Robotic Inhibition Assay for Determination of HMG-CoA Reductase Inhibitors in Human Plasma

Wei Fang,* Lida Liu, John Y.-K. Hsieh, Jamie Zhao, Bogdan K. Matuszewski, John D. Rogers, and Michael R. Dobrinska

Drug Metabolism Department, Merck Research Laboratories, West Point, Pennsylvania

The cholesterol-lowering drug simvastatin (SIMV, Zocor[®]) reduced heart attacks by 42% in patients who had high cholesterol levels and suffered from heart disease. Upon oral administration, SIMV is quickly hydrolyzed to its β -hydroxyacid and other acid metabolites, which are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. A Tecan-based enzyme inhibition assay has been developed to improve the existing Zymark-based assay for the determination of both active and total concentrations of HMG-CoA reductase inhibitors in human plasma. A Tecan Genesis 200 robotic workstation equipped with eight probes and customized hardware was utilized to achieve higher sample throughput and improve assay reproducibility and mechanical stability.

The developed enzyme inhibition assay was validated over two concentration ranges of 0.4–20 ng equivalent/mL, and 2–50 ng equivalent/mL. Intra- and interday precision data (coefficient of variation (CV)) for both concentration ranges were less than 9%, with an accuracy of 93–107%. The interday precision for the determination of quality control (QC) samples was less than 2% and 8%, respectively. The respective interday QC accuracy values were 93–103% and 97–104%. Good linearity across the two concentration ranges was observed, with acceptable reproducibility. This improved enzyme inhibition assay has been utilized to analyze human plasma samples from several clinical studies. *J. Clin. Lab. Anal.* 16:209–215, 2002. © 2002 Wiley-Liss, Inc.

Key words: Robotic Inhibition Assay; HMG-CoA reductase inhibitors; cholesterol-lowering drug

INTRODUCTION

The cholesterol-lowering drug simvastatin (SIMV, Zocor[®]) has been proven to help save lives by reducing coronary mortality, and to help prevent heart attacks and strokes (1–7). More than 50% of the cholesterol present in the human body originates from body biosynthesis. To lower cholesterol levels, its biosynthesis must be reduced. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a key rate-limiting enzyme in the cholesterol biosynthetic pathway. SIMV, itself an inactive precursor, is rapidly hydrolyzed after oral administration to its β -hydroxyacid metabolite, which is a potent inhibitor of HMG-CoA reductase. In addition, SIMV and its β -hydroxyacid metabolite are metabolized to several other active metabolites and inactive lactone metabolites. Therefore, the enzyme inhibition assay was designed to measure the composite of all active species in plasma (active inhibitors) as well as the sum of active species plus their corresponding lactone counterparts (inactive inhibitors), measured following base-hydrolysis of plasma sample (8,9).

A manual enzyme inhibition assay has been used to determine the concentration of HMG-CoA reductase inhibitors in human plasma (1). However, the manual enzyme inhibition assay is very difficult to perform reproducibly and reliably because of the multi-liquid transfer steps required and imprecise incubation-time control. Over the last 14 years, a single-tip Zymark robot has been used to perform the enzyme inhibition assay in our laboratories (10,11). Although this robot relieves the analysts from performing some tedious, repetitive, and time-consuming tasks, some drawbacks, such as low sample throughput, frequent analyst intervention, and mechanical instability, still exist. Recently, a 96-well plate technology was introduced

*Correspondence to: Wei Fang, Drug Metabolism Department, Merck Research Laboratories, Sumneytown Pike, 26-372, West Point, PA 19486.

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successfully into the field of bioanalysis. A Tecan Genesis 200 (Tecan US, Research Triangle Park, NC) robotic workstation equipped with eight probes, along with 96-well plates and some customized hardware, was utilized to improve the speed and reliability of the enzyme inhibition assay. As a result, not only was the sample throughput increased fourfold, but the reproducibility and mechanical stability were also greatly improved. Full details of this Tecan-based assay are presented herein.

