Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 1011–1016
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of methodologies based on HPLC and Raman spectroscopy for monitoring the stability of lovastatin in solid state in the presence of gallic acid

M.G. Orkoula [a], C.G. Kontoyannis [b,c,*], C.K. Markopoulou [d], J.E. Koundourellis [d]

[a] *Department of Materials Science, University of Patras, Patras, Greece*
[b] *Institute of Chemical Engineering and High Temperature Chemical Processes, FORTH, P.O. Box 1414, GR-26504, Patras, Greece*
[c] *Department of Pharmacy, University of Patras, Patras, Greece*
[d] *Laboratory of Pharmaceutical Analysis, School of Pharmacy, Aristotelian University of Thessaloniki, Thessaloniki 54124, Greece*

Received 15 November 2003; received in revised form 2 March 2004; accepted 11 March 2004

Available online 28 July 2004

Abstract

Methodologies based on FT-Raman spectroscopy and HPLC were developed for monitoring the stability of lovastatin in the solid state in the presence of gallic acid, a natural antioxidant. A Raman calibration curve was constructed using the area of the strong but overlapping vibration mode of lovastatin at 1645 cm^{-1} and of the gallic acid at 1595 cm^{-1}. Mixtures of the active ingredient with the antioxidant were heated in the presence of atmospheric air up to 120 °C. The molar ratios of lovastatin and gallic acid in the artificially oxidized mixtures were determined from their Raman spectra using the calibration curve. The results were compared to those obtained from the application of the HPLC methodology and found to match satisfactorily. The HPLC analysis was based on a reserved-phase Zorbax C_g, 10 μm (4.6 mm × 25 cm i.d.) column, using a gradient elution program by varying the proportion of solvent A acetonitrile 100% to solvent B 0.1% v/v phosphoric acid, and a programmable diode array detection at 225 nm. The Raman methodology was simpler and non-destructive for the sample but yielded only molar ratios as opposed to the HPLC technique where the moles of the both ingredients were determined.

Keywords: Raman spectroscopy; Lovastatin; Gallic acid; Quantitative analysis; HPLC; Oxidation

1. Introduction

Lovastatin, a cholesterol-lowering agent, is among the drugs that are susceptible to oxidative degradation [1,2], and storage under nitrogen in a cold place is recommended [3]. The stabilization of such drugs in tableted powder mixes is accomplished by the addition of antioxidants such as butylated hydroxyanisole. In a recent publication [4], the efficiency of numerous natural antioxidants in stabilizing lovastatin was investigated and the respected oxidation kinetic curves for the solid state were reported. The determination of lovastatin in the binary mixtures of the drug with the antioxidants, before and after the artificial

* Corresponding author. Tel.: +30-2610997727; fax: +30-2610997658.
E-mail address: cgk@iceht.forth.gr (C.G. Kontoyannis).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.03.006

oxidation, was done using quantitative HPLC analysis as proposed by both the European Pharmacopoeia and USP [5,3]. The identification of lovastatin, according to USP 23, can be accomplished by matching the infrared absorption and/or ultraviolet absorption spectrum with that of "USP lovastatin RS" [3].

Usage of HPLC for quantitative purposes is not only destructive for the sample but also time consuming and cumbersome since extensive sample preparation is needed [6–10]. An effort to devise a rapid methodology for verification of both identity and content of drug formulations containing lovastatin was reported several years ago by using the mid-infrared spectroscopy [11]. Although the methodology described in that publication was easier than the usage of HPLC, sample preparation was also required since the particle-size uniformity had to be controlled carefully as scattering is greater at shorter wavelengths. Therefore, an analytical technique capable of non-destructive and rapid quantitative analysis of lovastatin in the presence of antioxidants is needed.

