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ORIGINAL RESEARCH

Analysis of five HMG-CoA reductase inhibitors—atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin: pharmacological, pharmacokinetic and analytical overview and development of a new method for use in pharmaceutical formulations analysis and *in vitro* metabolism studies

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ABSTRACT: A specific, accurate, precise and reproducible high-performance liquid chromatographic (HPLC) method was developed and validated for the simultaneous quantitation of five 3-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase inhibitors, viz. atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin, in pharmaceutical formulations and extended the application to *in vitro* metabolism studies of these statins. Ternary gradient elution at a flow rate of 1 mL/min was employed on an Intertisl ODS 3V column (4.6 × 250 mm, 5 μm) at ambient temperature. The mobile phase consisted of 0.01 M ammonium acetate (pH 5.0), acetonitrile and methanol. Theophylline was used as an internal standard (IS). The HMG-CoA reductase inhibitors and their metabolites were monitored at a wavelength of 237 nm. Drugs were found to be 89.6–105.6% of their label's claim in the pharmaceutical formulations. For *in vitro* metabolism studies the reaction mixtures were extracted with simple liquid–liquid extraction using ethyl acetate. Baseline separation of statins and their metabolites along with IS free from endogenous interferences was achieved. Nominal retention times of IS, atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin were 7.5, 17.2, 21.6, 28.5, 33.5 and 35.5 min, respectively. The proposed method is simple, selective and could be applicable for routine analysis of HMG-CoA reductase inhibitors in pharmaceutical preparations as well as *in vitro* metabolism studies.

KEYWORDS: hyperlipidemia; statins; metabolism; HPLC analysis

INTRODUCTION

Hyperlipidemia, the elevation of lipid concentration in plasma, is the manifestation of a disorder in the synthesis and degradation of plasma lipoproteins. The main lipids that are of relevance in hyperlipidemia are cholesterol and triglycerides. Cholesterol plays a crucial role in maintaining cell membrane integrity and physiological functions of the body, including steroid hormone synthesis. On the other hand, high levels of cholesterol concentration are associated with pathological conditions such as atherosclerosis (Lacoste *et al.*, 1995; Hardman *et al.*, 1996), characterized by deposition of cholesterol in the arterial wall (Levine *et al.*, 1995; Williams and Tabas, 1995). Atherosclerosis of the coronary and peripheral vasculature is the leading cause of death worldwide (Murray and Lopez, 1997). The statistics show that 38–42% of deaths are related to cardiovascular diseases in Western and other developed countries (Page *et al.*, 1970; Kannel *et al.*, 1971; Anderson *et al.*, 1987; White, 1995). Lowering cholesterol levels can arrest or reverse atherosclerosis in all vascular beds and can significantly decrease the morbidity and mortality associated with atherosclerosis. Each 10% reduction in cholesterol levels is associated with ~20–30% reduction in the incidence of coronary heart disease (The Pravastatin Multinational Study Group for Cardiac Risk Patients, 1993; Scandinavian Simvastatin Survival Study Group, 1994; Byington *et al.*, 1995; Sheperd *et al.*, 1995; Sacks *et al.*, 1996; Downs *et al.*, 1998; The Long-Term Intervention with Pravastatin in Ischemic Disease Study Group, 1998).

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Abbreviations used: AV, atorvastatin; HMG-CoA, 3-hydroxy-3-methyglutaryl coenzyme A; LV, lovastatin; PV, pravastatin; RV, rosuvastatin; SV, simvastatin; IS, Internal standard; SVA, Simvastatin acid; DMSO, Dimethyl sulfoxide; CYP, Cytochrome P450; RSD, Relative standard deviation; CV, Coefficient of variation; LLOQ, Lower limit of quantification.

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The main methods of treating hyperlipidemia (or hypercholesterolemia) are dietary and lifestyle changes and administration of hypolipidemic drugs (Betteridge *et al.*, 1993). The principal groups of hypolipidemic agents are bile acid binding resins, fibric acid derivatives, 3-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), derivatives of nicotinic acid, probucol and the omega-3 marine triglycerides (Scandinavian Simvastatin Survival Study Group, 1994). Statins are the most effective among all these agents. Statins are proven to reduce coronary artery events and have fewer adverse effects than other hypolipidemic agents (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995; Byington *et al.*, 1995).

Presently several HMG-CoA reductase inhibitors are commercially available in the market. In this paper a brief overview of pharmacology, pharmacodynamics, pharmacokinetics, safety aspects and analytical aspects on atorvastatin (AV), lovastatin (LV), pravastatin (PV), rosuvastatin (RV) and simvastatin (SV) is discussed.

