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Clinical analysis and quantitation of MB-102, a novel fluorescence tracer agent, in human plasma†

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A rapid analytical method for the quantitation of MB-102 (a fluorescent tracer agent) in human plasma using HPLC with fluorescence detection has been developed and validated. This new plasma sample preparation method, 1/100 direct dilution with phosphate buffered saline instead of typical protein precipitation, improves both robustness and assay efficiency by eliminating the need for sample drying and the need for an internal standard. The validation results show that the new method is robust, specific, precise, accurate, and has a wide linear dynamic range (0.4 ng mL^{-1} to 400 ng mL^{-1}) with good recovery. The results from 59 clinical study subjects analyzed by both methods yield an excellent correlation with a slope of 1.015 and an R^2 of 0.994. Sample preparation using direct dilution is easier and faster than the protein precipitation method, and is excellent for determining the MB-102 content in human plasma. The concentration of MB-102 in plasma as a function of time is used to calculate the glomerular filtration rate using a standard pharmacokinetic model.

Introduction

Determination of the glomerular filtration rate (GFR) is widely accepted as the most reliable measurement of renal function.[1] The current clinical practice for assessing kidney function uses a single serum creatinine measurement to obtain an estimated GFR (eGFR) using equations of Ferguson and Waikar[2] and Inker *et al.*[3] However this methodology is not sensitive, often inaccurate, and time-delayed, and the results are affected by factors such as age, hydration, muscle mass, and diet.

An exogenous GFR tracer agent such as iohexol (Omnipaque™) can be used to measure the GFR,[4–6] but it requires laborious sample preparation and specialized post-processing, available at few clinical sites.

To overcome the deficiencies of the clinically employed eGFR methodology and the laborious research-grade measured GFR techniques, significant effort has been directed at finding exogenous fluorescent tracer agents that can be detected transdermally, and hence employed at the point-of-care.[7–10] To this end, MB-102, a fluorescent tracer agent has been designed and synthesized to have properties of an ideal GFR tracer agent with the necessary photo-physical properties.[11] Nonclinical studies have demonstrated negligible pathological concerns,[12] and efficacy in clinical studies has been reported.[13,14]

The structure of MB-102 (chemical name 3,6-diamino-2,5-bis {*N*-[(1*R*)-1-carboxy-2-hydroxyethyl]carbamyl}pyrazine) is shown in Fig. 1. This agent emits a broad fluorescence spectrum peaked at 556 nm when excited at 434 nm.

However, traditional sample preparation methods using human plasma are still laborious and time-consuming. The new method reported here involves preparing plasma samples from on-going human clinical studies by 1/100 direct dilution in 1× Phosphate Buffered Saline (PBS), and then analysing them by reverse phase HPLC using fluorescence detection. The concentration results obtained are then used to determine the measured GFR (mGFR) of the subjects in these studies using standard pharmacokinetic software.

The new method is less time-consuming and does not require setting up of additional laboratory equipment for sample drying. Because every step in the new method is quantitative, there is no need for an internal standard. Therefore, it is highly promising for clinical use at the point of care. It is shown to be sensitive, specific, precise and accurate, with a wide linear dynamic range for quantitation of MB-102 in plasma.

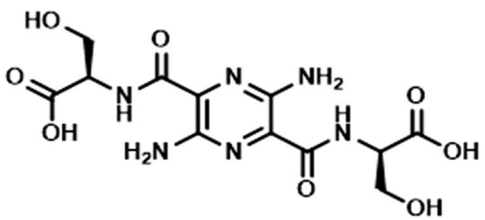

Fig. 1 Structure of MB-102.