In the present study, the feasibility of using FT-Raman spectroscopy for non-destructive monitoring of the lovastatin/gallic acid ratio, before and after oxidation experiments, was evaluated. The ratios obtained were compared to moles of lovastatin and the antioxidant, obtained from the application of an HPLC methodology that was also developed for this specific binary mixture. To the authors' knowledge, it is the first time that the Raman spectrum of lovastatin is reported.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of analytical USP-NF grade and were used without further purification. Lovastatin reference standard was kindly donated from Merck chemical company and gallic acid was supplied from Fluka. Gallic acid and lovastatin were characterized prior to their use by FT-IR Spectroscopy (EQUINOX 55, Bruker, Karlsruhe, Germany), while gallic acid was further characterized by FT-Raman spectroscopy (FRA-106/S FT-Raman, Bruker, Karlsruhe, Germany), and their spectra were found to match those reported in the literature [12–14]. Lovastatin and gallic acid were sized at 25 °C by dynamic light scattering (Mastersizer S, Malvern Instruments, U.K.) and found to be 14.9 and 23.8 μm respectively. Phosphoric acid and acetonitrile of HPLC ultra gradient grade, used for the mobile phase, were supplied from Merck. All solution preparations were made using deionized water, filtered by a "Millipore-Q plus 185" equipment.

2.2. Raman spectroscopy

A calibration curve was constructed by preparing carefully weighted mixtures of lovastatin and gallic acid. The mol percentage of lovastatin varied from 10% to 90%. The homogeneity of the powders was verified by obtaining five Raman spectra for each mixture, focusing the laser beam at randomly selected spots of the surface.

The Raman spectra were recorded using a FRA-106/S FT-Raman (Bruker) with the following characteristics: the laser excitation line used was the 1064 nm of a Nd:YAG laser, a secondary filter was used to remove the Rayleigh line, the scattered light was collected at an angle of 180°, the system was equipped with a liquid N_2-cooled Ge detector (D 418), the power of the incident laser beam was about 450 mW on the sample's surface, while the spot size at the focused beam was about 100 μm. Typical spectral line width was 0.5 cm^{-1}, while the recorded spectra were the average of 300 scans.

The artificial oxidation of the mixture was performed by placing the powders in aluminum cylindrical cups with 10 mm diameter and 4 mm height, having a cavity in the center of 2 mm diameter and 1 mm depth. The Raman spectra were recorded before and after the aluminum sample holders placing in a thermostated (±1 °C) oven with fresh air circulation.

2.3. HPLC measurements

2.3.1. High performance liquid chromatography: instrument and conditions

HPLC analysis was performed on a HP Series 1090 (Hewlett Packard) equipped with a binary pump delivery system, a degasser, an autosampler, and a HP diode-array UV-vis detector. Integration and data elaboration were performed by Chemstation software (Hewlett Packard). A reversed-phase column Zorbax

C_8 with particle size 10 μm (4.6 mm × 25 cm i.d.) was used. The mobile phase was degassed by filtering through a membrane filter Millipore HV 0.45 μm. The following gradient elution was carried out: mobile phase A, 0.1% v/v solution of phosphoric acid; mobile phase B, acetonitrile. The linear gradient elution system was: 100% A for the first 3 min with a flow rate of 1 ml/min, and then reached the 65% B in 7 min with a flow rate of 1.5 ml/min, standing at 65% B for 7 min. The gradient returned to 100% A, and a flow rate of 1 ml/min in 4 min, standing at a 100% for another 5 min as post-time. The quantification of lovastatin and gallic acid by diode array detector was performed at 225 nm. Comparing their retention time and UV spectrum with those of the reference standards the chromatographic peaks of the samples were identified. Of each sample, 50 μl was injected, with draw speed of 90 μl/min.

2.3.2. High performance liquid chromatography: sample and standard preparation

Stock solutions were prepared by dissolving lovastatin and gallic acid in a diluent 70:30 v/v CH_3CN/H_2O to obtain a concentration of about 35.2 μg/ml for gallic acid and 80.8 μg/ml for lovastatin. The standard solutions were prepared by dilution of the stock solutions in diluent to reach concentration ranges of 6.48–32.32 μg/ml and 2.28–14.08 μg/ml for lovastatin and gallic acid, respectively.

