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Simultaneous determination of timolol maleate, rosuvastatin calcium and diclofenac sodium in pharmaceuticals and physiological fluids using HPLC-UV

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ABSTRACT

A novel HPLC-UV method was developed for the simultaneous determination of timolol (TM), rosuvastatin (RST), and diclofenac sodium (DS) in pharmaceuticals, human plasma and aqueous humor using naproxen sodium as internal standard (IS). The target compounds were analyzed on Hypersil BDS C_{18} column (250 mm × 4.6 mm, 5 μm), applying 0.2% triethylamine (TEA) and acetonitrile (ACN) (40:60, v/v), in isocratic mode as mobile phase, pH 2.75 adjusted with 85% phosphoric acid at a flow rate of 1 ml/min. The column oven temperature was kept at 45 °C and the peak response was monitored at 284 nm after injecting a 50 μl sample into HPLC system. The direct liquid–liquid extraction procedure was applied to human plasma and bovine aqueous humor samples using mobile phase as an extraction solvent after deproteination with methanol. The different HPLC experimental parameters were optimized and the method was validated according to standard guidelines. The recoveries of the suggested method in human plasma were 98.72, 96.04, and 95.14%, for TM, RST, and DS, while in aqueous humor were 94.99, and 98.23%, for TM, and DS, respectively. The LOD values were found to be 0.800, 0.500, and 0.250 ng/ml, for TM, RST, and DS, respectively, while their respective LOQ values were 2.00, 1.50, and 1.00 ng/ml. The co-efficient of variation (CV) were in the range of 0.1492–1.1729% and 1.0516–4.0104%, for intra-day and inter-day studies, respectively. The method was found accurate in human plasma and bovine aqueous humor and will be applied for the quantification of these compounds in plasma, and aqueous humor samples using animal models and in pharmaceuticals.

1. Introduction

Timolol (TM) (Fig. 1), (*S*)-1-[(1,1-dimethyl)amino]-3-[[4-(4-morpholinyl9-1,2,5-thiadiazol-3-yl]oxy]-2-propanol, is a nonspecific β-adrenergic blocker. TM was the first β-blocker to be used as an antiglaucoma agent. None of the newer β-blockers were found to be as effective as TM. TM is administered as anti-hypertensive agent as well [1,2]. Quantitatively TM was determined by various spectrophotometric and HPLC techniques in bulk and finished pharmaceuticals and physiological fluids [3–7].

Rosuvastatin (RST) is a statin that lowers the blood cholesterol and is used for the treatment of hyperlipidemia (Fig. 1). It is a synthetic compound, a selective and competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyne A (HMG-CoA) reductase [8–10]. Chemically it is a bis[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl-(methyl-sulfonyl)amino]pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoicacid] calcium salt [3–5].

It is used to reduce the plasma LDL cholesterol, small dense LDL, total cholesterol, triglycerides and apolipoprotein B levels thus reduces the risk of cardiovascular events in hyperlipidemic and normocholesterolemic patients [11]. It also increases the level of HDL cholesterol to some extent [12]. Various analytical methods have been reported for the determination of RST including, spectrophotometric and HPLC-UV [13,14]. The simultaneous quantification of RST with gemfebrozil in human plasma [15], and with other statins in pharmaceutical preparations has been reported [16]. The simultaneous analysis of RST and atorvastatin in human plasma using HPLC-UV has been reported [14].

Diclofenac sodium (DS) is a member of NSAIDs class used as anti-inflammatory, analgesic and antipyretic (Fig. 1). DS is also used in the management of pain and ocular inflammation [17]. It stops synthesis of prostaglandins by inhibiting cycloxygenase enzyme. Chemically it is sodium 2-[2-(2,6-dichloroanilino) phenyl]acetate [18].

Several methods have been reported for the determination of DS such as spectroscopic [19], chromatographic [20,21] and the latest methods with LC–MS have been reported [22]. The simultaneous determination of DS with ketoprofen, naproxen, fenoprofen,

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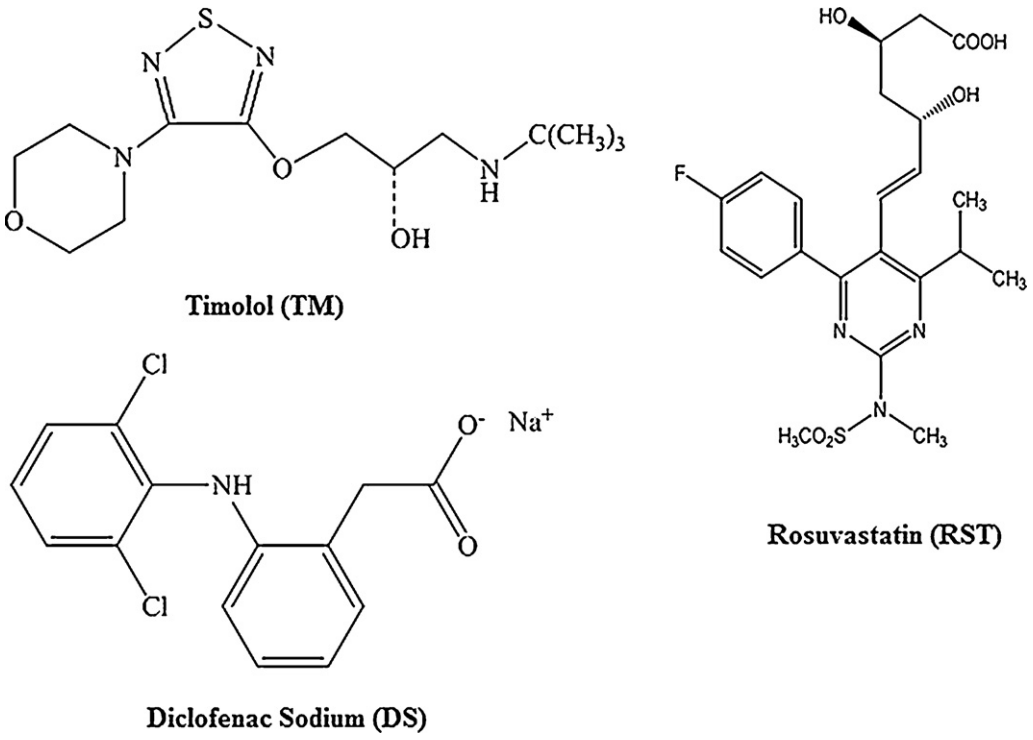

Fig. 1. Chemical structure of timolol maleate (TM), rosuvastatin (RST) and diclofenac sodium (DS).

flurbiprofen, ibuprofen, DS and mefenamic acid in pharmaceutical formulations and human plasma using HPLC-UV [23], and with other NSAIDs in aqueous preparation [24] and human plasma [22] using LC–ESI-MS has also been investigated.

