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Determination of simvastatin-derived HMG-CoA reductase inhibitors in biomatrices using an automated enzyme inhibition assay with radioactivity detection

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Abstract

A robust, automated enzyme inhibition assay method was developed and validated for the determination of HMG-CoA reductase inhibitory activities in plasma and urine samples following simvastatin (SV) administration. The assay was performed on Tecan Genesis 150 and 200 systems equipped with 8-probe and 96-well plates. Plasma samples containing HMG-CoA reductase inhibitors were treated with acetonitrile for protein precipitation before being incubated with HMG-CoA reductase, [^{14}C]-HMG-CoA, and NADPH for a fixed length of time at a fixed temperature. The product, [^{14}C]-mevalonic acid, was lactonized and separated from excess substrate via a small ion exchange resin column, and radioactivity was counted on a scintillation counter. HMG-CoA reductase inhibitors were measured before and after base hydrolysis. The two values obtained for each sample are referred to as 'active' and 'total' HMG-CoA reductase inhibitor concentrations. Simvastatin acid (SVA), the β-hydroxy acid of SV, was used as a standard to generate a calibration curve of HMG-CoA reductase activity versus SVA concentration (ng/ml). Three calibration ranges, 0.4–20, 2–50, and 5–100 ng/ml, in human and animal plasma and urine were validated. The assay precision was less than 8.5% CV in plasma and less than 10.4% in urine. The assay accuracy was 93.6–103.0 and 98.1–103.9% for the 0.4–20 and 2–50 ng/ml calibration ranges, respectively, in human plasma, and was 97.3–105.1, 94.4–105.2, and 90.2–95.7% for calibration range 5–100 ng/ml in rat plasma, dog plasma and human urine, respectively.

Keywords: Simvastatin; Hypercholesterolemia; HMG-CoA reductase inhibitors; Enzyme inhibition assay; Automation

1. Introduction

1.1. Background

The relationship between hypercholesterolemia and coronary artery disease is well established. In humans, biosynthesis of cholesterol plays an im-

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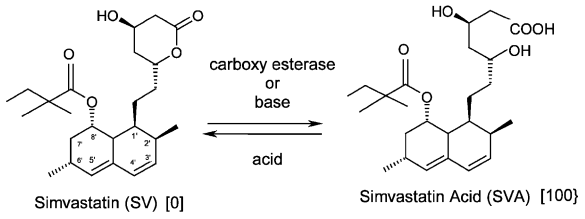


Fig. 1. Structure of simvastatin (SV) and simvastatin acid (SVA).

portant role in triggering the onset of hypercholesterolemia, and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in this bioprocess. Following oral administration, simvastatin (SV, Fig. 1) [1], an inactive precursor, is hydrolyzed in vivo to the corresponding β-hydroxy acid (simvastatin acid (SVA), Fig. 1). The latter is a potent inhibitor of HMG-CoA reductase, and thus, of de novo cholesterol synthesis. In hypercholesterolemic patients, SV is generally well tolerated and causes substantial reduction in serum concentrations of low-density lipoprotein cholesterol [2–5].

In vitro and in vivo metabolism of SV in both animals and humans has been investigated [5–8]. The metabolic profile of SV shows a very complex scheme with several of the open-acid form metabolites possessing HMG-CoA reductase inhibitory potency. Specific assay methods measuring SV and SVA in biological samples using HPLC, GC/MS and LC/MS/MS have been reported [9–17]. Attempts to measure other major active metabolites by these methods have not been successful because the synthetic metabolites are unstable. The enzyme inhibition assay, which measures overall inhibitory activity from all active and potentially active metabolites, is therefore, a preferred assay method for pharmacokinetic, bioavailability, and drug interaction studies. The very early version of this method for assay of human and animal plasma was performed manually [18]. Later, a semi-automated method was developed using a combination of Tecan model RSP 5051 and Zymark XP automation systems [19] and used to provide bioanalytical support for drug development of HMG-CoA reductase inhibitors in Merck Research Laboratories. These early robotic systems were equipped with a single probe, and a maximum of 40 samples including the standard and quality control samples could be analyzed per analytical run. Assays with much higher sample throughput became possible recently thanks to the advances in multi-probe robotics and 96-well plate sample handling format. Automated assay procedures were recently developed on 8-probe Tecan Genesis robotics. The procedures have significantly increased the sample throughput for this multiple-step, lengthy assay method. A brief description of the early stage instrument set-up for the automated assay procedure has been reported [20]. In this paper, we describe the details of the development, optimization, validation and application of this Tecan Genesis-based high throughput assay method for the quantitation of SV-derived HMG-CoA reductase inhibitors in various biomatrices including human, dog, and rat plasma, as well as human urine.

1.2. Principle of the assay

The basis of this assay procedure is the inhibition of HMG-CoA reductase by SVA and other active metabolites of SV. In this assay, if no inhibitors are present, ^{14}C-HMG-CoA is converted to ^{14}C-mevalonic acid and then to ^{14}C-mevalonolactone after lactonization. An increase in the concentration of the inhibitory species will, therefore, result in a decrease in the amount of ^{14}C-mevalonolactone detected. The concentration of HMG-CoA reductase inhibitors in study samples can be determined by interpolation from a standard curve with the HMG-CoA reductase activity (in percent) plotted against the log of the concentration of SVA. Reductase activity (measured in percent) at each spiked inhibitor concentration was calculated as counts per minute (cpm) ^{14}C-mevalonolactone obtained at that concentration divided by the cpm obtained for a plasma blank. The sample concentrations are expressed as SVA-equivalent concentrations. Concentrations of HMG-CoA reductase inhibitors in the biomatrices are measured before and after base hydrolysis. The two values obtained for each sample are referred to as ‘active’ and ‘total’ HMG-CoA reductase

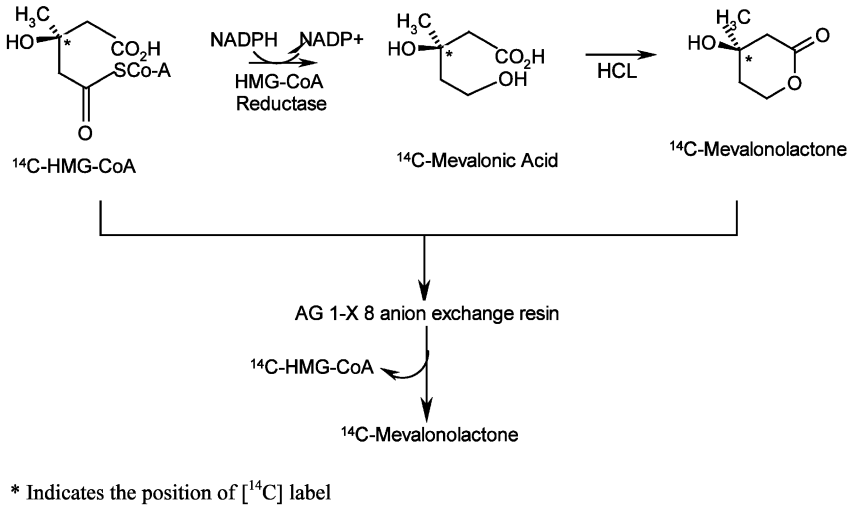


Fig. 2. Chemistry of HMG-CoA reductase assay.

inhibitor concentrations. Base hydrolysis ensures that any lactones present in the samples are converted to their corresponding hydroxy acids and, therefore, quantified in the assay if active. The difference between the active and total concentrations would be an indication of the quantity of lactone, or other hydrolyzable species, which are potential HMG-CoA reductase inhibitors present in the biomatrices. Hydrochloric acid was utilized to quench the assay and lactonize the product, mevalonic acid, to mevalonolactone, which elutes from an anion exchange resin column with aqueous solution and is isolated from the excess HMG-CoA substrate bound to the resin. Fig. 2 illustrates the chemistry of this assay.

