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Optimization of RPLC Conditions for Quantitative Analysis of Atorvastatin and Rosuvastatin in Pharmaceutical Dosage Form

Zehra Üstün[1], Aybike Talay[1], Ebru Çubuk Demiralay[1]*

[1]*Süleyman Demirel University, Department of Chemistry, Faculty of Science and Literature, 32260 Isparta, Turkey*

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**Corresponding author e-mail: ebrucubukdemiralay@gmail.com, ebrucubuk@sdu.edu.tr*

Abstract: In this study, a simple reverse phase liquid chromatographic (RPLC) method has been developed and subsequently validated for simultaneous determination of atorvastatin (ATV) and rosuvastatin (RSV). The column used was X Terra C18, (250 mm x 4.6 mm I.D., 5µm) with flow rate of 1 mL min^{-1} using photodiode array detection at 244 nm. The described method was linear over a concentration range of 3-13 µg mL^{-1} and 4-14 µg mL^{-1} for the assay of ATV and RSV respectively. Losartan was used as internal standard (IS) in the experiment. The limit of detection (LOD) values for ATV and RSV were found to be 0.133 and 0.221 µg mL^{-1} respectively. Limit of quantification (LOQ) for ATV and RSV were found to be 0.473 and 0.670 µg mL^{-1}, respectively. The results of the study showed that the proposed RPLC method is useful for the routine determination of ATV and RSV and in their pharmaceutical dosage form.

Keywords: Method optimization, validation, simultaneous determination, atorvastatin, rosuvastatin

İlaç Dozaj Formunda Atorvastatin ve Rosuvastatinin kantitatif analizi için RPLC koşullarının optimizasyonu

Özet: Bu çalışmada, atorvastatin (ATV) ve rosuvastatinin (RSV) eş zamanlı tayini için ters faz sıvı kromatografi (RPLC) metodu geliştirilmiş ve valide edilmiştir. Ayırmada X Terra C18 (250 mm x 4,6 mm, 5µm) kolon kullanılmış ve 244 nm' de yürütülen çalışmada akış hızı 1 mL dakika^{-1} olarak belirlenmiştir. Tanımlanan metodun doğrusal aralığı ATV için 3-13 µg mL^{-1}, RSV için 4-14 µg mL^{-1}olarak belirlenmiştir. Deneyde iç standart (IS) olarak Losartan kullanılmıştır. ATV ve RSV için dedeksiyon limiti (LOD) değerleri sırasıyla 0,133 ve 0,221 µg mL^{-1} olarak hesaplanmıştır. Kantitasyon limiti (LOQ) değerleri ise ATV ve RSV için sırasıyla 0,473 ve 0,970 µg mL^{-1} olarak tayin edilmiştir. Elde edilen sonuçlara bakılarak, önerilen RPLC metodu ATV, RSV ve bunların farmasötik dozaj formlarının rutin analizlerinde kullanılabilir olduğu gözlemlenmiştir.

Anahtar Kelimeler: Metot optimizasyonu, validasyon, eş zamanlı tayin, atorvastatin, rosuvastatin

INTRODUCTION

Hypercholesterolaemia plays a crucial role in the development of atherosclerotic diseases in general and coronary heart disease in particular. The risk of progression of the atherosclerotic process to coronary heart disease increases progressively with increasing levels of total serum cholesterol or low density lipoprotein (LDL) cholesterol at both the individual and the population level [1].

Statins are a group of drugs used primarily in lowering cholesterol. The discovery of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (HMG-CoA), called statins, was a breakthrough in the prevention of hypercholesterolemia and related diseases [2]. Different types of statins are currently available: natural statins, semi-synthetic statins and fully synthetic statins. Rosuvastatin (RSV) is a fully synthetic statin and has been billed a "superstatin" because of its pronounced ability to reduce low-density lipoprotein cholesterol levels and increase high-density lipoprotein cholesterol compared with existing agents. The development of a concise synthetic strategy for rosuvastatin is therefore highly desired [3].

