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Dedicated to the memory of
Professor Maria Brezeanu (1924–2005)

NON-MISCIBLE SOLVENT LARGE VOLUME INJECTION – HPLC/DAD METHOD FOR DETERMINATION OF BUTYLATED HYDROXYANISOLE IN LOVASTATIN AND SIMVASTATIN PHARMACEUTICAL FORMULATIONS

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A simple and sensitive RP-HPLC method for the determination of 2 and 3-tert-butyl 4- hydroxyanisole (BHA) as antioxidants in pharmaceutical formulations containing Lovastatin and Simvastatin is described. Sample preparation is based upon the selective extraction of BHA with i-octane followed by the direct injection of the extract onto the chromatographic column. High injection volumes of the samples containing the target analyte dissolved in i-octane are allowed, without observing any focusing phenomena, resulting in an enhanced sensitivity. The method was tested for precision (repeatability and intermediate reproducibility), sensitivity, accuracy and robustness. UV spectrometric detection at 291 nm was used, allowing identification and detection limits in the ppm range (BHA concentration with respect to the analysed solid material). The proposed method can be successfully used in routine assay of BHA in pharmaceutical formulations of Lovastatin or Simvastatin, as well as for monitoring the BHA content during stability studies.

INTRODUCTION

In many pharmaceutical products, the active drug may undergo oxidative degradation, resulting in serious limitations in terms of shelf-life. Antioxidants are chemical substances able to preclude or to significantly slow the oxidation reactions. Their action consists in the generation of stable radicals inhibiting the first step of the oxidative degradations.

Synthetic phenols such as 2 and 3-tert-butyl 4-hydroxyanisole (BHA) and tert-butyl hydroquinone (TBHQ) are widely used as antioxidants in both pharmaceutical and food industries.[1,2] The content of BHA, individually or combined with other antioxidants, in products for human use, is strictly regulated in the European Community (EU) by means of the 95/2/EC directive.

A literature survey shows that BHA has been determined in high variety of matrices (lard, margarines, edible oils, cosmetics, capsaicinoids, chewing gum, wine, vinegar, biscuits, plasma) using electrometric techniques,[3-10] fluorescence spectrometry,[12,13] gas chromatography with different detection systems, including mass spectrometry,[14-17] or micellar electrokinetic chromatography.[18] HPLC methods are often used, mainly with UV spectrometric detection[1,19-22] and electrometric detection.[23-25] In order to increase sample throughput at routine scale, flow injection methods for the assay of BHA have been also proposed.[26-31]

The action of BHA as antioxidant for hydroxymethylglutaryl coenzyme A reductase inhibitors namely Lovastatin and Simvastatin was already proved.[32] Until now, many pharmaceutical formulations with Lovastatin or Simvastatin containing BHA as antioxidant have been authorized on different markets.

However, when using HPLC for the assay of BHA, the real analytical challenge consists in the selection of an appropriate solvent selectively isolating the target analyte against the active drugs and a sensitive detection, rather than the chromatographic separation itself. Isolation of BHA against the active

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drug is mandatory when considering its high excess with respect to the antioxidant (usually 400 to 1100 folds). Nonselective extraction leads to column overloading with the active drug or a lack of sensitivity due to the subsequent reduction of the injection volume. Some other limitations on the injection volume may be induced by the nature of the extraction solvent and its compatibility with the mobile phase composition at the beginning of the chromatographic run, in order to avoid solvent focusing phenomena. Relative high sensitivity is also required because BHA should also be monitored during the stability studies carried out on the respective pharmaceutical formulations. At the end of the testing stability periods, BHA concentration in the pharmaceutical product may be strongly reduced relative to the initial values (already limited at 0.02% by the EU regulations) due to its consumption related to the antioxidant activity. Identification limits in the ppm range (with respect to the analysed solid material) are thus imposed for the correct assay of BHA in drugs, close to or after their expiry date.

The method described further relies on the assay of BHA in pharmaceutical formulations containing Lovastatin and Simvastatin, using extraction in i-octane followed by the injection of a large volume of this extract directly onto the chromatographic column, without any other additional preparation. The method is simple, sensitive, robust, precise and accurate.