EXPERIMENTAL

Chemicals and Reagents

Simvastatin acid ammonium salt (I; Fig. 1) and human HMG-CoA reductase (partially purified) were obtained from Merck Research Laboratories (West Point, PA). Human HMG-CoA reductase (6.44 mg/mL, specific activity 2.4 mmole/min/mg) was stored at -70°C as 10- μL aliquots. ^{14}C -HMG-CoA (20 $\mu\text{Ci/mL}$) was purchased from New England Nuclear (Boston, MA). Acetonitrile (HPLC grade), potassium hydroxide (KOH, certified ACS grade), phosphoric acid (H_3PO_4 , concentrated, HPLC grade), potassium hydrogen phosphate (K_2HPO_4 , certified ACS grade), hydrochloric acid (HCl, reagent ACS grade), and potassium dihydrogen phosphate (KH_2PO_4 , HPLC grade) were purchased from Fisher Scientific (Springfield, NJ). Water (high purity for HPLC, GC) was obtained from Baxter (Franklin Lakes, NJ). Dithiothreitol (DTT), and NADPH (1 mg/vial) were commercially available from Sigma (St. Louis, MO). Triton X-100 (high purity, 10% aqueous) and AG 1-X 8 anion exchange resin (100–200

mesh, formate form) were purchased from Pierce (Rockford, IL) and Bio-Rad (Hercules, CA), respectively. Human control plasma (drug free) was purchased from Biological Specialty (Colmar, PA).

All 96-well collection plates (0.5, 1.2, and 2.2 mL) and removable cartridges (20 μm PE frit) were purchased from either Orochem Technologies (Westmont, IL) or Matrix Technologies (Hudson, NH). Polypropylene test tubes (7 mL, 13×75 mm; 15 mL, conical) and mini scintillation vials (6 mL) were purchased from Sarstedt (Newton, NC). Cryotubes (3.6 mL) were purchased from Nalge Nunc (Rochester, NY).

Instrumentation

A Tecan Genesis 200 robotic workstation equipped with eight probes was utilized for sample preparation and enzyme inhibition. A Gilson ASPEC-XL4 (Middleton, WI) with modified racks was used for scintillation cocktail addition. Liquid scintillation counters (LS 6000 and 6500; Beckman, Fullerton, CA) were used for measuring sample radioactivity. Beckman Allegra 6KR and Qiagen 4-15C (Qiagen, Valencia, CA) centrifuges were used for centrifuging the test tubes and 96-well plates, respectively. SPE Dry 96 (Jones Chromatography, Lakewood, CO) was used to evaporate solvent inside the 96-well plates.

Sample Preparation

The plasma samples were thawed at room temperature, vortex-mixed vigorously for 30 sec on a vortex mixer, and centrifuged for 10 min at 3,000 rpm ($2,500 \times g$). The samples were then loaded onto the

Fig. 1. Total and active inhibitors produced by in vivo metabolism and chemical hydrolysis of HMG-CoA inhibitor (SIMV).

Fig. 2. Assay quantitation principle.

sample racks of the Tecan's platform, and the Tecan Genesis 200 system performed the following tasks:

1. Pipet 25 μL of 0.25 M acetic acid into each well of the 2.2-mL 96-well plate.
2. Aliquot 250 μL of plasma sample and pipet into each well of the plate.
3. Add 500 μL of acetonitrile to each well of the plate. Seal the plate manually with the lid, vortex-mix for 1 min, and then centrifuge for 20 min at 3,000 rpm ($2,500 \times g$).
4. Aliquot 300 μL of the supernatant and pipet into each well of the 1.2-mL 96-well plate labeled as "active or A," with each well containing 10 μL of 1 M phosphate buffer (pH 7.4). Or, aliquot 300 μL of the supernatant and pipet into each well of the 1.2-mL 96-well plate labeled as "total or T," with each well containing 10 μL of 0.5 N potassium hydroxide. Seal the plate manually with the lid, vortex-mix, and centrifuge the plate for 5 min at 3,000 rpm ($2,500 \times g$).
5. Add 10 μL of 0.25 N H_3PO_4 to each well of the 1.2 mL plate and let it sit for 15 min at room temperature.
6. Place both "active" and "total" plates containing plasma extract under the heated (37°C) N_2 of SPE Dry 96.
7. Add 200 μL of Triton-500 into each well of both the "active" and "total" plates. Seal the plate manually with the lid, sonicate for 5 min, vortex-mix for another 5 min, and then centrifuge for 20 min at 3,000 rpm ($2,500 \times g$).