Atorvastatin (ATV) is used along with a proper diet to help lower "bad" cholesterol and fats (such as LDL, triglycerides) and raise "good" cholesterol (HDL) in the blood. It works by reducing the amount of cholesterol made by the liver. Lowering "bad" cholesterol and triglycerides and raising "good" cholesterol decreases the risk of heart disease and helps prevent strokes and heart attacks. In addition to eating a proper diet (such as a low-cholesterol/low-fat diet), other lifestyle changes that may help this medication work better include exercising, losing weight if overweight, and stopping smoking [4].

Reversed phase liquid chromatography (RPLC) technique is used in method development and in the understanding of the physicochemical phenomena of acid-base species. Knowledge of the dissociation constants (pK_a) in hydro-organic media used as mobile phases can be very useful in explaining the chromatographic behavior of analytes. Information about these values is necessary to choose the optimal chromatographic conditions for development of analytical methods for determination of active pharmaceutical ingredients (API) [5]. Various techniques employed include a derivative spectrophotometric method, UV spectroscopy, HPTLC, enzyme inhibition with radioactivity detection, as well as a number of HPLC methods. Most of the the analytical techniques for ATV and RSV described in the literature are based on the RPLC determination of these pharmaceutically active ingredients alone in pharmaceutical formulations with another active drug substance and biological samples [6-12]. A simultaneous determination of these drugs in pharmaceuticals was described by Gomes et.al. [12]. However, no references have been found for optimization study using chromatographic behaviours of ATV and RSV in pharmaceutical formulations. The difference of this study, combined effect of acetonitrile content and pH of the mobile phase on the retention behavior of ATV and RSV were used. The range of pH values used should be broad enough including the pK_a of the pharmaceutically active ingredients. The experimental region was selected in such a way that the retention factors of the these pharmaceutically active ingredients would stay within the limits $1<k<10$. The primary goal of the present work was to develop and validate more precise, accurate and reliable method for the determination of ATV and RSV in pharmaceutical dosage forms which would be suitable for routine use in clinical practice with regards to the wide range of concentration in tablet samples and large number of samples needed to be analyzed in short time.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and reagents were of analytical grade. ATV, RSV and losartan (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (organic modifier), potassium hydrogen phthalate (standard buffer), sodium hydroxide were obtained from Merck (Darmstadt, Germany). Ortho-phosphoric acid (min. 85%) was obtained from Riedel-de Haen (Germany).

Apparatus

The chromatographic equipment used consisted on a Shimadzu HPLC system (Shimadzu Technologies, Kyoto, Japan) equipped with a pump (LC-20AD), a diode array dedector (DAD, SPD-M20A), a column oven (CTO-10AS VP) and a degasser system (DGU-20A3). Investigated compounds were separated on X Terra C18 analytical column (250mm x 4.6mm I.D.,5 µm) with isocratic elution. pH measurements of the mobile phase were carried out with a Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach, Switzerland) using M-T combination pH electrode. The pH values of the mobile phases were measured against a 0.05 mol kg^{-1} potassium hydrogen phthalate solution as primary standard reference, dissolved in the appropriate acetonitrile-water medium in accordance with IUPAC rules [13].

Chromatographic Procedure

In this study, the mobile phases used were acetonitrile-water mixtures at 50% (v/v). At each mobile phase, different pH values were studied, spread over thepH range from 2.5 to 8.0 adjusted by the addition of 1.0 M sodium hydroxide solution containing 25 mM phosphoric acid of the mobile phase.

For each compounds the retention time values (t_R) were determined from three separate injections for every prepared mobile phase. Retention factors for each compound and mobile phase were calculated using the expression $k = (t_R-t_0)/t_0$. The dead time (t_0) was measured by injecting uracil solution (Sigma, USA, 0.1%, in water), which was established for each mobile phase and pH studied. The flow rate of the mobile phase was 1.0 mL min^{-1}. The column temperature was kept at 25^{o}C and the injection volume was 20 µL. The compounds studied had different optimal wavelengths (for ATV and RSV 244 nm, for losartan (IS) 215 nm).

Commercial Tablet Formulation

The commercially available tablet formulation. ALVASTIN® (produced by Ali Raif, İstanbul) containing 20 mg ATV and COLNAR® (produced by AstraZeneca, İstanbul) containing 5 mg RSV were analyzed using the proposed methods.