EXPERIMENTAL

Reagents and materials

All solvents (acetonitrile, methanol, ethanol, acetone, n-pentane, n-hexane, i-octane) were HPLC grade from Merck (Merck KGaA, Darmstadt, Germany). 2-tert-butyl hydroxyanisole, Eur. Ph. quality (less than 10% 3-tert-butyl hydroxyanisole content), was obtained from the same source. Lovastatin and Simvastatin (cat. no. EPL070000 and EPS 0650000, batches 1 and 1a, respectively) are Eur. Ph. Certified reference standards (Council of Europe, Strasbourg, France). Different pharmaceutical products (tablets) containing Lovastatin and Simvastatin were authorized drugs and were obtained from the local market. Phosphoric (d = 1.71 g/mL), formic (d = 1.22 g/mL) and glacial acetic acids were p.a. grade from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). Amorphous colloidal silica, lactose, magnesium stearate, cellulose and starch were pharmaceutical grade substances. Water was Milli Q grade, having a conductivity of 0.055 μS/cm and a total organic carbon (TOC) content less than 30 ppb (Millipore GmbH, Eschborn, Germany). Volumetric glassware (volumetric flasks, pipettes and cylinders) were Volac class A batch certified (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and have been gravimetrically verified in house.

Apparatus and operational parameters

Experiments were carried out on an Agilent 1100 liquid chromatograph with auto sampler (large volume injection option) and diode array detector (Agilent Technologies, Waldbronn, Germany). The system is twice a year operationally qualified using its software built-in procedures.

Zorbax Eclipse XDB-C8, 150 mm length, 4.6 mm internal diameter and 5 μm particle size was used as the chromatographic column (serial USRK017651, Agilent Technologies). Column validation made before starting the experiments revealed a reduced plate height ($\bar{h}$) of 2.19. Construction of the Van Deemter curve was made for fluoranthene, having uracil as dead time marker.

The mobile phase is composed from acetonitrile and an aqueous 0.1% (v/v) phosphoric acid solution. Gradient elution was used, having the following profile: 40% acetonitrile from 0 to 14 min, stepwise increase at 100% acetonitrile in 0.01 min; 100% acetonitrile for 6 min; back to the initial composition in 1 min. The starting isocratic part from the elution program allows separation of the 2 and 3-tert-butyl 4-hydroxyanisole isomers. The step profile up to 100% acetonitrile was introduced for a fast elimination of the residual coextracted statins and the extraction solvent, respectively. However, this step is essential for the reproducibility of the method, as showed in the following section. Column equilibration between successive runs should be at least 5 min. A flow rate of 1.5 mL/min was used. The mobile phase and the column were maintained at 25°C using the Agilent column thermostat Peltier based heater G1316A.

Detection was made at 291 nm (± 2 nm) with a reference wavelength of 480 nm, allowing selective identification of BHA. If statine (Lovastatin or Simvastatin) observation is needed, an additional 238 nm detection channel is necessary.

RESULTS AND DISCUSSION

Solid – liquid extraction and solvent evaporation

According to the literature[33], BHA, Lovastatin and Simvastatin are soluble in alcohols, acetonitrile and acetone. BHA is cited as being soluble in aliphatic hydrocarbons, while statins exhibit less solubility in such media. A preliminary approach to the sample preparation procedure was oriented towards the extraction of

both BHA and the corresponding statin from the pharmaceutical formulation in a solvent, followed by evaporation to dryness under nitrogen and selective removal of the target analyte with an appropriate solvent. Some inconsistent results obtained during the experiments lead to the conclusion that evaporation (even made at room temperature and under careful operation) of solutions containing BHA may induce the loss of the analyte by steaming. Consequently, 20 ppm solutions of BHA in different solvents were prepared, aliquots were evaporated at room temperature under continuous nitrogen flow and residues were retaken in the mobile phase and injected onto the chromatographic column. The recoveries of BHA are presented in Fig. 1.

