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Analytical method for monitoring concentrations of cyclosporin and lovastatin in vitro in an everted rat intestinal sac absorption model

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Abstract

Cyclosporin A (CSA) and lovastatin (LV) are lipophilic drugs, which show poor and erratic absorption when administered perorally. The permeability of these compounds can be increased transiently by altering the membrane characteristics of the absorptive epithelium by the use of sorption promoters (SPs). In the present work a simple validated HPLC method utilizing an isocratic mobile phase with short retention times for CSA and LV was developed in order to monitor their concentrations in Kreb's Ringer bicarbonate (KRB) solution in vitro in intestinal sac absorption model. The same method was utilized to determine the apparent permeability coefficients and absorption profiles of CSA and LV by a modified Wilson–Wiseman method. Drugs were analysed by a reversed-phase HPLC method using a Shim-pack C_{18} column. An isocratic mobile phase containing acetonitrile and water in the proportions 70:30 and 80:20 was used for the HPLC analysis of CSA and LV, respectively. The flow-rate was 2 ml/min and quantitative determinations were carried out at 215 nm at 70 °C for CSA. In the case of LV the flow-rate was 1 ml/min and detection was done at 238 nm at 25 °C. The method was found to be specific as none of the proposed SPs, components of KRB or intestinal sac artefacts interfered with the drug peaks. Recovery studies and intra- and inter-day variations were within statistical limits. The limits of detection were 250 and 10 ng/ml and the limits of quantitation were 400 and 30 ng/ml for CSA and LV, respectively. The calibration curve was found to be linear in concentration range of 0.5–6 μg/ml for CSA and 0.05–0.4 μg/ml for LV. The proposed method was found to be rapid and selective and hence can be applied for continuous monitoring of CSA and LV in vitro in intestinal sac absorption studies.

Keywords: Cyclosporin; Lovastatin

1. Introduction

The origin of the biopharmaceutic classification system (BCS), which categorises drug molecules on the basis of solubility and permeability, has had a strong impact on drug research, development and regulatory approvals [1]. This new concept has not only generated the need for extensive database regarding solubility and permeability coefficients of pre-existing molecules, but it has also made the determination of these values an imperative step in the basic drug development process. Drug molecules with low solubility and poor permeability through biomembranes (Class IV drugs) are the most difficult to formulate in conventional drug delivery systems.

Cyclosporine A (CSA or cyclosporin A) and

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lovastatin (LV) are two highly lipophilic drug molecules that are poorly absorbed on oral administration [2,3]. The inadequate bioavailability of these drugs is attributable to extremely low aqueous solubility, high molecular mass, poor membrane permeability, *p*-glycoprotein efflux associated exsorption and metabolism by the polymorphic intestinal and hepatic cytochrome P450 [3–5]. Apparently, absorption of such molecules can be increased by coadministration of absorption enhancers [6], which act by increasing solubility as well as permeability while decreasing enzymatic metabolism [7].

We aim to develop a simple isocratic reversed-phase HPLC method capable of quantifying the absorption enhancement parameters of CSA and LV in vitro in a rat everted intestinal sac absorption model. Utilizing the same mobile phase constituents, i.e. water and acetonitrile, but varying their proportions and the temperature of the column, an isocratic reversed-phase HPLC method is developed with short run times and minimum sample preparation, that can specifically detect drug peaks in the presence of absorption enhancers, intestinal sac artefacts and components of physiological salt solution, i.e. Kreb's Ringer bicarbonate (KRB) medium.

CSA has been analysed in biological fluids by HPLC methods and radioimmunoassays [8,9], whereas LV has been estimated in biological fluids by HPLC [10], enzyme inhibition assays [11] and gas chromatography–mass spectrophotometry (GC–MS) [12]. All these procedures are either time consuming or require expensive and elaborate equipment.

2. Experimental

2.1. Materials

CSA and LV were gift samples supplied by Dabur Research Foundation (India) and Dr. Reddy's Laboratories (India). HPLC grade solvents (J.T. Baker, USA) and analytical grade reagents (Ranbaxy Labs., India and Loba, India) were used in this study. Sorption promoters were purchased from Sigma (USA) and designated as SP-1 and SP-2. Purified water, obtained by reverse osmosis (USF ELGA) and filtered through a 0.45-μm membrane, was used throughout the study.

2.2. Chromatographic system and instrumentation

Shimadzu HPLC system was used for purpose of analysis in the study and it consisted of an LC-10AT VP solvent pump, DGU-14AM on-line degasser, autoinjector SIL-10AD VP with temperature control, CTO-10AS VP column oven and SPD-10AVP UV–Vis spectrophotometric detector. Shimadzu CLASS-VP software was used for data acquisition and mathematical computations. A Stuart scientific thermostat with stirrer (SM 26) and external thermal probe (capable of precise temperature control ±1 °C) were employed in the in vitro everted intestinal sac absorption studies, Minisart® NML (0.45 μm) Sartorius filters and Vortex-Genie 2 cyclomixer were used for sample preparations. The chromatographic conditions used in the analyses are outlined in Table 1.