PHARMACOLOGY, PHARMACODYNAMICS AND PHARMACOKINETICS OF STATINS

Statins specifically and competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is an early rate-limiting step in cholesterol biosynthesis in the body. The statins have an affinity for the enzyme HMG-CoA reductase that is approximately three orders of magnitude greater than that of the natural substance HMG-CoA (Moghadasian, 1999). These agents are highly effective in reducing total cholesterol and the low-density lipoprotein levels in several forms of hypercholesterolemia (Mabuchi *et al.*, 1981, 1983; Bilheimer *et al.*, 1983; Arad *et al.*, 1992; Davignon *et al.*, 1992). LV is a natural product which is derived from the fungus *Aspergillus terreus* (Alberts *et al.*, 1980; Hoffman *et al.*, 1986). LV, PV and SV are structurally similar. PV and SV are produced by semi-synthetic processes from LV and mevastatin, respectively (Endo *et al.*, 1976). AV and RV are totally synthetic molecules and have structures distinct from the fungi-derived HMG-CoA reductase inhibitors. AV, LV and SV are relatively lipophilic compounds, while PV and RV are more hydrophilic as a result of a polar hydroxyl group and methane sulfonamide group, respectively (McTavish and Sorkin 1991; McTaggart *et al.*, 2001). The chemical structures of AV, LV, PV, RV and SV are shown in Fig. 1. The chemical structures of statins govern their water solubility, which in turn influences their absorption, distribution, metabolism and excretion (Serajuddin *et al.*, 1991; Lennernas and Fager, 1997; White, 2002; Schachter, 2005). The similarities and differences of pharmacodynamics and pharmacokinetics of a few HMG-CoA reductase inhibitors have recently been compared (Lennernas and Fager 1997; Schachter, 2005). The pharmacokinetics of HMG-CoA reductase inhibitors has been summarized in several reviews (Henwood and Heel, 1988; Mauro and MacDonald, 1991; McTavish and Sorkin, 1992; Blum, 1994; Plosker and McTavish, 1995; Haria and McTavish, 1997; Lea and McTavish, 1997; Lennernas and Fager, 1997; White, 2002; Schachter, 2005). In addition, Sirtori (1993) and Garnett (1995) reviewed the HMG-CoA reductase inhibitors tissue distribution and drug interactions, respectively. Within the recommended dosage ranges, the relationship between responses, expressed as percentage reduction in LDL cholesterol, and dose is log–linear (Pedersen and Tobert, 1996). The statins as a group are generally very well tolerated, with gastrointestinal complaints such as diarrohea, pain, constipation and flatulence being the most commonly reported adverse effects along with rashes, dizziness, pruritis and headache (Tobert, 1988; Scandinavian Simvastatin Survival Study Group, 1994; Blum, 1994). AV, PV and RV are administered as the active hydroxy form. These drugs are absorbed from the gut and undergo extensive first-pass metabolism in the liver. LV and SV are prodrugs that are administered as inactive lactone forms. Their lactone forms are absorbed from the gut and hydrolyzed to the active β-hydroxy acid form in the liver (Tang and Kalow, 1995). All statins are absorbed rapidly following administration, reaching peak plasma concentration within 4 h (Pan *et al.*, 1990; Warwick *et al.*, 2000). Food intake increases the bioavailability of LV and decreases the one of PV and AV, whereas the bioavailability of SV and RV is not affected. Serum protein binding of AV, RV and PV was 98, 88 and 50%, respectively. These drugs are excreted mainly in the feces via the bile, with a smaller portion excreted in the urine (Halstenson *et al.*, 1992; Pentikainer *et al.*, 1992; Quion and Jones, 1993). Both LV and SV and their β-hydroxy acid metabolites are highly (95%) bound to plasma proteins. The metabolites undergo extensive first-pass metabolism in the liver and are mainly excreted in the bile; about 85% of administered dose has been recovered from the feces as metabolite and about 10–15% from the urine, mainly as inactive forms (Mauro, 1993; Tang and Kalow, 1995). The CYP3A4 primarily metabolizes AV, LV and SV; RV is chiefly metabolized by CYP2C9, whereas PV, unlike other statins, is not significantly metabolized by the CYP system and is mainly eliminated unchanged and by phase-I reactions. In addition to CYP3A4, CYP2C9 and 2D6 are also involved in the metabolism of established statins (Transon *et al.*, 1996; McCormick *et al.*, 2000; Fujino *et al.*, 2004). Lipophilic statin drugs are known to be much more susceptible to oxidative metabolism by the CYP450 system

