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Characterization of MB-102, a New Fluorescent Tracer Agent for Point-of-Care Renal Function Monitoring

Jeng-Jong Shieh [1,*], I. Rochelle Riley [1], Thomas E. Rogers [1], Lung-Fa Kao [2], Richard B. Dorshow [1]

[1] MediBeacon Inc., St. Louis, Missouri 63132
[2] Department of Chemistry, Washington University, St. Louis, Missouri 63130

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ABSTRACT

MB-102 is a fluorescent tracer agent designed for measurement of point-of-care glomerular filtration rate (GFR) and is currently in clinical studies. MB-102 possesses a strong UV absorbance at 266 nm and 435 nm, and broad fluorescent emission at ~560 nm when excited at ~440 nm. The MB-102 formulation is stable at 2°C-8°C for >3 years. The pK_a's of the 2 acid groups are 2.71 and 3.40. Both X-ray crystallography and HPLC confirmed the D, D chirality of MB-102 in solid, in solution, and in the drug formulation. Initial safety and toxicity was published previously [Bugaj and Dorshow, 2015], which enabled the commencement of clinical studies. *In vitro* studies showed that 4.1% of MB-102 is bound to human plasma proteins, compared to 6.0% for the accepted standard GFR agent iohexol. The blood-to-plasma ratio for MB-102 was 0.590, illustrating minimal distribution of MB-102 into red blood cells. The manufacture of MB-102 under good manufacturing practice yields the designed molecular structure at high purity (>95% wt/wt).

Introduction

Determination of glomerular filtration rate (GFR) is widely accepted as the most reliable measure of renal function,[1] but there is a growing medical need for an accurate point-of-care measurement of GFR to assess function due to either acute or chronic kidney injury. The current common clinical practice for assessing kidney function measures the serum creatinine level to obtain an estimated GFR (eGFR), calculated based on the equations of Ferguson and Waikar,[2] Inker et al.,[3] or Cockcrof-Gault.[4] However, serum creatinine is not a sensitive marker of renal function, has a time-delayed response to changes in renal function, and its value can depend on factors other than renal function such as age, hydration, muscle mass, and diet. The eGFR equations themselves are ensemble averages and do not necessarily reflect an individual patient. For these reasons, it is well established in the nephrology clinical community that eGFR based on serum creatinine and the estimation equations are a poor surrogate for true GFR.[5]

Exogenous GFR tracers such as iohexol (Omnipaque™) do provide a valid GFR assessment.[6] Iohexol has the necessary properties of negligible protein binding, negligible *in vivo* metabolism, distribution into the extracellular fluid compartment, and excretion intact by glomerular filtration without tubular renal secretion or reabsorption.[7,8] The measurement procedure for GFR is obtaining blood samples drawn from the subject at multiple time points (usually over 6 h or more) and then analyzing the plasma for iohexol content using various multistep analytical methods. GFR is then determined from the concentration versus time profile using standard pharmacokinetic modeling. However, most clinical facilities do not have the analytical capability to do the necessary laboratory work, and the multiple blood draws plus pharmacokinetic analysis render this methodology time-consuming and not point-of-care. Thus, the poor surrogate of eGFR continues to be the clinically amenable standard in GFR measurement.

To overcome the deficiencies of this exogenous GFR tracer method, significant effort has been directed at finding an exogenous fluorescent agent that can be detected transdermally,[9-12] eliminating the need for blood draws and complex laboratory analytical procedures. MB-102, a fluorescent tracer agent, was designed to be excreted *in vivo* through glomerular filtration, and with the necessary photophysical properties and other chemical and physical characteristics necessary for a robust exogenous GFR agent.[13,14]

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* *Correspondence to:* Jeng-Jong Shieh (Telephone: +1-314-735-0970).
E-mail address: jjshieh@medibeacon.com (J.-J. Shieh).

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MB-102 has been evaluated in over 20 formal toxicology, safety, and pharmacology studies necessary for advancement to human clinical trials. No toxicological effects nor safety concerns were reported.[15,16] Several clinical studies have been performed with intravenous administration of MB-102 and transdermal fluorescence detection of MB-102 from a light source—photodetector module placed directly on the skin. Results validate that this methodology is a clinically amenable point-of-care true measurement of GFR.[17,18]

Herein we report on the chemical and photophysical properties of MB-102 as determined in the GMP-manufactured active pharmaceutical ingredient (API) and the formulated product for intravenous administration. The biological properties of MB-102 in the physiological matrix, such as percentage binding to plasma proteins, and degree of partition into blood cells are also reported here.

The structure of MB-102 (3,6-diamino-2,5-bis{N-[(1R)-1-carboxy-2-hydroxyethyl]carbamoyl}pyrazine) is shown in Figure 1. MB-102 has a broad fluorescence spectrum, with a peak at ~560 nm when excited at ~440 nm.

Experimental

Chemicals

MB-102 was manufactured under good manufacturing practice (GMP) by the scheme shown in Figure 2. The key intermediate, MB-301, was coupled with 2 D-serine benzyl esters to form a benzyl protected reaction intermediate which was then converted to MB-102.[13]

MB-301 and 2 positional isomers, CMI-154-71 and CMI-143-179, were obtained from Chem-Master International, Hauppauge, NY.

MB-102 dosing solution: 18.6 mg/mL manufactured under GMP by AMRI, Glasgow, Scotland.

1× phosphate-buffered saline (PBS; without calcium or magnesium) was obtained from Mediatech Inc.

MeOH was obtained from Fisher Scientific (Fair Lawn, NJ; A456-4, Lot 156744).

Acetonitrile (ACN; Fisher, Optima® Grade), 0.1% trifluoroacetic acid (TFA) in water (Fisher, Optima® Grade), and 0.1% TFA in ACN (Fisher, Optima® Grade) were all obtained from Fisher Scientific.

Milli-Q DI water (18.2 MΩcm @ 25°C, 3 ppb TOC) was obtained using a Milli-Q purification system (Millipore Q-POD® Remote Dispense [Millipore SAS 67210 Molsheim]).

Quantitative analyses of MB-102 in plasma have been described in detail.[19] The HPLC conditions used in each of the characterization studies are described in the corresponding sections below.

