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Fast screening of lovastatin in red yeast rice products by flow injection tandem mass spectrometry

Fenhong Song*, Aref El-Demerdash, Shwn-Ji Susie H. Lee, Robert E. Smith

Total Diet and Pesticide Research Center, U. S. Food and Drug Administration, 11510 W 80th St., Lenexa, KS 66214, USA

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ABSTRACT

Drug adulteration in dietary supplement materials is a world-wide problem and poses a regulatory challenge. Red yeast rice is a product used by consumers to lower blood levels of cholesterol. While most current methods to analyze red yeast rice are based on HPLC separation with a photo-diode array detector and/or a mass spectrometry detector, which takes 20–40 min analysis time per sample, we developed a method to do fast screening of the active compound lovastatin by direct infusion into a mass spectrometer. This method takes under 1 min per analysis on the instrument. By using multiple reaction monitoring with five product ions, all the ion ratios of the analyte in the samples are compared with those from the standards for qualitative analysis. The results from this method were compared to the result from the liquid chromatography tandem mass spectrometry, which uses retention time and one ion ratio as the confirmation criteria. No false positives or false negatives were found among the 12 samples tested. The method also seems to be effective in measuring the lovastatin in red yeast rice semi-quantitatively. This kind of method could be adapted to the screening of other dietary supplement products.

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1. Introduction

Drug adulteration in dietary supplement material is a world-wide problem and poses a big regulatory challenge. Most of the current available methods to detect drug adulteration are based on liquid chromatography with a photo-diode array detector and/or a mass spectrometry detector [1,2]. While these methods use chromatography to separate the target compound from other interfering compounds in the matrix, we attempted to use the improvements in scan speed, sensitivity, and high selectivity of the new triple quadrupole mass spectrometer with multiple reaction monitoring to distinguish the targeted analytes from other compounds in the matrix by flow injection without chromatographic separation [3,4]. This type of fast screening method, if it is proven to be effective, will be very useful for regulatory agencies.

Red yeast rice, used in Chinese medicine for many years to improve blood circulation, is a product used by consumers. It is believed that the active ingredients are a class of compounds called monacolins [5,6]. Among these compounds, monacolin K or lovastatin, is an inhibitor of hydroxymethylglutaryl coenzyme A (HMG, CoA) reductase, which is a rate-limiting enzyme in cholesterol biosynthetic pathway [7]. Lovastatin is an FDA approved drug manufactured originally by Merck & Co Inc. (White house Station, NJ) under the trade name of Mevacor. When consumers take red yeast rice without knowing there are other drug ingredients in it, this may cause some safety concern. So, lovastatin in red yeast rice products is being monitored at FDA laboratories. In this work we used commercially available red yeast rice dietary supplements as model systems to see if they could be screened for the presence of lovastatin by flow injection tandem mass spectrometry (FI–MS/MS). The semi-quantitative flow injection results were compared to those obtained with quantitative LC–MS/MS. Finally, the red yeast rice samples were screened for the possible presence of simvastatin, which is a potential adulterant.

2. Materials and methods

The measurement was performed on an Agilent 6460 LC–MS/MS system (Santa Clara, CA, USA) with an electrospay source equipped with Agilent 1200 LC system. The LC system consists of a pump, a vacuum degasser, a temperature controlled column compartment, and a refrigerated autosampler set at 4 °C. For the FI–MS/MS method, the flow from the autosampler was connected to the electrospray ion source by using a guard column (Inertsil ODS-3, GL Sciences Inc., Tokyo, Japan) in the column compartment set at 45 °C. Isocratic chromatographic elution was used with 10:90 A:B (v/v) where A was 4 mM of ammonium formate plus 0.05% formic acid in water, and B was 4 mM of ammonium formate plus 0.05% formic

* Corresponding author. Tel.: +1 913 752 2125; fax: +1 913 752 2122.
E-mail address: fenhong.Song@fda.hhs.gov (F. Song).