2.3. Preparation of standard stock solutions

The primary stock solution (1 mg/ml) of CSA was prepared in acetonitrile. This was further diluted with KRB to prepare calibration standards of CSA in concentration range of 0.5–6 μg/ml. In the case of LV, 500 μl of primary stock solution (1 mg/ml in acetonitrile) was diluted to 10 ml with acetonitrile to prepare calibration standards covering the concentration range 0.05–0.4 μg/ml. The KRB used in all the solutions was freshly prepared, sonicated and filtered through a 0.45-μm membrane filter. For the purpose of analysis, 100 μl of calibration standard solution was injected into the HPLC system and the area under the peak was monitored.

2.4. Assay characteristics

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using aliquots of primary stock solutions and KRB as diluent. Test solutions of five different concentrations were prepared from primary stock solutions and analysed by the proposed HPLC method to determine the recovery in KRB. The intra- and inter-day variations were determined by analysis of the same calibration

Table 1
HPLC parameters for determination of CSA and LV in KRB solution

Parameter	CSA	LV
Method	Reversed-phase HPLC	Reversed-phase HPLC
Mobile phase	Isocratic composition acetonitrile–water (70:30, v/v)	Isocratic composition acetonitrile–water (80:20, v/v)
Column	C_{18} Shim-Pack (CLC-ODC-M) 250 mm×4.6 mm I.D. 5 μm particle size	C_{18} Shim-Pack (CLC-ODC-M) 250 mm×4.6 mm I.D. 5 μm particle size
Flow-rate	2 ml/min	1 ml/min
Detection	UV detector, 215 nm	UV detector, 238 nm
Column temperature	70 °C	25 °C
Injection volume	100 μl	100 μl

standards prepared within same day and between different days by the same analyst.

2.5. Specificity

2.5.1. Non-interference by components of KRB, SPs, intestinal sac artefacts and probable degradation during in vitro everted intestinal sac studies

The drug solutions in KRB were spiked with 0.25% (w/v) of the sorption promoters SP-1 and SP-2. A 100-μl volume of solution was injected into the HPLC system to test for possible interference by the KRB components. The interference by intestinal sac artefacts was checked by performing in vitro everted intestinal sac studies as outlined in Section 2.6. Any probable interference, which may occur due to possible chemical or enzymatic degradation, was also monitored in these experiments.

2.5.2. Non-interference of LV by lovastatin acid (LVA)

LV possesses a lactone ring, which undergoes hydrolytic cleavage in aqueous solutions to produce the dihydroxyacid form LVA. To examine the specificity of the proposed HPLC method towards LV in the presence of LVA, LV was chemically converted into LVA by employing the method of Keyomarsi et al. [13] and a mixture of LV and LVA was injected into the HPLC system. An accurately weighed amount of LV (52 mg) was dissolved in 1.04 ml of ethanol (95%, v/v) by addition of 813 μl of 1 *M* NaOH. The resulting solution was neutralized by addition of 1 *M* HCl to a pH of 7.2 and the final volume was made up to 13 ml with distilled water to obtain a 10 m*M* solution of LVA. An aliquot of 100 μl from this solution (filtered through a 0.45-μm Minisart NML Sartorius filter) was added to 900 μl of 50 ng/ml LV solution in KRB, cyclomixed for 30 s and injected in HPLC system.

2.6. In vitro everted intestinal sac absorption studies

All the animal studies were done after approval of the protocol by the Institutional Animal Ethical Care committee (IAEC) of NIPER. In vitro absorption studies were performed using everted rat jejunal segments [14]. Subsequent to animal sacrifice by excessive ether inhalation, the intestine was rapidly removed and placed in a beaker containing cold KRB solution (continuously aerated). The jejunal segments (approximately 6 cm) were used in the present studies ($n=3–6$). The details of everted rat intestinal sac absorption method had been described elsewhere [14]. Drug solution was withdrawn from the serosal compartment of the jejunal membrane at different time points up to 2 h and immediately

replaced with fresh KRB to keep the volume of the serosal solution constant. The permeability of CSA and LV was obtained by plotting the cumulative amount of drug permeated through the sac versus time. The slope of linear portion of the graph was taken as permeation flux (F, $\mu g\ min^{-1}$) [15]. The apparent permeability coefficient (APC) was calculated using following formula

$$APC = \frac{F}{SA \times IDC}\ cm\ min^{-1} \quad (1)$$

where SA is the surface area of the barrier membrane (cm^2) and IDC is the initial drug content in the mucosal solution (μg).

3. Results and discussion

3.1. Range and linearity

KRB solution is a standard physiological solution, which is used to dissolve the drugs and observe their transport characteristics across biological membranes [14]. A major difficulty in the estimation of CSA and LV in KRB is their limited aqueous solubilities, which are 6.6 and 0.4 $\mu g/ml$, respectively [16,17]. Thus, the upper limit of calibration range cannot exceed beyond their respective solubilities.