Determination of UV/Vis and Fluorescence Properties

The experimental procedure was briefly described previously.[13] Details are as follows. MB-102 was dissolved in 1× PBS to form a 2 mM stock solution. The UV absorbance properties were determined on a 100 μM solution in PBS using a UV-3101PC UV-Vis-NIR scanning spectrophotometer system from Shimadzu. The fluorescence properties (λ_{ex}, λ_{em}, and intensity maximum at λ_{em}) were determined on a 10 μM solution in PBS using a Fluorolog-3 spectrofluorometer system from Jobin Yvon Horiba.

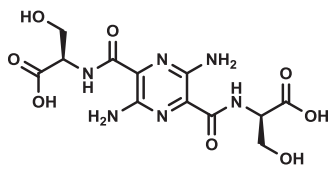

Figure 1. MB-102 showing 2 chiral centers, both in the D-form.

Determination of pK_a

A Sirius GLpK_a system (SN9902164) and Refinement Pro software (v.2.23.21) were used. The titration was made using 0.7 mg/mL MB-102 in an aqueous phase. The assay was run from high to low starting at a pH of 11.0 and ending at a pH of 1.8.

Determination of Chiral Purity

MB-102 was prepared by coupling D-serine benzyl ester with MB-301. The chiral purity of MB-102 API was analyzed using a RegisPack column (250 mm × 4.6 mm, 5-μm particle size, Cat. # 783104, Ser. # 89,928), monitored at 264 nm with a normal phase solvent of hexane/ethanol/methanol (80/10/10 v/v/v) + 0.1% TFA in isocratic mode at 1.5 mL/min flow rate (65~66 bar column pressure). A total volume of 25 μL of sample was injected for analysis. The enantiomer of MB-102, named MB-101, has 2 chiral centers in the L, L configuration and was synthesized by the synthetic scheme shown in Figure 2, but starting with L-serine benzyl ester. MB-101 was used as a reference compound in this study. In the study, 5.7 mg of MB-101 powder, 15.5 mg of MB-102 API, and 22.4 mg of MB-102 drug formulation solution were added to 5.0 mL of 200 proof ethanol in a 20.0-mL scintillation vial. The solution was sonicated in a water bath for 5 min. One milliliter of supernatant was transferred to a 1.5-mL polypropylene Eppendorf vial and centrifuged at high speed (>4000 rpm) for 10 min. The supernatant was transferred to an amber HPLC vial for HPLC analysis.

Positional Isomers of MB-102 and MB-301—NMR Analysis

Nuclear magnetic resonance (NMR) experiments were performed at 25°C on an Agilent DD2 600 MHz spectrometer with 5-mm cryogenic probe. Proton spectra were obtained with an 8116-Hz spectral width collected into 16 K data points with 10.0-s pre-acquisition delay and 7.2-μs pulse width. Carbon-13 spectra were obtained with 37,878-Hz spectral width collected into 64 K data points with a 1.5-s pre-acquisition delay and 7.4-μs pulse width. Chemical shifts were measured in parts per million downfield from an internal standard (IS) TSP. COSY spectra were recorded using a 1.5-s delay and 7.6-μs pulse width. A total of 360 × 2048 data matrix with 8 scans per t1 increment were collected. Sine-bell functions were used in weighting the t2 and t1 dimensions. After 2-dimensional Fourier transformation, the spectra were displayed in a 2048 × 2048 frequency domain representation.

Positional Isomers of MB-102 and MB-301—HPLC Analysis

The analysis was conducted using a Waters Alliance e2695 Separation Module with 2998 PDA Detector and 2475 FLR detector using an analytical column—Phenomenex Luna LC Column 250 × 4.6 mm 5 μm C18 (2) 100Å. The column was at room temperature using 2 mobile phases: mobile phase A (MPA): 0.1% TFA/H_2O and mobile phase B (MPB): 0.1% TFA/ACN. UV detection was at 264 nm and 434 nm and fluorescence detection was at 539 nm when excited at 397 nm. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The HPLC gradients are 0-3 min, 90% A; 3-20 min, linear gradient to 10% A; 20-20.1 min, 90% A; and 20.1-30 min 90% A to ready the system for the next injection.

Stability in Drug Formulation

Three batches (batch 1, 2, and 3) of MB-102 formulation manufactured in GMP were stability tested for shelf life determination at the site. These batches were manufactured in different scale. The shelf life determination was carried out at 2°C-8°C (storage

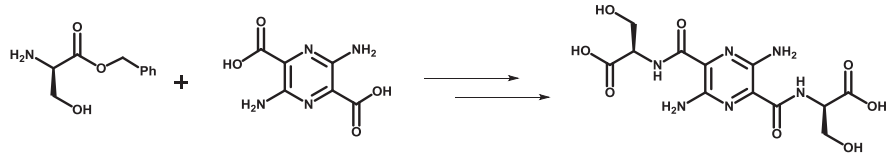

Figure 2. Abbreviated reaction scheme for synthesis of MB-102 from MB-301.

condition), at 25°C/60%RH, and at 40°C/75%RH, for an extended period of time. The MB-102 drug product will be expired when the peak area of total MB-102-related impurities sum up to ≥4.0% of total peak area of MB-102 drug product by HPLC analysis.

Experimentally, at each time point, samples under study were removed from the conditioning chamber, diluted to 0.4 mg/mL, and analyzed by HPLC for peak area of MB-102 and impurities detected by UV at 264 nm. The MB-102 formulated at 18.6 mg/mL in PBS appeared to be very stable when stored at 2°C-8°C. For batch 1 and batch 2, MB-102 drug stored at 2°C-8°C has near zero but negative slope for up to 24 months using linear regression analysis of data. Shelf life of these 2 batches stored at 2°C-8°C cannot be determined. The Arrhenius equation shown below was employed to determine the degradation rate at 2°C-8°C based on the degradation rate of MB-102 stored at 25°C/60%RH and 40°C/75%RH, respectively.

As shown in Figure 3, the rate changes (k_3, k_2) for batch 2 stored at 25°C/60%RH for 24 months and at 40°C/75%RH for 6 months can be determined experimentally. These degradation rates were used to calculate the activation energy (E_a) of degradation of this batch based on the Arrhenius equation illustrated below.

$$\mathrm{Log}(k_3 / k_2) = [(-E_a / 2.303R) \times (1/T_3) - (1/T_2)]$$

where k_3 is the slope of linear regression analysis of degradation rate stored at 40°C/75%RH, and k_2 is the slope of linear regression analysis of degradation rate stored at 25°C/60%RH. R is the universal gas constant (0.00831 kJ/mole).