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Materials and methods

Chemicals

The dosing solution of 18.6 mg mL^{-1} MB-102 in PBS was manufactured under GMP by AMRI (Glasgow, Scotland). 1× PBS (Phosphate Buffered Saline) without calcium or magnesium was obtained from Mediatech Inc. (21-040-CM, Lot 22716007). MeOH was obtained from Fisher Chemical (A456-4, Lot 156744). Acetonitrile (ACN), (Optima® Grade), 0.1% trifluoroacetic acid in water (Optima® Grade), and 0.1% trifluoroacetic acid in acetonitrile (Optima® Grade) were all obtained from Fisher Scientific. Milli-Q DI water (18.2 MQ cm@25 °C, 3 ppb TOC) was obtained using a Milli-Q purification system [Millipore Q-POD® Remote Dispense (Millipore SAS 67210 Molsheim)].

Sample preparation

Plasma samples were obtained from on-going clinical studies conducted by MediBeacon Inc. at several clinical research facilities. A blank plasma sample (K_2EDTA) was purchased from Zen-Bio Inc. (Research Triangle Park, North Carolina). All plasma samples were stored at −80 °C until used.

Preparation of calibration standards and quality controls

For sample preparation by direct dilution, calibration standards containing 0.4, 1.0, 2.0, 4.0, 10.0, 16.0, 40.0, 100.0, 200.0, and 400.0 ng mL^{-1} and quality controls at low, mid, and high levels of MB-102 (see Table 2) were prepared in 1% human plasma in PBS on the day they were to be used. The MB-102 dosing solution (18.6 mg mL^{-1}) used in the human clinical study was diluted 1/100 with 1% human plasma in PBS to form a stock solution of 186 μg mL^{-1} MB-102. This stock solution was diluted with 1% human plasma in PBS to form a calibration standard working stock of 2000 ng mL^{-1} MB-102. A quality control (QC) working stock at 1000 ng mL^{-1} was also prepared from the 186 μg mL^{-1} MB-102 stock by dilution with 1% plasma in PBS. The low, mid, and high levels of quality controls were prepared from this QC working stock by dilution with 1% human plasma in PBS.

For sample preparation by protein precipitation, calibration standards containing 0.4, 1.0, 2.0, 4.0, 10.0, 16.0, 40.0, 100.0, 200.0, and 400.0 ng mL^{-1} and quality controls at low, mid, and high level of MB-102 were prepared as follows. Ten thousand fold concentration of MB-102 in 1× PBS were prepared for each standard and QC. These stock solutions were diluted 1/100 with plasma to make the working calibration standards and QCs. An aliquot of 200 μL of each working standard and QC was stored in a 600 μL Eppendorf polypropylene vial at −80 °C until use. Using the HPLC method described below, these working calibration standards and QCs were qualified and certified for the analysis of unknown samples.

Preparation of samples for analysis

To prepare plasma samples by the direct dilution method, 10 μL of plasma sample (after thawing to room temperature and mixing thoroughly by mild vortexing) was mixed with 990 μL of 1× PBS in an amber HPLC vial, capped, mixed on a rotating plate for about 10 minutes, and then centrifuged at 1000 rpm for two minutes before being placed into the HPLC sample chamber for analysis.

For plasma samples using our revised protein precipitation method, calibration standards, quality controls, and unknown samples were prepared using standard protocols. On the day of sample analysis, calibration standards, quality controls and unknown samples were removed from the −80 °C freezer and thawed to room temperature without any heating aids. After mixing mildly by vortexing, 50 μL of plasma was mixed with 200 μL of methanol containing 4.5% of 1× PBS (v/v). The mixture was vortexed at high speed for at least 10 seconds and then centrifuged at over 4000 rpm for 10 minutes. The entire supernatant was transferred to a second container and mixed thoroughly. From there 50 μL of supernatant was mixed in a 1.5 mL amber HPLC vial containing 950 μL of 1× PBS. The mixture was vortexed at high speed for 5 seconds, and then placed into the HPLC sample chamber for analysis. With this procedure, the need for drying down the supernatant after plasma protein precipitation was eliminated. A portion of the supernatant was diluted directly with 1× PBS for HPLC analysis, so that the internal standard commonly added after protein precipitation but prior to reconstitution was not required.