The selected drugs in this study are usually co-prescribed in clinical practice. Till date no single method has been reported for simultaneous determination of TM, RST and DS in pharmaceuticals and physiological fluids, i.e. plasma, serum and aqueous humor. Therefore it is necessary to develop the sensitive and validated HPLC-UV method for the simultaneous determination of these drugs in human plasma samples. It is required to quantify these drugs with a single sensitive HPLC-UV method. The aim of this novel suggested method is to determine simultaneously the above drugs in the presence of naproxen sodium as an IS in pharmaceutical preparations and physiological fluids (plasma, serum and aqueous humor). The method was found accurate, rapid and sensitive in comparison with other reported methods [4,13]. The method was validated according to standard guidelines with optimization various experimental conditions [25,26]. This method will be applied for the determination of pharmacokinetic and drug–drug interaction studies of these drugs in human and animal models.

2. Materials and methods

2.1. Materials and reagents

Timolol maleate (TM) purity 97.5% (Schazo Pharma. Pvt. Ltd. Lahore), rosuvastatin (RST), purity 98.4% (Feroz Sons Laboratories Pvt. Ltd. Nowshera), diclofenac sodium (DS) purity 99.0% (Medicraft Pharma. Pvt. Ltd. Peshawar), were the kind gift of local pharmaceuticals. Ciprofloxacin, levofloxacin, naproxen sodium, atenolol, propanolol, and atorvastatin were obtained from Fluka (Sigma–Aldrich, Oslo, Norway). Methanol, acetonitrile, diethyl ether, tetrahydrofuran, chloroform, dichloromethane, n-hexane, ethanol, phosphoric acid, and triethylamine (HPLC grade), were purchased from Sigma–Aldrich (Oslo, Norway). Purified water was prepared using a Millipore ultra-pure water system (Milford, USA).

2.2. Instrumentation

Perkin-Elmer HPLC system (Norwalk, USA), consisted of a pump (series 200), on-line vacuum degasser (series 200), autosampler (series 200), Peltier column oven (series 200), linked by a PE Nelson network chromatography interface (NCI) 900 with UV/VIS (series 200). The whole HPLC system was controlled by Perkin-Elmer Totalchrom Workstation Software (version 6.3.1). The data was acquired and quantified by this software.

2.3. Preparation of standard stock solutions

Stock solutions of analytes and IS (ciprofloxacin, levofloxacin, naproxen sodium, atenolol, propanolol, and atorvastatin), each (1 mg/ml) were prepared in acetonitrile and stored in amber glass vials at −20 °C until analysis. Working standards solutions were prepared in volumetric flasks (10 ml), using mobile phase in the concentration range of 0.05–2 μg/ml of each analyte, keeping IS concentration 1 μg/ml in each sample. The calibration curves were constructed at seven concentrations levels for the standard solutions of each analyte. Similarly, a 1:1 mixture containing 1 μg/ml of each analyte and IS was also prepared.

2.4. Sample preparation

2.4.1. Plasma sample

Blood samples were collected from human volunteers, at Department of Pharmacy, University of Peshawar (Pakistan), in ethylenediamintetraacetic acid (EDTA) glass tubes and centrifuged at 1600 × *g* for 10 min at 4 °C. The study was approved by the concerned ethical committee. The plasma was collected and stored at −20 °C until analysis. For sample preparation the plasma was first thawed at room temperature and a volume (200 μl) was spiked

with the respective volume in the range of 10–400 μl of standard stock solution (50 μg/ml), of TM, RST and DS each to prepare their respective dilutions in the range of 0.05–2 μg/ml at 0.05, 0.100, 0.250, 0.500, 1.00, 1.50, and 2 μg/ml for each analyte. The equal volume (10 μl) of IS (1 mg/ml), was added to each sample to make its concentration 1 μg/ml in each sample and vortexed for 3 min. The liquid–liquid extraction procedure was applied as given in Section 2.4.3.

2.4.2. Aqueous humor sample

Aqueous humor was collected in borosilicate glass tubes from the bovine eyes. The collected sample was stored in screw capped air tight glass vials at −20 °C till analysis. At the time of analysis aqueous humor was thawed and a volume (200 μl), was spiked with the respective volume in the range of (10–400 μl), of the standard solutions of TM, and DS each to prepare their respective dilutions in the range of 0.05–2 μg/ml at 0.05, 0.100, 0.250, 0.500, 1.00, 1.50, and 2 μg/ml for each analyte. An equal volume (10 μl) of IS (1 mg/ml) was added to each sample to make its concentration 1 μg/ml in each sample and vortexed for 3 min. The extraction was carried out as given in Section 2.4.3.

2.4.3. Liquid–liquid extraction

Sample (200 μl) was transferred to plastic eppendorf tube (ca ≈2 ml), and spiked with each analyte in its respective concentration range and a constant amount of IS (1 μg/ml) was added to each sample. The samples were vortex-mixed for 3 min and methanol (600 ml) was added for de-proteination. Extraction was carried out with 1 ml of mobile phase. The samples were then centrifuged for 5 min at 2000 × *g* and 4 °C. After centrifugation the clear supernatant was transferred to eppendorf tube and the volume was made to 1 ml with mobile phase. Sample (50 μl) of the supernatant was injected into HPLC for analysis.

Calibration curves were constructed for all the analytes in the range of 0.05–2 μg/ml at 0.05, 0.100, 0.250, 0.500, 1.00, 1.50, and 2 μg/ml for each analyte using naproxen sodium (1 μg/ml) as IS in mobile phase, spiked plasma and spiked aqueous humor and different columns like Hypersil BDS C_{18} column (250 mm × 4.6 mm, 5 μm); Symmetry C_8 (250 mm × 4.6 mm, 5 μm); Discovery HS C_{18} column (150 mm × 4.6 mm, 5 μm), Summetry C_8 column (150 mm × 3.9 mm, 5 μm) were evaluated for separation of drugs in the mixture.

2.5. Chromatographic conditions

The HPLC analysis of the studied compounds was performed using ACN:0.2% TEA (60:40, v/v), pH 2.75 adjusted with 85% phosphoric acid, as mobile phase pumped at flow rate of 1 ml/min, in isocratic mode on Hypersil BDS C_{18} column (250 mm × 4.6 mm, 5 μm). The column oven temperature was kept at 45 °C and the peak response was monitored at a wavelength of 284 nm. The sample (50 μl) was injected into HPLC system and the data was acquired using Perkin-Elmer Totalchrom Workstation Software (version 6.3.1).

2.6. Chromatographic conditions and experimental parameters optimization

2.6.1. Selection of stationary phase (column)

Different particulate reversed-phase chromatographic columns (stationary phases) such as; Hypersil BDS C_{18} column (250 mm × 4.6 mm, 5 μm); Symmetry C_8 (250 mm × 4.6 mm, 5 μm); Discovery HS C_{18} column (150 mm × 4.6 mm, 5 μm), Summetry C_8 column (150 mm × 3.9 mm, 5 μm), protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA), pre-column guard cartridge were tried for the analysis of TM, RST and DS.

2.6.2. Mobile phase composition

The mobile phase composition was optimized using various organic solvents including methanol, acetonitrile, tetrahydrofuran, and water in different composition in isocratic mode for the analysis of the above mentioned compounds. The mobile phase composition that resulted in a better resolution and shorter analysis time of the studied compounds was selected as mobile phase for the simultaneous analysis.

2.6.3. Mobile phase flow rate

The mobile phase flow rate was adjusted in isocratic mode for the analysis of studied analytes after applying various flow rats in the range of 0.9–2 ml/min.

