyzability of metoprolol. Metoprolol’s dialytic clearance \((87 \text{ ml/min})\) is only 9% of its reported total clearance in patients with ESKD \((31)\). Although this may not warrant a supplemental dose postdialysis, dialytic clearance shows an additional route of elimination for metoprolol that is not observed in healthy patients or patients with CKD. As for atenolol, the A-V difference method applied by Flouvat \textit{et al.} \((32)\) produced a clearance of 43 ml/min for patients with ESKD on a coil kidney dialysis with cuprophane membrane. Using the same equation, high-flux polysulfone dialyzers in our study revealed a substantially higher dialytic clearance of 167 ml/min. When the recovery clearance equation was applied, the dialyzability of atenolol at 72

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**Figure 1.** \(\beta\)-Blocker plasma concentration-time profiles of patients with ESKD during hemodialysis. Plasma concentration-time profiles of atenolol (A), metoprolol (B), bisoprolol (C), and carvedilol (D) during hemodialysis in patients with ESKD. Each subject received a single oral dose of a \(\beta\)-blocker 4 hours before dialysis onset. Plasma concentrations of \(\beta\)-blockers were determined using ultraperformance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. Dashed curves represent individual pharmacokinetic profiles, and mean pharmacokinetic curves are displayed in color. Results are presented as mean±SD, with \(n=8\) for all treatment groups.
ml/min was still higher than the value determined by Flouvat et al. (32). Furthermore, atenolol’s dialytic clearance (72 ml/min) is five- to nine-fold higher than its total clearance (8–14 ml/min) in patients with ESKD (33,34). Carvedilol’s larger volume of distribution, decreased water solubility, and extensive protein binding (98%) suggested minimal or low dialytic clearance (35,36). Similar to earlier findings by Miki et al. (37), we found that carvedilol displayed very minimal dialytic elimination. A low dialyzability was similarly expected for bisoprolol after consulting the dialysis of drugs guideline and peer-reviewed articles (Table 1) (19–22). Our study indicates that bisoprolol is cleared during hemodialysis, regardless of the calculation method used. Kanegae et al. (38) found a comparable dialytic clearance of 51 ml/min using the A-V method for patients who were also prescribed polysulfone-based dialyzer membranes. This finding is contrary to previous estimates of dialyzability for bisoprolol, but it is reflective of its intermediate physicochemical properties compared with carvedilol and atenolol (39,40). Although its total clearance ranges from 70 to 84 ml/min in patients with ESKD not on dialysis (38,41), the dialytic clearance of bisoprolol (44 ml/min) is still over 1.5-fold higher than its kidney-mediated excretion (28 ml/min) in these patients.

Only atenolol and metoprolol were expected to be dialyzable; however, the results of our study allow us to definitively state that bisoprolol is cleared during hemodialysis. If dosing is not carefully monitored, patients taking dialyzable drugs may have inadequate β-adrenergic receptor blockade, resulting in suboptimal treatment. Although the amounts of drugs that we recovered in dialysate may seem unsubstantial relative to their initial dose, the bioavailability of these β-blockers is an important consideration to determine the significance of hemodialysis on drug clearance. For example, approximately 50% of atenolol is absorbed systemically, with values as low as 12%–30% in patients with ESKD (33,34). Hence, the 3.7 mg of atenolol recovered in dialysate (7.5% of the initial dose) can be a significant amount and warrant changes for how atenolol is prescribed to patients on dialysis. Accordingly, atenolol, metoprolol, and bisoprolol may require postdialysis dosing to ensure that adequate plasma concentrations are maintained. Conversely, carvedilol removal by dialysis is negligible. Because kidney-mediated excretion accounts for <2% of its elimination, plasma levels of carvedilol do not accumulate in kidney impairment (42). These findings suggest that no dosage adjustments are required for carvedilol in patients on hemodialysis (37,43). Although current clinical practice guidelines outlined by the National Kidney Foundation make no recommendations on which β-blocker should be prescribed to patients with CKD (44), selection of carvedilol over other β-blockers may be considered.

The strongest evidence in support of carvedilol as opposed to other β-blockers is from its proven efficacy in patients with ESKD. Carvedilol is the only β-blocker that has been tested in prospective, randomized clinical trials in patients on hemodialysis. Cice et al. (45) showed that year-long administration of carvedilol reduces left ventricular volumes and improves overall cardiac function for patients on hemodialysis with dilated cardiomyopathy. This cohort was followed for another 12 months and showed a significant reduction in all-cause mortality, cardiovascular mortality, and all-cause hospitalizations compared with
Although the dialyzers were all modern high-flux dialyzers. Patients enrolled in our study used different dialyzers. Drug that is dialyzable after a single dose is also expected to quantify dialytic clearance. Another limitation of our study design is that metoprolol and bisoprolol overdose. The last important consideration pertains to factors that can contribute to interindividual variability in clearance. Hepatic drug metabolizing enzymes involved in clearance of metoprolol, bisoprolol, and carvedilol have genetic polymorphisms. For example, individuals can be categorized from poor to ultrarapid metabolizers of metoprolol on the basis of their CYP2D6 genotype (47). Hence, poor metabolizers of metoprolol may have a larger component of their dose available for removal by hemodialysis.