Based on the data shown in Figure 3 and MS Excel® calculations, the E_a (89.00374 kJ/mole) was determined and used to calculate the degradation rate (k_1 = 0.00531) of the batch stored at 2°C-8°C. With this degradation rate, this batch will last more than 598 months before expiration. The approach using Arrhenius equation of the degradation rates at 25°C/60%RH and 40°C/75%RH to project the shelf life at 2°C-8°C worked well and also verified. In batch 3, the degradation rate of MB-102 stored at 2°C-8°C was determined to be 0.05670 from the linear regression analysis of HPLC data. This result compared extremely well with the degradation rate of 0.05702 derived from the Arrhenius equation treatment of analytical data from 25°C/60%RH and 40°C/75%RH.

Comparison of Human Plasma Protein Binding of MB-102 and Iohexol

Materials Used

Human plasma (K2EDTA, Bioreclamation, Inc.)

Centrifree® Centrifugal Filter (Ultrace® regenerated cellulose; NMWL 30,000 amu, Lot R5JA31736).

MB-102: 1.15 mg/mL prepared in house, aliquoted, and stored at −80°C until use.

Iohexol: Omnipaque 300 at 647.1 mg/mL, 788.05 mM, lot number 12884913 obtained from GE Healthcare.

Sulfamethoxazole obtained from Fluka (lot number 048K0124).

(S)-(-)-Warfarin obtained from Sigma (lot number 1385525V).

Analytical Procedure

MB-102 solution (3 μM) and iohexol (175 μM) were studied together, using sulfamethoxazole (1 μM) and warfarin (1 μM) as controls.

In this study, MB-102, iohexol, sulfamethoxazole, and warfarin were first incubated with human plasma for 60 min, then the free portion was separated from the bound by ultrafiltration using Centrifree® Centrifugal Filter (Ultrace® regenerated cellulose; NMWL 30,000 da, Lot R5JA31736). The filtrate (human plasma water) was analyzed for MB-102 and iohexol using a set of external calibration standards.

Following the same procedures of incubation and filtration as the plasma protein binding study, the recovery of MB-102 and iohexol in 1× PBS was examined for potential nonspecific binding of MB-102 (99.4% recovery, $N = 3$, SD = 0.2) and iohexol (99.5% recovery, $N = 3$, SD = 1.3). The stability of MB-102 and iohexol in 1× PBS and in plasma, as well as sulfamethoxazole and warfarin in plasma, was also examined and checked for the recovery of MB-102 (99.9% in PBS, $N = 3$, SD = 1.9% and 101% in plasma, $N = 3$, SD = 2), iohexol (98.8% in PBS, $N = 3$, SD = 0.6% and 101% in plasma, $N = 3$, SD = 2.0), sulfamethoxazole (104% in plasma, $N = 3$, SD = 7), and warfarin (102% in plasma, $N = 3$, SD = 3).

In this study, the incubation and filtration procedures were the same for all of the compounds tested whereas the analysis procedure was different. The procedure to prepare human plasma water (filtrate after ultrafiltration) for analytical work involves several steps.

(1) Spike the stock solution (100× concentration) of article in study into 4-mL human plasma (K2EDTA, Bioreclamation, Inc.) pre-equilibrated at 37°C to achieve the desired concentration and mix gently for 1 minute.
(2) Incubate the compound under study in plasma in a 5% CO_2 chamber at 37°C for a minimum of 60 min.

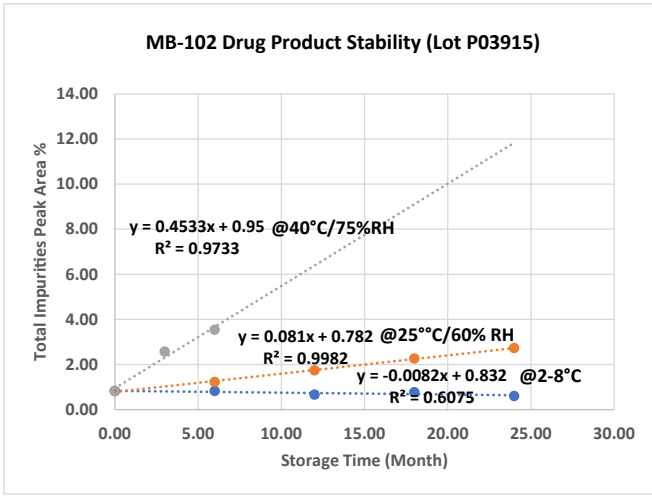

Figure 3. Determination of the stability of MB-102 formulation stored at 4°C.

(3) Centrifuge 800 μL of each incubated solution in 3 replicates through a Centrifree® Centrifugal Filter at 2900 rpm (1000 RCF) at 37°C for 30 min to collect the filtrate.
(4) Analyze the filtrate collected using respective analytical procedure for each compound.

To determine the recovery of MB-102 and iohexol through the incubation and filtration steps, 100 μL of each solution after mixing and before incubation and filtration was retained and analyzed to serve as reference for recovery determination. The blank plasma filtrate was collected for preparing calibration standards, quality controls, and blanks, using the plasma samples processed through the same procedures as that of the compound under study. The stock solutions of MB-102 at 300 μM in PBS, iohexol at 17,500 μM in PBS, sulfamethoxazole at 100 μM in 50% ACN/water, and warfarin at 100 μM in 50% ACN/water were used. The analytical procedure of each compound is described below.