2.6.4. Column oven temperature

The column oven temperature significantly affects the elution and resolution of different compounds. The column oven temperature was therefore evaluated in the range of 25–50 °C to show its effects on the analysis of above mentioned compounds.

2.6.5. Internal standard

Different compounds including ciprofloxacin, levofloxacin, naproxen sodium, atenolol, propanolol, and atorvastatin were tried to be used as IS. The compound that showed better compatibility, best recovery and shorter analysis time was selected as IS for the suggested method.

2.6.6. Sample size

The sample loop size was evaluated in the range of 20–50 μl to adjust the sample size and minimize the problems like column loading and lack of sensitivity of the mentioned compounds.

2.6.7. Detector wavelength

For simultaneous determination of TM, RST, and DS using naproxen sodium as IS the detector's wavelength was evaluated in the range of 280–300 nm. The wavelength that resulted in the optimal sensitivity and better resolution was chosen as the wavelength for simultaneous analysis of studied compounds.

2.7. Validation of the method

The proposed method was validated according to standard guidelines [27]. The precision, specificity, sensitivity, linearity, recovery, robustness, stability of solutions and system suitability parameters were evaluated. The proposed method was validated with respect to the following parameters.

The specificity of the suggested method was tested through separation of studied compounds in the mobile phase, 1:1 mixture (containing 1 μg/ml of each analyte and IS), plasma and aqueous humor samples spiked with appropriate concentration of each analyte.

Percent recovery was tested to measure the accuracy of the suggested method. The % recovery was determined from spiked plasma and aqueous humor samples at two selected concentration levels of each analyte keeping IS concentration same in each sample. Recovery was calculated according to the following equation:

$$\text{Recovery} = \frac{[C] \times 100}{[A]} \quad (1)$$

where [*A*] is the peak area response ratios of the analytes with reference to IS in the mobile phase; *C* is the peak area response ratios of the analytes with reference to IS in spiked plasma/aqueous humor.

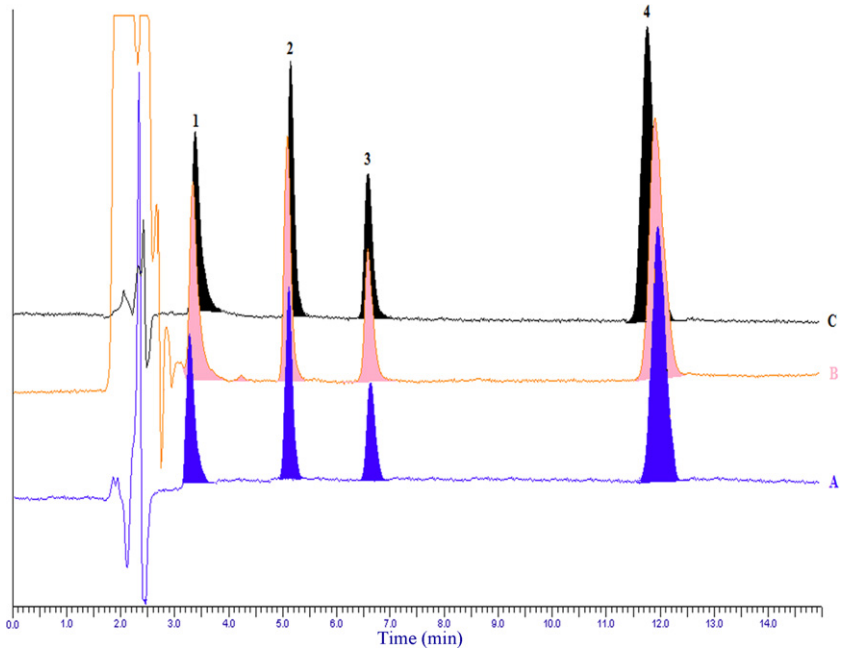

Fig. 2. Overlay of different representative chromatograms. A = spiked aqueous humor, B = spiked plasma sample and C = standard solution. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at column oven temperature of 45 °C using mobile phase ACN:0.2% TEA in the ratio of 45:55 v/v and flow rate of 1.0 ml/min.

The linearity of the proposed method was determined from the calibration curves constructed at seven concentration levels. Calibration curves were constructed for all the analytes in the mobile phase, spiked plasma and spiked aqueous humor samples by plotting their peaks response ratios (ratios of peak areas of analytes to IS) with respect to their respective spiked concentrations using a linear least squares regression analysis. The slope (m), intercept (b), and correlation coefficient (r) were calculated from their respective regression equations.

Precision of the method was determined through injection repeatability and analysis repeatability of spiked plasma and aqueous humor samples. Injection repeatability was assessed through injecting 10 times the same plasma and aqueous humor spiked with 1 μg/ml of each analyte into the HPLC system. The retention time and peak area of each analyte were expressed as mean, standard deviation (SD), and covariance (%RSD) as precision of the suggested method. Analysis repeatability was evaluated from the analysis of five spiked samples prepared from the same plasma/aqueous humor spiked with 1 μg/ml of each analyte, and the results were expressed as mean, standard deviation (SD), and covariance (%RSD) of the recovered amount. Intra-day and inter-day studies were carried out on spiked plasma/aqueous humor samples at 8:00, 16:00, and 24:00 h, for 1 week at alternate days to assess the intermediate precision. The results were expressed as mean, standard deviation (SD), and covariance (%RSD) of the recovered amount. The recovered amount was calculated in the form of concentration by the following equation:

$$C = \left(\frac{X}{Y}\right) \times \left(\frac{A}{B}\right) \times C_s \times F_D \quad (2)$$

where X and Y are peak areas of the analyte in plasma/aqueous humor samples and 1:1 mixture (1 μg/ml of each analyte and IS), respectively; A and B are peak areas of the IS in plasma/aqueous humor samples and 1:1 mixture (1 μg/ml of each analyte and IS), respectively; C_s is the concentration of analyte in the 1:1 mixture; F_D is the dilution factor.

The limit of detection (LOD) and limit of quantification (LOQ) for all the analytes were quantified at a concentration whose signal-to-noise ratio (S/N) was three and ten, respectively. For LOD and LOQ evaluation dilutions of the analytes were prepared in the ranges of 0.5–5 ng/ml and 5–20 ng/ml for all the analytes. The LOD and LOQ were then determined from the peaks by the software at signal-to-noise ratio (S/N) of three and ten, respectively.

The robustness of the proposed method was tested through small deliberate changes in the various chromatographic conditions, like mobile phase composition (±2%), column oven temperature (±5 °C), detector wave length (±2 nm) and flow rate of mobile phase (0.2 ml/min).

Stability studies of standard solutions and spiked plasma/aqueous humor samples stored at 25 °C, 4 °C and −20 °C were carried out for 1 month. The % stability was calculated by the following equation.

$$\%\,\text{Stability} = \frac{S_t}{S_0} \times 100 \quad (3)$$

where S_t is stability of analyte at time t, and S_0 is stability at initial time.