HPLC method

Analyses were performed on a Waters Acquity UPLC H Class Chromatography System, equipped with a column heater, a sample heater/cooler, a vacuum degasser, an autosampler, a fluorescence detector, and a pump capable of delivering binary gradients. A HPLC analytical column, Phenomenex Luna C18 (2), 4.6 × 250 mm, 5 μm, 100 Å (Phenomenex, Cat. no. 00G-4252-E0, S/N H15-133556), and a Security Guard Cartridge C18, 4 × 3 mm ID, 5 μm (Phenomenex, Cat. no. KJ0-4282) were used for the analysis. Waters Empower 3 software equipped with the UPLC system was used for the assay setup, analysis monitoring, and data processing. Two mobile phases, Mobile Phase A: 0.1% trifluoroacetic acid in water (Fisher, Optima® Grade) and Mobile Phase B: 0.1% trifluoroacetic acid in acetonitrile (Fisher Scientific, Optima® Grade), were used. The column temperature was set to 30 °C, the autosampler temperature was set at 5 °C, the excitation wavelength was set at 434 nm and the emission wavelength was set at 556 nm. The counting rate was set at 5 point per sec and the PMT gain was set at 50. The flow cell temperature was set at ambient temperature. Ten μL of sample were injected and MB-102 eluted at about 3.6 minutes.

Table 1 HPLC gradient conditions

Time (min)	Flow (mL min^{-1})	% *A*	% *B*	Gradient curve
0.00	1.1	85	15	6
5.00	1.1	40	60	6
5.05	1.6	10	90	6
6.75	1.6	10	90	6
6.80	1.1	85	15	6
10.00	1.1	85	15	6

Table 1 shows the gradient conditions developed for maximum resolution of plasma components.

Method validation

Using this HPLC method and sample preparation by direct dilution, a validation study was conducted to measure precision, accuracy, linearity, reproducibility, plasma and sample solution stability, freeze–thaw stability, lowest limits of quantitation, and specificity. The acceptance criterion was ±15% data deviation from the normal value, except at the lowest limit of quantitation (LLOQ) where ±20% was used.[16]

Specificity and selectivity

The interference of plasma components with MB-102 was investigated by comparing chromatograms of plasma samples that were blank to those spiked with MB-102. The LLOQ was determined to be at least 10 times the noise level on blank samples, while the lowest limit of detection (LLOD) was determined to be three times the noise level on blank samples.

Accuracy and precision

Three replicates of samples at five concentrations of MB-102 in 1% plasma/PBS were run and each analyzed for accuracy. For precision determination, ten replicates of a 200 ng mL^{-1} sample were injected to quantitate the precision.

Recovery

Recovery was determined at three different levels of MB-102 by comparing plasma samples before and after spiking with MB-102. The percentage of MB-102 recovered from the spike was determined as the percentage of recovery.

Plasma sample stability and plasma solution stability

MB-102 stability in human plasma upon long term storage (frozen at −80 °C) and short-term (bench top ambient temperature with or without light exposure, and 2–8 °C) storage of the plasma sample in 1× PBS solution was evaluated. Plasma samples were tested for three freeze (−80 °C) and thaw (room temperature) cycles. The stability of MB-102 in 1% plasma in 1× PBS at room temperature with or without light exposure, at 2–8 °C, and at auto-sampler temperature (5 °C) was evaluated in three replicates at time intervals of 24 and 48 hours.

Results and discussion

HPLC chromatogram of MB-102

Fig. 2 below illustrates an overlay of HPLC chromatograms of MB-102 (3.3 ng mL^{-1} in 1% human plasma in 1× PBS and in 1× PBS) and a blank control (1% human plasma in 1× PBS and in 1× PBS) with excitation at 434 nm and monitored at an emission wavelength of 556 nm. The 1% of plasma proteins present in the blank control solution does not have a peak at 3.62 min that would interfere with MB-102 for either detection or quantitation. The comparison of spiked MB-102 in 1% plasma in 1× PBS and spiked MB-102 in 1× PBS indicates a minimal matrix effect.