MB-102. Samples were prepared by diluting 1/100 with 1× PBS and mixed thoroughly for HPLC analysis. HPLC analysis used a Phenomenex Luna C18 (2) 100Å (4.6 × 250 mm, 5 μm) analytical column and an Opti-Lynx C18 (4.6 × 3 mm, 5 μm) guard column with

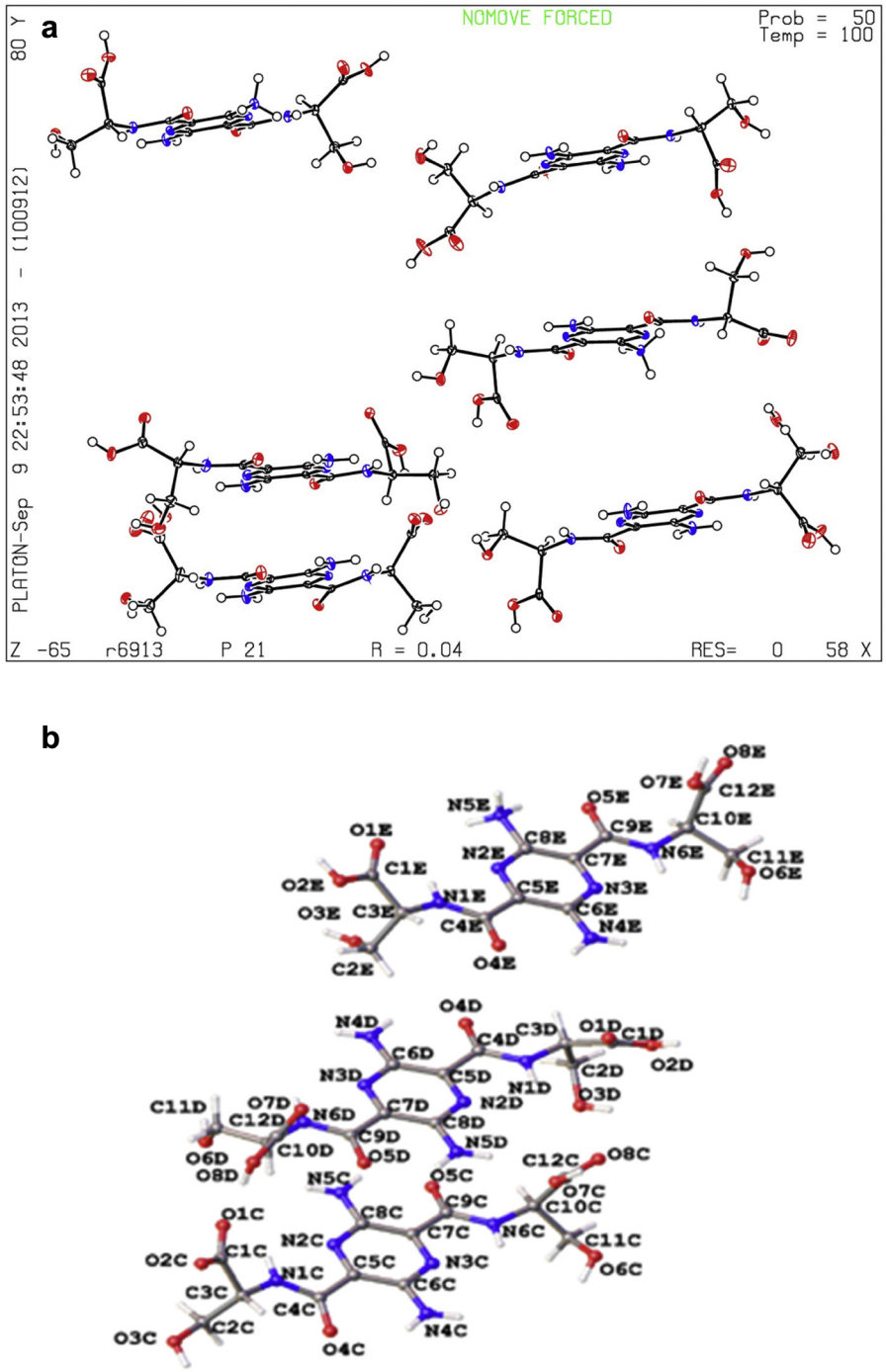

Figure 4. Crystal structure. (a) Molecular packing of MB-102 in a unit cell. (b) Charge distribution of MB-102 molecules in a unit cell.

fluorescence detection excited at 439 nm and emitted at 557 nm. The HPLC gradient conditions were 0-3 min at 90% MPA (0.1% TFA/H2O) and 10% MPB (0.1% TFA/ACN), 3.0-20.0 min linear gradient to 10% MPA, 90% MPB. The column was re-equilibrated to the initial condition of 90% MPA and 10% MPB from 20.1-30 min. Then 10 μL of sample was analyzed at a flow rate of 1.0 mL/min with the flow cell temperature at 30°C and autosampler temperature at 15°C. The column was maintained at ambient temperature. The calibration standards of 3 nM, 6 nM, 15 nM, 30 nM, 45 nM, and 60 nM in the respective matrix were used. MB-102 eluted at 8 min under these conditions.

Iohexol. The sample was diluted 1/5 with ACN containing 40 μg/mL of theobromine to precipitate plasma proteins. After centrifugation, the supernatant was collected, dried down by nitrogen steam at 40°C, and then reconstituted in PBS for HPLC analysis. The HPLC analysis used a Phenomenex Prodigy ODS-3V 100Å (4.6 × 250 mm, 5 μm) analytical column and an Opti-Lynx C18 (4.6 × 3 mm, 5 μm) guard column with UV detection at 254 nm. The HPLC conditions were isocratic with a mobile phase of 3% ACN/12% methanol/85% aqueous containing 20 mM sodium acetate, pH 6.0 and 0.65 mM tetrabutylammonium bromide at a flow rate of 1 mL/min. The column temperature was ambient, and autosampler temperature was 15°C. A 10 μL of sample was injected and iohexol eluted at 5 min, whereas theobromine eluted at 7.4 min. Calibration standards of 350, 262.5, 175, 87.5, 35, and 17.5 μM were used for quantitative analysis.