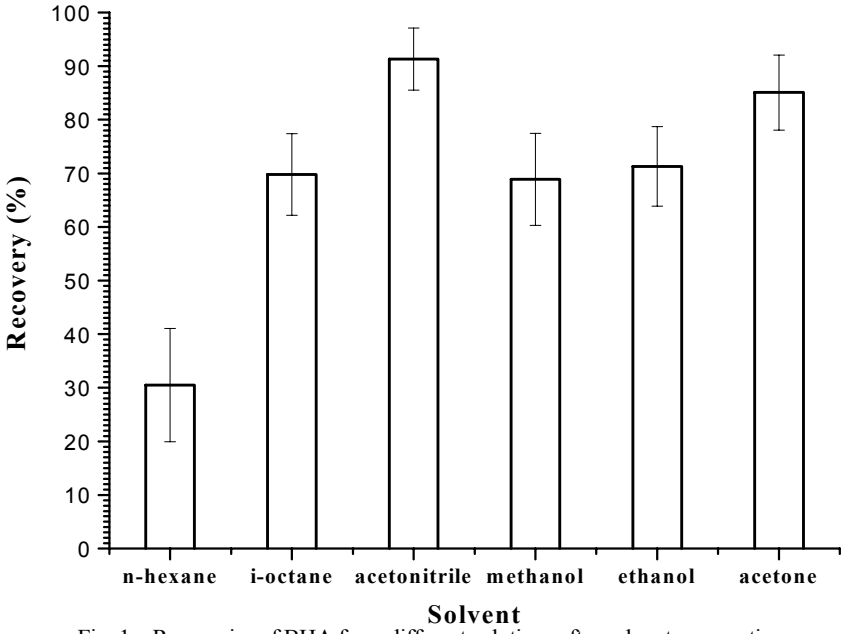

Fig. 1 – Recoveries of BHA from different solutions after solvent evaporation (evaporation was made at room temperature under nitrogen flow).

Although acetonitrile ensures the highest recovery of BHA, co-extraction of statins represents the major difficulty.

Solid–liquid extraction and direct injection

It became obvious that solvent evaporation should be avoided during preparation procedures on samples containing BHA. Extraction, filtration and direct injection of the resulting solution should thus be considered as a rugged solution. In pharmaceutical formulations the concentration of BHA is limited up to 0.02% (w/w). Considering therapeutic dosages of Lovastatin and Simvastatin (20/40 mg per tablet or 10/20 mg per tablet, respectively), it results that the excess of the active drug with respect to BHA is 400 to 1100 folds higher. Such a procedure generates potential problems in terms of column overloading with the active drug and focusing phenomena on injection, leading to peak asymmetry or splitting.

A quasi selective removal of BHA from statin containing pharmaceutical formulations can be achieved by aliphatic solvents. However, injection of such solvents that are not miscible with the mobile phase should be studied in detail. Repetitive injections of a 20 ppm solution of BHA in i-octane, at different volumes ranging from 1 to 500 μL were made. Focusing phenomena appear only for injection volumes higher than 400 μL. Up to this volume, peak symmetry is rigorously conserved. However, a linear decrease of the absolute retention time of the BHA peak with the increase of the injection volume was also observed, as presented in Fig. 2.

When repeating injection of 100 μL aliquots from the 20 ppm BHA solution in i-octane, reproducible retention was obtained without any variation trend. Results are given in Fig. 3.