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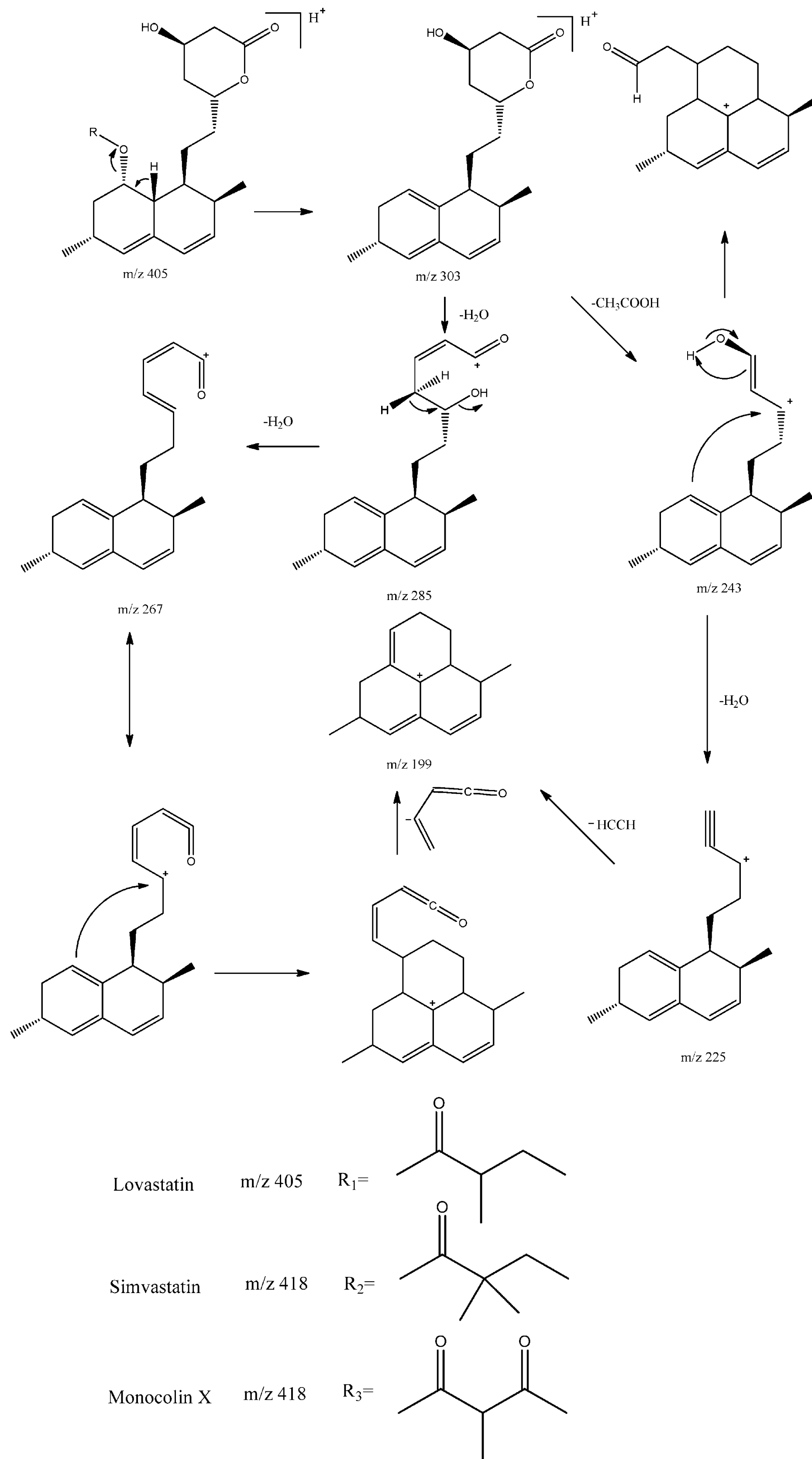

Fig. 1. Proposed fragmentation pathways of statin example shown.

Table 1
Mass spectrometer parameters for lovastatin product ion FI–MS/MS.

Transition name	Precursor ion	Product ion	Dwell time (ms)	Frag[b] (V)	CE[c] (V)
Transition 1[a]	405.1	285.1	160	80	0
Transition 2	405.1	267.2	160	80	5
Transition 3	405.1	243.2	160	80	5
Transition 4	405.1	225.3	160	80	8
Transition 5	405.1	199.4	160	80	8

[a] Transition 1 is the quantifier ion transition.
[b] Fragmentor potential.
[c] Collision energy.

acid in methanol (B) at a flow rate of 0.4 ml/min with a stop time of 0.65 min. The source parameters were set as follow: dry gas temperature = 350 °C, drying gas flow = 8 l/min, nebulizer flow = 35 psi, sheath gas temperature = 350 °C, sheath gas flow = 11 l/min, capillary potential = 3000 V, nozzle voltage = 500 V. The fragmentor potentials and collision energies of the product ions were optimized using standard reference (Table 1). The spectra were obtained in positive ion mode.

The entire instrument system and date acquisition were controlled by mass hunter software. Methanol was high purity grade (Burdick and Jackson, Morristown, NJ, USA). Ammonium formate and lovastatin reference standard were ACS grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA). The standard solutions were prepared by proper dilution of the stock solution of lovastatin in methanol to 5 ng/ml, 20 ng/ml, 100 ng/ml, 500 ng/ml and 2.0 μg/ml in 75:25 methanol/water (v/v). Dietary supplement materials were purchased from local grocery stores or from Internet web sites. The tablets or capsule samples, five of each, ranged from 603 to 994 mg (samples 1–4 and 7–11, half dose, containing 600 mg of red yeast rice) and 1.65 g (sample 5, a full dose containing 1200 mg of red yeast rice) were combined. Tablet samples and raw red yeast rice samples (about 10 g) were ground into fine powder. A sample size of half dose (corresponding to about 600 mg of read yeast rice) was weighed for each sample and then mixed with 25 ml of 75:25 methanol/water (v/v). It was sonicated for 30 min, and then centrifuged at 4000 rpm at 22 °C for 5 min. The supernatant was separated for subsequent qualitative measurement and further diluted 100 fold with 75% of methanol/water for the semi-quantitation study. After filtrated with 0.2 μm membrane, it was injected on to the mass spectrometer. The matrix spiked samples were prepared at 1.0 μg/ml, 250 ng/ml, and 100 ng/ml by addition of 10 μl of the corresponding standards to 990 μl of the matrix blank of sample 6 after 100 fold dilution.