Analysis of Tablets

Ten tablets from the sample to be analyzed were accurately weighed and grind until just reduced to a fine powder. An accurately weighed amount of the powder equivalent to one tablet was transferred into a 100 mL volumetric flask. Approximately 50 mL of

acetonitrile was added and the content of the flask was sonicated for 15 min. The solution in the flask was completed to volume with acetonitrile. After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate and adding the appropriate IS solution, diluting them with mobile phase to obtain the final solution. The amounts of ATV and RSV were calculated from the corresponding regression equations.

Recovery Studies from Tablets

To keep an additional check on the accuracy of the method developed, recovery experiments were performed by adding the known amount of pure pharmaceutically active ingredients to preanalyzed samples of tablets. Known amounts of the pure pharmaceutically active ingredients and a constant level of an internal standard were added to preanalyzed tablet solution and the mixtures were analyzed. The percent recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. After three repeated experiments, the average recovery percentage of these compounds was calculated for each compound. Thus, the effect of common tablet formulation excipients on chromatograms (e.g., tail, broadening, etc.) was investigated. Recovery experiments also showed the reliability and suitability of the proposed method.

RESULTS AND DISCUSSION

One important criterion in the effectiveness of an optimization procedure is the minimum number of initial experiments that is required in order to predict the optimum conditions for separation. The aim of this study was to identify the influence of RPLC conditions on the separation and the retention factor of ATV and RSV based on a relationship between mobile phase pH and retention factors ($1<k<10$) to find out the optimum separation condition for simultaneous determination of the ATV, RSV and internal standard (losartan) (Figure 1) in pharmaceutical formulation.Preliminary experiments indicated that the X Terra C_{18} (250 mm, 4.6mm I.D., 5 μm) reversed phase column provides efficient and reproducible separation of studied compounds at 25 ^{o}C. Hence, X Terra C_{18} column was selected for method optimization and validation. The retention factors were obtained over a pH range of 2.5-8.0 in order to determine chromatographic behaviour of these substances using the RPLC method. The studied compounds are weak acids with pK_a from 5.5 to 6.0, so the eluent pH should be below 5.5 because at this pH all of them exist in undissociated form and could be separated on a C18 column. Thus, pH 4.5 was selected as optimum pH value with best peak shape and retention values. The influence of mobile phase pH on analyte retention is presented in Fig. 2. As an example, in Fig. 2, the experimental data obtained at 50% (v/v) acetonitrile-water binary mixture (k vs pH) are plotted for ATV and RSV, showing the sigmiodal curves.