Enzyme Inhibition Assay

Before the start of the enzyme inhibition assay, 30 μL of 3.0 N HCl was added to the first two wells of the "active" and "total" plates for blank samples. The Tecan Genesis 200 system executed the following tasks:

1. Pipet 40 μL of plasma extract from the sample plate to the 0.5-mL 96-well plate situated on top of the heating block at 37°C .
2. Add 30 μL of enzyme solution to each well and incubate for 27 min.
3. Add 30 μL of substrate diluent to each well and incubate for 27 min.
4. Add 30 μL of 3.0 N HCl to each well and incubate for 27 min. (At the end of this step, the generated ^{14}C -mevalonic acid (Fig. 2) is lactonized.)
5. Transfer 100 μL of the incubation solution into each removable cartridge packed with 0.8 mL of anion exchange resin suspension.
6. Add 0.4 mL $\times$ 2 of Triton-10,000 and collect the eluent into a 6-mL scintillation vial.

Principle of the Enzyme Inhibition Assay

Plasma HMG-CoA reductase inhibitors (simvastatin acid (I) and its acid metabolites) were indirectly determined by the activities of the HMG-CoA reductase. The activities of the HMG-CoA reductase were measured by incubating the enzyme with ^{14}C -labeled

substrate (^{14}C -HMG-CoA) and NADPH for a fixed length of time. The product, ^{14}C -mevalonic acid (Fig. 2), was then lactonized and separated from excess substrate using an anion exchange cartridge, followed by counting in a liquid scintillation counter. The log concentrations of inhibitors have a linear relationship with the activities (%) of the enzyme. The percent activities of the enzyme are expressed as the ratios of the counts of ^{14}C -mevalonic lactone obtained from all the inhibitors involved to the counts obtained for human control plasma. To determine simvastatin (the lactone form) or its lactone metabolites, the plasma extracts have to be hydrolyzed first at room temperature, followed by incubation with HMG-CoA reductase.

Precision, Linearity, Accuracy, and Selectivity

The precision of the assay was determined by replicate analyses ($n=5$) of human control human plasma containing I at all the concentrations utilized for constructing the calibration curves. All results are expressed as ng equivalents of the free acid form of SIMV per mL of plasma. The linearity of each standard curve was established by plotting percent activities of HMG-CoA reductase against the log concentrations of I. The percent activities of the reductase at each concentration were calculated as counts per minute (cpm) of ^{14}C -mevalonic lactone obtained at that concentration divided by the cpm obtained from human control plasma. The unknown sample concentrations were calculated from the linear regression of the standard curves. The accuracy of the method was

expressed as $[(\text{mean analyzed concentration})/(\text{nominal concentration})] \times 100$. Assay selectivity was assessed by analyzing the blank control and the subjects' pre-dose plasma samples.

RESULTS

System Performance Validation

The system layout of the Tecan Genesis 200 robotic workstation is shown in Fig. 3. Liquid transfer performance from each of the eight probes was verified gravimetrically. Eight replicate aliquots of 10, 25, 40, and 100 μL of deionized water were transferred from reagent trough to a 96-well plate. Each plate contained 12 removable strips with eight wells on each strip. Each aliquot was pipetted into a well from each strip. Each strip was weighed before and after liquid transfer. Table 1 shows good precision (coefficient of variation (CV) $<3.2\%$) and acceptable accuracy (87–101%) of transferring liquid volume from 10 μL to 100 μL by the Tecan Genesis 200 system.