2. Experimental

2.1. Instruments

Tecan Genesis RSP 150/8 and Genesis RSP 200/8 robotic systems from Tecan, US (Research Triangle Park, NC) were used for sample preparation and enzyme incubation, respectively. Liquid scintillation counters model LS-6500 from Beckman Coulter (Fullerton, CA) were used for the measurement of radioactivity of ^{14}C-labeled mevalonolactone, the final product of the assay. Centrifuge model 4-15C from Sigma (Osterode am Harz, Germany) and nitrogen solvent evaporators model SPE Dry-96 from Jones Chromatography (Lakewood, CO) were used during sample preparation. Phoenix robotic workstation from J-Kem Scientific, Inc. (St. Louis, MO) was used for transferring scintillation cocktail. Waterbath model Isotemp 20135 from Fisher Scientific Inc. (Pittsburgh, PA) was used to control the temperature during enzyme incubation.

2.2. Materials

SV, ^{14}C-SV, and SVA were synthesized at Merck Research Laboratories (Rahway, NJ). The purity of SVA, used as standard, was $>$ 99.6%. Human control plasma was purchased from Biological Specialty (Lansdale, PA). Human HMG-CoA reductase 2.4 μmole/mg per min (6.44 mg/ml) was partially purified at Merck Research Laboratories. DL-dithiothreitol (DTT), nicotinamide adenine dinucleotide phosphate reduced form (NADPH, sodium salt), and DL-3-hydroxy-3-methylglucotaryl coenzyme A (HMG-CoA. sodium salt) were obtained from Sigma Chemical (St. Louis, MO). Triton $\times$ 100 was purchased from Pierce Co. (Rockford, IL). Acetonitrile, monopotassium phosphate, di-potassium phosphate, potassium hydroxide, hydrochloric acid, and

Table 1
The composition of substrates in different calibration ranges

Calibration range (ng/ml)	[^{14}C]-HMG-CoA volume (μl)	HMG-CoA		H_2O volume (ml)
		Concentration (mg/ml)	Volume (μl)	
0.4–20	320	0.5	20	1.4
2–50	160	2.5	80	1.4
5–100	320	0.5	20	1.4

water, (all HPLC grade), were purchased from Fisher Scientific. AG 1 × 8 anion exchange resin, formate form was from Bio-Red (Hercules, CA). Acetic acid (glacial) was purchased from Mallinckrodt (Paris, KY). DL-3-[glutaryl-3-^{14}C]-hydroxyl-3-methylglutaryl Coenzyme A, (57.6 mCi/mmol) was purchased from Perkin Elmer Life Science Product, Inc. (Boston, MA). Scintillation cocktail was obtained from Beckman Instrument. 96-Well plates, 1.2, 0.5-ml, their lids, and 2.25-ml well plate with 20 μm frit were purchased from Orochem Co. (Westmont, IL); 2.2-ml was purchased from Matrix Co. (Hudson, NH). Liquid scintillation vial (6-ml) was purchased from Sarstedt, Inc (Newton, NC).

2.3. Preparation of reagents

All of the reagents were prepared with Triton (a surface activating reagent) to ensure complete transfer of liquids.

Enzyme stock solution was prepared by dilution of 10 μl of partially purified human HMG-CoA reductase to 16 ml with enzyme diluent consisting of NADPH (0.25 M), potassium phosphate buffer (pH 7.4; 0.26 M), DTT (20 mM), and Triton (0.4%). The enzyme stock solution was stored at −70 °C in appropriate aliquots: ∼350 μl for the calibration range 2–50 ng/ml, and ∼120 μl for the calibration ranges 0.4–20 and 5–100 ng/ml. Each aliquot of the enzyme stock solution in cryovials was further diluted with 5.6 ml of enzyme diluent to prepare enzyme working solution before use.

A [^{14}C] labeled HMG-CoA substrate stock solution was prepared by mixing HMG-CoA, [^{14}C]-HMG-CoA and water and stored as aliquots at −70 °C. Table 1 lists the volumes and concentrations of the components in each aliquot for various calibration ranges. For daily assay, each aliquot of substrate stock solution was diluted with 4 ml of substrate diluent consisting of NADPH (0.25 M), potassium phosphate buffer (pH 7.4; 0.26 M), and Triton (0.4%) to make substrate working solution before use.

Hydrochloric acid (3 N) was made by mixing hydrochloric acid (6 N) with equal volume of Triton (× 500) aqueous solution (1:1, v/v) before the assay.

Anion exchange columns were prepared by wetting each of the 2.25-ml well (20 μm frits) with methanol and water first, then 0.8 ml of a slurry of AG 1 × 8 anion exchange was added to make the final 0.4 ml of anion exchange resin package. Slurry of AG 1 × 8 anion exchange resin was a mixture of resin–Triton solution (× 10 000) (1:1, v/v).

2.4. Preparation of calibration standards and quality control samples

A 1-mg/ml (all concentration values are expressed as free-acid equivalent of SVA) standard of stock solution of SVA was prepared by dissolving an accurately weighed amount of SVA sodium salt in appropriate volume of acetonitrile–water (60:40, v/v) solvent. A separate 1 mg/ml SVA solution in acetonitrile–water (60:40, v/v) was prepared for use as the quality control (QC) stock solution. This 1 mg/ml standard stock solution was diluted with the same solvent to obtain 10, 5, 2.5, 1.0, 0.5 and 0.2 μg/ml SVA working solutions. These working solutions were used to prepare calibration standards at concentrations 50, 25, 10, 5 and 2 ng/ml of SVA in human plasma for the 2–50 ng/ml calibration range. QC working solutions at concentrations of 4, 2 and 0.8

μg/ml were subsequently prepared which were used to make quality control samples at concentrations of 40, 20 and 8 ng/ml of SVA in human plasma.