3. Results and discussion

The suggested method is novel in the sense that simultaneous determination of TM, RST, and DS was carried out for the first time using naproxen sodium as an IS. The experimental parameters of the method were optimized and the method was validated according to standard guidelines [27]. All the analytes were separated applying the proposed method in standard mixtures, plasma samples, and aqueous humor samples as presented in Fig. 2. The suggested method was found accurate and quite specific for the simultaneous analysis of these compounds in plasma and aqueous humor samples. Complete separation of the target compounds was achieved in 7 min using the proposed method.

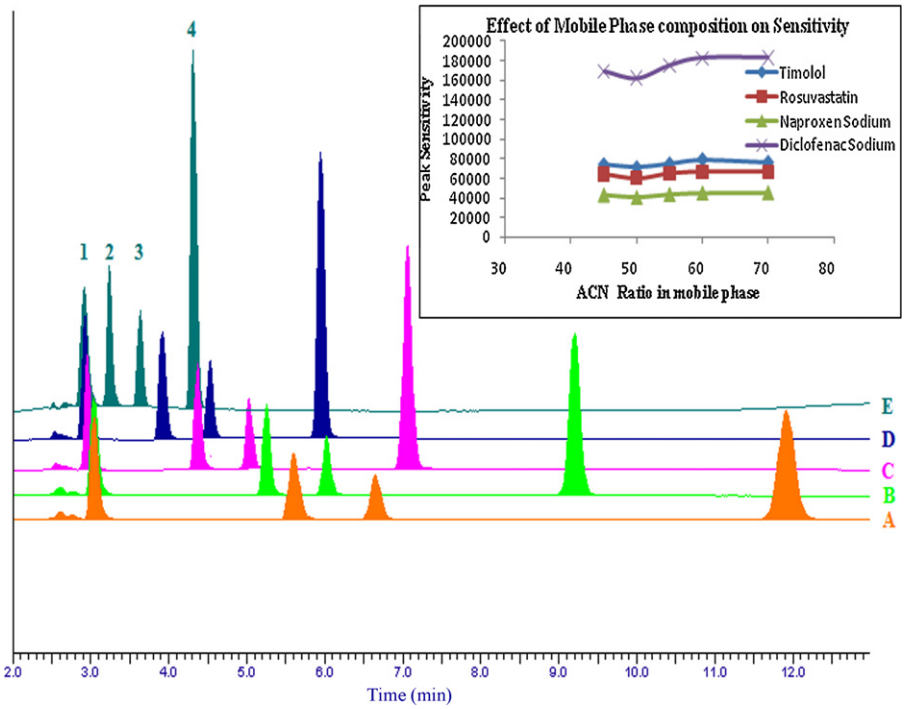

Fig. 3. Influence of ACN ratios in the mobile phase on the elution of different analytes. A = having 45% ACN, B = having 50% ACN, C = having 55% ACN, D = having 60% ACN and E = having 70% ACN. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at column oven temperature of 45 °C at a flow rate of 1.0 ml/min.

3.1. Optimization of HPLC experimental parameters

Different experimental parameters were optimized in the specified ranges to choose the optimum mobile phase, stationary phase, mobile phase flow rate, detector's wavelength, column oven temperature and pH.

The mobile phase comprised of ACN:0.2% TEA in the ratio of 60:40 (v/v), was selected for the analysis of the above mentioned compounds. The retention times of the studied compounds decreased with increasing the ratio of acetonitrile (ACN) in the mobile phase. The overall analysis time decreased significantly with increasing the ACN content. The effect of ACN on the retention time of DS was greater in comparison with TM and RST. Increasing the ratio of ACN above 60% resulted in the co-elution of TM peak with the solvent front although the analysis time decreased appreciably as presented in Fig. 3.

The optimal stationary phase that resulted in better separation and resolution of the studied compounds among the various tested stationary phases was selected for the simultaneous determination of above mentioned analytes. The better separation was achieved on Hypersil BDS C_{18} column among the tested columns. The poor resolution of TM was obtained as TM was coeluted with the peak of solvent front in case of other tested columns.

Similarly, flow rate of mobile phase greatly affected the analysis of the studied analytes. Although run time decreased significantly at higher flow rates along with better resolution of the peaks, however; sensitivities of the analytes decreased as shown in Fig. 4. The flow rate greatly affected the retention of DS in comparison with other analytes. Co-elution of TM with solvent front resulted at higher flow rates. The flow rate 1 ml/min was chosen as optimal flow rate for the simultaneous analysis of these compounds.

Peak sensitivities were not affected significantly by column oven temperature, however, run time was decreased and resolution of the peaks was increased at higher temperature. The optimal temperature for the simultaneous analysis was chosen 45 °C as shown in Fig. 5.

Higher sensitivities and better resolution of the analytes were achieved at pH 2.75 of the mobile phase among the different tested pH of the mobile phase as shown in Fig. 6. The sensitivity of DS was greatly affected by pH of the mobile phase as compared with other analytes. Variation in the retention times of the analytes was observed at different pH as shown in Fig. 6.

Detector's wavelength has been selected after recording the sensitivities of the analytes at various wavelengths. The greater sensitivities of TM, RST and DS were recorded at 284 nm. Small changes in the retention times of the analytes have been observed as evident in Fig. 7.

IS that has shown better sensitivity, recovery, stability and compatibility with other analytes was chosen among the various tested compounds. Naproxen sodium was used as IS in these studies as it resulted in better recovery and good compatibility in comparison with other tested compounds.

3.2. Sample preparation

Standard stock solutions of TM, RST, DS and naproxen sodium were prepared in acetonitrile and working dilutions were prepared in the mobile phase on daily basis. Extraction from plasma and aqueous humor samples was carried out using different organic solvents. De-proteination was carried out using methanol and the analytes were then extracted applying various organic solvents. The mobile phase resulted in better recoveries of the analytes as compared with other solvents used for extraction. The supernatant separated after centrifugation was directly injected into HPLC system and complete separation of all the target analytes was achieved.

3.3. Method validation

The suggested method was validated in terms of selectivity, sensitivity, recovery, precision, and robustness according to standard guidelines.