Optimization of Resin Volume and Elution Volume

An experiment was conducted to ensure full retention of the analyte on the anion exchange resin cartridge by first loading 100 μL of a mixture consisting of 23% ^{14}C -labeled substrate, 54% Triton-500, and 23% 3N HCl onto each removable cartridge and then eluting with different volumes (0.1–0.4 mL) of Triton-10,000. No breakthrough of the analyte was observed when >0.3 mL of anion exchange resin was packed into each

Fig. 3. Tecan Genesis 200 layout for enzyme inhibition assay.

cartridge. Based on these experiments, 0.4 mL of resin volume was chosen for analyte retention throughout the assay validation and application. The volume required for analyte elution was also investigated. The highest and most reproducible recovery was observed with the use of 2×0.9 mL of Triton-10,000 solution.

Assay Validation

The intraday precision (defined as the CV), with five replicates at all the concentrations on two different standard curve lines, was less than 9% and the accuracy was within 96–106% of the nominal concentration (Table 2). Good linearity was obtained for both standard curve lines (0.4–20 ng/mL and 2–50 ng/mL).

Interday stability data were obtained by analyzing quality control (QC) samples containing 2, 8, and 16 ng equivalent (low standard curve) and 8, 20, and 40 ng equivalent (high standard curve) added to 1 mL of human control plasma. Two sets of QC samples at each concentration were prepared and assayed on 3 consecutive days. All measured concentrations were within 7% of the nominal values, with precision better than or equal to 7.5% (Table 3).

TABLE 1. Tecan Genesis 200 system performance data

Water transfer volume (μ L)	Accuracy ^a (%)	Precision ^b C.V. (%)
10	87.2	3.2
25	94.5	2.3
40	97.6	1.5
100	100.8	0.9

^aAccuracy is expressed as $[(\text{mean weight}/d_{\text{H}_2\text{O}})/(\text{nominal volume})] \times 100$, $d_{\text{H}_2\text{O}}$: density in g/cm^3 .

^b $n = 24$.

Application and Comparison

Plasma samples collected at 0.5–24 hr postdose from two subjects were analyzed using both Tecan-based and Zymark-based assays on the same day in order to compare the results generated by the two different procedures. A good agreement was observed between the two assays (Fig. 4).

DISCUSSION

A summary of the comparison of the two assay procedures is presented in Table 4. At the beginning of the sample preparation step, all the plasma samples were required to be homogeneous before aliquoting and analysis. In the Zymark-based assay, all the sample tubes were first loaded into a sample rack for vortex-mixing, and then loaded individually into centrifuge buckets. After centrifugation, each sample tube was put back into the sample rack. This procedure required all the sample tubes to be handled individually and manually, and to be carefully labeled. However, in the Tecan-based assay, customized sample racks were used. Each rack holds 16 samples in a 4×4 format, and fits into a Beckman centrifuge bucket. An efficient and more organized way of processing a large number of sample tubes was achieved using the customized sample racks, as there was no need to transfer and label the sample tubes.

The Tecan Genesis 200 workstation equipped with eight probes replaced the Zymark robot equipped with a single tip for all liquid transfers. Not only was the speed of sample transfers and reagent additions increased by 400%, but mechanical reliability was improved in the Tecan-based assay as well.

During the protein precipitation and enzyme incubation steps, the Tecan-based assay takes advantage of the 96-well plate and eight-probe pipetting technologies, achieving higher speed of sample processing without requiring the labeling of all sample containers.

TABLE 2. Intraday precision and accuracy data for the determination of active inhibitor in human plasma

Low Curve (0.4–20 ng equivalent/mL)			High Curve (2–50 ng equivalent/mL)		
Nominal concentration (ng equivalent /mL)	Accuracy ^a (%)	Precision ^b CV (%)	Nominal concentration (ng equivalent/mL)	Accuracy ^a (%)	Precision ^b CV (%)
0.4	99.5	4.5	2	104.8	8.1
1	96.6	4.2	5	95.7	5.1
5	95.6	4.7	10	96.2	8.8
10	105.3	3.1	25	106.4	5.0
20	97.9	4.0	50	98.7	1.5

^aAccuracy is expressed as $[(\text{mean analyzed concentration})/(\text{nominal concentration})] \times 100$.