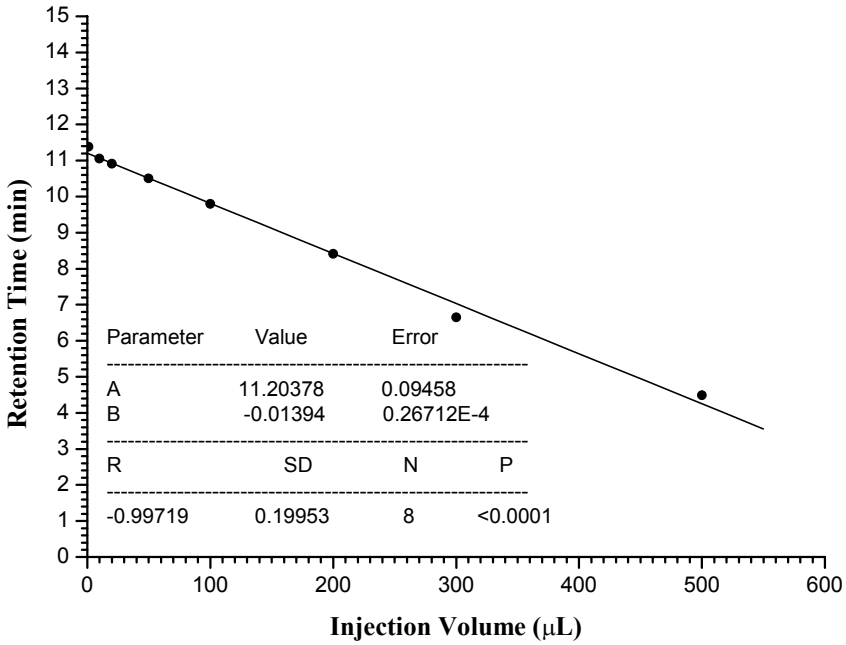

Fig. 2 – Reduction of the absolute retention time characterizing the BHA peak after injection of increasing volumes of a solution of the analyte in i-octane. (conditions are given in the text and in the Experimental section.)

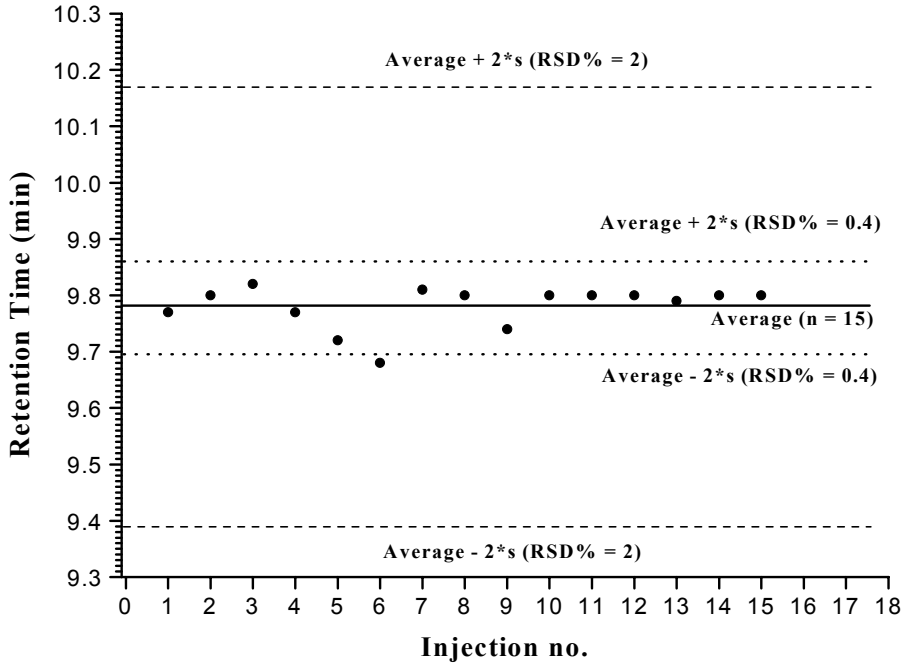

Fig. 3 – Reproducibility of the retention data for repetitive injections of 100 μL portions from a BHA solution in i-octane. (conditions are given in the text and in the Experimental section.)

The explanation of such unexpected behavior should be related to the intrinsic hydrophobic character of the analyte and of the solvent, expressed as the logarithm of the octanol/water partition coefficient (log P). Calculations made according to the method of Meylan et all[34,35] generate a 4.08 value for i-octane and 3.5 for BHA. This means that i-octane is more hydrophobic than BHA and consequently, its elution from the system should be slower. Immediately after injection, i-octane will saturate the stationary phase along a portion situated in the head of the column, due to the high interaction with octadecyl chains and the lack of miscibility with the mobile phase. Therefore, the chromatographic partition of BHA will start only after the exit from the stationary phase zone saturated with i-octane. Considering BHA retention linearly depending upon the column length (about 0.76 min absolute retention is produced by 1 cm of the column length) and the previously determined regression

equation relating retention time to the injection volume (t_R = -0.0139 × V_{inj} + 11.204), it results that 10 μL of i-octane "reduces" the available column length by about 0.18 cm. In order to have a better insight on the phenomena, five repetitive 100 μL injections of a 20 ppm BHA solution in i-octane were carried out, applying only an isocratic elution (the step increase of the acetonitrile percentage in the mobile phase was eliminated from the gradient elution profile).