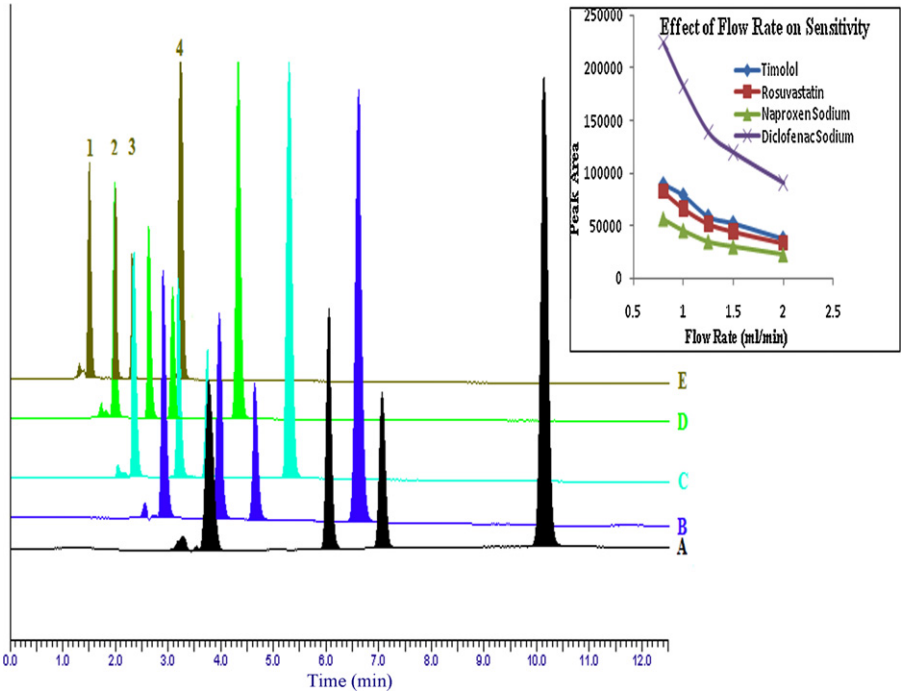

Fig. 4. Influence of flow rate of the mobile phase on the elution of different analytes. A = at 0.8 ml/min, B = at 1.0 ml/min, C = at 1.25 ml/min, D = at 1.5 ml/min and E = at 2 ml/min. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at column oven temperature of 45 °C using mobile phase ACN:0.2% TEA in the ratio of (60:40, v/v).

The target compounds were separated in standard mixtures, plasma and aqueous humor samples using the proposed method with no extraneous peak. The method was found quite suitable for the analysis of the above studied analytes.

The linearity of the method was evaluated from the calibration curves of standard mixtures and spiked plasma and aqueous humor samples constructed at seven concentration levels of all the analytes in the range of 0.05–2 μg/ml. Calibration curves of standard mixtures of TM, RST, and DS and their spiked plasma samples and aqueous humor samples are shown in Fig. 8a and b, respectively. The regression equation and their respective correlation co-efficient (r) are given in Table 1.

Accuracy of the proposed method was determined on the basis of percent recovery at three concentration levels (0.05, 1

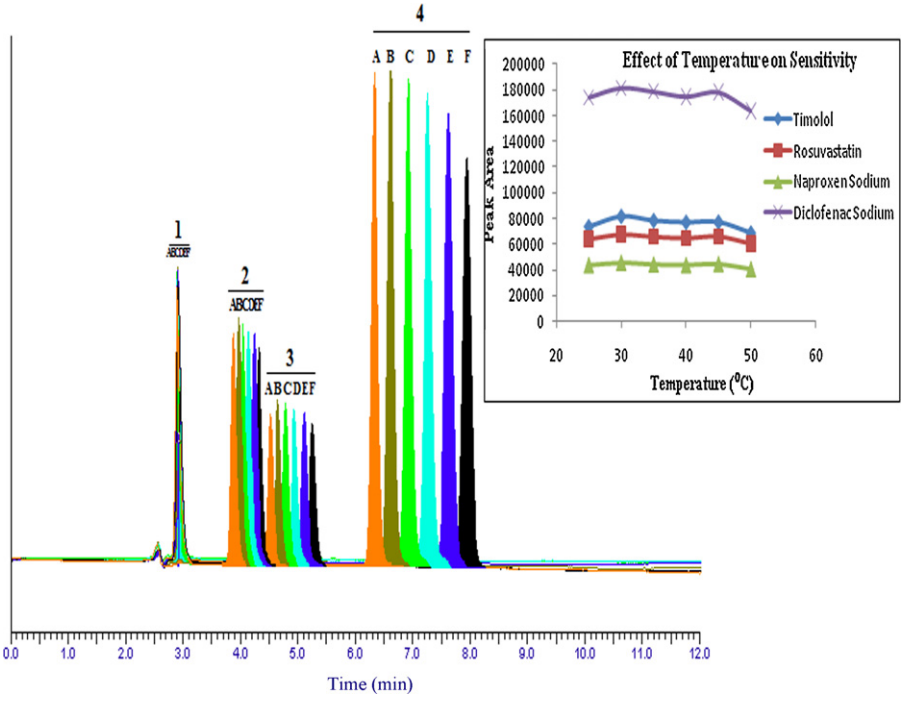

Fig. 5. Influence of column oven temperature on the elution of different analytes. A = at 50 °C, B = at 45 °C, C = at 40 °C, D = at 35 °C, E = at 30 °C and F = 25 °C. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at a flow rate of 1.0 ml/min using mobile phase ACN:0.2% TEA in the ratio of (60:40, v/v).

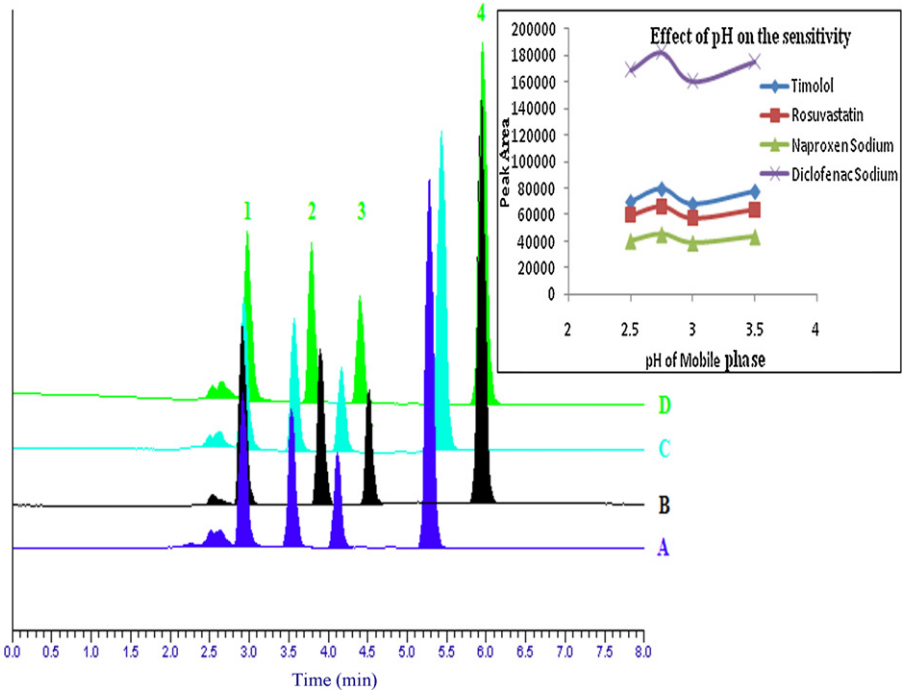

Fig. 6. Influence of various pH of the mobile phase on the elution of different analytes. A = at pH 2.5, B = at pH 2.75, C = at pH 3.0 and D = at pH 3.5. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at column oven temperature of 45 °C using mobile phase ACN:0.2% TEA in the ratio of (60:40, v/v), and flow rate of 1.0 ml/min.

and 2 μg/ml), for TM, RST, and DS. The results are shown in Table 1.

The precision of the method was evaluated through injection repeatability, analysis repeatability, and intra-day, inter-day studies as shown in Table 2. The intra-day co-efficient of variation (% CV) was in the ranges of 0.183–0.635, 0.949–1.867, and 0.940–1.910 for TM, RST, and DS, respectively. Similarly, their respective values for inter-day studies were in the range of 2.182–4.010, 1.070–3.685, and 1.052–2.158 for TM, RST, and DS, respectively.