The calibration range of CSA was taken between 0.5 and 6 $\mu g/ml$ and for LV between 0.05 and 0.4 $\mu g/ml$ in order to monitor permeation across the absorptive membrane. It is anticipated that in presence of SPs the solubilities of these drugs may increase to some extent. The drug permeating through the membrane and reaching serosal side still continues to be well within concentration range of the analytical method. Furthermore, the serosal solution is withdrawn and replaced by fresh KRB at different intervals of time which keeps the drug within the concentration ranges.

The standard curve was constructed on 3 consecutive days and the regression parameters, i.e. slope, intercept and correlation coefficient were calculated in each case (Table 2). In the case of CSA the mean value of the slope was found to be $1.3139 \cdot 10^{-5}$ [relative standard deviation (RSD) = 1.86%] and the correlation coefficient was >0.9931 in all the experiments, confirming the linear relationship between the concentration of the drug and the area under the peak. Similarly to CSA, the LV regression statistics show concordance in the values of regression parameters (Table 2). The mean slope value was $1.3181 \cdot 10^{-3}$ (RSD = 1.74%) and correlation coefficient was at least 0.9997 in all cases.

Table 2
Regression statistics of CSA and LV ($n = 3$)

Drug	Day	Slope	Intercept	Correlation coefficient
CSA	1	$1.3085 \cdot 10^{-5}$	0.03252	0.9931
	2	$1.3406 \cdot 10^{-5}$	0.02400	0.9999
	3	$1.2926 \cdot 10^{-5}$	0.03757	0.9999
LV	1	$1.3085 \cdot 10^{-3}$	5.2274	0.9997
	2	$1.2963 \cdot 10^{-3}$	5.4856	0.9998
	3	$1.3421 \cdot 10^{-3}$	5.4524	0.9998

3.2. LOD and LOQ

The LOD of CSA was 250 ng/ml (RSD = 0.68%) and for LV it was 10 ng/ml (RSD = 2.6%). The lowest concentration of CSA quantifiable by this method was 400 ng/ml (RSD = 1.37%). LV is measurable at a concentration of 30 ng/ml (RSD = 0.99%) (Table 3) by the present methodology. It can be detected up to 5 ng/ml in biological fluids by using enzyme inhibition assays [11]; but it is non-specific and provides the numerical values of total HMG-CoA reductase inhibitors.

3.3. Accuracy and precision

The accuracy is the exactness of the analytical method or the closeness of the agreement between the value (which is accepted either as a conventional true value or an accepted reference value) and the value found. To confirm the accuracy of the pro-

Table 3
LOD and LOQ values of CSA and LV ($n = 6$)

Drug	Method parameter	Parameter value (ng/ml)	RSD (%)
CSA	LOD	250.00	0.68
	LOQ	400.00	1.37
LV	LOD	10.00	2.60
	LOQ	30.00	0.99

posed method, recovery experiments were carried out by standard addition technique in which quality control samples were prepared by spiking the KRB with known amount of drug to obtain three different concentration levels within the calibration curve range. The spiking was done in a manner similar to preparation of standard solutions for calibration range elucidated before.

CSA was analysed at three concentration levels—1.5, 3 and 5 μg/ml. Each level was determined in replicates ($n=6$) and the amount of drug was found by the assay method. The results are reported in Table 4. The RSD values in all the cases were <2%, which implies that the method is accurate.

LV was analysed at concentrations of 0.15, 0.25 and 0.35 μg/ml ($n=9$). Similarly to CSA the data fall within the conventional acceptance criteria of lower variations and deviations (value <2% for the in vitro samples) [18].

Precision is the degree of repeatability of an analytical method under operation and is usually expressed as the RSD for a statistically significant number of samples. To determine intra- and inter-day precision of the assay, replicate ($n=3$) sets of calibration samples were analysed within the same day and between different days by the same analyst. The RSD of the assay results was determined and the results are shown in Table 5. The range of RSD values was 0.14–1.86% (intra-day variation) and 0.02–1.80% (inter-day variation) for CSA in concentration range of 0.5–6 μg/ml. LV exhibited RSD values between 0.31 and 1.81% (intra-day variation) and 0.52 and 1.99% (inter-day variation) in a concentration range of 0.05–0.4 μg/ml. No distinct pattern of variability was observable between concentration of drug and inter- and intra-day variations. The RSD values of both the drugs showed equal variability of the method. However, for both drugs variations were higher at lower concentration levels.