Our study has some limitations that should be considered. The recovery clearance method is accepted as the gold standard for determining dialyzability. This method requires a sensitive assay to measure low drug concentrations in large volumes of dialysate. Despite concentrating samples 100-fold, some dialysate samples from patients taking carvedilol were below our limit of quantification. However, the negligible dialytic clearance of carvedilol likely resulted in virtually no drug for detection. The precision of our carvedilol assay was slightly above our acceptable range. At very low concentrations of carvedilol, this will introduce a small amount of variability in the calculation of clearance. In addition, it is unknown whether β-blockers bind to dialysis membranes or the dialysate collection barrel, which can result in an underestimated recovery clearance value. When calculating AUC of β-blockers, we determined total concentration (free plus protein bound). This may also result in an underestimated dialytic clearance. Another limitation of our study design is quantifying β-blockers after a single oral dose. Although a drug that is dialyzable after a single dose is also expected to be cleared when taken chronically, future studies should examine dialytic clearance for patients at steady state. Patients enrolled in our study used different dialyzers. Although the dialyzers were all modern high-flux dialyzers, the type of dialyzer may have introduced a small amount of variability in our clearance calculation. Lastly, our study did not investigate the interdialytic pharmacokinetics of the four β-blockers. Previous studies have shown that the t1/2 and kidney clearances of these drugs on nondialysis days return to values that are comparable with those of patients with ESKD not yet on RRT (32,37,48–50).

In conclusion, we have experimentally determined the dialytic clearance of the most commonly prescribed β-blockers. Our data show that atenolol and metoprolol are markedly dialyzed compared with carvedilol. Despite previous literature suggesting that bisoprolol would have little to no dialyzability, we show that it is, in fact, cleared during hemodialysis in patients using modern high-flux dialyzers. Definitive dialytic clearance data presented in this study can help design prospective trials to determine whether outcomes are superior with poorly dialyzable β-blockers compared with highly dialyzable β-blockers.

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Disclosures

None.

References


Beta-blocker dialyzability in chronic hemodialysis patients

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Supplemental Material

Beta-blocker Extraction and Liquid Chromatography

Blood samples were centrifuged at 2000g for 10 minutes within one hour of collection. Plasma was separated from blood cells and subsequently stored with dialysate samples at -80°C until analysis. We determined total plasma (free and protein-bound) and dialysate concentrations of atenolol, metoprolol, bisoprolol, and carvedilol using solid phase extraction (SPE) followed by ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QToFMS). Beta-blockers were extracted from plasma and dialysate samples with SPE cartridges (C18, Strata-X Polymeric Reversed Phase 33 µm) obtained from Phenomenex (Torrance, CA) conditioned according to manufacturer’s specifications. Deuterated beta-blockers (atenolol-d7, metoprolol-d7, bisoprolol-d7, and carvedilol-d3) at 50 ng/mL were used as internal standards for drug quantification. Plasma, dialysate, and internal standards were passed across the SPE cartridges and washed with 1 mL of nano-pure water followed by 1 mL of 20% methanol in water. Analytes were eluted into clean glass test tubes with 1 mL of methanol solution containing 0.1% trifluoroacetic acid. Eluents were dried in a 40°C water bath using an Organomation N-EVAP™ nitrogen evaporator (Berlin, MA) and then reconstituted in mobile phase. To ensure adequate compound detection, plasma extractions containing carvedilol were concentrated by a factor of 10, while plasma samples containing other beta-blockers did not require concentration. Dried eluents from dialysate extractions were concentrated by a factor of 100. For analyte separation,
reconstituted samples were injected at a volume of 5 µL with a flow rate of 0.7 mL/min on a Phenomenex Kinetex C8 column (1.7 µm particle size, 50 x 2.1 mm). The Waters ACQUITY UPLC™ I-Class system (Waters, Milford, MA) autosampler maintained the column temperature at 40°C. Water (A) and acetonitrile (B), both containing 0.1% formic acid, were used as the mobile phase solutions for compound elution. The UPLC elution parameters were as follows: 0.00–0.20 min, 2% B; 0.20–1.50 min, 2–80% B; 1.50–2.50 min, 80% B; and 2.51–3.51 min, 2% B.

**Beta-blocker Analysis with Mass Spectrometry**

We conducted mass spectrometry using a Waters Xevo™ G2S-QToFMS, and measured beta-blockers in positive electrospray ionization. The capillary and cone voltages were set at 0.5 kV and 40 V, respectively, and a source temperature of 150°C was maintained. The desolvation gas flow was 1200 L/h at a temperature of 650°C, and the cone gas flow was 50 L/h. We acquired data in centroid mode using an MS^E^ method. Samples were acquired in positive polarity with extended dynamic range and the analyzer mode set to resolution. Both functions 1 (low energy collision) and 2 (high energy collision) of the centroid method acquired data within a mass range of 50-1200 Da and a scan time of 0.05s. We set collision energy for function 1 of the MS^E^ method at 0V, while for function 2 it was ramped from 15–50 V. For function 3, lockspray was acquired to maintain accurate mass detection and reproducibility. The lockmass consisted of leucine-enkephalin (1ng/µL) set at a flow rate of 10 µL/min. Acquisition of data was controlled by Waters MassLynx v4.1 software and peak integration of sample chromatograms were conducted with QuanLynx software (Waters, MA, USA).