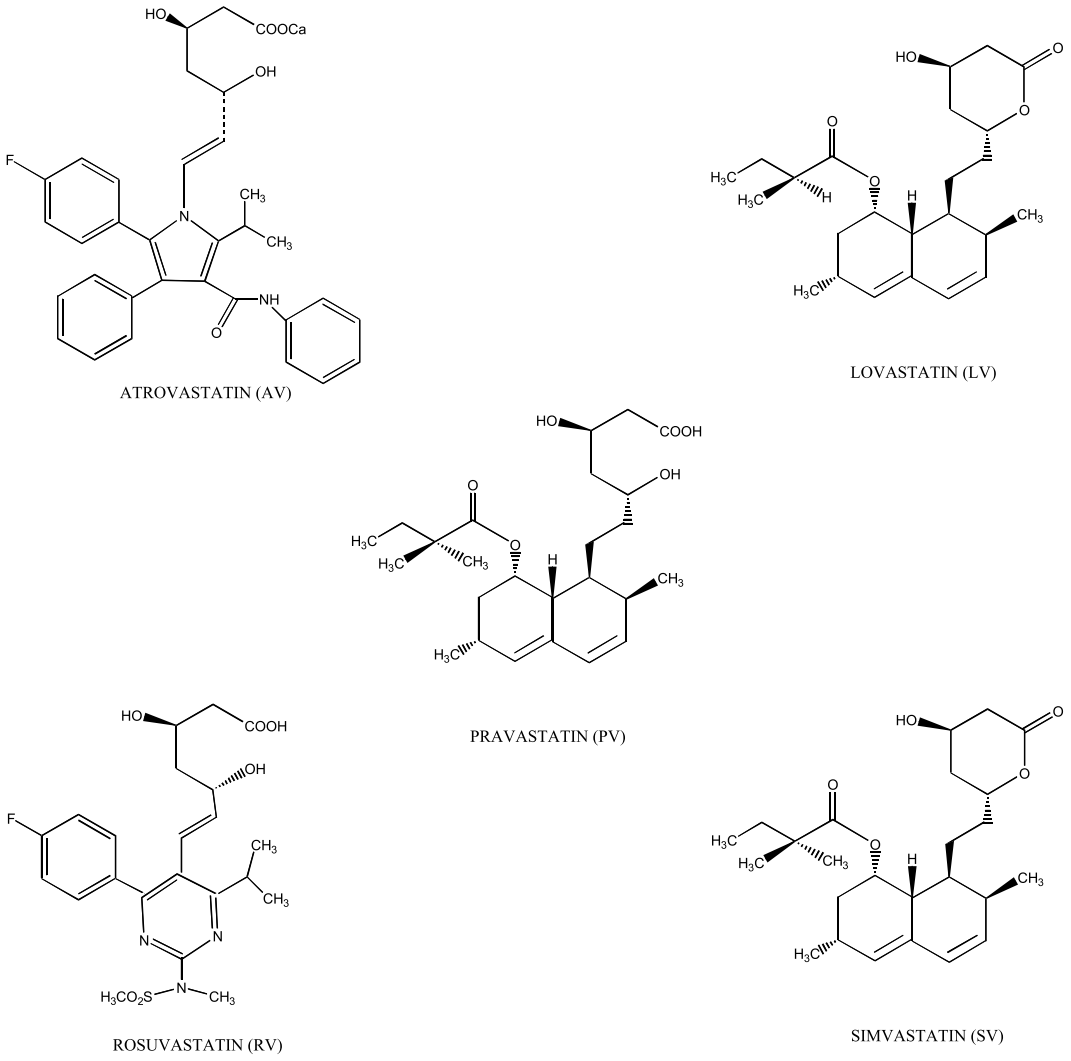

Figure 1. Structural representation of atorvastatin (AV), lovastatin (LV), pravastatin (PV), simvastatin (SV) and rosuvastatin (RV).

(Schachter, 2001). All these statins are eliminated mostly as metabolites, except PV, and have active metabolites that significantly contribute to their lipid-lowering effect. There is indirect evidence that those active metabolites of LV, SV (Garnett, 1994) and AV (Jones, 1996) with longer terminal half-lives than the parent compound exist, but none of these have been characterized.

SAFETY ASPECTS

Statins are mainly considered for long-term use and often constitute part of a multiple-drug regime, which commonly leads to drug interactions. It is now recognized that the stains metabolized by the CYP450 system are more likely to produce muscle toxicity because of the risk of drug interactions with many drugs that inhibit CYP450, notably the CYP3A4 isoform (Muscari *et al.*, 2002; Sica and Gehr, 2002); drug interactions may increase plasma levels of statins, with a consequent increased risk of toxic effects. Besides the common adverse effects, all statins harbor the risk of myopathy and fatal rhabdomyolysis. The withdrawal of cerivastatin (CV) from the clinical use in 2001 heightened scrutiny of these effects, although all available data indicate that the increased incidence of rhabdomylosis reported for CV appears to be specific to this agent (Staffa *et al.*, 2002). Usually, the frequency of myopathy is low but the incidence increases when statins are used in combination with agents that share common metabolic pathways. As statins do not differ in their pharmacodynamic property, the difference in their pharmacokinetic profiles constitutes the rationale for choosing a specific statin suitable for combination therapy (Igel *et al.*, 2001).

ANALYTICAL ASPECTS

Several analytical methods have been developed for the quantitation of each HMG-CoA reductase inhibitor. Among all the reports, only one report published has included the simultaneous analysis of three statins, LV, PV and SV (Morris *et al.*, 1993). Recently, Erturk *et al.* (2003) reviewed the analytical methods for quantitative determination of LV, SV, PV, AV and fluvastatin in biological samples. In the following section we will briefly review the various gas chromatography (GC), HPLC and liquid chromatography–mass spectrometry (LC-MS) analytical methods reported for the quantitation of statins used in the present study.

Atorvastatin

Gibson *et al.* (1997) used a GC-MS method for quantitation of AV with a limit of quantification (LOQ) of 0.1 μg/mL. Serum concentrations of AV acid, lactones and its two metabolites (and their lactones, respectively) were quantified by LC-MS/MS from human plasma (Kantola *et al.*, 1998; Bullen *et al.*, 1999; Jemal *et al.*, 1999; Mazzu *et al.*, 2000) and dog and rat plasma (Bullen *et al.*, 1999).