For sample preparation, about 10 mg (containing 8.47 mg lovastatin and 1.59 mg gallic acid, 70 mol% lovastatin—30 mol% gallic acid) of each sample were accurately weighed and transferred to 100-ml volumetric flask. After appropriate dilutions, the final concentrations in each sample were 20.3 μg/ml lovastatin and 3.82 μg/ml gallic acid.

3. Results and discussion

3.1. Development of the Raman analytical methodology

The intensity of a Raman line depends on a number of factors including the incident laser power, the frequency of the scattered radiation, the absorptivity of the materials involved in the scattering, and the response of the detection system. Thus, the area under the Raman peak, $A(\nu)$, can be represented as: [15]

$$A(\nu) = I_0 K(\nu) C \quad (1)$$

where I_0 is the intensity of the excitation laser line, ν is the Raman shift, and $K(\nu)$ is a factor which includes the frequency dependent terms: the overall spectrophotometer response, the self-absorption of the medium, and the molecular scattering properties. C is the concentration of the Raman active species.

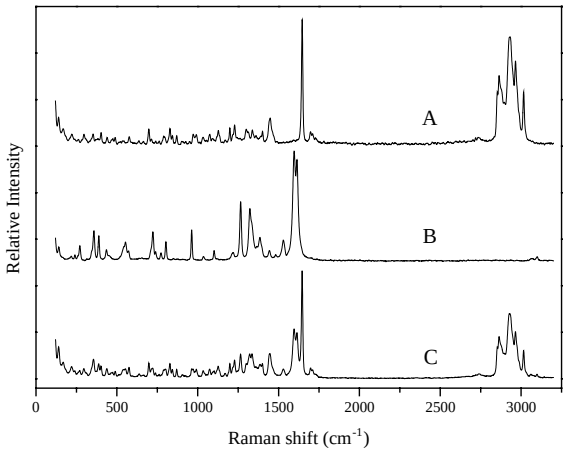

Fig. 1. Raman spectra of lovastatin (A), gallic acid (B), and their 70–30 binary mixture (C).

The Raman spectra of lovastatin and gallic acid powder can be seen in Fig. 1A and B, respectively. The more intense vibrations of the aromatic ring at 1595 cm^{-1} for gallic acid and 1645 cm^{-1} for lovastatin were used for the quantitative analysis. The spectrum of the 70–30 mol% of lovastatin/gallic acid binary mixture can be seen in Fig. 1C.

The peak analysis software "PeakFit"v.4.0 (Jandel, Scientific Software), using a statistical fit of both Gaussian and Lorentzian type of peaks, was employed for the deconvolution of the overlapping bands (Fig. 2). The peak areas of the deconvoluted bands were used for the construction of the calibration curve.

For a lovastatin–gallic acid mixture, Eq. (1) can be rewritten for the 1645 cm^{-1} peak of lovastatin and the 1595cm^{-1} peak of gallic acid as follows:

$$\frac{A_L}{A_G} = \frac{k_L}{k_G} \times \frac{x_L}{x_G} \quad (2)$$

where A_L and A_G are the deconvoluted peak areas for lovastatin and gallic acid, respectively, K_L and K_G the