Table 4
Recoveries of CSA and LV in KRB solutions ($n=6$ for CSA and $n=9$ for LV)

Concentration (μg/ml)	Recovery (%)	RSD (%)
CSA		
1.5	99.25	1.97
3.0	100.36	1.07
5.0	98.97	1.70
LV		
0.15	99.93	1.36
0.25	99.42	1.46
0.35	100.19	0.51

Table 5
Precision of the method ($n=3$)

Concentration (μg/ml)	Intra-day variation RSD (%)	Inter-day variation RSD (%)
CSA		
0.5	1.23	1.80
1.5	0.14	0.02
3.0	1.47	1.14
5.0	1.86	1.67
6.0	1.07	1.05
LV		
0.05	1.81	1.99
0.15	0.60	0.57
0.25	0.77	1.22
0.35	0.31	0.72
0.40	1.17	0.52

3.4. Specificity

The specificity is the ability of analytical method to measure accurately and specifically the analyte of interest in the presence of the other components that might be expected to be present in the sample matrix. The mean retention time of CSA was found to be 6.94 min (RSD = 1.59%). The drug peak was not interfered with by any of the components of KRB and sorption promoters to be used during in vitro absorption studies. Representative chromatograms of CSA with (0.25%, w/v) SP-l, (0.25%, w/v) SP-2 and intestinal artefacts leached out during in vitro everted intestinal sac absorption are shown in Fig. 1.

The HPLC analysis of CSA presents a special case of peptide analysis because of its lipophilic nature and solvent dependent conformation. The cumulative effect of these two facts is that it exhibits a solvent dependent lipophilic pattern due to variation in its conformation and hydrogen bonding capacities. This results in formation of separate conformers in biphasic solvent system, which are difficult to separate [8,19–21]. It has been reported that the retention time of small peptides (less than 20 amino acid

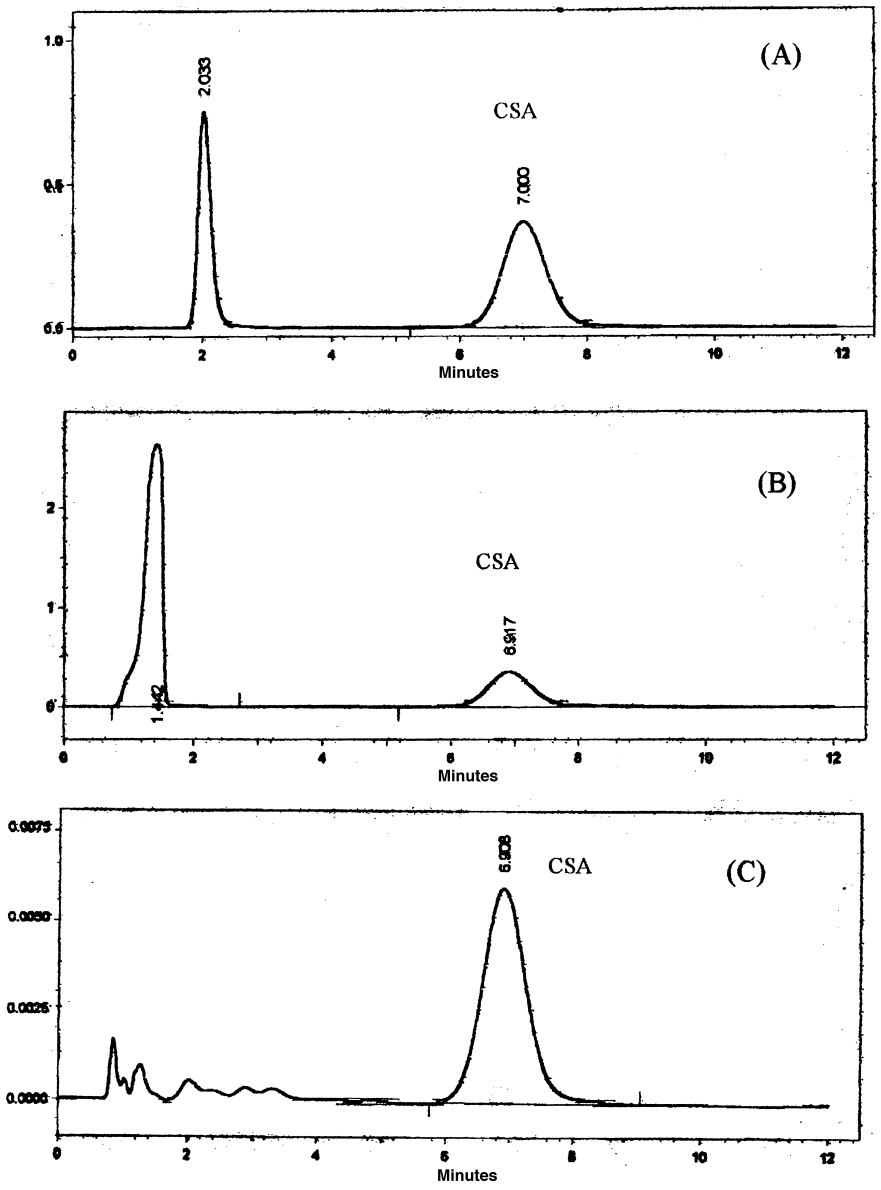

Fig. 1. Representative chromatograms of CSA in presence of (A) 0.25% SP-1 (B) 0.25% SP-2 and (C) intestinal sac artefacts.

residues) in reversed-phase analysis is influenced by the number, type of amino acid residues, molecular structure and conformation [9].