Lovastatin

Morris *et al.* (1993) have reported the simultaneous determination of LV with PV and SV in plasma using GC with chemical ionization mass spectrometry. The simultaneous estimation of LV and its hydroxy metabolite in mouse and rat plasma was reported by Wu *et al.* (1997) on LC-MS/MS. In addition to these GC and LC-MS methods several HPLC methods were reported (Stubbs *et al.*, 1986; Wang-Iverson *et al.*, 1989; Zhao *et al.*, 1997; Choi *et al.*, 1998; Ye *et al.*, 2000; Zheng *et al.*, 2000). Recently, an HPLC-MS/MS method was developed by Korfmacher *et al*. (1995) for the determination of LV and hydroxyacid in dog plasma.

Pravastatin

PV and its metabolites were quantified on GC-MS following derivatization either with chemical ionization (Funke *et al*., 1989) or electron impact ionization detection (Cai *et al*., 1996). Simultaneous determination of PV with LV and SV in plasma using GC with chemical ionization mass spectrometry was also reported (Morris *et al.*, 1993). Several HPLC methods have been developed for estimation of PV using either UV detection without derivatization (Whigan *et al*., 1989; Iacona *et al*., 1994; Otter and Mignat, 1998; Sigurbjornsson *et al*., 1998; Li *et al*., 1999, 2001; Siekmeier *et al.*, 2000; Bauer *et al.*, 2005) or laser-induced fluorescence detection following derivatization (Dumousseaux *et al*., 1994). In addition, several HPLC-MS/MS methods were reported for quantitation of PV alone (Jemal *et al.*, 1998; Hedman *et al.*, 2003; Zhu and Neirinck 2003) or along with its metabolites from human plasma (Kawabata *et al.*, 1998; Mulvana *et al.*, 2000) and also from rat plasma and mouse, rat, monkey and human serum (Jemal *et al.*, 1998). All these LC-MS/MS methods provide good specificity and sensitivity.

Rosuvastatin

Only two reports are available on quantitation of RV in human plasma (Hull *et al.*, 2002, 2004). Quantification of RV alone (Hull *et al.*, 2002) and along with its *N*-desmethyl metabolite (Hull *et al.*, 2004) was reported in human plasma by automated solid-phase extraction using tandem mass spectrometric detection.

Simvastatin

Two GC-MS methods have been reported for quantitation of SV and simvastatin acid (SVA) with (Takano *et al.*, 1990) or without derivatization (Morris *et al.*, 1993; Cai *et al.*, 1999). Several HPLC methods with UV (Carlcucci *et al.*, 1992; Tan *et al.*, 2000) or fluorescence (Ochiai *et al.*, 1997) detection were also reported. In a few publications the quantification of SV and SVA with LC-MS/MS has been reported (Jemal *et al.*, 2000; Zhao *et al.*, 2000; Yang *et al.*, 2003).

SCOPE

Hitherto there has been no bioanalytical method reported in literature for simultaneous estimation of AV, LV, PV, RV and SV on HPLC. Development of an assay that has a generic application for quantitative determinations of HMG-CoA reductase inhibitors has significant utility. We believe simultaneous determination of statins offers the following advantages: (a) ease and convenience of clinical routine monitoring; (b) applicability to routine *in vitro* metabolism studies and drug–drug interaction studies; (c) simultaneous analysis of pharmaceutical dosage forms. Our aim was to develop a generic method that allowed for the determination of statins without the need for the development of separate and distinct method for each statin having diversified structural features and difference in solubility profile. To the best of our knowledge, the quantitation of the chosen statins specifically in pharmaceutical formulations and *in vitro* metabolism studies has not been performed. In order to have a universal acceptance and ensure ease of applicability, we have used a UV detector with no special assembly of detection systems for measurement of statins.

EXPERIMENTAL

Chemicals and reagents. AV, LV, PV, RV, SV (Fig. 1), desmethyldiazepam and 4-hydroxytolbultamide were synthesized by the Medicinal Chemistry Group, Dr Reddy's Laboratories Ltd (DRL), Hyderabad and were characterized using chromatographic and spectral techniques by Central Instrumentation Laboratory, DRL, Hyderabad. Purity was found to be more than 99% for all the compounds. Theophylline (internal standard, IS) and NADPH sodium salt were purchased from Spectrochem, Mumbai, India. Tablets of AV (Lipvas), LV (Lostatin), PV (Prastatin), RV (Rosuvas) and SV (Simlip) were purchased from Okasa Pharma Pvt Ltd, Goa, Dr Reddy's Laboratories Ltd, Hyderabad, Emcure Pharmaceutical Ltd, Dapodi, Pune, Ranbaxy Laboratories Ltd, Dewas, Delhi and Lupin Ltd, Mumbai, respectively. Diazepam, tolbutamide, testosterone, ticolopidine, quinidine and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co., St Louis, MO, USA. Bufuralol, hydroxybufuralol, ketoconazole, 6β-hydroxytestosterone and sulfaphenazole were procured from Ultra Fine Chemicals, Manchester, UK. Acetonitrile, methanol (HPLC grade), potassium chloride, magnesium chloride, sucrose, ammonium acetate and glacial acetic acid (analytical reagent grade) were purchased from Qualigens, Glaxo (India), Mumbai, India. All aqueous solutions including the buffer for the HPLC mobile phase was prepared with Milli Q (Millipore, Milford, MA, USA) grade water. Human liver microsomes (protein concentration 20 mg/mL) and human lymphoblast-expressed CYPs, viz. CYP2C9, 2C19, 2D6 and 3A4, were procured from Gentest Corporation (Woburn, MA, USA).