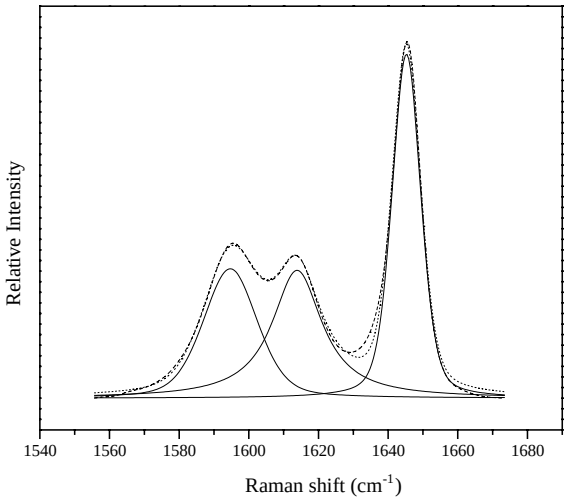

Fig. 2. Deconvolution of the 1595 cm^{-1}, 1615 cm^{-1} peaks of gallic acid and 1645 cm^{-1} peak of lovastatin. Solid line (—): deconvoluted peaks; dash line (---): recorded spectrum of the mixture; dot line (···): spectrum line corresponding to the cumulative area of the deconvoluted peaks.

frequency factors for the 1645 cm^{-1} peak of lovastatin and the 1595 cm^{-1} peak of gallic acid, respectively, and x_L and x_G the molar fractions of lovastatin and gallic acid in the mixture, respectively.

A plot of the ratios A_L/A_G versus x_L/x_G, as expected, yielded a straight line, Eq. (3).

$$\frac{A_L}{A_G} = 0.61 \cdot \frac{x_L}{x_G} \quad (3)$$

The correlation coefficient, r, was 0.997, while the standard deviation for the slope was found to be 0.02. The detection limits (*DL*), at 99.9% confidence level, were calculated to be 1.86 mol % for lovastatin and 2.2 mol % for gallic. *DL* is defined as [16]:

$$DL = t \times s_b \times \sqrt{\frac{N+1}{N}} \quad (4)$$

where t is the statistical parameter t, which is often called *Student's t*, defined as: $t = (x-\mu)/s$, where s is the standard deviation of the measurements and $(x-\mu)$ represents the absolute deviation from the mean value; s_b is the standard deviation of the blank measurements; N is the number of blank measurements. The standard deviation of the blank measurements for lovastatin was calculated as the s_b of ten A_L/A_G values that were extracted from 10 gallic acid spectra, i.e. from spectra

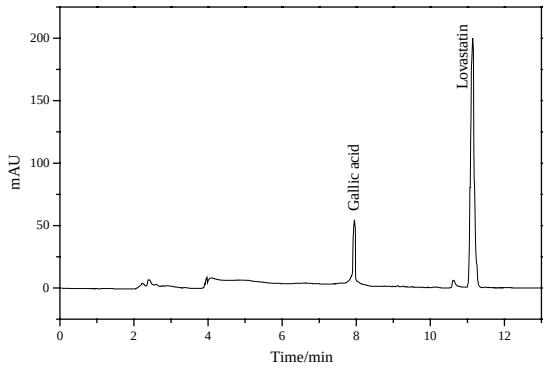

Fig. 3. Gradient high performance liquid chromatogram of gallic acid t_R 7.91 min, 3.66 μg/ml and lovastatin t_R 11.12, 20.33 μg/ml.

with no lovastatin. Similarly, the s_b for the gallic acid was determined from 10 lovastatin spectra.

3.2. HPLC results

3.2.1. Chromatographic separation

In the present investigation a binary gradient elution system was developed because the advantage of such systems is that the bandwidths can be approximately constant both for early- and late-elution analysis. Two mobile phases, one containing methanol and the other acetonitrile, were tested. Methanol has been found to have a similar effect on the elution of the analytes, but it was not as effective as acetonitrile. Phosphoric acid and acetonitrile or methanol (>50% v/v) in the mobile phase were necessary for the elution of lovastatin in a reasonable retention time with no peak broadening or tailing. At the same time, the presence of these solvents even in small quantities caused the elution of gallic acid together with the solvent front. Therefore, a system consisting of gradient time/elution program with mobile phases: (A) acetonitrile, (B) 0.1% v/v phosphoric acid, was applied. Also the usage of a Zorbax column C_8 10 μm, (25 cm × 4.6 mm i.d.), which has less lipophilic properties than the suggested C_{18} 5 μm (250 × 4.6 mm i.d.) [4] was also considered important for the resolution of this problem (Fig. 3).