To prepare human plasma standards for 0.4–20 ng/ml calibration range, 2, 1, 0.5, 0.1, and 0.04 μg/ml standard working solutions were used to prepare plasma calibration standards at concentrations 20, 10, 5, 1 and 0.4 ng/ml of SVA; QC working solutions at concentrations 1.6, 0.8 and 0.2 μg/ml were prepared and used to make plasma QC samples at concentrations 16, 8 and 2 ng/ml.

For calibration range 5–100 ng/ml used for animal plasma or human urine, 10, 5, 2.5, 1, and 0.5 μg/ml standard working solutions were used to prepare calibration standards at concentrations 100, 50, 25, 10 and 5 ng/ml of SVA in human urine and animal plasma; QC working solutions at concentrations 8, 4 and 2 μg/ml were prepared and used to make QC samples in human urine and animal plasma at concentrations 80, 40 and 20 ng/ml of SVA.

All of the stock and working solutions were stored at −20 °C and brought to room temperature before use. All the QC samples were stored as 1-ml aliquots at −70 °C freezer.

2.5. Sample preparation

Plasma samples containing HMG-CoA reductase inhibitors were treated with acetonitrile for protein precipitation. The extraction solutions of the plasma samples then underwent pH adjustment to a value of 7.4 or base hydrolysis for active or total inhibitory assay, respectively. Urine samples were mixed with Triton solution and phosphate buffer to dilute protein concentration and to adjust pH value to 7.4 first, then this solution was used for determination of active inhibitors after further dilution with Triton solution or underwent base hydrolysis for determination of total inhibitory activity. The sample preparation was completed on a Tecan Genesis RSP/150 robotic system with eight probes and with 96-well plate format (Fig. 3). GEMINI software was utilized to program all of the liquid transfer steps.

For the 0.4–20 and 2–50 ng/ml calibration ranges, to each of the 2.2-ml wells in a 96-well plate (plate# 2, Fig. 3), the following reagents were

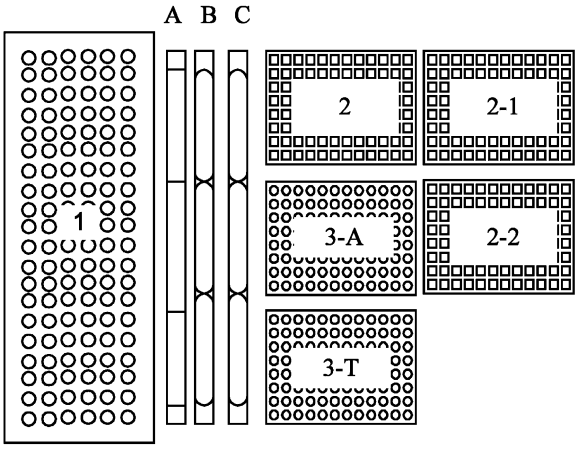


Fig. 3. Arrangement of racks on Tecan Genesis RSP 150/8 for sample preparation.

added sequentially: 25 μl of acetic acid (0.25 M), 250 μl of plasma sample (thawed and centrifuged for 10 min at 3000 rpm), and 500 μl of acetonitrile. The purpose of adding acetic acid is to adjust the pH value of the plasma sample to ∼7 to prevent the hydrolysis of SV and other lactone metabolites. The plate containing the sample mixture was sealed with a 2.2-ml plate lid, vortexed on a Multi Vortexer for 1 min, and centrifuged at 4000 rpm for 20 min. Two 300-μl aliquots of the supernatant were transferred into two separate 1.2-ml 96-well plates (plate# 3-A and 3-T, Fig. 3). The samples in plate# 3-A were treated with 10 μl of potassium phosphate buffer (1 M) for the determination of active inhibitory activity, the samples in Plate# 3-T were treated with base hydrolysis for the measurement of total inhibitory activity: 10 μl of potassium hydroxide (0.5 M) was added (pH should be ∼12) followed by incubation for 20 min; then 10 μl of phosphoric acid (0.25 M) was added to adjust the pH value back to 7.0. The supernatant in both plates was evaporated to dryness on a solvent evaporator SPE Dry-96 with nitrogen at 37 °C for about 40 min.

For the calibration range 5–100 ng/ml used for dog and rat plasma samples (150 μl volume size), the volumes of reagents were adjusted to: 15 μl of acetic acid (0.25 M) and 300 μl of acetonitrile. The supernatant thus obtained was allocated as 100 μl

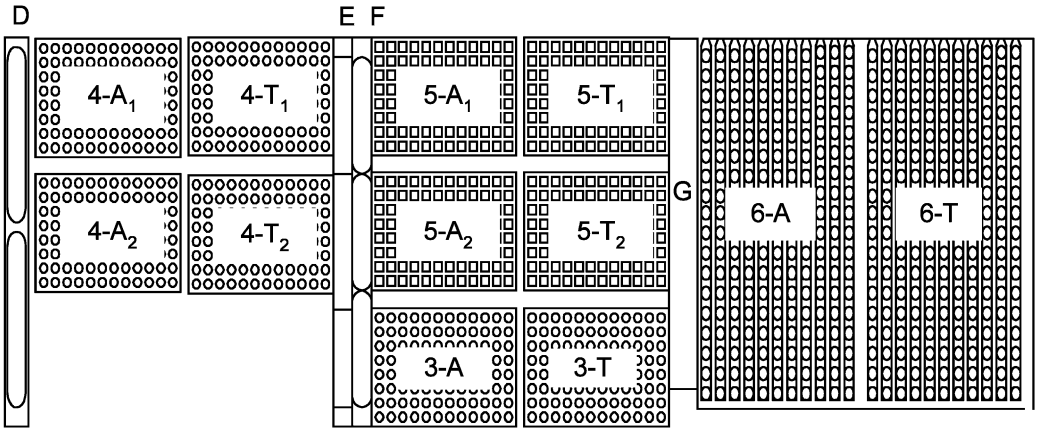


Fig. 4. Arrangement of racks on Tecan Genesis RSP 200/8 for enzyme inhibitory assay.

aliquots for each of the active and total inhibition assays.

For the calibration range 5–100 ng/ml for human urine, in plate# 2 (Fig. 3) 150 μl of thawed and centrifuged urine specimen was mixed with 150 μl of phosphate buffer (pH 7.4; 1 M) to adjust the pH value to ~7 and then dilute with 150 μl of Triton × 500 solution. For the active inhibitor assay, in plate# 3-A (Fig. 3), 50 μl of the treated urine sample was mixed with 20 μl of potassium phosphate buffer (pH 7.4; 1 M) and 230 μl of Triton × 500 to make a total volume of 300 μl; for the total inhibitor assay, in plate# 3-T (Fig. 3), 50 μl of the treated urine sample was hydrolyzed for 20 min with 20 μl of potassium hydroxide (0.5 M), then neutralized with 20 μl of 0.25 M phosphoric acid to pH 7.0, and finally mixed with 210 μl of Triton × 500 to make a total volume of 300 μl. These sample solutions were ready for enzyme assay directly without the need for solvent evaporation and reconstitution steps.