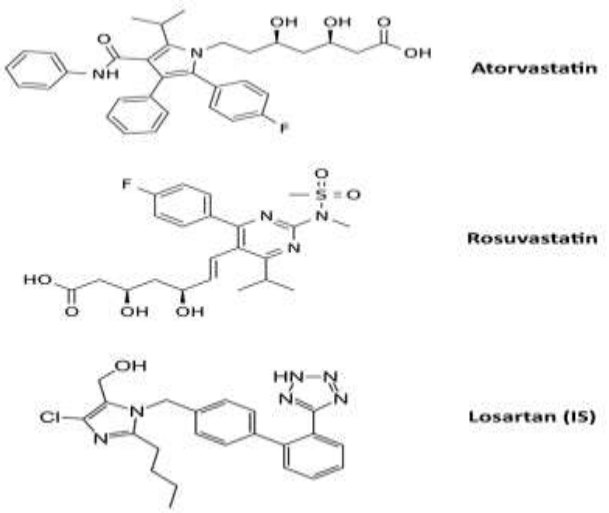

Figure 1. Chemical structures of studied compounds

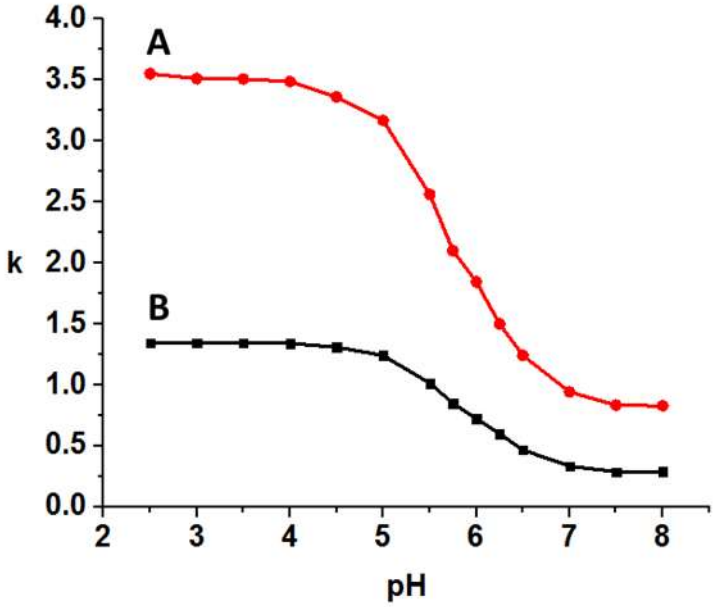

Figure 2. Values of retention factor values vs. pH of the mobile phase for A) ATV B) RSV on the X Terra C18 column at 25°C using the acetonitrile-water (50:50, v/v) binary mixture. The theoretical results are indicated as continuous lines and the plotted points are experimental results

The selectivity, efficiency and retention data were calculated with Purnell equation (Table 1). It can be achieved when the acetonitrile content in the mobile phase is 50% (v/v) at pH 4.5, in which all solutes are well separated in an analysis time of about 11 min. Under these conditions, the appropriate retention factor ($1<k<10$), selectivity ($\alpha \geq 1.15$), resolution ($R_s \geq 1.5$) and retention time were obtained. The proposed RPLC method provides a simple procedure to simultaneous analysis of investigated pharmaceutically active ingredients in pharmaceutical formulations by DAD detection.

Table 1. Retention, selectivity, and separation factor values for rosuvastatin, atorvastatin and losartan (IS), at 50% ACN at pH 4.5

Compounds	k_2	α	$k_2/1+k_2$	(α-1)/α	(¼)√N	R_s
Losartan (IS)/Rosuvastatin	2.073	1.544	0.352	0.675	22.504	5.347
Atorvastatin/Losartan (IS)	3.471	1.675	0.403	0.776	24.567	7.682

A widely used technique of quantitation involves the addition of an internal standard to compensate for errors in the analytical measurements. Hence, the quantitative determination of ATV and RSV was carried out using internal standard method. This method compensates for variations in physical parameters, especially inaccuracies in pipetting and injecting mL volume, requiring significant extraction, pretreatment or preparation steps. Losartan was chosen as the IS because it showed a shorter retention time with a better peak shape and a better resolution from the investigated compounds peak. According to United States Pharmacopoeia (USP 34) method [14], system suitability tests are an integral part of a liquid chromatographic method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. System suitability tests were carried out on freshly prepared standard stock solutions of studied compounds. The parameters include tailing factor, retention factor, theoretical plate number, retention time, tailing factor, selectivity, and RSD% of peak height or area for repetitive injections. System suitability test results are shown in Table 2. The chromatographic conditions described ensured adequate retention and resolution for all analytes. The results obtained from the system suitability tests satisfy the USP requirements. Using the described analytical method, an optimal resolution of the analytes was achieved. A typical chromatogram is shown in Figure 3. ATV, RSV and IS were well separated in a total duration of 11 min, with good peak resolutions, sharpness, and symmetry.

Table 2. System suitability test results

Parameter	Reference Range	RSV	Losartan (IS)	ATV
Retention Factor (k)	1-10	1.343	2.073	3.471
Selectivity Factor (α)	≥1.15	-	1.544	1.675
Resolution (Rs)	≥ 1.5	-	5.347	7.682
Tailing Factor (TF)	≤2	1.154	1.043	1.090
Theoretical plate number (N)	> 2000	7067	8190	9652