The period between two successive injections was set to 13.2 min (12.5 min for the chromatographic run and 0.7 min for the automated injection). In the resulting chromatograms, a continuous shift of the retention time characterizing the BHA peak was observed. The linear dependence between the absolute retention time of the BHA peak and the period elapsed from the initial injection moment is described by the equation t_R = -0.1024 × t + 9.857, characterized by a correlation coefficient of -0.9991. Starting from the fourth injection, peak symmetry was drastically altered. It seems obvious that retention time of the BHA peak in the second chromatogram is similar to the retention time of the peak resulting after a single injection of 200 μL sample solution, retention time in the third chromatogram corresponds to the retention of the peak obtained after a single injection of a 300 μL sample and so on. These results clearly show that in the isocratic conditions applied to the separation, the zone in the stationary phase saturated with i-octane is not moving within the column (i-octane sticks to the column head). When doing the following injection, the column length available for BHA partition is proportionally reduced by generating a second i-octane saturated stationary phase zone (additive effect). One can conclude that the elution gradient is not only useful for the residual statin elution, but also for the migration of the i-octane saturated zone.

Validation of the analytical procedure

The influence of the aliphatic solvent was the first considered parameter. Extracting 500 mg portions of solid mixtures containing Lovastatin or Simvastatin, commonly used pharmaceutical excipients (cited in the Experimental section) and spiked with BHA amounts at 0.02% (w/w) concentration level with 5 mL aliquots of n-pentane, n-hexane and i-octane, followed by direct injection of the corresponding supernatants resulting after centrifugation, allows the calculation of similar extraction yields. Positive errors are enhanced with the increase of the solvent volatility. i-Octane was finally preferred as the extraction solvent, providing an averaged recovery of 86.2 % (s = 2.08 for n = 10). Relationship between extraction yield and the volume of i-octane used for 500 mg solid samples containing 100 μg of BHA is given in Fig. 4.

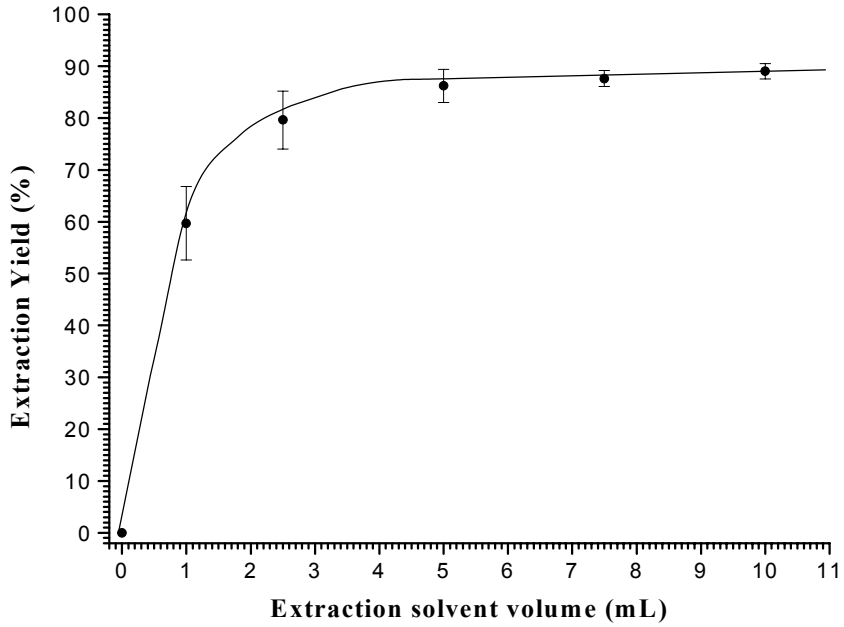

Fig. 4 – Extraction yields of BHA from solid mixtures containing statines and commonly used pharmaceutical excipients with respect to the solvent (i-octane) volume used during extraction. (conditions are given in the text and in the Experimental section.)