LOD and LOQ values were determined at precision and accuracy of ~20% variations for the evaluation of sensitivity of the suggested method. The respective chromatograms of LOD and LOQ are given in Fig. 9. The respective values are given in Table 1.

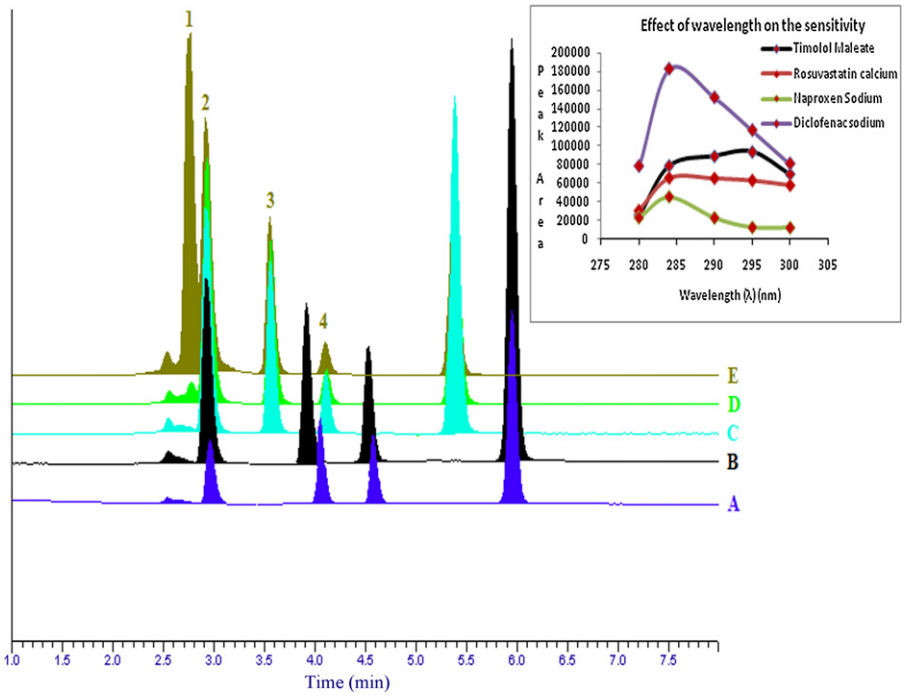

Fig. 7. Influence of various detector wavelengths on the elution of different analytes. A = at 280 nm, B = at 284 nm, C = at 290 nm, D = at 295 nm and E = at 300 nm. *Peaks*: 1, timolol; 2, rosuvastatin; 3, naproxen sodium; 4, diclofenac sodium. The chromatograms were obtained at column oven temperature of 45 °C using mobile phase ACN:0.2% TEA in the ratio of 60:40 (v/v), and flow rate of 1.0 ml/min.

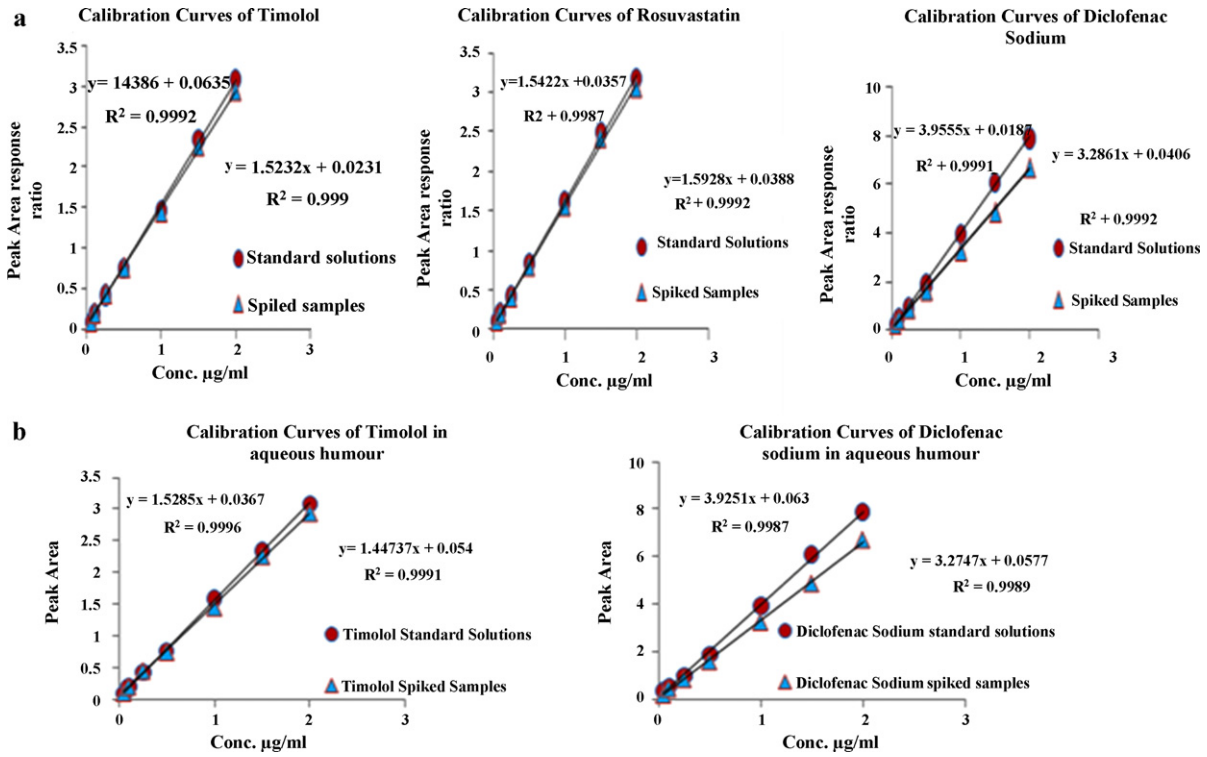

Fig. 8. (a) Calibration curves of timolol, rosuvastatin and diclofenac sodium standard solutions and spiked plasma samples. (b) Calibration curves of timolol and diclofenac sodium standard solutions and spiked aqueous humor *Note*: Each point is a mean of triplicate injections.