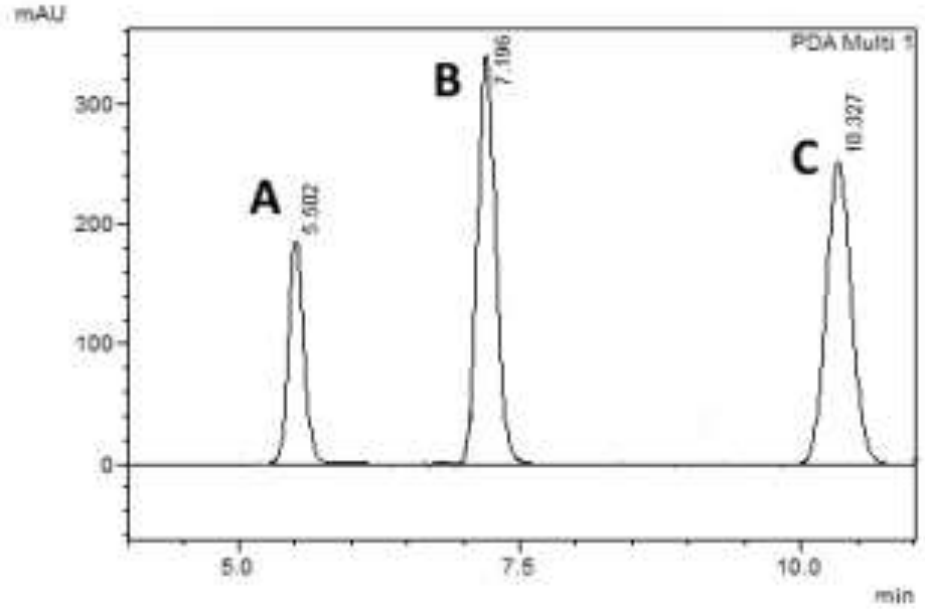

Figure 3. The representative chromatogram obtained analysis of standard mixture. A) RSV, B) Losartan (IS) and C) ATV

Calibration plots for proposed method were evaluated and checked by analyzing standard solutions at six concentration levels, ranging from 4.0 to 14 μg $mL^{-1}$for RSV, at seven concentration levels, ranging from 3.0 to 13 μg $mL^{-1}$for ATV. The calibration curve and equation for compounds were calculated by plotting the peak area ratios of compounds to IS vs. concentration of the compounds (Table 3). Limit of detection (LOD) and limit of quantitation (LOQ) were measured for pharmaceutically active ingredients. These parameters were determined according to 3.3:1 and 10:1 signal/noise ratios. Accuracy, precision, and reproducibility of the proposed method were evaluated by performing replicate analysis of the ATV and RSV in mobile phase.

Table 3. Statistical evaluation of the calibration data of atorvastatin and rosuvastatin by RPLC

Calibration parameters	Atorvastatin	Rosuvastatin
Linearity range (μg mL^{-1})	3-13	4-14
Slope	0.488	0.355
Intercept	0.064	0.042
S.E. of slope	0.002	0.003
S.E. of intercept	0.020	0.027
Correlation coefficient	0.999	0.999
Detection limit (μg mL^{-1})	0.133	0.221
Quantitation limit (μg mL^{-1})	0.403	0.670

Intra-day and inter-day variations of the method were determined using five replicate injections of two concentrations and analyzed on the same day and three different days.

Within calibration curves, two different concentrations were prepared in assayed with related calibration curves to determine intra-day and inter-day variability.Precision, accuracy and reproducibility of the intra-day and inter-day were determined as the RSD% and mean value. The intra-day variation was found between 0.070 and 0.522, RSD% values. Inter-day precision was determined by replicate analysis over 3-day period and RSD% values were found between 0.594 and 1.000.

The applicability of the proposed method was tested by the determination of ATV and RSV in tablet dosage forms. Development method was applied to the direct determination of these compounds in their tablet dosage form, using the related calibration straight line without any sample extraction or evaporation other than filtration and adequate dilution steps. The results obtained are satisfactorily accurate and precise as indicated by the excellent recovery% and RSD% < 2 (Table 4). Tablets common excipients, such as talc, lactose, starch, gelatin or magnesium stearate did not interfere with the assay. No interfering peaks were found in the chromatogram (Figure 4). To study accuracy of the method, recovery experiment was carried out by applying the standard addition method. Each of addition was repeated three times. The accuracy was expressed as the percentage of analytes recovered by the assay. Table 4 lists the recoveries of the drugs from spiked concentrations. Chromatograms is shown in Fig 5. The results indicate the method is highly accurate for determination of ATV and RSV.