Extraction of BHA from solid samples was achieved by ultrasonication. The ratio of 5 mL of i-octane for each 100 μg of BHA in the solid sample represents a compromise allowing a higher extraction yield obtained with the lower solvent consumption and without a major dilution of the sample. In order to avoid errors related by accidental solvent vaporization, filtration was replaced by centrifugation (4500 rpm, 5 min). Injections of 100 μL aliquots from the supernatant provide chromatograms as illustrated in Fig. 5.

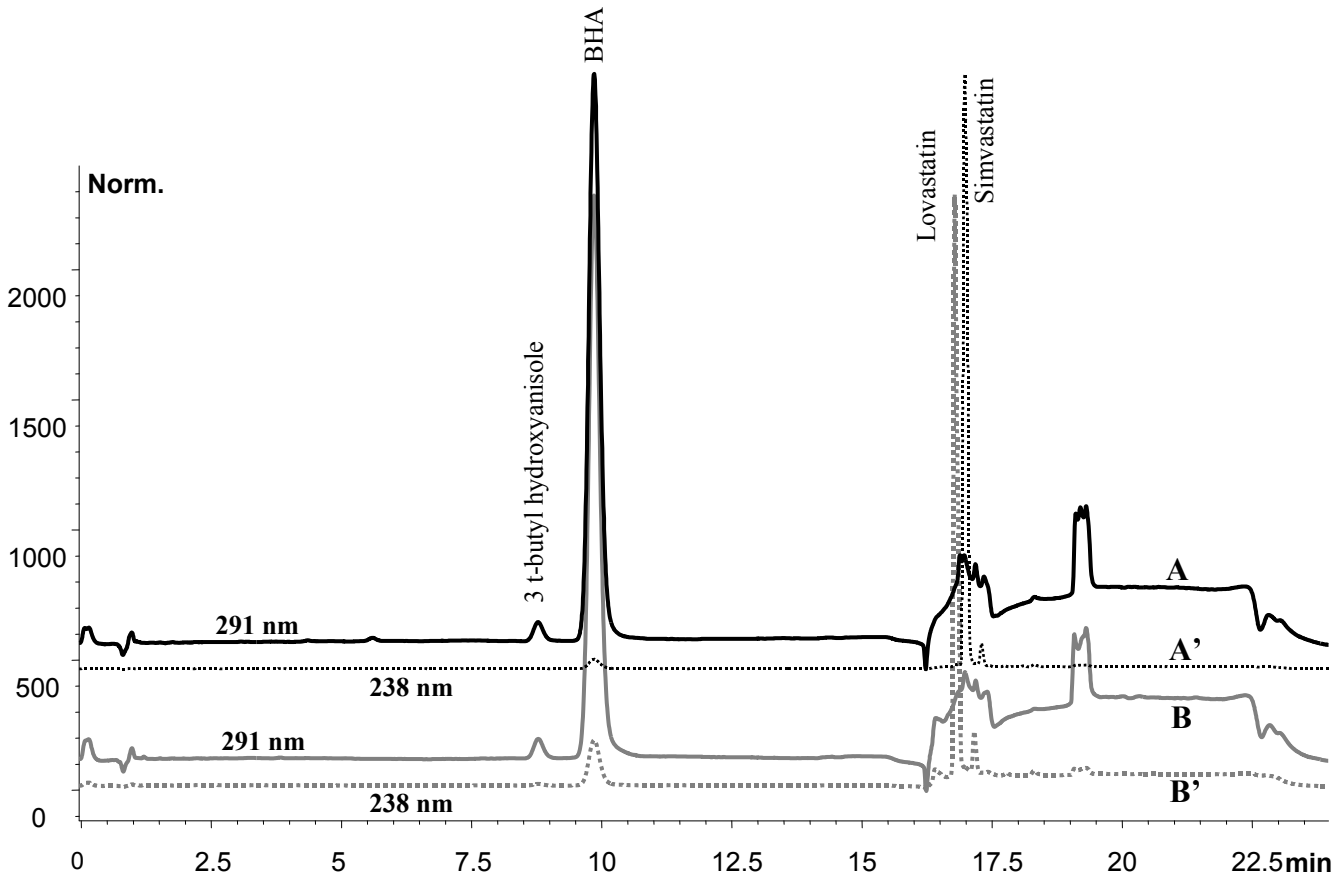

Fig. 5 – Chromatograms resulting after injection of the BHA extracts from pharmaceutical formulations containing Lovastatin or Simvastatin in i-octane. (A' and B' are chromatograms monitored at 238 nm for observing residual coextracted active drugs; A and B are chromatograms monitored at 291 nm used for assaying BHA in solid pharmaceutical formulations; separation conditions are given in the Experimental section.)

It was proved that any duration of the ultrasonication period during extraction higher than 15 min will produce a quantitative recovery of BHA.

Applying the whole method (extraction and chromatographic separation) repeatedly on solid mixtures (obtained after crushing and subsequent homogenization of commercially available tablets containing Simvastatin or Lovastatin as active drugs and BHA as antioxidant, respectively), the relative standard deviations calculated for the BHA peak areas fall in the 0.6% range (n=10). The same process repeated during five different experimental sessions on the same solid mixtures produces relative standard deviations for BHA peak areas around 1.5%. Other antioxidants, such as citric acid or n-propyl gallate, existing in the tested solid mixtures do not interfere. No trends were observed for the peak area experimental values during repeatability and intermediate reproducibility procedures, respectively.

Linearity of the method was studied for solid mixtures containing Lovastatin or Simvastatin and other common pharmaceutical excipients, spiked with BHA at different concentrations ranging from 0.003% (w/w) to 0.04% (w/w). The linear regression equation is characterized by a correlation coefficient of 0.9995. The quantitation limit (LOQ) calculated with a confidence of 95%, is around 0.0016% (w/w) and detection limit (LOD) is placed in the 0.0005% (w/w) range. One can conclude that the application of the proposed method on solid samples containing BHA at the maximum accepted level (0.02%) allows a precise observation of the analyte consumption down to 8% from its initial concentration.