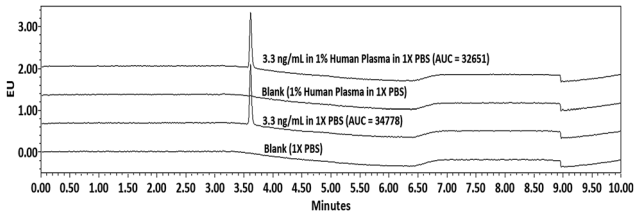

Fig. 2 Overlay of HPLC chromatograms of MB-102 at 3.3 ng mL^{-1} in 1% plasma, 3.3 ng mL^{-1} in 1× PBS and their corresponding blank controls.

Method validation

The results of method validation are summarized in Table 2. The MB-102 standard calibration showed a very good linear response for concentrations ranging from 0.4 ng mL^{-1} to 400 ng mL^{-1} with an r^2 of 0.9997 using a linear regression and a weighing of $1/X$. As shown in Table 2, the slope of the linear curve is 1.30×10^4 with a RSD of 1.61 in three replicates. The LLOQ at 0.4 ng mL^{-1} has a signal/noise ratio of 19.48, a USP tailing of 0.937, and a USP plate count of 36 606 as shown in Fig. 3.

The assay precision (99.2% recovery with 0.45% RSD), and assay accuracy (ranging from 101.2% to 95.7% recovery for the five levels studied) are excellent. A stability check after three freeze–thaw cycles indicated that the plasma was very stable when stored at −80 °C. The sample solution stability when stored at 4 °C or at room temperature without light for up to 48 hours was also very stable. There is a small degradation (1–2%) of MB-102 in solution when stored at room temperature with light for 48 hours. Spike recovery studies at three different levels of MB-102 in plasma indicated that all three levels of spike recovery were within ±15% of the amount spiked. To determine the purity of the MB-102 peak eluted at 3.62 minutes for the unknown sample, the plasma sample was protein precipitated, the supernatant was dried down and reconstituted into PBS, and analyzed using HPLC with UV absorbance monitored at 445 nm (in PDA mode). The MB-102 PDA peak purity from a spiked MB-102 in PBS was compared to that of a 30 minute post-dose plasma sample collected from a subject in the clinical study. The results (Fig. 4 and 5) indicated that the MB-102 peak from the clinical study that eluted at 3.62 minutes and absorbed at 445 nm was more than 90% pure.

The overall data from the validation study indicated that this HPLC method with fluorescence detection is precise and accurate and suitable for assaying the amount of MB-102 in human plasma.

Method transfer

To test the intra-lab robustness of the new method, it was transferred to a GMP-certified bioanalytical lab for use in the analysis of plasma samples from clinical studies. As a check on the robustness of the method transfer, the plasma samples of

Table 2 Accuracy, precision, plasma stability, sample solution stability and spike recovery of MB-102