The HPLC analysis of cyclosporin was performed at a column temperature of 70 °C because of extreme peak broadening at lower temperatures. This phenomenon can be explained by considering principles of basic chromatography promulgated by Giddings [21] and sophisticated molecular dynamic simulations of different conformational forms of CSA [19]. There is an improvement in column efficiency for CSA and cyclosporin D (CSD), i.e. increase in the number of theoretical plates and decrease in height equivalent to theoretical plates (HETP), due to an increase in the column temperature [9]. This effect is due to the influence of temperature on the viscosity

of the mobile phase, where an elevation in column temperature reduces the viscosity of mobile phase and could result in reduction in column pressure. Alternatively, the improvement in efficiency with increasing temperature may also be due to the fact that CSA and CSD are relatively high-molecular-mass polypeptides of a unique cyclic form. As a result, they might have low diffusion or penetration at low temperatures. The increase in column temperature results in increase of mass transfer or diffusion of the solute to the stationary phase [21]. Giddings reported that both of these effects collectively decrease the HETP [21].

A more specific explanation could be provided by conformational analyses of cyclosporin. It is reported that the extreme peak broadening at lower temperatures could be related to the difficulty in resolving several conformers of CSA completely [20]. The crystal structure of CSA and the structure of isolated CSA in non-polar solvents, as determined using two-dimensional NMR techniques are similar. The basic features of dominant conformer, as illustrated in Fig. 2 (structure I), are the presence of stable intramolecular H-bonds [NH----O=C] leading to the formation of a stable twisted β-sheets and a type II′ β-turn at Sar3 and MeLeu4 [19]. In contrast to the situation in non-polar solvents where a single conformer dominates, NMR spectra of CSA in polar solvents such as DMSO, THF–LiCl and ethanol–water suggest the coexistence of various stable conformers. CSA in non-polar solvents adopts a folded conformation stabilized by intramolecular H-bonds between the strong H-bond donor amide groups and the strong H-bond acceptor carbonyl groups. In contrast to this, CSA in polar solvents can adopt more open conformations, which expose its polar groups to the polar solvent following cleavage of the internal H-bonds. Thus, large flexible molecules can adapt their conformation in order to expose to the environment those groups that can best interact with it (a ‘chameleonic’ behaviour) [19]. Fig. 2 (structure II) shows X-ray average structure of CSA in water over 150–200 ps trajectories. In the proposed HPLC method, reversed-phase column C_{18} (octadecyl groups on silica support) is used with a 70:30 combination of the polar solvents acetonitrile and water. In practice, which conformer of CSA predominates at this composition, structure I or II is difficult to assign based on data available (and is matter of detailed analysis using centrifugal partition chromatography followed by computer aided molecular simulations); but as proposed by Tayar et al. [19], CSA should predominantly be in the unfolded state with polar groups exposed to mobile phase and hydrophobic C_{18} column. This means that drug will tend to have less affinity with column and elute fast. This can be further substantiated by observations of Mereish and Ueda [9], according to which, when the flow-rate of mobile phase is increased from 0.5 to 2.0 ml/min the retention time decreases from 27.0 to 6.0 min. Undoubtedly, the best explanation is the combination of Giddings basic principles of chromatographic separations [21] and idiosyncratic solvent dependent conformational propensity of CSA.

The peak of LV was well resolved without any interference by SP-1, SP-2 and intestinal sac artefacts (Fig. 3). Mean retention time of LV was found to be 6.90 min (RSD = 1.99%). LV undergoes reversible conversion into a dihydroxyacid derivative known as lovastatin acid (LVA) in vitro and in vivo (Fig. 4). This necessitates establishing whether the method is able to resolve the two compounds as separate peaks or it gives a single peak (total HMG-CoA reductase inhibitory activity). Thus, when LV was converted into LVA and a mixture of the two drugs was injected into HPLC system, two separate peaks corresponding to LV and LVA were observed (Fig. 3). This implies that method is LV specific and can be applied for estimation of LVA separately. Furthermore, chromatograms from in vitro everted intestinal sac absorption studies show no signs of appearance of any other degraded product.

As a result of the poor aqueous solubility of these drugs, suspensions of drugs in KRB were used as mucosal reservoir solutions. Even if the drug degrades in solution, there is a sufficient amount of solid drug, which immediately replaces the degraded drug content and maintains the drug at constant saturation solubility level. This is acceptable to our objective of maintaining constant initial concentration in mucosal solution. The only point of inspection is whether the degraded products (if at all any possible enzymatic or chemical degradation ensues) interfere with drug peak. It is evident from chromatograms in Fig. 1 and Fig. 3 that no such interferences were observed.