2.6. Enzyme incubation procedure

GEMINI software was utilized to program the liquid transfer for the enzyme assay. Fig. 4 shows the arrangement of racks aligned on Tecan Genesis RSP 200/8 robotic system. For the enzyme inhibition assay, the 'active' and 'total' plates (plate#3-A and plate#3-T in Fig. 3) containing the dried residue were placed onto the Tecan Genesis RSP 200/8 robotic system (Fig. 4). After the addition of Triton × 500 solution (200–300 μl expected for plasma samples to be assayed with calibration range 0.4–20 and 2–50 ng/ml; 600 μl expected for plasma samples to be assayed with calibration range 5–100 ng/ml), the plate# 3-A and 3-T were vortexed for 5 min for reconstitution and then centrifuged at 4000 rpm for 20 min. The purpose of centrifugation was to reduce the bubbles on the surface of solution caused by vortexing and also to prevent well-crossover caused by the drops of the solution on the sealing lid of the plates during vortexing. At this step the samples were ready for assay. For the active inhibitory activity assay, 40 μl of the sample solutions in plate# 3-A were duplicately transferred into plate# 4-A_1 and 4-A_2 (Fig. 4). For the total inhibitory activity assay, 40 μl of the sample solutions in plate# 3-T was transferred into plate# 4-T_1 and 4-T_2 (Fig. 4). The temperature of the plates was maintained at 37 °C using a water bath/circulator. The following reagents were added into each sample sequentially with a 30 min waiting time programmed by the software between each two consecutive additions: enzyme working solution 30 μl, substrate working solution 30 μl, and hydrochloric acid (3 N) 30 μl. After the addition of hydrochloric acid, the product of the assay, mevalonic acid, was lactonized to mevalonolactone. The assay solutions were then loaded onto 2.25 ml-well frit plates packed with 0.4 ml of AG

Table 2
Mean ($n = 5$) measured concentrations (%CV, %RE) of SV acid in biomatrices standards in human, dog, and rat during enzyme inhibition assay validation

Species/matrix	Calibration range (ng/ml)	Spiked SVA concentration (ng/ml)				
		Mean ($n = 5$) measured concentration (%CV, %RE)	Mean ($n = 5$) measured concentration (%CV, %RE)	Mean ($n = 5$) measured concentration (%CV, %RE)	Mean ($n = 5$) measured concentration (%CV, %RE)	Mean ($n = 5$) measured concentration (%CV, %RE)
Human/plasma	0.4–20	0.4	1	5	10	20
		0.40 (4.4, 0.6)	0.97 (4.2, −3.5)	4.83 (4.7, −3.5)	10.47 (3.1, 4.7)	19.36 (4.1, −3.2)
Human/plasma	2–50	2	5	10	25	50
		2.2 (6.0, 9.0)	4.6 (6.1, −8.0)	9.6 (4.0, −4.4)	26.7 (2.2, 6.9)	50.0 (3.0, 0)
Dog/plasma	5–100	5	10	25	50	100
		5.24 (7.8, 4.8)	9.66 (8.1, −3.4)	23.88 (4.7, −4.5)	52.66 (4.6, 5.3)	99.70 (2.8, 0.3)
Rat/plasma	5–100	5	10	25	50	100
		5.50 (9.1, 9.9)	9.48 (11.3, −5.2)	23.33 (2.8, −6.7)	50.06 (6.1, 0.1)	110.37 (5.3, 10.4)
Human/urine	5–100	5	10	25	50	100
		5.42 (5.7, 8.4)	9.58 (7.2, −4.2)	22.56 (7.1, −9.8)	52.28 (7.8, 4.6)	103.82 (5.7, 3.8)

n, Number of replicates; CV, coefficient of variation; %RE, percent relative error.

Table 3
Mean ($n = 15$) measured concentrations (%CV, %RE) of SV acid in biomatrices quality controls in human, dog, and rat during enzyme inhibition assay validation

Species/matrix	Calibration range (ng/ml)	Nominal SVA concentration (ng/ml)		
		Mean ($n = 15$) measured concentration (%CV, %RE)	Mean ($n = 15$) measured concentration (%CV, %RE)	Mean ($n = 15$) measured concentration (%CV, %RE)
Human/plasma	0.4–20	2 1.87 (5.8, −6.4)	8 8.16 (4.0, 2.0)	16 16.48 (3.0, 3.0)
Human/plasma	2–50	8 7.85 (8.5, −1.9)	20 20.78 (3.6, 3.9)	40 41.44 (2.0, 3.6)
Dog/plasma	5–100	20 18.89 (5.0, −5.6)	40 40.79 (7.2, 2.0)	80 84.13 (4.8, 5.2)
Rat/plasma	5–100	20 19.45 (5.1, −2.7)	40 40.61 (5.6, 1.5)	80 84.09 (5.5, 5.1)
Human/urine	5–100	20 18.05 (7.9, −9.8)	40 38.40 (9.4, −4.0)	80 76.59 (10.4, −4.3)

n, Number of replicates; CV, coefficient of variation; %RE, percent relative error.

1-X8 resin inside each well, and mounted on a 2.2 ml-well collection plate (5-A_1, 5-A_2, 5-T_1, and 5-T_2, Fig. 4). By washing the AG 1-X8 resin twice with 0.8 ml of Triton × 10 000, the mevalonolactone was eluted into the collection plate and then the solutions were transferred into the scintillation vials placed in the Beckman scintillation vial racks (6-A and 6-T, Fig. 4). The vial racks were then placed onto a Phoenix robotic workstation equipped with a 6-probe setup, and scintillation cocktail (3.4 ml) was added. The vials were then counted for radioactivity for 2 min on a Beckman scintillation counter. A control biomatrix sample was used as background (blank) which was processed along with standards and samples but the sequence of reagents addition was: hydrochloric acid, enzyme, and then substrate. A standard calibration curve was constructed by plotting enzyme activity versus log concentration of SVA (expressed as free acid). The enzyme activity was expressed as $A = (B_x - B_0)/(B - B_0)100\%$ where $A =$ activity in percent, $B_x =$ count (cpm) of the mevalonolactone produced with inhibitors, $B_0 =$ background count of the reagents and $B =$ counts of the mevalonolactone produced without presence of inhibitors. A program 'EIA-RIA' provided along with the scintillation counter by Beckman Coulter was utilized to calculate the HMG-CoA reductase inhibitor concentrations in calibration standards, QC samples and study samples. Each sample was assayed in duplicate and the inhibitor concentration of the sample was expressed as the average of the two values.