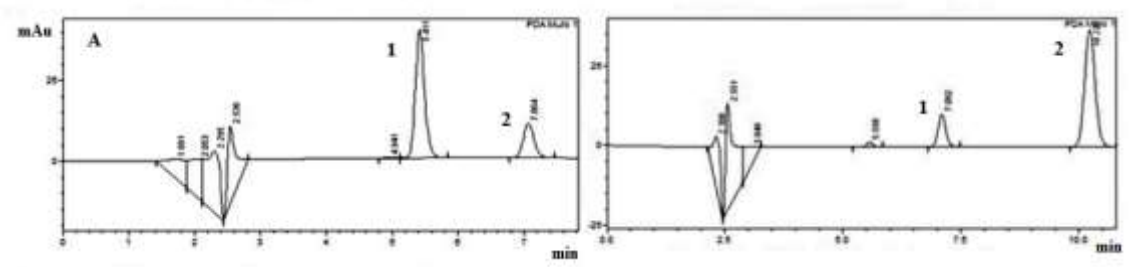

Figure 4. Chromatograms of Tablet Solution (A) 1. RSV (6 µg mL^{-1}), 2. Losartan(IS) (1µg mL^{-1}) B) 1.Losartan(IS) (1µg mL^{-1}), 2. ATV (5µg mL^{-1}),

Table 4. Results of the assay and the recovery analysis of compounds in pharmaceutical dosage form

Sample No	Label claim (mg/tab)	Amount of Atorvastatin in tablets (mg)	Recovery%	Label claim (mg/tab)	Amount of Rosuvastatin in tablets (mg)	Recovery%
1	20.000	19.971	99.854	5.000	4.922	98.439
2		19.922	99.610		4.987	99.734
3		19.858	99.290		5.044	100.877
Mean		19.917	99.585		4.984	99.684
SD		0.057	0.283		0.061	1.220
%RSD		0.284	0.729		1.224	1.083

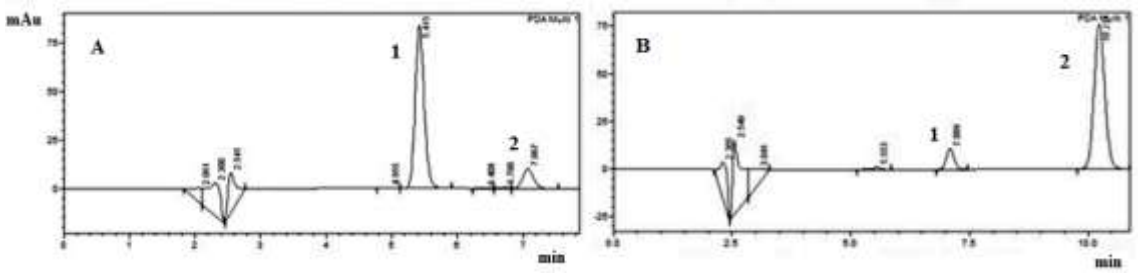

Figure 5. Chromatogram of Recovery Solutions A) pharmaceutical dosage form spiked with each pharmaceutically active ingredient at 1. RSV (12 µg mL^{-1}), 2. Losartan(IS) (1µg mL^{-1}) B) pharmaceutical dosage form spiked with each pharmaceutically active ingredient at 1. Losartan(IS) (1µg mL^{-1}), 2. ATV (10 µg mL^{-1}).

CONCLUSION

This paper represents the first study dealing with the assignment of optimum chromatographic condition of ATV and RSV using chromatographic behaviour at fixed proportion of acetonitrile-water binary mixture and different pH values of mobile phase. A rapid and reliable isocratic RPLC method for the determination of ATV and RSV was developed and validated. Statistical analysis proves that the method is precise for the analysis of these compounds in pharmaceutical formulations without any interference from the excipients. Method validation produced excellent results for linearity, precision, accuracy, limit of quantitation and limit of detection. The statistical parameters of this method showed good results. The recovery studies revealed excellent accuracy and high precision of the method. The RPLC method was found to give better results. Therefore the proposed method could be applied for routine analysis of ATV and RSV.

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Diğer yazar e-posta adresleri:

Zehra Üstün: zehra_ustun@hotmail.com

Aybike Talay: aybiketalay@hotmail.com