Chromatography. The HPLC system consisted of a Waters 2695 Alliance (Milford, MA, USA) separation module attached to a Waters® 2996 photodiode array (PDA) detector. A C_{18} Inertsil® ODS 3V column (4.6 × 250 mm, 5 µm, GL Sciences Inc., Tokyo, Japan) was used for the analysis. The ternary mobile phase system, consisting of reservoir A (0.01 M ammonium acetate buffer, pH 5.0–acetonitrile; 90:10), reservoir B (0.01 M ammonium acetate buffer, pH 5.0–acetonitrile; 5:95) and reservoir C (0.01 M ammonium acetate buffer, pH 5.0–methanol; 10:90), was run as per the gradient program (Rao *et al*., 2003) with a total flow rate of 1 mL/min through the column to elute the analytes. The eluate was monitored by the PDA detector (scan range 200–400 nm) and data integration was carried out by Millennium[32] software (version 4).

Standard solutions. Composite stock solution of AV, LV, PV, RV and SV (5 mg/mL) was prepared by dissolving appropriate amounts of the compounds in DMSO. Similarly IS stock solution (1 mg/mL) was prepared in DMSO. These stock solutions were stored at approximately 5°C, and were found to be stable for several weeks. A working IS solution (100 µg/mL) was prepared in methanol. A series of standard solutions was prepared by appropriate dilution of the above-mentioned stock solution in methanol to reach a concentration range of 0.1–100 µg/mL. In each sample 10 µL of

Table 1. Recoveries and precision of the proposed method in pharmaceutical formulations

Excess drug added to the analyte (%)	Theoretical content (µg)	Recovery (%)	% RSD
AV			
0	40	97.70	2.03
80	72	97.93	2.13
100	80	98.78	1.75
120	88	98.73	1.44
LV			
0	20	98.15	2.37
80	36	97.42	3.77
100	40	100.99	2.18
120	44	100.98	0.52
PV			
0	20	103.50	1.80
80	36	96.67	1.56
100	40	103.57	1.33
120	44	114.46	1.09
RV			
0	20	100.75	3.71
80	36	98.56	4.23
100	40	98.04	4.83
120	44	98.91	1.17
SV			
0	20	97.60	7.60
80	36	96.99	3.79
100	40	101.43	1.91
120	44	101.21	3.24

Table 2. Precision of the proposed method in pharmaceutical formulations

Compound[a]	Intra-day precision (SD of areas)	% RSD	Inter-day precision (SD of areas)	% RSD
AV	4.91	5.21	4.86	5.18
PV	4.31	4.76	4.79	5.33
LV	5.08	3.57	3.94	3.54
RV	3.74	4.04	1.85	2.00
SV	2.78	3.10	2.16	2.36

[a] Average of three concentrations 72, 80 and 88 μg/mL (for AV) and 36, 40 and 44 μg/mL for PV, LV, RV and SV.

100 μg/mL of IS were added. The peak–area ratio of each statin to that of the IS was plotted against the corresponding concentration to obtain a calibration graph.

Assay of statins in pharmaceutical formulations. To determine the content of each statin (used in the present study) simultaneously in conventional tablets (label claim: 20 mg of LV, PV, RV, SV and 40 mg of AV per tablet), 10 tablets each of AV, LV, PV, RV and SV were weighed, their mean weight determined and ground to a fine powder using a glass mortar and pestle. An accurately weighed powdered sample containing 125 mg of each statin was transferred into a 25 mL volumetric flask. The volume was adjusted with methanol and the resultant solution was sonicated for 10–15 min and filtered through a 0.45 μm nylon filter (Millipore, Milford, USA). An aliquot of 100 μL of the filtrate was transferred to a 10 mL volumetric flask and diluted to 10 mL, and the volume was made up to the mark with methanol. From the resulting solution, 100 μL solution were mixed with IS solution (10 μL of 100 μg/mL of theophylline) and injected onto the column.