2.7. Validation procedures

The intra-assay precision and accuracy of the method were determined by analyzing five replicates of plasma or urine standards at all concentrations to construct the calibration curve. Three calibration ranges were validated. The initial inter-day precision and accuracy were determined by analyzing five replicates of the quality control samples for three consecutive assays running at concentrations 2, 8, and 16 ng/ml for calibration range 0.4–20 ng/ml; 8, 20, and 40 ng/ml for calibration range 2–50 ng/ml; 20, 40, and 80 ng/ml for calibration range 5–100 ng/ml. The accuracy was calculated by (mean observed concentration)/(spiked concentration) × 100%, and the precision was calculated by the coefficient of variation (%CV = 100% × S.D./mean). Tables 2 and 3 list the initial intra-day and inter-day assay results.

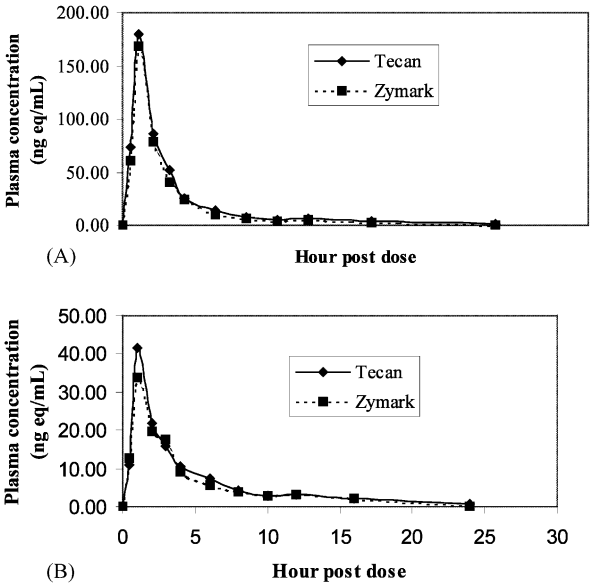


Fig. 5. Plasma concentration of total (A) and active (B) HMG-CoA reductase inhibitors in a healthy subject receiving 80-mg single dose of SV.

3. Results and discussion

The method has been successfully applied to determine SV-derived active and total HMG-CoA reductase inhibitory activities in human plasma and urine, and animal plasma. Comparing with the Zymark based protocol [19], this method increased throughput by four times, and decreased the radioactive hazard waste by 40%. A cross validation between Zymark-based and Tecan-based assay methods was performed by assaying a group of human plasma samples collected from randomly selected subject receiving oral dose of 80-mg SV. As showed in Fig. 5, the two sets of results generally showed very good match and the deviations between the two sets of values were well within our acceptance criterion ($< 20\%$).

During the course of the development of this Tecan Genesis-based automatic method, the instrument set-up and calibration, the selection of proper syringe size and probe was needed to achieve better performance. The washing parameters for cleaning the fixed probes were evaluated by a carryover test for the reagents and analytes separately. To make this enzyme inhibition assay suitable with the 96-well plate format, the volumes of ion exchange resin and eluting solution were re-evaluated. The concentration of enzyme working solution was also re-adjusted. Because of the narrow linear range in this HMG-CoA reductase inhibition assay, samples with expected inhibitor concentrations above the upper limit of calibration range were pre-diluted and the dilution parameters have been optimized. In addition, the -70 °C storage stability of HMG-CoA reductase inhibitors in plasma, the specificity of the assay, and the analyte recovery from the protein precipitation were also evaluated.

3.1. Instrument set-up and calibration

In the early development stages of the enzyme inhibition assay [20], a single Tecan Genesis RSP 200/8 was used to perform both the protein precipitation and the enzyme incubation portions of the assay. Due to the small internal diameter of the probes used with the robotic workstation (to ensure the accuracy and precision for the small volume pipetting required by the enzyme incubation), in routine daily assay it was observed that the probe tips on the Tecan occasionally became clogged by un-precipitated suspensions in plasma samples during protein precipitation step. In the present set-up, the protein precipitation and the enzyme incubation steps are performed on two separate Tecan robotic systems. This allowed the probes with a larger internal diameter to be used for plasma sample transferring during protein precipitation, thus overcoming the probe-clogging problem. The additional space created by using two separate robotic system workstations also increased throughput by 10% and allowed for easier usage by the analysts.

The precision and accuracy of liquid transfer by Tecan Genesis robotics were measured by the gravimetric method. The liquid handling parameters were set according to the Genesis Application Software GEMINI. Deionized water was transferred at specified volumes to individual pre-weighed wells in 96-well format. The transferred liquid was then weighed on an analytical balance. For accuracy purposes, the calibration range from 10 to 800 μl was divided into two

Table 4
Mean ($n = 8$) measured volume (%CV, %RE) of deionized water in calibration of Tecan Genesis RSP150/8

Calibration rang (μl)	Desired volume (μl)				
	Mean ($n = 8$) measured volume (%CV, %RE)	Mean ($n = 8$) measured volume (%CV, %RE)	Mean ($n = 8$) measured volume (%CV, %RE)	Mean ($n = 8$) measured volume (%CV, %RE)	Mean ($n = 8$) measured volume (%CV, %RE)
10–250	10	25	40	100	250
	10.0108 (3.2, 0.1)	25.0576 (1.7, 0.2)	40.4227 (0.5, 1.1)	101.5021 (0.4, 1.5)	254.3395 (0.6, 1.7)
200–800	200	300	500	600	800
	196.9784 (0.4, −1.5)	294.1484 (0.3, −1.9)	495.4901 (0.7, −0.9)	601.6449 (0.9, 0.3)	803.0793 (0.2, 0.4)

n, Number of replicates; CV, coefficient of variation; %RE, percent relative error.

sections: one calibration range from 10 to 250 μl and another calibration range from 200 to 800 μl. The representative calibration results from Tecan Genesis RSP 150/8 were as follows: the accuracy was from 100.1 to 101.7 and 98.5 to 100.4% of nominal values for calibration range 10–250 and 200–800 μl, respectively, and the corresponding precision ($N = 8$) was from 0.38 to 3.21 and 0.21 to 0.89% CV, respectively (Table 4).

3.2. Syringes and probes for robotic system

Both Tecan Genesis RSP 150/8 and 200/8 systems were equipped with 1-ml syringes and fixed probes. The standard stainless steel probes were installed on Tecan Genesis RSP 150/8 for sample preparation and 384-well small probes were installed on the Genesis RSP 200/8 for enzyme incubation. For sample preparation procedures, the standard probe with proper inner diameter can reduce the probe-clogging caused by un-precipitated suspensions in plasma samples. For enzyme incubation procedures, several different types of probes were tested and the 384-well small probe was selected because of the suitability of its length for this robotic system and its reliability in this assay condition.

3.3. Carryover

Carryover of reagents caused by the fixed probe during liquid transfer was evaluated. Human control plasma samples (drug free) were utilized to run the enzyme incubation portion of this evaluation. If carryover of substrate or enzyme occurs, it will cause increasing concentrations of these reagents, which will result in an increasing amount of assay product, ergo, increased scintillation counts. The scintillation counts of [^{14}C]-mevalonolactone from the 96-wells are listed in Table 5. The precision of the counts from each of the 12 columns were from 1.1 to 2.6%. No increasing trends from the first column to the 12th column were found, indicating no apparent reagent-carryover occurred under this assay condition.