The peak asymmetry for lovastatin was very close to an ideal value of 1.0, whereas gallic acid yields a value of 1.2. The resolution factor *Rs*, between the two chromatographic peaks, was 13.2 and was calculated from the equation $Rs = 2(t_2 - t_1)/W_1 + W_2$, where t_2

Table 1
HPLC parameters using the gradient elution system and detection, quantitation limits of the compounds determined

Active ingredients	t_R (min)	k′[a]	Detection limit (LOD) (μg ml^{-1})	Quantitation limit (LOQ) (mg ml^{-1})
Lovastatin	11.12	1.8	0.59	1.97
Gallic acid	7.91	1.0	0.82	2.72

[a] $t_o = 3.91$ min.

and t_1 are the retention times of the two components and W_1 and W_2, which are the peak widths at the base of the two respective peaks, were obtained by extrapolation of the relatively straight sides to the baseline. Other characteristic numerical values of the chromatographic parameters t_R and k' are shown in Table 1.

3.2.2. Linearity and quantitative analysis

Six working solutions for each analyte in the range of 6.48–32.32 μg/ml for lovastatin and 2.28–14.08 μg/ml for gallic acid were prepared. Analysis was performed in duplicate to determine the linearity of the assay. The regression lines were calculated by the method of least squares of peak areas versus analyte concentrations. The equations corresponding to the regression lines of the analytes were:

$$\text{Lovastatin, } y = 76.59x + 70.90 \quad r = 0.9998 \qquad (5)$$

$$\text{Gallic acid, } y = 44.56x + 25.67 \quad r = 0.9991 \qquad (6)$$

They were consistently linear in the already mentioned range for both compounds. The minimum detectable level, LOD, and minimum quantitation level, LOQ, defined as: LOD $= 3S_{y/x}/m$, LOQ $= 10S_{y/x}/m$, respectively, where $S_{y/x}$ is the residual standard deviation and m is the calculated slope of the corresponding calibration [17], can be found in Table 1. From a mixed standard solution of the analytes, five replicates were injected onto the column and the relative standard deviation (%RSD) was calculated. This for lovastatin (20.33 μg/ml) was 0.89%, and for gallic acid (3.66 μg/ml) 1.58%.

4. Comparison of HPLC and Raman results

Based on the calibration curve, Eq. (3), the Raman spectra of two solid mixtures of lovastatin and gallic acid in a ratio of 70–30 mol %, the first heated at 80 °C for 45 min and the second at 120 °C for 15 min, yielded the results shown in Table 2 along with the respective results obtained from the application of the HPLC analytical methodology.

From Table 2, it is apparent that FT-Raman spectroscopy is equally reliable in determining the molar ratio of lovastatin/gallic acid as the commonly used HPLC. The relative advantages of FT-Raman spectroscopy is the minimal sample preparation and the rapid determination since for the recording of an FT-Raman spectrum, approximately 5 min are sufficient. On the other hand, the recording of an HPLC chromatogram, including the preparation of the apparatus and the solvent, is more time consuming. Furthermore, the application of the FT-Raman technique is non-destructive for the specimen. The disadvantage of the described Raman methodology is that it yields relative molar ratios and not the absolute number of moles as opposed to the HPLC technique.