Changes in column temperature are not affecting the selectivity of the chromatographic separation. However, changes of ± 5°C generate retention data outside the normal variation interval. Column thermostatization is required for obtaining high retention reproducibility. Retention of BHA exponentially increases by the increase of the content of the aqueous constituent of the mobile phase during the first isocratic step of the elution program. It can be considered that variations in the volumetric proportion of the aqueous component in the mobile phase ranging from 59% to 60.4% still generate retention data in the normal variation interval. Modifying the concentration of the phosphoric acid in the aqueous component of the mobile phase from 0.05% (v/v) to 0.2% (v/v) does not affect the selectivity and generates retention data in the normal variation interval. When phosphoric acid is replaced by formic or acetic acids, no influences on selectivity or retention data were observed. It seems obvious that the chromatographic method is not affected by small variations of the operational parameters, proving inherently its robustness.

CONCLUSIONS

A method for assaying BHA in solid pharmaceutical formulations containing Lovastatin or Simvastatin is proposed. The analyte is selectively extracted with i-octane and the extract is directly loaded to the chromatographic column. Commonly used pharmaceutical excipients (amorphous colloidal silica, lactose, magnesium stearate, cellulose and starch) and antioxidants (citric acid and n-propyl gallate) do not interfere. Large volume injection (up to 200 μL) of the extract is allowed without generating irreproducibility of retention data or affecting peak symmetry. LOQ is situated in the 0.0016% (w/w) range. The method is simple, selective, precise and robust. It can be applied for routine assaying of BHA in pharmaceutical formulations containing statins on release as well as for the stability studies (monitoring the decrease of BHA concentration down to 8% from its initial value).

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