To evaluate the carryover of the analyte by the fixed probes, a pooled human plasma sample collected following SV administration was utilized to determine the concentration of HMG-CoA reductase inhibitors. If there is carryover of the analyte using the fixed probes, there will be an increasing concentration of inhibitors, which will result in decreasing amount of assay product, ergo, decreased scintillation counts. This evaluation results, the scintillation counts of [^{14}C]-mevalonolactone from this assay, are listed in Table 5. The precision ($n = 8$) of each column was from 0.8 to 3.5% in the 12 columns of the 96-well plate. No decreasing trend was found from first column to 12th column which indicates that no apparent analyte carryover occurred in this assay.

3.4. Ion exchange resin

The anion exchange resin binding HMG-CoA selectively was used to separate [^{14}C]-mevanololacton from the unreacted [^{14}C]-HMG-CoA. To

Table 5
Mean ($n = 8$) scintillation counts (%CV) of enzyme incubation product mevalonolactone from individual enzyme incubation (w/w inhibitors) of each column on 96-well plate

Column number of 96-well plate, mean ($n = 8$) scintillation counts (%CV) of mevalonolactone from individual column of 96-well plate											
1	2	3	4	5	6	7	8	9	10	11	12
Without inhibitor											
7940 (1.6)	7964 (1.1)	7856 (1.4)	7890 (2.2)	7773 (2.8)	7884 (1.9)	7854 (2.1)	7866 (2.6)	7766 (1.6)	7870 (2.1)	7943 (1.8)	7875 (1.6)
With inhibitor											
3091 (2.8)	3101 (3.5)	3122 (2.4)	3183 (3.3)	3118 (2.2)	3177 (2.7)	3148 (0.8)	3202 (1.8)	3139 (1.9)	3185 (2.1)	3189 (1.7)	3185 (2.4)

CV, coefficient of variation.

minimize the amount of anion exchange resin needed for this separation, three volumes of the anion exchange resin were evaluated. To each column containing 0.8, 1.5, or 2 ml of resin slurry, 100 μl of enzyme incubation solution containing 1.167 nmol of [^{14}C]-HMG-CoA (the normal assay amount) was added. Each column was then washed twice with 2 ml of Triton × 10 000. The eluting solutions from each resin column were mixed with 10 ml of scintillation cocktail in a 20-ml scintillation vial separately and counted on the scintillation counter for 2 min. Four ml of Triton × 10 000 solution (radioactivity free) mixed with 10 ml of scintillation cocktail was used as a background. It was found that the scintillation counts of solutions eluted from three different anion exchanger resin volumes were all close to those of the background. This indicates that no [^{14}C]-HMG-CoA went through the resin, suggesting that 0.8 ml of anion exchanger resin slurry (0.4 ml of resin) was sufficient to bind all of the excess substrate.

3.5. Eluting solution and scintillation cocktail addition

The volume of eluting solution used on the anion exchange columns was evaluated. One hundred μl of incubation solution was added to each column packed with 0.8-ml of resin slurry. The incubation solution was obtained by incubating 40 μl Triton × 500 with 30 μl enzyme and 30 μl substrate solution at 37 °C for 30 min, followed by the addition of 30 μl hydrochloric acid for lactonization of mevalonic acid. The purpose of using inhibitor-free Triton × 500 instead of SV extract solution was to obtain the maximum amount of incubation product. These columns were then washed with different volumes of Triton × 10 000 solution and the eluents were mixed with same amount of scintillation cocktail and counted on scintillation counter. A reference was obtained by mixing the 100 μl of incubation solution with scintillation cocktail directly and counted. The results showed that when using 0.8 ml of Triton × 10 000 to wash the resin twice, the number of the counts of the combined eluents were similar to that of the reference. So a total volume

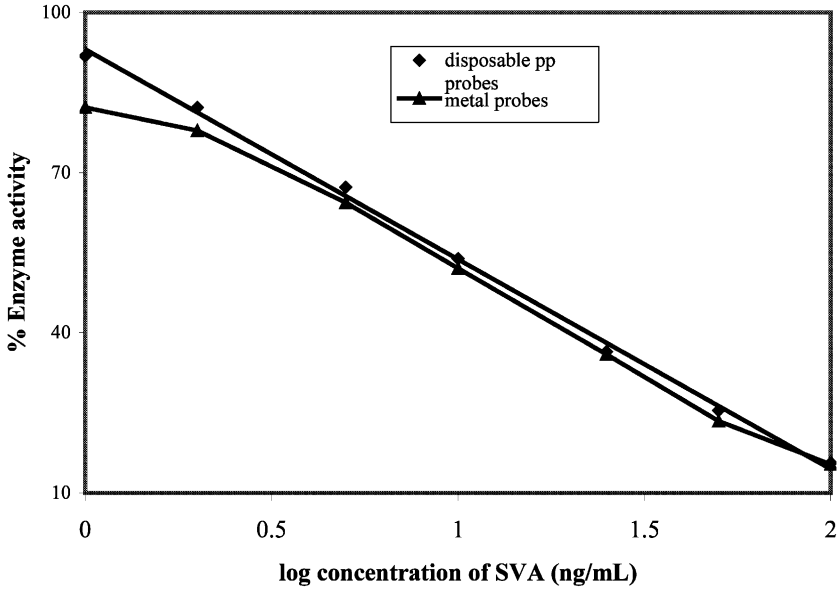


Fig. 6. Comparison of linearity of enzyme inhibition assay on Tecan and on Zymark robotic systems.

of 1.6 ml eluting solution was adequate for the complete elution of the enzyme incubation product mevalonolactone. To save space on the robotic workstation, 6-ml scintillation vials were used to replace the 20-ml vials used in previous semi-automated method [20].