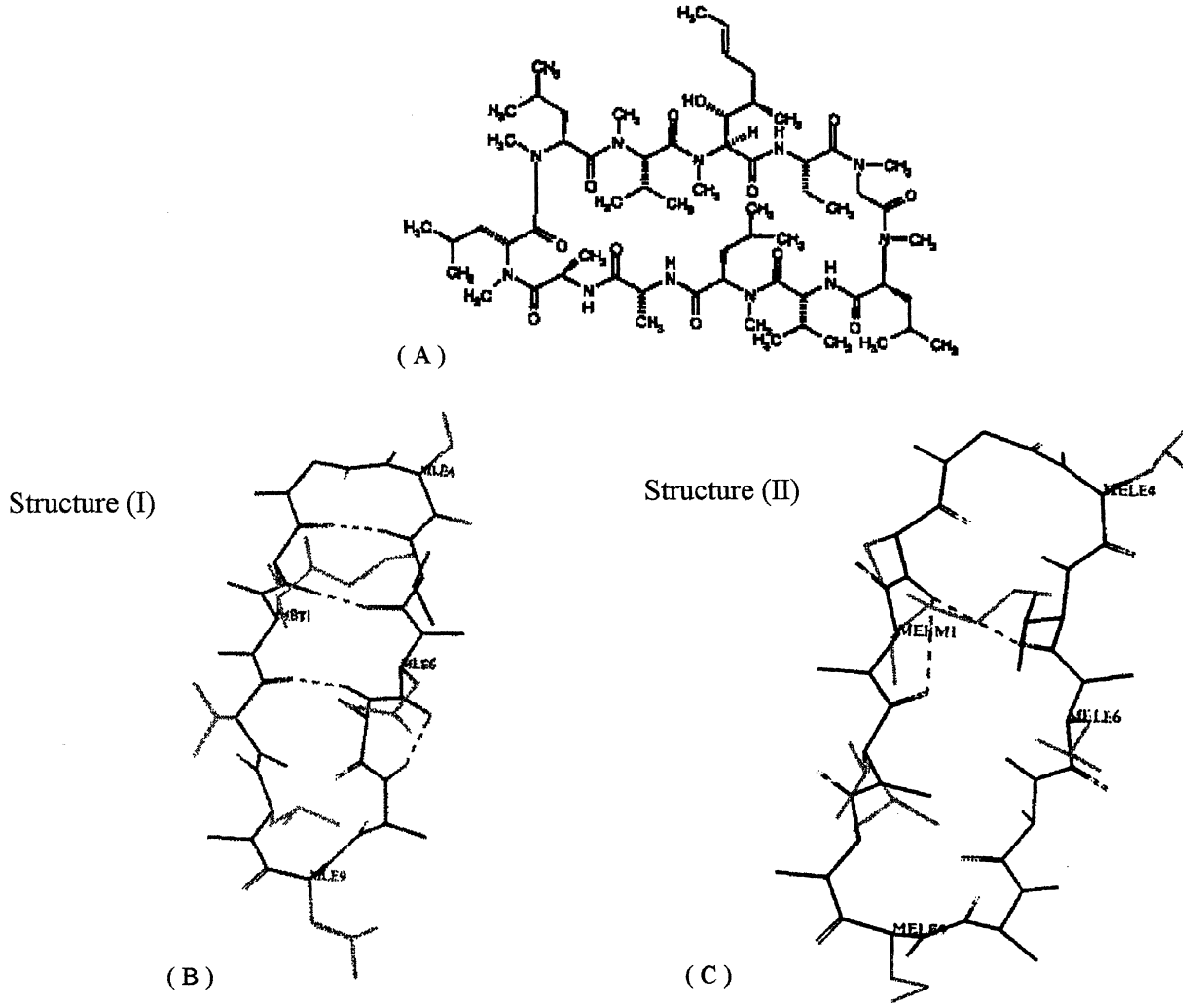

Fig. 2. Environment dependent conformations of CSA (A). Structural formula of CSA (B). X-ray structure of CSA (dominant conformer in crystal and non-polar solvent) (C). X-ray structure in water. Reprinted with permission from [19], Copyright (1993) American Chemical Society.

3.5. In vitro everted intestinal sac absorption studies

The drug samples obtained at different time intervals from in vitro absorption studies were analysed by a HPLC method. The cumulative amount of drug permeated in μg through the mucosal membrane of the sac was plotted against time (min). The slope of linear portion of the graph was taken as permeation flux (F, μg min^{-1}) and the APC values of the drugs were calculated using Eq. (1). The amount of drug absorbed in μg per unit area of sac is plotted against time (min) to obtain absorption profiles of CSA and LV (Fig. 5). The APC values of CSA and LV are $0.96(\pm0.04)\cdot10^{-4}$ and $6.91(\pm0.35)\cdot10^{-6}$ cm min^{-1}, respectively.