Although the focus of this work was to investigate the suitability of Raman spectroscopy as an alternative

Table 2
Raman and HPLC analytical results obtained from a 70 mol % lovastatin—30 mol % gallic acid mixture ($x_L/x_G = 2.333$) after artificial oxidation

Oxidation conditions	Raman results: L/G mol % ratio $n = 5$	HPLC results: L* moles/ml $n = 3$	HPLC results: G* moles/ml $n = 3$	HPLC results: L/G mol% ratio $n = 3$	Difference HPLC and Raman (%)
80 °C, 45 min	2.33 ± 0.15	50.27 × 10^{-9}	21.86 × 10^{-9}	2.30 ± 0.06	1.3
120 °C, 15 min	2.48 ± 0.15	50.38 × 10^{-9}	21.31 × 10^{-9}	2.48 ± 0.06	0

G = gallic acid, L = Lovastatin, n = number of measurements.

technique to HPLC and not an extensive investigation of the suitability of gallic acid as antioxidant together with lovastatin, it is worth noting that comparing the resulted x_L/x_G value for the sample that was heated at 80 °C for 45 min (Table 2) with the initial x_L/x_G ratio of the mixture, 2.33, no difference was observed. On the other hand, heating the sample at 120 °C for 15 min yielded a x_L/x_G ratio larger than 2.33, suggesting that the molar fraction of the gallic acid in the mixture was reduced. This implies that gallic acid behaved as antioxidant and was "sacrificed" while the concentration of the active ingredient remained stable.

5. Conclusions

The FT-Raman spectroscopy proved to be a useful tool for the determination of the molar ratio of lovastatin and gallic acid in their binary mixture in solid state. The analytical results were found to be in accordance with the respective results obtained from the developed HPLC methodology. The Raman methodology was faster as well as non-destructive for the sample, but yielded only molar ratios and not the absolute number of moles as opposed to the HPLC technique. Based on these findings, the application of FT-Raman spectroscopy for monitoring the stability of drugs in the presence of antioxidants can be envisaged.

References

[1] M.J. Kaufman, Pharm. Res. 7 (1990) 289–292.
[2] G.B. Smith, L. DiMichelle, L. Colwell, G. Dezeny, A. Douglas, R. Reamer, T. Verhoeven, Tetrahedron 49 (1993) 4447–4462.
[3] USP 23, NF-18, United States Pharmacopeial Convention, Inc., 1995, p. 907.
[4] S. Javernik, S. Kreft, B. Strukel, F. Vrecer, Pharmazie 56 (2001) 738–740.
[5] European Pharmacopoeia 1997, third ed., Supplement 2001, p. 1070.
[6] D.J. Mazzo, S.E. Biffar, K.A. Forbes, C. Bell, M.A. Brooks, J. Pharm. Biomed. Anal. 6 (1988) 271–276.
[7] R. Kysilka, V. Kren, J. Chromatogr. 630 (1993) 415–417.
[8] P. Sharma, H. Pal Sihgh Chawla, R. Panchagnula, J. Chromatogr. B. 768 (2002) 349–359.
[9] G. Morovjan, G. Szakacs, J. Fekete, J. Chromatogr. A. 763 (1997) 165–172.
[10] L.Y. Ye, P.S. Firby, M.J. Moore, Therap. Drug Monit. 22 (2000) 737–741.
[11] J. Ryan, S. Comptaon, M. Brooks, D. Compton, J. Pharm. Biomed. Anal. 9 (1991) 303–310.
[12] The Aldrich Library of Infrared Spectra, second ed., C.J. Pouchert (Ed.), Aldrich Chemical Co., USA, 1978.
[13] R. Monaghan, A. Alberts, C. Hoffman, G. Schomberg, Patent USP 4,231,938 11/1980.
[14] http://www.sigmaaldrich.com/.
[15] D. Strommen, K. Nakamoto, in Laboratory Raman Spectroscopy, J. Wiley, New York, 1984, p. 71.
[16] D.A. Skoog, D.M. West, F.J. Holler, in: Fundamentals of Analytical Chemistry, Saunders College Publishing, 1992, p. 53.
[17] J.C. Miller, J.N. Miller, in: Statistics for Analytical Chemistry, 1986, p. 98 (Chapter 4).