MB-102		Measured concentrations		
Concentration (ng mL^{-1})	Number of observations	Mean (ng mL^{-1})	RSD (%)	Accuracy (%)
380.0	3	384.7	0.73	101.2
160.0	3	159.6	0.80	99.7
60.0	3	59.3	0.76	98.8
20.0	3	19.8	1.46	99.2
3.0	3	2.9	3.20	95.7
Injection precision				
200.0	10	198.5	0.45	99.2
Linearity				
0.4	3	0.4	4.30	105.0
1.0	3	1.0	3.39	101.0
2.0	3	2.0	1.44	100.0
4.0	3	4.0	3.40	100.3
10.0	3	9.9	2.08	98.7
16.0	3	15.4	2.33	96.4
40.0	3	39.8	1.20	99.6
100.0	3	99.2	1.35	99.2
200.0	3	199.2	0.45	99.6
400.0	3	402.4	0.49	100.6
Slope	3	1.30×10^4	1.61	
Intercept	3	-4.20×10^2	−165.5	
R^2	3	0.9999	0.01	
3 freeze–thaw cycles				
1.76	3	1.79	1.09	101.7
8.81	3	8.81	1.79	100.0
61.87	3	62.34	1.82	100.8
Sample solution stability at 4 °C for 48 hours				
1.76	3	1.75	2.56	99.4
8.81	3	8.66	2.06	98.3
61.87	3	60.84	3.47	98.3
QCs prepared at three days stored at 4 °C for 48 hours				
3.7	1	3.74	N/A	101.1
37.9	1	37.55	N/A	99.1
372.6	1	373.50	N/A	100.2
QCs prepared at three days stored at RT without light				
3.7	1	3.87	N/A	104.6
37.9	1	37.15	N/A	98.0
372.6	1	372.58	N/A	100.0
QCs prepared at three days stored at RT with light				
3.7	1	3.76	N/A	101.6
37.9	1	36.40	N/A	96.0
372.6	1	364.59	N/A	97.9
Recovery of MB-102 spiked into plasma				
0.6	3	0.67	4.24	111.7
6.0	3	6.14	5.48	102.3
60.0	3	60.25	2.9	100.4

12 subjects were also analyzed internally in our lab. All 15 plasma samples collected at 0, 5, 10, 15, 30, 60, 90, 120, 180, 240, 300, 360, 480, 600, and 720 minutes post injection from each subject were used. A comparison of the results (data shown in Appendix A) from these subjects obtained in-house with those from the contract lab is shown in Fig. 6. A linear regression of the correlation indicates a slope of 1.02 with an R^2 of 0.99. The results indicated that the method is robust and thus easily transferred between laboratories.

Next, the revised method of plasma sample preparation by protein precipitation for HPLC analysis was compared to the direct dilution method. As described in the experimental

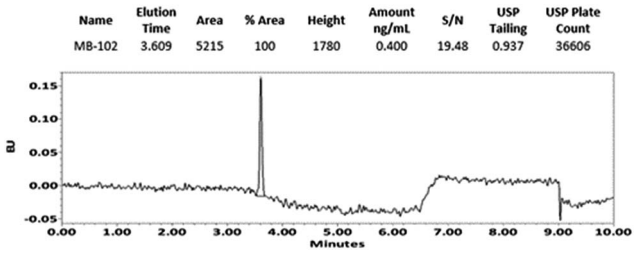

Fig. 3 The signal/noise, USP tailing factor, and USP plate count of MB-102 at 0.4 ng mL^{-1} in 1% plasma in PBS.

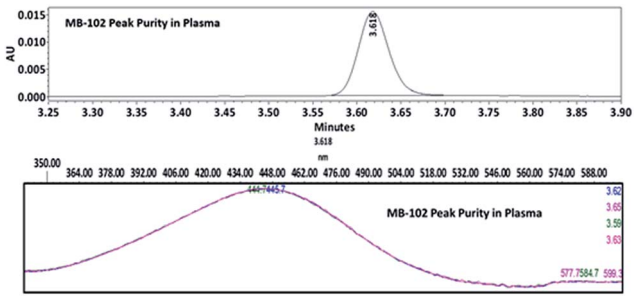

Fig. 4 Peak purity of MB-102 spiked into 1× PBS.

section, plasma samples from the same 12 subjects were analyzed using the revised protein precipitation method. The correlation plot of MB-102 concentration as determined by the direct dilution method *vs.* by the revised protein precipitation method is shown in Fig. 7. The results (Appendix A) with a correlation slope of 1.01 and an R^2 of 0.99 strongly support the validity of the new method.

In another test of the direct dilution method, results (data shown in Appendix B) from that method performed by the contract lab were compared to those from the protein precipitation method performed internally for 15 samples for each of 59 subjects from the clinical study. The correlation plot (Fig. 8) showed a correlation slope of 1.02 with an R^2 of 0.99.