4. Conclusions

The proposed HPLC method is a simple isocratic reversed-phase method which can analyse CSA and

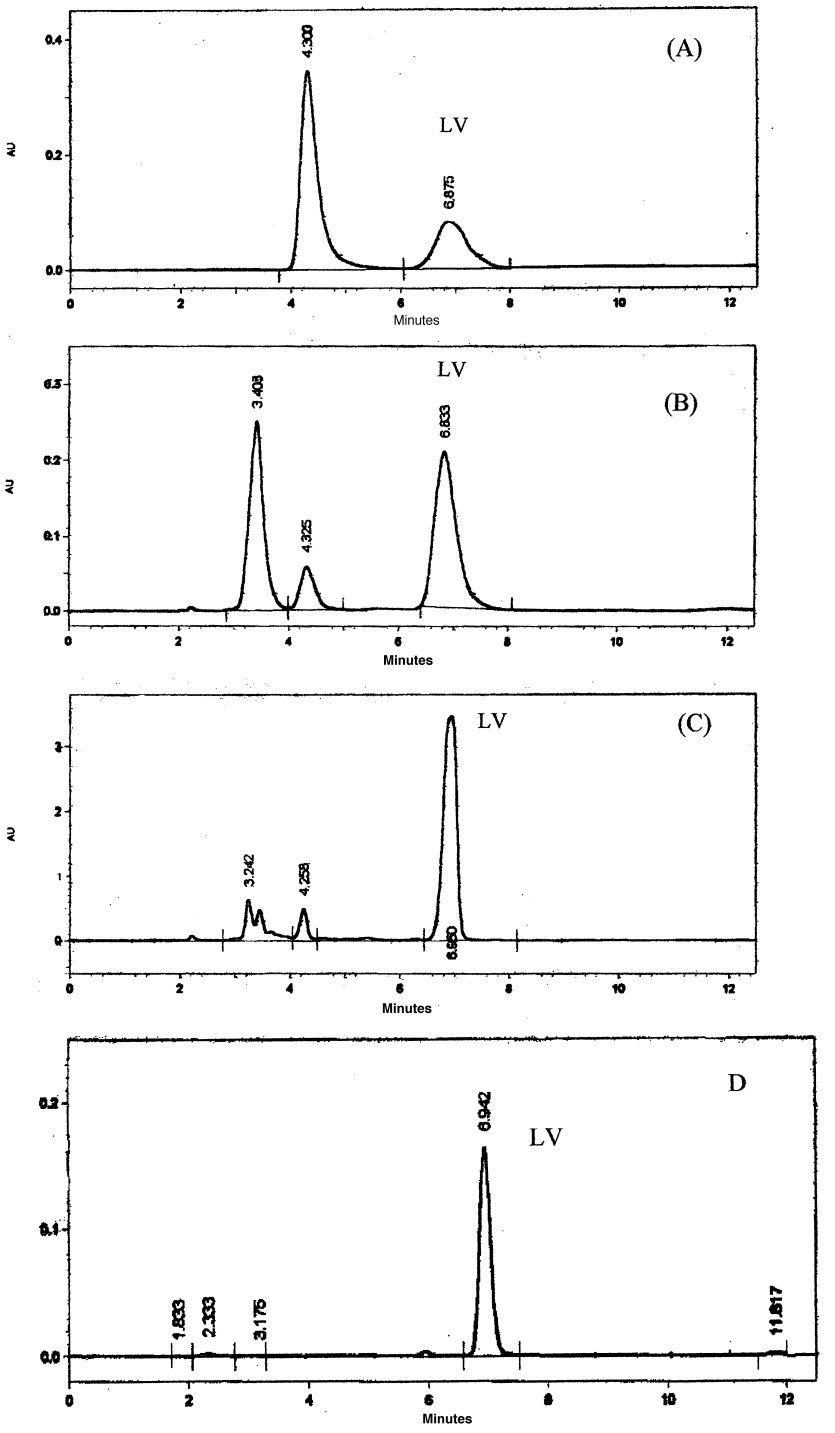

Fig. 3. Representative chromatograms of LV in presence of (A) LVA (B) 0.25% SP-1 (C) 0.25% SP-2 and (D) intestinal sac artefacts.

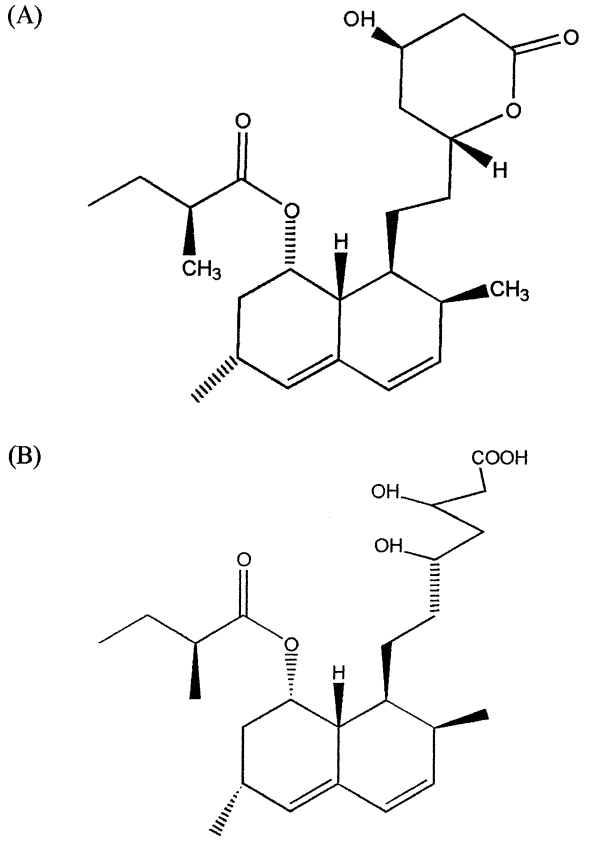

Fig. 4. Structural formula of (A) lovastatin (LV) and (B) lovastatin acid (LVA).