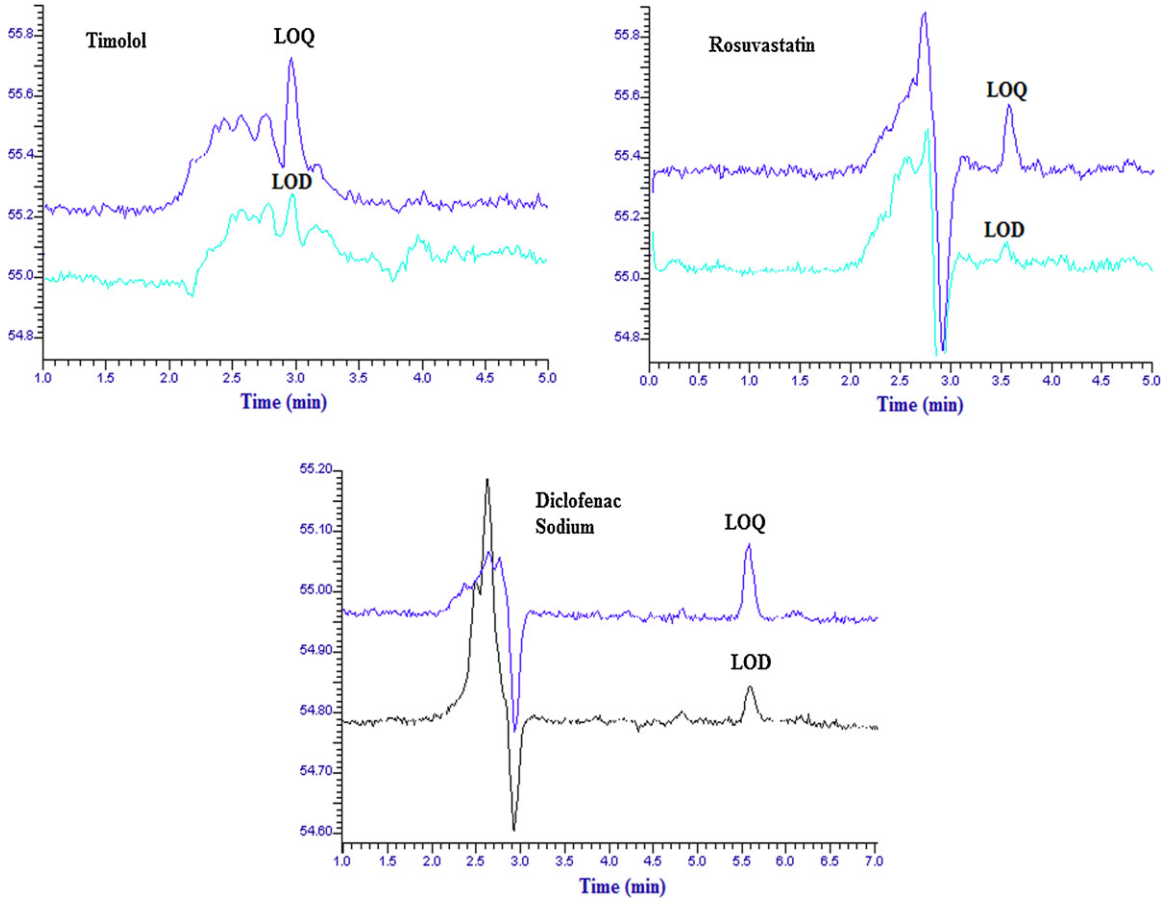

Fig. 9. Chromatograms representing LOD and LOQ values of timolol, rosuvastatin and diclofenac sodium.

Table 1
Validation parameters of the proposed method.

Parameters	Analytes		
	Timolol (mean ± SD; %RSD)	Rosuvastatin (mean ± SD; %RSD)	Diclofenac sodium (mean ± SD; %RSD)
Linearity			
Calibration range (μg/ml)	0.05–2 μg/ml	0.05–2 μg/ml	0.05–2 μg/ml
Standard solution			
Regression equation	$y = 1.523x + 0.023$	$y = 1.592x + 0.038$	$y = 3.955x + 0.01$
Correlation coefficient (R^2)	0.999	0.999	0.999
Spiked plasma			
Regression equation	$y = 1.483x + 0.063$	$y = 1.542x + 0.035$	$y = 3.286x + 0.040$
Correlation coefficient (R^2)	0.999	0.998	0.999
Standard solutions			
Regression equation	$y = 1.528x + 0.036$		$y = 3.925x + 0.063$
Correlation coefficient (R^2)	0.999		0.998
Spiked aqueous humor			
Regression equation	$y = 1.443x + 0.054$		$y = 3.274x + 0.057$
Correlation coefficient (R^2)	0.999		0.998
Accuracy (% recovery)			
Spiked sample (0.05 μg/ml)	95.17 ± 1.24; 1.30	97.15 ± 1.03; 1.06	94.77 ± 0.96; 1.01
Spiked sample (1.0 μg/ml)	98.30 ± 1.01; 1.08	94.99 ± 1.11; 1.17	96.31 ± 0.73; 0.75
Spiked sample (2.0 μg/ml)	97.25 ± 0.33; 0.34	94.03 ± 0.12; 0.13	98.99 ± 0.19; 0.20
Accuracy (amount recovered)			
Spiked sample (0.05 μg/ml)	0.049 ± 0.0010; 2.04	0.048 ± 0.0008; 1.67	0.047 ± 0.0009; 1.91
Spiked sample (1.0 μg/ml)	0.986 ± 0.0122; 1.23	0.951 ± 0.0113; 1.19	0.965 ± 0.0119; 1.23
Spiked sample (2.0 μg/ml)	1.945 ± 0.0101; 0.52	1.891 ± 0.0090; 0.48	1.981 ± 0.0106; 0.54
Percent recovery (relative) (% recovery ± SD; %RSD) (spiked human plasma) spiked sample (1.0 μg/ml)	98.72 ± 1.19; 1.20	96.04 ± 3.09; 3.22	95.14 ± 1.19; 1.25
Percent recovery (absolute) (% recovery ± SD; %RSD)	(92.65 ± 2.32; 2.50)	(94 43 ± 1.58; 1.67)	(93.54 ± 1.38; 1.47)
Percent recovery (relative) (% recovery ± SD; %RSD) (spiked aqueous humor) spiked sample (1.0 mcg/ml)	94.99 ± 0.98; 1.03	96.04 ± 3.09; 3.22	98.23 ± 1.13; 1.15
Percent recovery (absolute) (% recovery ± SD; %RSD)	92.56 ± 1.72; 1.86	94.25 ± 1.49; 1.58	95.85 ± 1.52; 1.59
Repeatability			
Injection repeatability			
Spiked sample (2.0 mcg/ml)	2.9 ± 0.02; 0.69[a]	3.8 ± 0.01; 0.26[a]	6.2 ± 0.02; 0.32[a]
Spiked sample (2.0 mcg/ml)	27437.33 ± 109.20; 0.40[b]	28328 ± 110.49; 0.3[b]	77533.33 ± 136.55; 0.18[b]
Analysis repeatability			
Spiked sample (2.0 mcg/ml)	1.91 ± 0.02; 1.04	1.90 ± 0.01; 0.53	1.92 ± 0.02; 0.76
Sensitivity			
Limit of detection (ng/ml)	0.800	0.500	0.250
Limit of quantification (ng/ml)	2.000	1.500	1.000

[a] Retention time (min) of the analyte.
[b] Peak area of the analyte.

Stability studies were conducted at room temperature (25 °C), and at freezer temperature (−20 °C), for the standard mixtures and spiked plasma samples of TM, RST, and DS and aqueous humor samples spiked with TM, and DS for 1 week, respectively. The results obtained have shown that these compounds are stable at freezer temperature. The TM and DS standard solutions as well as spiked samples were degraded at room temperature.

Ruggedness of the proposed method was determined through small deliberate changes in various experimental parameters and the resulted changes in the peak area and retention times were found non-significant.

Table 2
Intra-day and inter-days studies.