Hemolysis

Several samples with hemolysis were observed in the clinical study, so the effects of blood hemolysis on the direct dilution

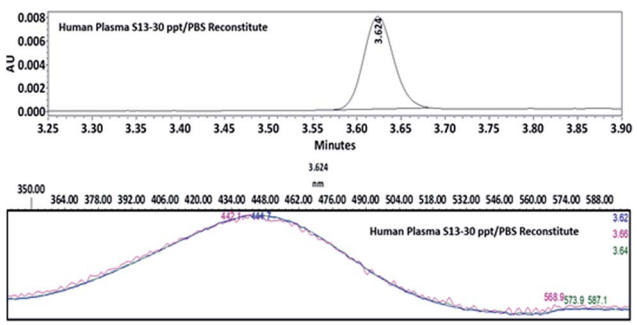

Fig. 5 Peak purity of MB-102 in plasma sample S13-30 after sample preparation.

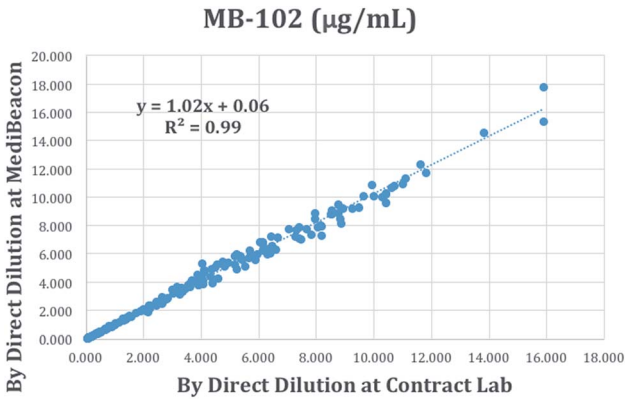

Fig. 6 Correlation of MB-102 in plasma obtained at the contract lab and at MediBeacon.

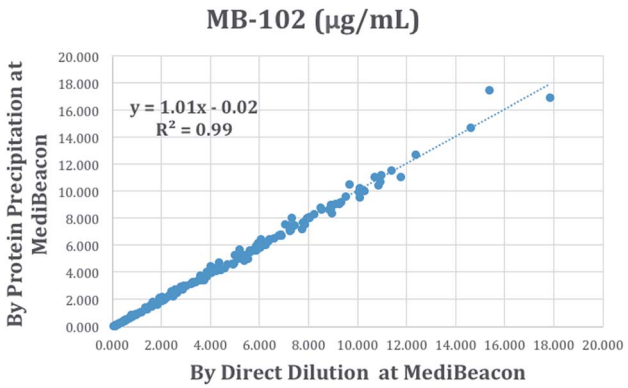

Fig. 7 Correlation of MB-102 in plasma obtained by direct dilution *vs.* by protein precipitation, both conducted at MediBeacon.

method for MB-102 quantitation in human plasma were investigated. As a reference, a color chart[15] showing the percentage of blood hemolysis in plasma is illustrated in Fig. 9. Using this chart, more than 90% of the plasma samples received from the clinical study had a hemoglobin content of 50 mg dL^{-1}

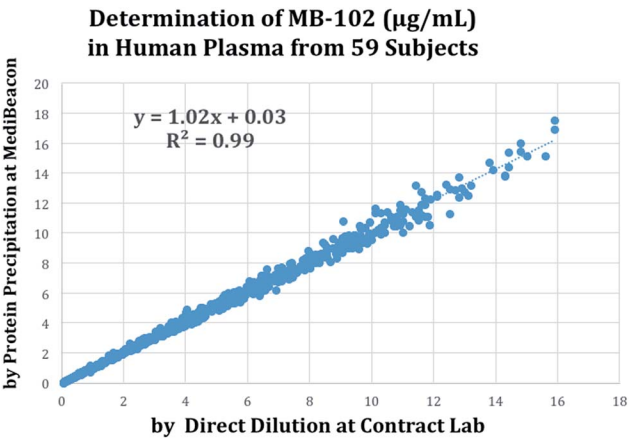

Fig. 8 Correlation of MB-102 in plasma obtained by direct dilution at the contract lab *vs.* by protein precipitation at MediBeacon.