Sulfamethoxazole. Samples (50 μL) were protein precipitated with 150 μL of ACN containing 250 ng/mL of tolbutamide. After mixing and centrifuging at 3000 rpm for 10 min at 4°C, 100 μL of supernatant was mixed with 100 μL of Milli-Q water thoroughly for HPLC-tandem mass spectrometry (MS/MS) analysis. The HPLC-MS/MS used a Phenomenex Kinetex C18 (2.1 × 250 mm, 2.6 μm) and a Thermo Javelin Filter (2.1 mm) linked to an AB Sciex API 4000 mass spectrometer equipped with a TurboIonSpray (ESI) probe. The mobile phases were MPA of 0.1% formic acid in water and MPB of 0.1% formic acid in ACN with gradient conditions of 0.0-0.5 min of 100% MPA; 0.5-0.6 min of 60% MPA, 40% MPB; 0.6-1.8 min of 50% MPA, 50% MPB; 1.8-2.1 min of 100% MPB; 2.1-2.6 min of 100% MPB; then back to initial conditions of 100% MPA from 2.61-3.4 min. The ion spray voltage was set at 450 and source temperature at 450°C. Gases used were CAD (7), CUR (10), GS1 (25), and GS2 (25). The product ions (M/Z 254.1 → 92.0 and 108.2) of sulfamethoxazole and the product ion (M/Z 271.2 → 155.2) for tolbutamide (IS) were monitored using a set of calibration standards of 100, 200, 400, 600, 800, 1000, and 1200 nM for quantitative analysis of human plasma samples and human plasma water samples.

Warfarin. The sample was prepared in a manner similar to that for sulfamethoxazole. A set of calibration standards of 400, 600, 800, 1000, and 1200 nM warfarin were used for plasma analysis. Another set of calibration standards of 100, 75, 50, 25, and 10 nM warfarin in human plasma water for the analysis of unknown samples. The product ion of (M/Z 309.1 → M/Z 251.1 and 163.0) for warfarin and product ion of (M/Z 271.2 → M/Z 155.2) for tolbutamide were monitored for quantitative analysis.

Data analysis of MB-102 was done by comparing the observed peak area to the standard curve using linear regression analysis with 1/X weighing using LabSolutions software. For iohexol, sulfamethoxazole, and warfarin, the observed analyte-to-IS peak area ratios for the test article and control substances were calculated and compared against standard curves using linear regression analysis with 1/X weighing using LabSolutions software for iohexol or Analyst 1.6.2 9 (AB SCIEX, Framingham, MA) for sulfamethoxazole and warfarin. The mean and standard deviation of the replicate concentrations were calculated using MS Excel 2010 (Microsoft, Redmond, WA). Protein binding following ultrafiltration was calculated using the following formula: fraction unbound = concentration of ultrafiltrate/concentration of plasma at 0 h. The percent bound was then 100 × (1- fraction unbound).

Stability in PBS or plasma following incubation for 1 hour was calculated as 100× (concentration at 1 h/concentration at 0 h).

Finally, recovery of MB-102 or iohexol through the Centrifree® Centrifugal Filters was calculated using the following formula: percent recovery = 100 × (concentration of ultrafiltrate/concentration at 0 h).

Determination of the In Vitro *Blood-to-Plasma Ratio of MB-102 in Male Human Blood*

Materials Used

MB-102 (1.15 mg/mL) stock solution prepared in house.

Chloroquine (as the diphosphate salt, Lot BCBM9716) obtained from Sigma-Aldrich (St. Louis, MO).

(±)-Verapamil (as the hydrochloride salt, Lot LRAA5980) obtained from Sigma-Aldrich.

Tolbutamide obtained from Sigma-Aldrich.

TFA, dimethyl sulfoxide, and ACN were purchased from Fisher Scientific.

PBS, pH 7.4, and formic acid were purchased from Sigma-Aldrich.

Milli-Q water prepared on-site as previously described was used.

Analytical Procedure

A spiking solution of MB-102 was prepared at 300 μM. Tubes of whole blood received refrigerated on ice packs were immersed in warm water (~37°C) to equilibrate to 37°C.

In the study, 0.5 mL of prewarmed whole blood in 1.5-mL Eppendorf tubes was spiked with 5 μL of 300 μM MB-102, 100 μM chloroquine, or 100 μM verapamil to yield final concentrations of 3 μM MB-102, 1 μM chloroquine, or 1 μM verapamil. The blood samples, after mixing, were placed in a 5% CO_2 humidified incubator maintained at 37°C for 60 min. After incubation, 100 μL of whole blood sample was transferred to a 1.5-mL Eppendorf tube. The remaining volume was subjected to centrifugation for 8 min at 10,000 rpm. The plasma supernatant was transferred to individual tubes. Samples were prepared for bioanalysis as noted below.

MB-102 Analysis

For the analysis of whole blood samples, standards, blanks, and quality control samples were prepared in blank 1% human plasma

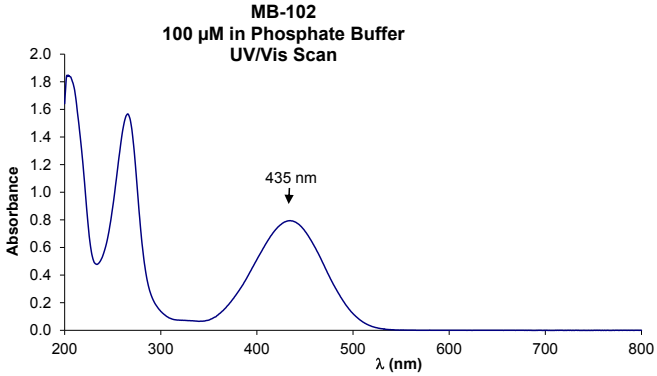

Figure 5. UV/Vis scan of 100 μM MB-102 in phosphate-buffered saline.

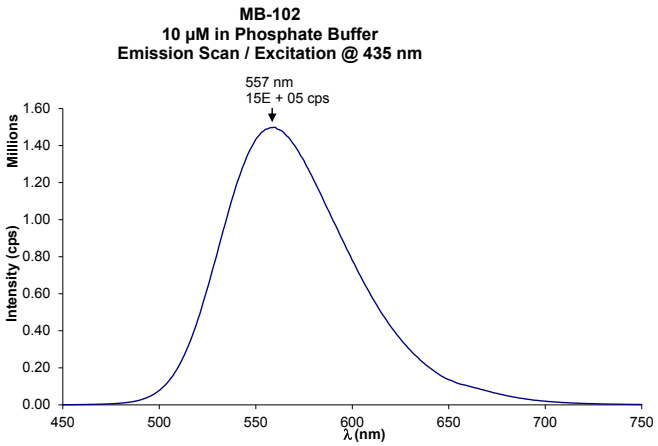

Figure 6. Fluorescence emission spectrum of 10 µM MB-102 in phosphate-buffered saline.

in PBS. To 100 µL of whole blood sample, 100 µL Milli-Q water was added. The tubes were mixed by vortex to lyse blood cells. Tubes were then subjected to centrifugation for 10 min at 10,000 rpm. Ten microliters of supernatant were transferred to a vial containing 990-µL blank PBS for analysis.