Spiked concentration (μg/ml)	Concentration recovered (μg/ml)			
	Intra-day (mean ± S.D)	%RSD	Inter-day (mean ± S.D)	%RSD
Timolol				
1.0	0.9386 ± 0.0095	0.6357	0.9166 ± 0.0368	4.0104
1.5	1.5098 ± 0.0063	0.4170	1.4527 ± 0.0543	3.7410
2.0	1.8933 ± 0.0035	0.1834	1.8552 ± 0.0405	2.1821
Rosuvastatin				
1.0	0.9499 ± 0.0111	1.1729	0.9071 ± 0.0334	3.6859
1.5	1.4332 ± 0.0054	0.3779	1.4171 ± 0.0205	1.4473
2.0	1.8675 ± 0.0028	0.1492	1.8421 ± 0.0197	1.0708
Diclofenac sodium				
1.0	0.9404 ± 0.0100	1.0586	0.9093 ± 0.0196	2.1580
1.5	1.4800 ± 0.0104	0.7052	1.4395 ± 0.0284	1.9763
2.0	1.9166 ± 0.0146	0.7622	1.8767 ± 0.0197	1.0516

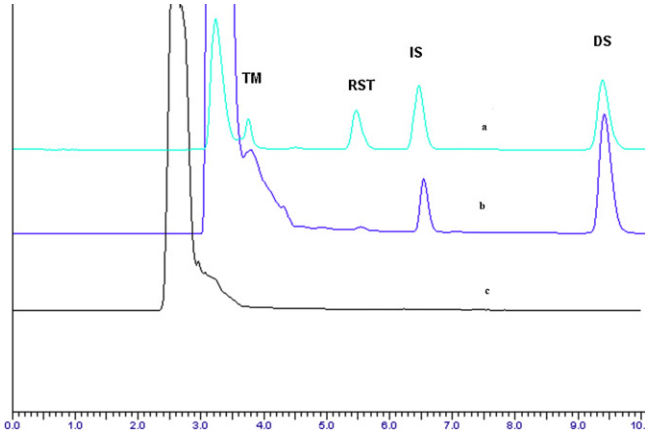

Fig. 10. Chromatograms representing timolol (TM), rosuvastatin (RST), and diclofenac sodium (DS) in: a, blank plasma; b, real plasma; c, 1:1 mixture (1 μg/ml of each analyte and IS).

4. Application of the method

The proposed validated HPLC-UV method was applied for the simultaneous assessment of TM, RST, and DS using naproxen sodium as IS in human plasma (Fig. 10b), and bovine aqueous humor. This method is a part of biochemical analysis of plasma and aqueous humor samples that will be collected from animal models. The method will be applied for the pharmacokinetic analysis of these compounds in animal models later on.

5. Conclusion

The developed suggested method is novel, robust and easy to automate. The method was validated according to standard guidelines and various experimental parameters were optimized for the simultaneous determination of TM, RST, and DS in human plasma and TM and DS in aqueous humor. Extraction was carried out with mobile phase preceded by protein precipitation with methanol. The complete separation of all targets peaks was achieved in 7 min using naproxen sodium as an IS. The proposed method will be applied for the pharmacokinetic studies of these analytes in physiological fluids (plasma, serum and aqueous humor) using animal models. This method can also be applied for the simultaneous quantification of these compounds in pharmaceuticals and in routine laboratory practice.

References

[1] T. Ishizaki, K. Tawara, Y. Oyama, H. Nakaya, J. Clin. Pharmacol. 18 (1978) 519.
[2] P. Lund-Johansen, Acta Med. Scand. 199 (1976) 263.
[3] M.L. Satuf, J.C.H. Robles, c.C. Goicoechea, A.C. Olivieri, Anal. Lett. 32 (1999) 2019.
[4] N. Erk, J. Pharmaceut. Biomed. 28 (2002) 391.
[5] M.C.F. Ferraro, P.M. Castellano, T.S. Kaufman, J. Pharmaceut. Biomed. 34 (2004) 305.
[6] S.P. Kulkarni, P.D. Amin, J. Pharmaceut. Biomed. 23 (2000) 983.
[7] D.J. Mazzo, J. Chromatogr. 299 (1984) 503.
[8] W.V. Brown, H.E. Bays, D.R. Hassman, J. McKenney, R. Chitra, H. Hutchinson, E. Miller, Am. Heart J. 144 (2002) 1036.
[9] P.H. Jones, M.H. Davidson, E.A. Stein, H.E. Bays, J.M. McKenney, E. Miller, V.A. Cain, J.W. Blasetto, Am. J. Cardiol. 92 (2003) 152.
[10] A.G. Olsson, F. McTaggart, A. Raza, Cardiovasc. Drug Rev. 20 (2002) 303.
[11] J. Shepherd, S.M. Cobbe, I. Ford, C.G. Isles, A.R. Lorimer, P.W. Macfarlane, J.H. McKillop, C.J. Packard, N. Engl. J. Med. 333 (1995) 1301.
[12] J.W. Blasetto, E.A. Stein, W.V. Brown, R. Chitra, A. Raza, Am. J. Cardiol. 91 (2003) 3.
[13] M.S.A.B.I.N. Sultana, J. Clin. Chem. Soc. 55 (2008) 1022.
[14] Y. Shah, Z. Iqbal, L. Ahmad, A. Khan, M.I. Khan, S. Nazir, F. Nasir, J. Chromatogr. B 879 (2011) 557.
[15] S. Vittal, N.R. Shitut, T.R. Kumar, M.C.A. Vinu, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 1252.
[16] M.K. Pasha, S. Muzeeb, S.J.S. Basha, D. Shashikumar, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 282.
[17] M. Lafranco Dafflon, V.T. Tran, Y. Guex-Crosier, C.P. Herbort, Graefe's, Arch. Ophthalmol. Chic. 237 (1999) 289.
[18] A.R. Sallmann, Am. J. Med. 80 (1986) 29.
[19] Y.C. deMicalizzi, N.B. Pappano, N.B. Debattista, Talanta 47 (1998) 525.
[20] Y.M. El-Sayed, M.E. Abdel-Hammed, M.S. Sueliman, N.M. Najib, J. Pharm. Pharmacol. 40 (1988) 727.
[21] C. Arcelloni, R. Lanzi, S. Pedercini, G. Molteni, Fermo, A. Pontiroli, R. Paroni, J. Chromatogr. B 763 (2001) 195.
[22] M.E. Abdel-Hamid, L. Novotny, H. Hamza, J. Pharmaceut. Biomed. 24 (2001) 587.
[23] Y. Sun, K. Takaba, H. Kido, M.N. Nakashima, K. Nakashima, J. Pharmaceut. Biomed. 30 (2003) 1611.
[24] F.I. Farré, M.L.F.M. Ginebreda, A.T.L. Olivella, L.M.B.D. Vilanova, J. Chromatogr. A 1–2 (2001) 187.
[25] A. Khan, M.I. Khan, Z. Iqbal, Y. Shah, L. Ahmad, S. Nazir, D.G. Watson, J.A. Khan, F. Nasir, Talanta 84 (2011) 789.
[26] A. Khan, M.I. Khan, Z. Iqbal, Y. Shah, L. Ahmad, D.G. Watson, J. Chromatogr. B 878 (2010) 2339.
[27] N.A. Épshtein, Pharm. Chem. J. 38 (2004) 212.