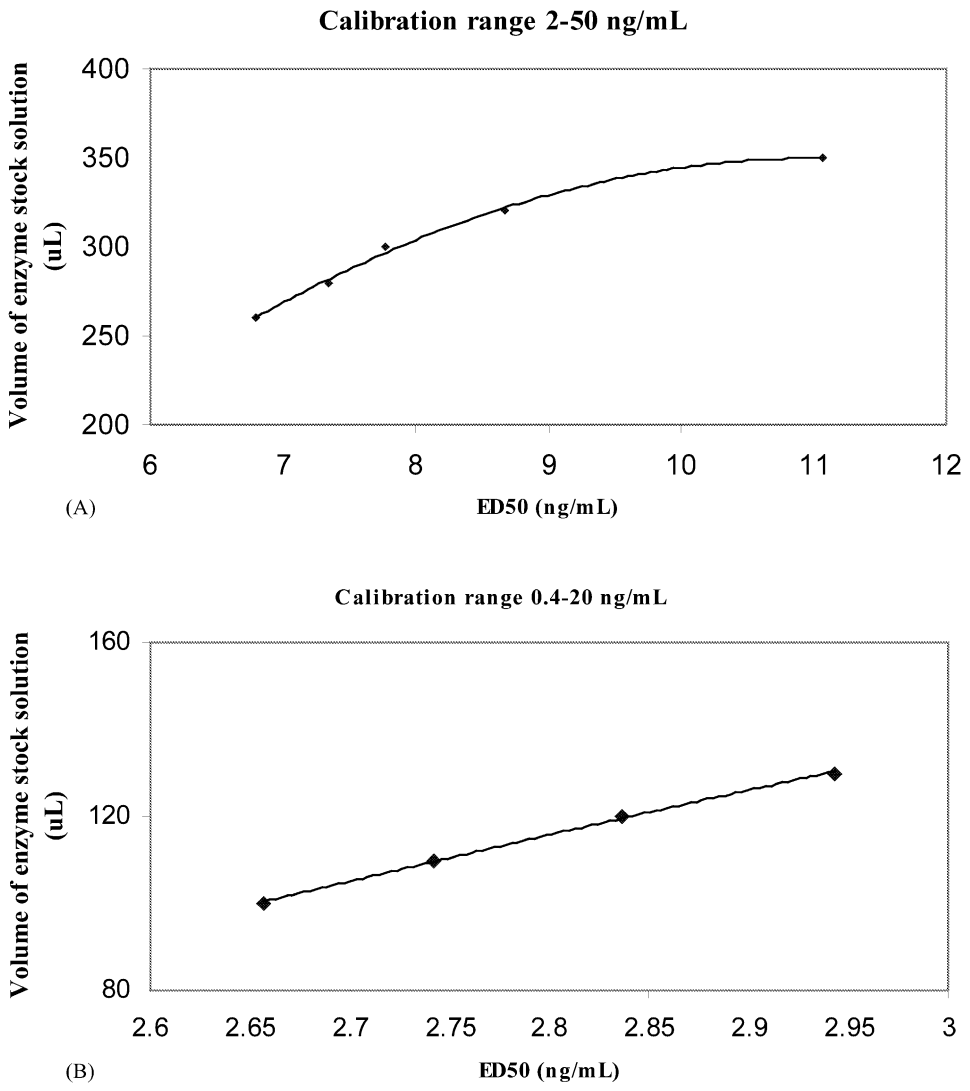
The addition of scintillation cocktail into the isolated mevalonolactone for the measurement of its radioactivity can be completed on Tecan robotic system by installation of a multi-port valve. Because of the glutinosity of the scintillation cocktail and the delicacy of the HMG-CoA reductase inhibitory assay, a modified 6-probe Phoenix Robotic Workstation was utilized for the transfer of scintillation cocktail. The workstation was equipped with six probes for convenient scintillation cocktail transfer into vials on an 18-vial rack.

3.6. Specificity

The assay specificity was evaluated to determine the impact of endogenous materials in the biomatrices on the reproducibility of the assay. Different sources ($N \geq 5$) of human and animal plasma and human urine were assayed together with neat reconstitution solution as the blank reference. The scintillation counts of ^{14}C-mevalonolactone resulting from different biomatrices showed similar values and were similar to those derived from the blank, indicating that no intrinsic HMG-CoA reductase inhibitory activity exists in those biomatrices which may cause interference with the assay.

3.7. Assay calibration range and linearity

Due to the nature of the enzyme inhibition assay, the linear dynamic range for HMG-CoA reductase inhibitor determination is very narrow (up to 50-fold). To accomplish the needs for assaying samples collected following SV doses ranging from 10 to 80 mg, three calibration ranges were established with lower limits of quantitation (LOQs) of 0.4, 2 and 5 ng/ml, respectively. When the obtained concentration of a study sample was higher than the upper limit of quantitation, this sample would be diluted and re-assayed. The calibration linearity of the enzyme assay was adjusted by varying the volume of enzyme stock solution. It was found that with the same concentrations of enzyme and substrate, the scintillation counts of assay product were different between the Tecan robotic system used in this method and the Zymark workstation used in previous method,



*ED_{50}: effective dose of 50% inhibition

Fig. 7. Volume of enzyme stock solution vs. *ED_{50} of SVA (ng/ml) in calibration range 2–50 ng/ml (A) and 0.4–20 ng/ml (B).

thus the linearity on Tecan Genesis was different from that on the Zymark workstation when following Zymark-based assay condition (Fig. 6). One possible reason for this difference was the adsorption of reagents onto the fixed metal probes of the Tecan system versus the disposable polypropylene tips used on the Zymark workstation. Thus a higher concentration of enzyme was used for the assay on the Tecan Genesis system. Fig. 7 shows the volume of enzyme stock solution versus ED_{50} of SVA on Tecan Genesis robotic system. The mean percent inhibition values for SVA on the HMG-CoA reductase activity in different calibration ranges and different matrices during method validation are listed in Table 6. Their correlation coefficients, slopes and intercepts were typically in the range 0.997 ± 0.003, -0.39 ± 0.02 and 0.8 ± 0.2, respectively.

Table 6
Mean ($n = 5$) percent (%CV) activity of HMG-COA reductase at various SVA concentrations during initial intra-day validation

Species	SV acid concentration (ng/ml)				
	Mean ($n = 5$) percent (%CV) activity	Mean ($n = 5$) percent (%CV) activity	Mean ($n = 5$) percent (%CV) activity	Mean ($n = 5$) percent (%CV) activity	Mean ($n = 5$) percent (%CV) activity
Dog plasma	5	10	25	50	100
	74 (1.8)	63 (2.1)	47 (1.6)	33 (1.9)	21 (2.3)
Rat plasma	73 (1.4)	62 (2.4)	48 (1.2)	36 (1.4)	23 (0)
Human urine	77 (1.7)	64 (3.6)	52 (2.6)	37 (2.2)	25 (3.5)
Human plasma	2	5	10	25	50
	82 (1.9)	65 (1.6)	52 (3.2)	32 (0.32)	20 (2.5)
Human plasma	0.4	1	5	10	20
	83 (1.2)	68 (1.4)	41(1.4)	27 (3.0)	17 (3.0)

n, Number of samples; CV, coefficient of variation.

3.8. Sample dilution

When a calculated concentration of HMG-CoA reductase inhibitors exceeded the upper limit of quantitation, the sample were diluted with corresponding control matrix (drug free) and reanalyzed. WORKLIST software provided by GEMINI was applied for the dilution program. When the dilution factor was equal to or less than 10, it was programmed as a one step dilution. When the dilution factor exceeded 10 then it was divided into two or three steps. To optimize the liquid handling parameters during the dilution process, human control plasma spiked with [^{14}C]-SV was used as the 'sample'. The diluted samples (final volume 250 μl) from different dilution factors were mixed with scintillation cocktail and counted on a scintillation counter. The reference was the scintillation counts of 250 μl of the [^{14}C]-SV spiked plasma sample (without dilution). Table 7 lists the dilution accuracy and precision after optimization. The accuracy was measured as the ratio of the dilution factor times counts of corresponding diluted samples versus the counts of the reference. The precision was calculated from eight replicate measurements for each dilution factor.