For the analysis of plasma samples, standards, blanks, and quality control samples were prepared in blank 1% human plasma in PBS. Each plasma sample from the *in vitro* incubations was diluted by transferring a 10-µL aliquot to a vial containing 990-µL blank PBS. All samples were analyzed without extraction as described below.

The HPLC system was a Shimadzu HPLC system equipped with a CBM-20A system controller, dual LC20AD binary pumps, DGU-20A$_3$ solvent degasser, SIL-20AC autosampler, CTO-20A column oven, and RF-20Axs Fluorescence detector (Shimadzu, Columbia, MD). Samples were placed in the autosampler rack maintained at 15°C. Ten microliters was injected, and then the injection needle was washed briefly with 10% methanol in 90% Milli-Q water (v/v) before resealing to the needle port. The chromatographic (HPLC) conditions used were the same as those described above for the analysis of MB-102 in plasma protein binding study. Concentrations were measured by linear regression analysis with 1/X weighting from standards prepared in 1% human whole blood in PBS or 1% human plasma in PBS as described above using LabSolutions software (Shimadzu, Columbia, MD).

Chloroquine and Verapamil Analysis

For the analysis of whole blood samples, the compounds under study were spiked into whole blood the same way as standards, blank, and quality control samples. For the analysis of plasma, the standards, blank, and control samples were prepared in blank human plasma. The blood samples were lysed with an equal volume of water and then centrifuged for 10 min at 10,000 rpm to collect supernatant. To extract the compounds, 50 µL of supernatants (lysed whole blood or plasma) was protein precipitated with 150 µL of ACN containing 250 ng/mL of tolbutamide as IS and centrifuged at 3000 rpm at 4°C for 10 min. The supernatant obtained was diluted 50% with water for LC-MS/MS analysis.

LC-MS/MS analysis used a Phenomenex Kinetex C18 column (2.1 × 50 mm, 2.6 µm) linked to an AB Sciex API 4000 mass spectrometer equipped with a TurboIonSpray (ESI probe). The chromatographic conditions used mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in ACN at a flow rate of 0.4 mL/min in a gradient mode (0.0-0.5 min 100% A; 0.5-0.6 min 55% A; 0.6-1.8 min 95% A; 2.1-2.6 min 0% A; 2.61-3.4 min 100% A). The multiple reaction monitoring transition of product ion M/Z (320.20 → 247.10) for chloroquine, M/Z (455.20 → 165.20) for verapamil, and M/Z (271.2 → 155.2) for tolbutamide were monitored for quantitative analysis. The analyte/IS peak area ratios for the test article and control substrates were calculated and compared against standard curves using linear regression analysis with a 1/X weighting using Analyst 1.6.2 (AB SCIEX). The mean and standard deviation of the replicate concentrations were calculated using MS Excel 2010 (Microsoft).

Results

Chemical and Physical Characterization for Purity Determination and Structure Elucidation of MB-102 API

The MB-102 API synthesized under GMP is an orange solid when viewed under ambient laboratory conditions and lighting. The

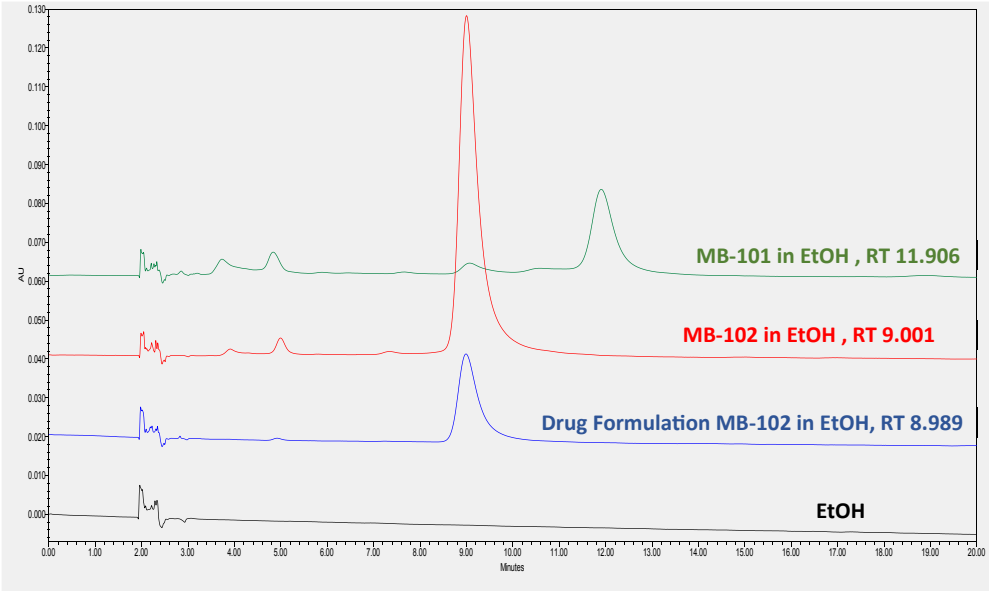

Figure 7. Overlay of MB-101, MB-102, and MB-102 drug formulation under normal phase separation using a RegisPack chiral column.

Table 1
Protein Binding of MB-102 to Human Plasma

Test Article	Protein Binding in Human Plasma				
	Fraction Unbound		Percent Bound		*N*
	Mean	SD	Mean	SD	
MB-102	0.959	0.024	4.1	2.4	3
Iohexol	0.940	0.010	6.0	1.0	3
Sulfamethoxazole	0.395	0.030	60.5	3.0	3
Warfarin	0.0118	0.0032	98.8	0.3	3

purity (% w/w) was greater than 95% after determination of moisture content by coulometric oven KF water analysis, residual solvents (methanol, ethanol, ACN, dichloromethane, and ethyl acetate) by gas chromatography, residue on ignition, and chromatographic peak area % purity by HPLC. The molecular structure of MB-102 was confirmed using infrared spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry. The empirical formula ($C_{12}H_{16}N_6O_8$) was confirmed experimentally via elemental analysis to be C (38.13%) versus theory 38.71%, H (4.35%) versus theory 4.33%, and *N* (22.33%) versus theory 22.57%. X-ray powder diffraction analysis indicated the API was crystalline. The specific rotation was determined to be −124.4° by <USP 781>. Differential scanning calorimetry and thermogravimetric analyses were conducted to examine the thermal properties of the API. A combination of these analytical assays was used to check for lot-to-lot reproducibility in manufacturing.