***In vitro* metabolism studies.** Incubations were performed in pooled human liver microsomes or with lymphoblast-expressed specific human CYP enzyme. Incubation mixture contained 430 μL of 100 mM potassium phosphate buffer (pH 7.4), 40 μL of protein (0.8 mg) and 5 μL of either single statin or two statins or statin + inhibitor or methanol (in the case of the blank). The reaction was initiated by adding 25 μL of NADPH (2 mM) after pre-incubation at 37°C for 2 min. The final volume of incubation mixture was kept at 500 μL. After a pre-determined incubation time (time varies from statin to statin), the reaction was terminated by the addition of 4.0 mL of ethyl acetate. IS (1 μg) was added and contents were vortex mixed and centrifuged at 3000 rpm for 10 min. The clear organic layer (3.2 mL) was drawn and evaporated to dryness under a gentle stream of nitrogen at 40°C (Zymark® Turbovap®, Kopkinton, MA, USA). The residue was reconstituted in 200 μL of mobile phase (A:B, 1:1) and 100 μL of this solution was injected onto HPLC. The metabolite(s) formation was measured when statins were incubated individually or two stains. Positive controls were run concurrently to ensure microsomal viability and included: hydroxylation of diclofenac (CYP2C9), hydroxylation of S-mephyntoin (CYP2C19), hydroxylation of bufuraolol (CYP2D6) and hydroxylation of testosterone (CYP3A4).

RESULTS AND DISCUSSION

Optimization of the experimental conditions

Preliminary experiments were carried out to optimize the experimental parameters affecting both the chromatographic separation of all the statins in the LC-column selected and their detection by UV. Each statin exhibited different maximum UV absorbance and, in order to detect all statins simultaneously with good sensitivity, 237 nm was selected as UV_{max}. The feasibility of different mixture of solvents such as acetonitirile and methanol using different buffers such as phosphate and formic acid, along with different flow-rates (in the range 0.5–1.0 mL/min), was tested for complete chromatographic resolution of all statins and IS. Versatility, suitability and robustness of the method were checked with various other C_{18} columns from various manufacturers, viz. Kromasil C_{18} (250 × 4.6 mm, 5 μm, Hichrom, Berkshire, UK) and Supelcosil LC-318 (250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) by running four replicates of each combination set comprising all statins along with IS under identical HPLC conditions. It was found that chromatographic resolution, selectivity and sensitivity were similar with all the columns (data not shown).

Specificity and chromatography

In the chosen completely optimized chromatographic conditions, specificity was indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank pooled human liver microsomes and pooled human liver microsomes with AV, LV, PV, RV, SV and IS. When single analytes were injected at the highest concentration in the chromatographic system, at the retention times of all analytes no interference was observed (data not shown). All analytes, viz. IS, AV, LV, PV, RV and SV, were well separated with retention time of 7.5, 17.2, 21.6, 28.5, 33.5 and 35.5 min, respectively. Figure 2 shows a typical overlaid chromatogram for the control pooled human liver microsomes (free

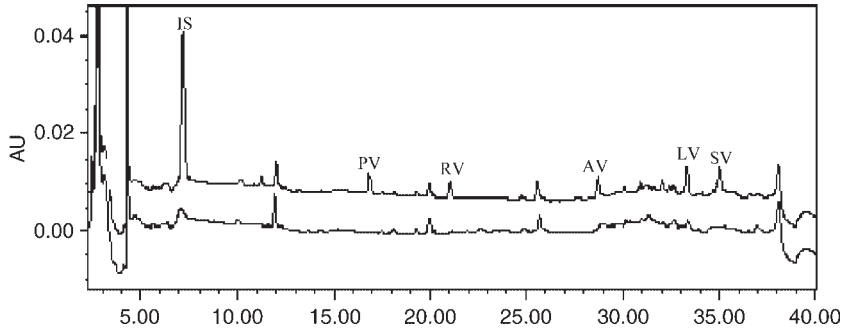

Figure 2. HPLC chromatograms of a 100 µL injection of (a) (lower chromatogram) pooled human liver microsomes, (b) (upper chromatogram) pooled human liver microsomes spiked with AV, PV, LV, RV and SV at LLOQ (0.1 µg/mL) and 1 µg/mL of IS.

of analyte and IS), pooled human liver microsomes spiked with AV, LV, PV, RV and SV at 0.1 µg/mL (LLOQ).

Calibration curve

Peak area ratios of each analyte to the IS were measured and acted as a surrogate for quantitation. A representative calibration graph of peak–area ratio (each analyte to IS) vs each analyte concentration in the range of 0.1–100 µg/mL for all statins was found to be linear. The average regression ($n = 3$) was 0.999 for all the analytes. The standard curve had a reliable reproducibility for each analyte across the calibration range. The lowest concentration with the RSD < 20% was taken as LLOQ (Shah *et al*., 1992) and was found to be 0.1 µg/mL for all statins used in the present study.

Assay of statins in pharmaceutical formulations

The accuracy of the proposed method was evaluated by recovery assays. Accuracy was determined by applying the described method to synthetic mixtures of excipients to which known amounts of each statin corresponding to 80, 100 and 120% of label claim had been added. The accuracy was then calculated as the percentage of each statin recovered by the assay. Mean recoveries (mean ± SD, $n = 4$) for AV, LV, PV, RV and SV were 100.7 ± 0.83, 104.4 ± 2.2, 99.3 ± 1.2, 97.8 ± 3.8 and 98.9 ± 1.4%, respectively. The results indicate satisfactory accuracy of the method for simultaneous determination of AV, LV, PV, RV and SV in the formulation.