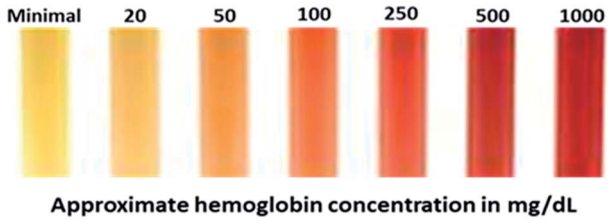

Fig. 9 Color chart of the percentage of blood hemolysis (*via* hemoglobin concentration, mg dL^{-1}) in plasma.

or less. Several of the plasma samples had hemoglobin content between 100 and 250 mg dL^{-1}. The MB-102 content of these samples was measured using both the direct dilution method and the revised protein precipitation method. The results shown in Table 3 and Fig. 10 are the samples with hemoglobin content between 50 mg dL^{-1} and 100 mg dL^{-1}. The slope (1.0194) of the correlation curve indicated that the impact on MB-102 quantitation was negligible when compared with the slope (1.0153) of the correlation curve shown in Fig. 8.

Only four out of approximately 875 samples had hemoglobin content between 100 mg dL^{-1} and 250 mg dL^{-1} as determined by the color chart. Analysis of these samples was not included in any further determination of concentration or GFR calculations.

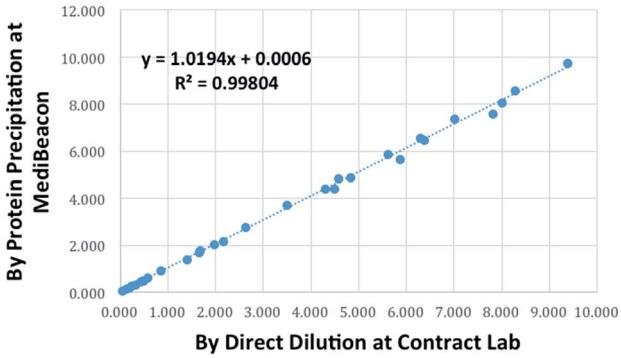

Fig. 10 Correlation of the MB-102 content of plasma samples with minor hemolysis prepared by direct dilution *vs.* by protein precipitation.

Time dependence of MB-102 concentration in plasma

The terminal slope of a semi-log plot of concentration as a function of time is proportional to the GFR. Fig. 11 compares the results of such a plot between MB-102 (determined using the direct dilution methodology) and iohexol (determined from the standard protein precipitation methodology) in two subjects from our last clinical study. Subject 1 has nominally normal renal function, so the agents are excreted from the body rather

Table 3 MB-102 of human plasma with minor hemolysis

Subject	Time (min)	MB-102 by direct dilution at the contract lab (μg mL^{-1})	MB-102 by protein precipitation at MediBeacon (μg mL^{-1})
2	5	7.010	7.386
2	30	6.290	6.541
2	60	4.840	4.853
2	90	3.500	3.719
2	180	1.690	1.777
6	720	0.062	0.061
8	720	0.133	0.137
11	60	4.300	4.410
13	5	9.390	9.731
15	240	0.854	0.903
28	600	0.487	0.463
30	60	6.380	6.450
30	120	5.870	5.637
35	360	0.475	0.488
38	10	8.290	8.570
39	60	7.820	7.590
40	300	1.410	1.384
40	480	0.574	0.587
40	600	0.318	0.312
40	720	0.195	0.191
41	600	2.160	2.164
42	30	8.010	8.081
42	60	5.610	5.861
42	90	4.590	4.841
42	240	1.670	1.678
43	600	0.436	0.437
43	720	0.247	0.259
49	240	4.500	4.404
51	480	2.630	2.770
52	600	1.970	2.014