Single-crystal Structure Determination of MB-102

The crystal structure of MB-102 was determined using single-crystal X-ray diffraction on a crystal grown by slow cooling of a MB-102 (2.0 mg/mL) solution in boiled water. The single crystal used for the study was monoclinic ($P2_1$ space group) with cell dimensions a = 8.7526 (4)Å, b = 19.1688(8)Å, and c = 26.3752(11)Å, α = 90°, β = 90.669° (3), γ = 90°, and unit cell volume 4424.9(3) $Å^3$. The crystal size was 0.388 × 0.206 × 0.094 mm^3. The refinement was using full-matrix least-square on F^2 down to final R indices R1 = 0.0416 with a goodness-of-fit of F^2 = 1.012. As shown in Figure 4, the unit cell lattice has 6 MB-102 molecules in the asymmetric unit, of which 3 molecules contain amine (N5) and 2 carboxylic acids; 1 molecule (suffix a) which is ammonium (N5), carboxylate and carboxylic acid; 1 molecule (suffix c) has amine group at N5, carboxylate and a carboxylic acid group (net 1− charge); and a 6th molecule (suffix e) has ammonium (N5) and 2 acid groups (net 1+ charge) giving an overall charge of the lattice of zero. This structure has a relatively small volume per non-H atom (14$Å^3$) which is not typical for organic compounds. The crystal structure confirmed the D, D chirality of MB-102.

UV/Vis and Fluorescence Properties

UV-Vis analysis detected absorbance maxima at 206 nm, 266 nm, and 435 nm as shown in Figure 5. The λ_{em} at 557 nm was determined when λ_{ex} was set at 435 nm as shown in Figure 6. The UV absorbance maximum at 435 nm is an important feature of MB-102 in that it exerts a broad band of fluorescence at 557 nm when excited at 435 nm. The excitation and emission profile of MB-102 provides a great avenue for quantitative analysis when in a biological matrix such as plasma or urine.

pK_a

The pK_a's of the 2 acid groups of MB-102 were determined to be 2.77 and 3.40. Determination of these 2 pK_a values provides guidance for formulation of MB-102 for various *in vitro* assays, cell-based assays, *in vivo* animal studies, and human clinical studies.

Chiral Purity

Figure 7 shows that MB-102, baseline separated from MB-101 (the L, L isomer), is eluted ahead of its enantiomer. The racemic compound (D, L = L, D configuration) of MB-102 was not detected in the drug formulation. The results also show that MB-101 has minor components of MB-102 (D, D enantiomer) and the racemic D, L/L,D form eluted between the D, D form and L, L form.

Positional Isomers of MB-102

MB-102 contains no positional isomers, based on the results of HPLC and NMR analysis of the MB-102 precursor, MB-301. More information about the determination of positional isomers can be found in the Supplemental Material.

MB-102 Stability in Drug Formulation

MB-102 was formulated into PBS as the drug dosing solution for human clinical studies. No other excipients are necessary for formulation. MB-102 in this formulation matches the API in terms of purity, photophysical properties, and chemical properties. The steps used to formulate MB-102 into PBS did not alter its chirality. The pH is easily adjusted to 7.2 ± 0.2. Thus, intravenous administration of this formulation is performed at physiological conditions.

Based on the data shown in Figure 3 and MS Excel® calculations following the Arrhenius equation, the activation energy, E_a = 89.00374 kJ/mole, was determined and used to calculate the degradation rate (k_1 = 0.00531) of MB-102 stored at 4°C (2°C-8°C) for this batch. With a slope of 0.00531 and an intercept of 0.82, it

Table 2
Blood-to-Plasma Ratio for MB-102, Chloroquine, and Verapamil

Test Article	Donor	Concentration (nM)		Blood-to-Plasma Ratio		
		Whole Blood[a]	Plasma	Individual	Mean	SD
MB-102	15SLMR1746	3000	5060	0.593	0.590	0.003
	15SLMR2520	3000	5090	0.589		
	15SLMR2521	3000	5110	0.587		
Chloroquine	15SLMR1746	1000	245	4.08	4.04	0.11
	15SLMR2520	1000	255	3.92		
	15SLMR2521	1000	243	4.12		
Verapamil	15SLMR1746	1000	1060	0.943	0.946	0.063
	15SLMR2520	1000	1130	0.885		
	15SLMR2521	1000	898	1.01		

[a] Nominal concentration.

will take 598.8 months for MB-102 formulation to have 4.0% total peak area of MB-102-related impurities.

Properties of MB-102 in Blood Matrixes

In the initial human clinical studies, MB-102 was simultaneously administered with iohexol in order to compare plasma clearance and hence plasma-determined GFR.[17,18] The Food and Drug Administration agreed that iohexol would be the standard of truth and thus the comparator for MB-102 in terms of it being a GFR tracer agent in humans. Therefore, the following *in vitro* studies were conducted with MB-102 and iohexol administered together.