^b $n = 5$.

TABLE 3. Interday QC precision and accuracy data for the determination of active inhibitor in human plasma

Run no.	Low curve (0.4–20 ng equivalent/mL)			High Curve (2–50 ng equivalent/mL)		
	LQC 2 ng eq./mL	MQC 8 ng eq./mL	HQC 16 ng eq./mL	LQC 8 ng eq./mL	MQC 20 ng eq./mL	HQC 40 ng eq./mL
Day 1	1.82	8.00	16.3	7.78	21.0	41.2
Day 2	1.90	8.35	16.7	8.55	21.3	41.0
Day 3	1.85	8.24	16.4	7.14	20.1	41.9
Mean	1.86	8.20	16.5	7.82	20.8	41.4
Precision ^a C.V. (%)	1.8	1.8	1.1	7.4	2.3	0.9
Accuracy ^b (%)	93.0	102.5	102.9	97.8	103.9	103.4

^an = 3.

^bAccuracy is expressed as [(mean analyzed concentration)/(nominal concentration)] × 100.

Fig. 4. Comparison of results generated by Tecan- and Zymark-based assays for the analysis of total inhibitors from two dosed subjects.

In the sample evaporation and concentration steps, the SPE 96 Dryer was used to replace the Zymark SpeedVac, resulting in a time-saving of more than 12 hr during the procedure.

After the isolation of the ¹⁴C-labeled mevalonic lactone product (Fig. 2), scintillation cocktail fluid was added to each vial. The customized capping device in the Tecan-based assay was designed to allow the capping of 44 scintillation vials at one time, as opposed to the manual capping required for each vial in the Zymark-based assay. In addition, the customized rotator and shaking devices replaced the manual shaking operation of the Zymark-based assay. The highly labor-intensive

sample preparation procedure employed in the Zymark-based assay was replaced with more simplified and efficient procedures.

Since the volumes of the anion exchange resin suspension and scintillation cocktail fluid used were cut from 1 mL to 0.4 mL, and 10 mL to 3.4 mL, respectively, in the Tecan-based method, elution solvent consumption and radioactive waste were also reduced.

Overall, the Tecan-based assay proved to be more efficient and reliable, with higher sample throughput, compared to the Zymark-based assay.

In conclusion, the Tecan Genesis 200 system has proved to be a reliable workstation for pipetting

TABLE 4. Comparison of the two robotic assay procedures

	Zymark-based assay procedure	Tecan-based assay procedure	Advantages of tecan-based assay
Sample transfer between containers	Tube to tube	Tube to 96-well plate	Higher speed
Reagent and supernatant transfer	Use 1 probe	Use 8 probes	Higher throughput
Sample preparation (mixing/dilution/centrifugation)	Handle each sample individually	Handle 16 samples at a time in an organized 4 × 4 rack	High throughput, less labeling and human error
Evaporation time	Overnight	1 h	Great time saving
Anion exchange resin volume	1 mL	0.4 mL	Less resin consumption
Scintillation cocktail volume	10 mL	3.4 mL	Reduced radioactive waste disposal
Scintillation vial capping	Individual and manual capping	Multi-vial (44 vials) capping	Less labor
Scintillation cocktail mixing	Manual shaking	Mechanical shaking	Less labor

reagents and performing plasma sample preparation and enzyme incubation steps. Using this system, an improved enzyme inhibition assay was validated over two concentration ranges of 0.4–20 ng equivalent/mL and 2–50 ng equivalent/mL for active and total HMG-CoA reductase inhibitors in human plasma. These assays have been successfully applied in several clinical studies. Higher sample throughput with a less labor-intensive sample preparation procedure was achieved by use of the Tecan Genesis 200 system equipped with some customized hardware and 96-well plate technology.

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