Table 4 MB-102 and iohexol in human plasma of subject 39 and subject 1

Subject 39			Subject 1		
Time (min)	MB-102/DD-C ($\mu g\ mL^{-1}$)	Iohexol ($\mu g\ mL^{-1}$)	Time (min)	MB-102/DD-C ($\mu g\ mL^{-1}$)	Iohexol ($\mu g\ mL^{-1}$)
5	8.540	97.7	5	10.300	167.000
10	8.750	162	10	8.820	222.000
15	9.630	184	15	7.410	202.000
30	7.950	151	30	5.660	158.000
60	7.820	133	60	3.900	101.000
90	6.140	116	90	2.770	71.300
120	5.710	105	120	2.160	58.000
180	5.350	96.3	180	1.310	38.900
240	4.950	91.4	240	0.885	25.100
300	4.480	84.4	300	0.634	18.300
360	3.990	77.9	360	0.403	12.400
480	3.550	66.9	480	0.193	6.760
600	3.020	61.9	600	0.093	3.260
720	2.650	55.7	720	0.057	1.980

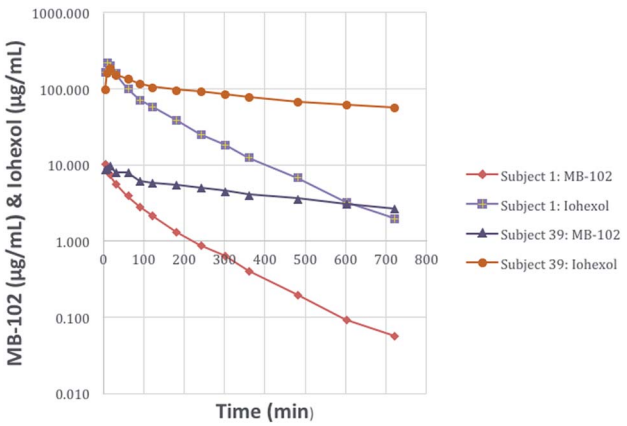

Fig. 11 Concentration *vs.* time for MB-102 and iohexol from subject 1 having normal renal function and subject 39 having Stage 4 chronic kidney disease (Table 4).

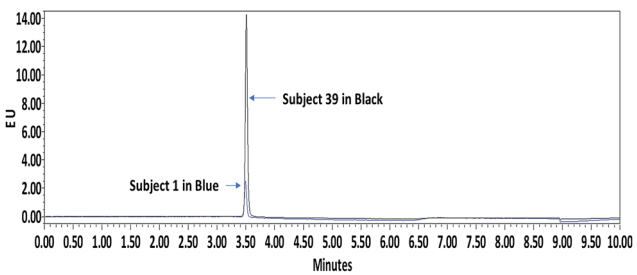

Fig. 12 HPLC chromatograms of subject 1 and subject 39 at 360 min post injection.

quickly. Subject 39 has Stage 4 chronic kidney disease, so the agents are excreted from the body rather slowly. In both subjects, the slope of the terminal phase was the same for both MB-102 time-dependent concentration and iohexol time-dependent concentration. Hence, the MB-102 determined GFR, using the direct dilution methodology to determine concentration, matched that of the known standard iohexol. The HPLC chromatogram comparison of subject 1 and subject 39 at 360 min post injection is illustrated in Fig. 12.

Conclusions

The new sample preparation and HPLC method reported herein using 100-fold direct dilution of plasma in 1× PBS for the measurement of MB-102 concentration is precise, accurate, and equivalent to the revised method of protein precipitation within experimental error. The determination of MB-102 concentration in plasma samples by the direct dilution method is also robust, less labor-intensive, and faster than the protein precipitation method. This new method enables rapid determination of the measured GFR using the exogenous tracer agent MB-102 in clinical trials, and offers great promise for more efficiency in diagnosing and monitoring kidney function.

Conflicts of interest

All authors are employed by MediBeacon Inc.

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