3.9. Parallelism

Since the concentration of HMG-CoA reductase inhibitors was expressed as SVA-equivalents, parallelism was evaluated to verify that other active inhibitors in the samples behave similarly to SVA upon dilution with blank matrix. Pooled post-dose plasma samples collected at different time points following oral SV dose and plasma samples spiked with SVA at 40 ng/ml were diluted with human control plasma with dilution factors of 2, 4, 8 and 16, respectively. The diluted samples were then assayed to determine their HMG-CoA reductase inhibitor concentrations. The results are plotted in Fig. 8 (enzyme activity vs. the log of dilution factor). The linear parallel curves show that parallelism was observed between SVA and other HMG-CoA reductase inhibitors in samples upon dilution.

3.10. Recovery

Recovery of analyte following protein precipitation was evaluated using SVA as a reference. SVA working solutions at three different QC concentrations were spiked into control human plasma

Table 7
Mean ($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma before (dilution factor = 1) and after dilution (dilution factor > 1)

Dilution factors					
Mean($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma	Mean($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma	Mean($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma	Mean($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma	Mean($n = 8$) (%CV, %RE) scintillation counts of the ^{14}C-SV in human plasma
1	2	5	10	
134 078 (0.3, 0)	68 505 (0.8, 2)	27 727 (0.7, 3)	14 009 (2.0, 4)	
1	20	50	100	200
115 679 (0.7, 0)	5698 (3.7, −1.5)	2309 (2.9, −0.2)	1195 (2.7, 3.3)	577 (4.2, −0.2)

n, Number of samples; CV, coefficient of variation; %RE, percent relative error.

before (pre-spiked) and after (post-spiked) protein precipitation and assayed. The recovery was calculated as the ratio of concentrations obtained from pre-spiked over that from post-spiked. The mean recovery measured at QC concentrations of 8, 20, and 40 ng/ml was 89.5%.

3.11. Stability

Human plasma samples, pooled from post-dose samples at different time points following an oral SV administration, were used to evaluate the stability of the active and total HMG-CoA reductase inhibitors. After 2 h room temperature bench top storage, no apparent change in the concentrations was observed for either the active or the total HMG-CoA reductase inhibitory activity. The active and total HMG-CoA reductase inhibitors were also found to be stable after three freeze/thaw cycles and for up to a storage period of 14 months at −70 °C.

3.12. Duplication

Since the assay procedure involves many steps and no internal standard is used, acceptable assay precision is maintained by assaying each blank, standard, QC, and study sample in duplicate. When the deviation between the two values is smaller than 10%, the average of the two duplicate values was reported as the final concentration for the sample. If the deviation between the two duplicate values is greater than 10% for a particular sample ('bad duplication'), then the assay result for this sample is considered not acceptable and re-analysis of the sample is needed. In case an error occurred to one of the eight probes or dilutors of the Tecan robotic system, out of range values could be observed for every eighth sample, causing bad duplication to the study samples involved. The observation of 'periodical' bad duplication values can be an indication of malfunction of one of the eight tips.

4. Conclusions

An automated, robust, and reliable enzyme inhibition assay was developed and fully validated

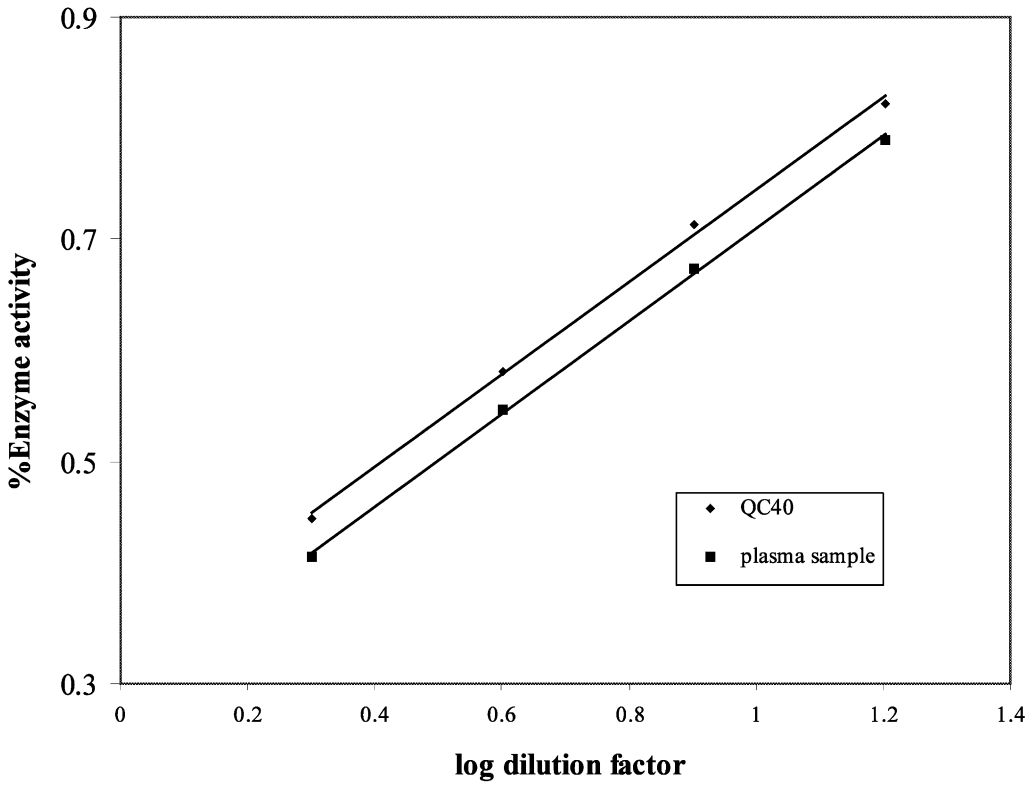


Fig. 8. Parallel study comparing the behavior of HMG-CoA inhibitors upon dilution in QC plasma sample vs. pooled plasma samples collected from SV dosed healthy subjects.

for the determination of SV-derived active and total HMG-CoA reductase inhibitor concentrations in various human and animal matrices. The assay was performed on Tecan Genesis RSP 150 and 200 robotic systems with 96-well plate format. Three calibration concentration ranges, 0.4–20, 2–50, and 5–100 ng eq/ml, have been validated and utilized to assay human and animal samples. The established assay method provided very good assay precision and accuracy, with higher throughput and less labor. It is much faster compared with the single-tip Zymark-based method previously used.

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