The recovery and precision of the proposed method was performed by spiking each statin in the selected formulation on different days (Tables 1 and 2). The intra-day and inter-day precision is shown in Table 2. The results indicated sufficient precision of the developed HPLC method.

The validated HPLC method was applied to the simultaneous determination of AV, LV, PV, RV and SV in tablets. The assay results, expressed as a percentage of the label claim, are shown in Table 3. The results indicate that the amount of each drug in the tablets corresponded to the required 90–110% of the label claim.

Table 3. Applicability of the proposed method for the determination of AV, LV, PV, RV and SV in tablets

Drug	Claimed (mg)	Found[a] (mg)	Content[b] (%)
AV	40	39.08	97.70
PV	20	20.70	103.50
LV	20	19.63	98.15
RV	20	20.15	100.75
SV	20	19.52	97.60

[a] Mean ± SD of four replications.
[b] Content (%) = found/claimed × 100.

In vitro metabolism studies

Figure 3 shows the *in vitro* metabolism of AV (upper panel) and PV (middle panel) individually and in combination (lower panel), when spiked in pooled human liver microsomes. Similarly, we have demonstrated the utility of the newly developed assay for *in vitro* metabolism studies with LV and RV individually and in combination (Fig. 4, upper, middle and lower panels, respectively). AV, LV, PV and RV showed similar *in vitro* metabolism to that reported in the literature. AV formed *para*-hydroxy-AV (AV-M1) and *ortho*-hydroxy-AV (AV-M2; Jones, 1996); LV formed 6′β-hydroxy-LV (LV-M1), 3″-hydroxy-LV (LV-M2), LV-hydroxyacid (LV-M3) and 6′-exomethylene-LV (LV-M4; Vyas *et al.*, 1990); PV yielded two major metabolites, dihydroxy-PV (PV-M1) and 3′-hydroxy-PV (PV-M2; Jacobsen *et al.*, 1997); RV metabolized into desmethyl-RV (RV-M1; McCormick *et al.*, 2000). We also performed the metabolism of selective statins with specific CYP450 and their inhibition with selective inhibitors.