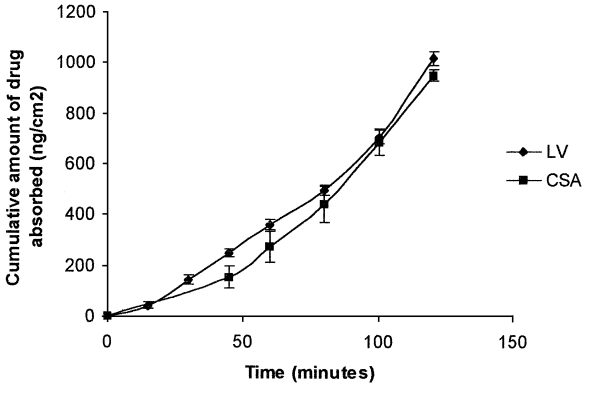

Fig. 5. In vitro absorption profiles of LV and CSA in rat everted intestinal sac model.

LV by using same mobile phase but with different proportions of components. It is capable of detecting low concentrations of CSA and LV in in vitro samples. The calibration curve was linear over the concentration ranges of 0.5–6.0 μg/ml for CSA and 0.05–0.4 μg/ml for LV ($R^2 > 0.99$ in both cases). There was no interference by the sorption promoters, intestinal sac artefacts or components of KRB. The method is accurate and precise as is evident from low RSD values in recovery studies, inter- and intra-day variations.

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References

[1] O. Pillai, A.B. Dhanikula, R. Panchagnula, Curr. Opin. Chem. Biol. 5 (2001) 439.
[2] B.D. Kahan, Transplantation 40 (1985) 457.
[3] H. Lennernas, G. Fager, Clin. Pharmacokinet. 32 (1997) 403.
[4] B.D. Kahan, Transplant. Proc. 31 (1999) 1635.
[5] A. Lindahl, R. Sandstrom, A.-L. Ungell, H. Lennernas, J. Pharm. Pharmacol. 50 (1998) 737.
[6] P. Sharma, H.P.S. Chawla, R. Panchagnula, Drugs Fut. 24 (1999) 1221.
[7] A.L. Ungell, A. Andreasson, K. Lundin, L. Utter, J. Pharm. Sci. 81 (1992) 640.
[8] C.Y. Yee, D.R. Salomon, in: W.E. Evans, J.J. Schentag, W.J. Juska (Eds.), Applied Pharmacokinetics—principles of Therapeutic Drug Monitoring, Applied Therapeutics, 1996, p. 28.1.
[9] K.A. Mereish, C.T. Ueda, Pharm. Res. 1 (1984) 245.
[10] R.J. Stubbs, M. Schwartz, W.F. Bayne, J. Chromatogr. 383 (1986) 438.
[11] A.W. Alberts, J. Chen, G. Kuron, V. Hunt, J. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, E. Harris, A. Patchett, R. Monaghan, S. Curie, E. Stapley, G. Albers-Schonberg, O. Hensens, J. Hirshfield, K. Hoogsteen, J. Liesch, J. Springer, Proc. Natl. Acad. Sci. USA 77 (1980) 3957.
[12] D. Wang-Iverson, E. Ivashkiv, M. Jemal, A.I. Cohen, Rapid Commun. Mass. Spectrom. 3 (1989) 132.
[13] K. Keyomarsi, L. Sandoval, V. Band, A.B. Pardee, Cancer Res. 51 (1991) 3602.
[14] H.H. Maurer, A.F.E. Rump, Drug Res. (Arzneim-Forsch) 41 (1991) 104.

[15] X. Boulenc, C. Roques, H. Joyeux, Y. Berger, G. Febre, Int. J. Pharm. 123 (1995) 13.
[16] S.D. Mithani, V. Bakatselou, C.N. TenHoor, J.B. Dressman, Pharm. Res. 13 (1996) 163.
[17] G.S. Brenner, D.K. Ellison, M.J. Kaufman, in: H.G. Brittain (Ed.), Analytical Profiles of Drug Substances and Excipients, Vol. 21, Academic Press, San Diego, 1992, pp. 277–305.
[18] M. Swartz, I.S. Krull, in: Analytical Method Development and Validation, Marcel Dekker, New York, 1997, pp. 24–48.
[19] N.E. Tayar, A.E. Mark, P. Vallat, R.M. Brunne, B. Testa, W.F. van Gunsteren, J. Med. Chem. 36 (1993) 3757.
[20] L.D. Bowers, S.E. Mathews, J. Chromatogr. 333 (1985) 231.
[21] J.C. Giddings, Anal. Chem. 35 (1963) 439.