Comparison of Human Plasma Proteins Binding of MB-102 and Iohexol

The results shown in Table 1 indicated that the percent unbound to plasma proteins for MB-102 was 95.9% (SD of 0.024), which is comparable to that of iohexol (94.4%, SD of 0.01). Two controls were also tested, sulfamethoxazole (39.5%, SD of 0.030) and warfarin (1.18%, SD of 0.0032), which agree with literature values.[20,21]

Comparison of Blood-to-Plasma Ratio of MB-102 and Iohexol in Male Human Blood

The blood-to-plasma ratio of individual samples was calculated using the formula below. Table 2 shows results for chloroquine (distributes predominantly to blood cells) and verapamil (distributes equally between blood cells and plasma), which were consistent with published values.[22] MB-102 showed a mean blood-to-plasma ratio of 0.590, indicating it was distributed predominantly to plasma, with minimal distribution to blood cells.

$$\text{Blood to plasma ratio} = \frac{\text{Concentration in whole blood}}{\text{Concentration in plasma}}$$

Discussion

The chemical composition and structure of MB-102 prepared in large scale are confirmed via numerous chemical and physical characterization studies. The single-crystal structure analysis confirms the D, D chirality of MB-102 in the solid state. The chirality of MB-102 in drug formulation is also confirmed by normal-phase HPLC analysis using a chiral separation column and compared to the L, L enantiomer (MP-101). The pK_a of the 2 acid groups were determined to be 2.77 and 3.40, indicating that MB-102 formulated in PBS at pH of 7.2 ± 0.2 is ionic in nature. MB-102 has strong UV absorbance at 206 nm, 266 nm, and 435 nm, and broad emission at 557 nm when excited at 435 nm. The intrinsic photophysical properties of MB-102 provide a robust method for quantitation in blood and urine as shown in our preclinical studies and in human clinical studies for GFR determination. MB-102 contains no detectable positional isomers in the API or in the formulation solution. This was confirmed by HPLC analysis and ^{13}C NMR analysis results of MB-102 precursor, MB-301. MB-102 formulation in PBS is very stable when stored at 4°C (2°C-8°C) which provides favorable conditions for commercialization of MB-102. *In vitro* studies of MB-102 exhibits minimal plasma protein binding (4.1%) when compared to iohexol (6.0%) in a codosing study. MB-102, in the blood matrix, presents predominately in the plasma (blood-to-plasma ratio of 0.590) and has minimal permeation into red blood cells.

Conclusion

From the analytical results reported herein, MB-102 has the necessary properties for use as a tracer agent for GFR determination. Its fluorescence property coupled with transdermal detection after bolus intravenous administration yields the first true measurement of GFR in real time and at the point-of-care.

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References

1. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis*. 2002;39:S1-S266.
2. Ferguson MA, Waikar SS. Established and emerging markers of kidney function. *Clin Chem*. 2012;58:680-689.
3. Inker LA, Schmid CH, Tighiouart H, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med*. 2012;367:20-29.
4. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron*. 1976;16(1):31-41.
5. Endre ZH, Pickering JW, Walker RJ. Clearance and beyond: the complementary roles of GFR measurement and injury biomarkers in acute kidney injury (AKI). *Am J Physiol Ren Physiol*. 2011;301:F697-F707.
6. Frennby B. Contrast media as markers of GFR. *Eur Radiol*. 2002;12:475.
7. Erley CM, Bader BD, Berger ED, et al. Plasma clearance of iodine contrast media as a measure of glomerular filtration rate in critically ill patients. *Crit Care Med*. 2001;29:1544.
8. Frennby B. *Use of Iohexol to Determine Glomerular Filtration Rate (Thesis)*. Lund, Sweden: University of Lund; 1996.
9. Rabito CA, Chen Y, Schomacker KT, Modell MD. Optical, real-time monitoring of the glomerular filtration rate. *Appl Opt*. 2005;44(28):5956-5965.
10. Yu W, Sandoval RM, Molitoris BA. Rapid determination of renal filtration function using an optical ratiometric imaging approach. *Am J Physiol Ren Physiol*. 2007;292:F1873-F1880.
11. Chinen LK, Galen KP, Kuan KT, et al. Fluorescence-enhanced europium-diethylenetriaminepentaacetic (DTPA)-monoamide complexes for the assessment of renal function. *J Med Chem*. 2008;51:957-962.
12. Schock-Kusch D, Sadick M, Henninger N, et al. Transcutaneous measurement of glomerular filtration rate using FITC-sinistrin in rats. *Nephrol Dial Transplant*. 2009;24:2997-3001.
13. Rajagopalan R, Neumann WL, Poreddy AR, et al. Hydrophilic pyrazine dyes as exogenous fluorescent tracer agents for real-time point-of-care measurement of glomerular filtration rate. *J Med Chem*. 2011;54:5048-5058.
14. Poreddy AR, Neumann WL, Freskos JN, et al. Exogenous fluorescent tracer agents based on pegylated pyrazine dyes for real-time point-of-care measurement of glomerular filtration rate. *Bioorg Med Chem*. 2012;20:2490-2497.
15. Bugaj JE, Dorshow RB. Pre-clinical toxicity evaluation of MB-102, a novel fluorescent tracer agent for real-time measurement of glomerular filtration rate. *Regul Toxicol Pharmacol*. 2015;72:26-38.
16. Dorshow RB, Bugaj JE. Next tier *in vitro* and *in vivo* nonclinical studies further elucidating the safety and toxicity profile of MB-102, a novel fluorescent tracer agent for measurement of glomerular filtration rate. *Regul Toxicol Pharmacol*. 2019;107:104417.
17. Dorshow RB, Debreczeny MP, Fink JC, Dowling TC. Initial clinical trial results of a real-time point-of-care glomerular filtration rate measurement utilizing a novel fluorescent tracer agent. *J Am Soc Nephrol*. 2015;26:259A.
18. Dorshow RB, Debreczeny M, Johnson JR, et al. Clinical study results of a real-time point-of-care glomerular filtration rate measurement. *J Am Soc Nephrol*. 2017;28:597.
19. Shieh J, Riley IR, Dorshow RB. Clinical analysis and quantitation of MB-102, a novel fluorescence tracer agent, in human plasma. *Anal Methods*. 2018;10:2376-2383.
20. Luden RC, Chao D. Effect of pirprofein on protein binding of warfarin and tolbutamide in human plasma. *J Pharm Sci*. 1981;70(12):1370-1371.
21. Wijkstorem A, Westerland D. Plasma protein binding of sulfadiazine, sulfamethoxazole and trimethoprim determined by ultrafiltration. *J Pharmaceutical Biomed Anal*. 1983;1(3):293-299.
22. "Blood to Plasma Ratio", *in vitro* ADME & PK, Cyprotex, an Evotec company. Available at: www.cyprotex.com/admepk/protein_binding/blood-to-plasma-ratio. Accessed December 2, 2019.