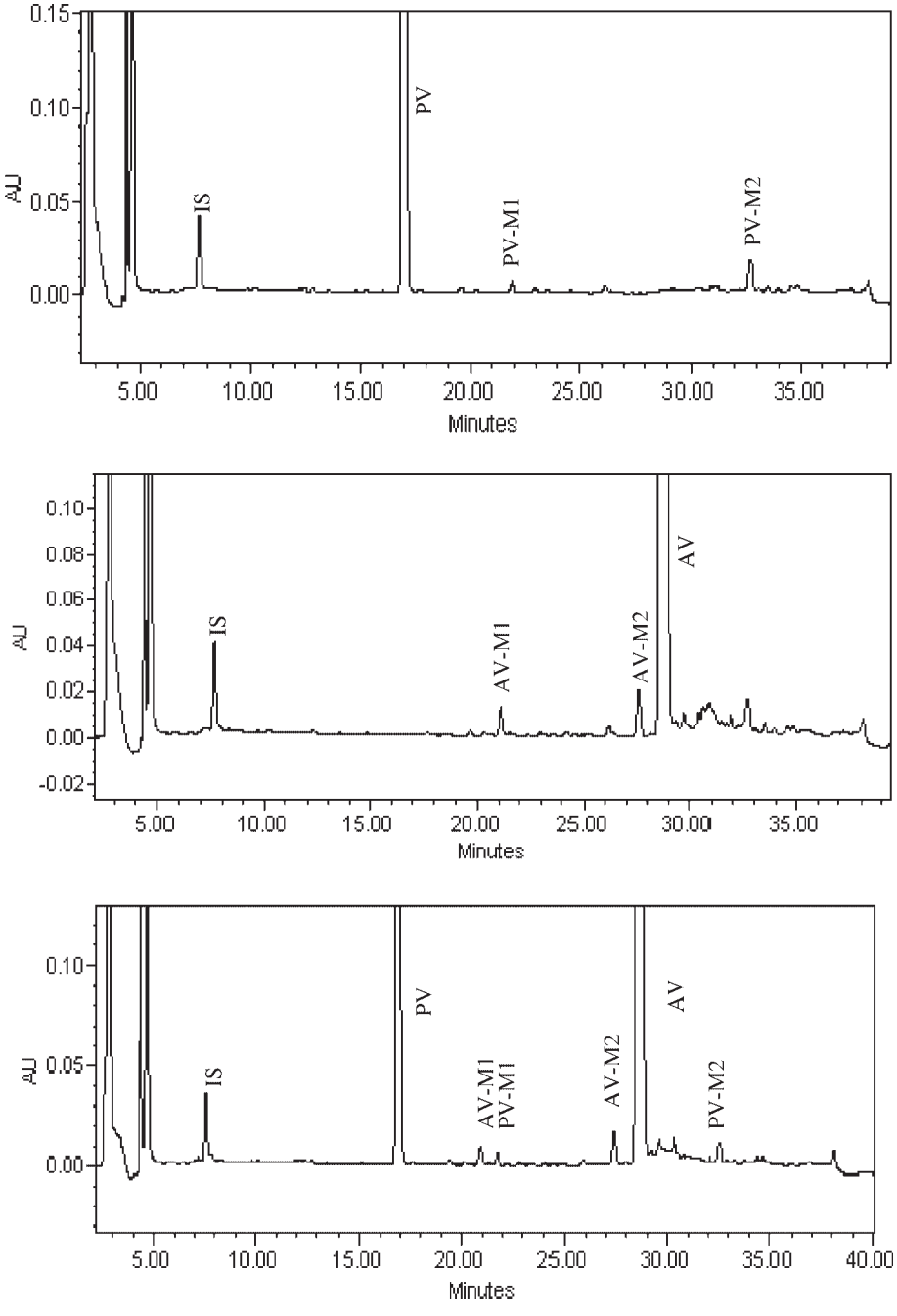

Figure 3. Representative HPLC chromatograms of pooled human liver microsomes spiked with AV (upper panel), PV (middle panel) and AV + PV (lower panel).

Microsomal viability was assessed with positive controls (data not shown). Chromatograms clearly indicate that the peaks of each parent statin and corresponding metabolites are well resolved when spiked individually/present in the combination set, and the method is quite useful for running the different combination sets continuously without having to change either the column or the associated HPLC conditions.

CONCLUSION

An HPLC-UV method utilizing optimized gradient elution with single wavelength has been developed for simultaneous analysis of five HMG-CoA reductase inhibitors, viz. AV, PV, LV, RV and SV in formulations and also *in vitro* metabolism studies. The validated method is specific, accurate, precise and reproducible.

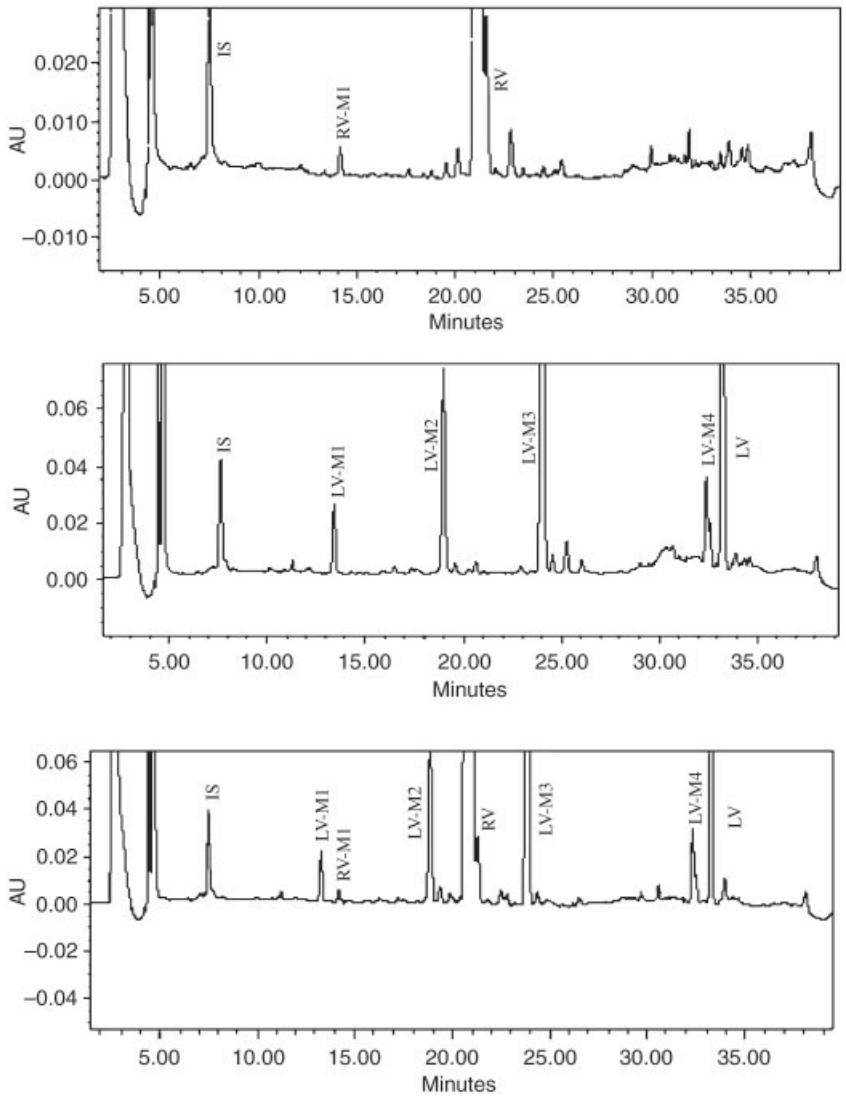

Figure 4. Representative HPLC chromatograms of pooled human liver microsomes spiked with LV (upper panel); RV (middle panel) and LV + RV (lower panel).

We have used the method successfully to study the *in vitro* metabolism of few statins in combination, viz. AV + PV and LV + RV, and demonstrated that this assay is easy to apply, effective and transferable.

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