For the LC–MS/MS method, an Intersil ODS-3 column (150 mm × 2.1 mm and 3.5 μm particle size, GL Sciences Inc., Tokyo, Japan) was used. Gradient elution was performed using the same mobile phase as above with a gradient program. It started with 50% of B, increased to 90% of B within 5 min and held for 8 min, then changed to 50% of B within 2 min. The flow rate was set at 0.25 ml/min and the stop time of the method is 20 min. The same source condition of the mass spectrometer as described above for the flow injection experiment was applied here with the exception of capillary potential = 3500 V. Multiple reaction monitoring (MRM) was performed by monitoring ions with *m*/*z* of 285.2, 225.2 and 199.2. A calibration curve was obtained by injecting the same five standards as above and fitting the data by linear regression.

3. Results and discussion

For the detection of drug adulteration in dietary supplement using liquid chromatography mass spectrometry a full product ion scan mode has been used in the studies for structural information or identification of unknown [8,9], although multiple reaction monitoring for targeted analysis has also been used [10,11]. Whenever MRM is used, two or three product ion transitions are typically monitored, and the ion ratios together with the retention time are compared for the identification. These methods take too much time for the high throughput needed for a screening method.

Direct flow injection tandem mass spectrometry has been tested for high-throughput analysis of pesticides in food matrices by monitoring two product ion transitions [3,4]. There are other fast detection methods based on mass spectrometry, such as direct analysis in real time (DART) [12] or desorption electrospray ionization (DESI) [13,14] have been developed in recent years to analyze several chemicals in complexes matrices. However, most laboratories currently do not have these ionization sources. In addition,

Table 2
Comparison of product ion ratios of lovastatin in standards and those found in the samples.

	Ion ratio 1 Transition 2	Ion ratio 2 Transition 3	Ion ratio 3 Transition 4	Ion ratio 4 Transition 5
Std 1	49.7	81.5	64.3	80.4
Std 2	48.7	82.1	65.5	82.1
Std 3	47.3	83.2	65.1	81.1
Std 4	48.3	83.3	66.7	81.6
Std 5	47.9	81.4	65.5	79.7
S1	46.4	81.3	63.2	82.1
S2	46.3	81.7	64.2	80.7
S3	49.2	87.0	66.4	84.9
S4	46.5	80.6	63.5	79.4
S5	47.0	82.7	67.8	83.6
S6	22.5		61.2	
S7	47.3	83.3	64.0	82.0
S8	46.6	80.8	65.1	79.1
S9	51.0	86.9	68.5	94.4
S10	46.7	83.2	65.3	80.1
S11	46.8	80.7	66.7	81.9
S12	48.1		125.3	

Table 3
Semi-quantification results from FI–MS/MS[a] and comparison with LC–MS/MS.[b]

	Average (μg/ml)	mg/per half dose	rsd%	μg/ml, LC–MS/MS	Ratio of FI–MS/MS to LC–MS/MS[c]
S1	0.092	0.230	3.2	0.107	0.863
S2	0.206	0.515	2.8	0.296	0.695
S3	0.219	0.548	4.0	0.302	0.727
S4	0.263	0.658	3.4	0.265	0.994
S5	0.187	0.468	5.3	0.280	0.667
S6[d]					
S7	0.085	0.213	6.2	0.106	0.803
S8	0.595	1.49	2.6	0.702	0.847
S9	0.072	0.180	8.2	0.089	0.812
S10	0.224	0.560	6.4	0.240	0.932
S11	0.336	0.840	6.2	0.383	0.878
S12[d]					

[a] Three injections per sample.
[b] One injection per sample.
[c] Average of three flow injection results.
[d] Below LOD.

quantitative analysis using FI–MS/MS with precisely controlled, well established sample introduction system is probably still better than using DART or DESI, in spite of continuous improvements of the later two. We decided to test flow injection mass spectrometry for fast screening of adulterated drugs in dietary supplement materials. In this study, we monitored five product ion transitions (Table 1) for detection of lovastatin in red yeast rice instead of just two or three transitions. So the confidence of the identification with mass spectrometric data itself is increased without retention time matching.

The parameters of the mass detector, shown in Table 1, were optimized for the major product ions, by using a lovastatin standard reference. A typical methanol/water mobile phase was used with the addition of ammonium formate to reduce possible sodium adducts of lovastatin. The fresh standard solutions were prepared and stored at −20 °C. No significant decomposition was noted within one month period.

Fragmentation pathways of lovastatin have been suggested [15]. Further details, including the structures of the product ions, mostly formed from neural loss of small molecules, are shown in Fig. 1.

A sample size of half of the recommended dose was extracted with 25 ml of 75:25 of methanol/water (v/v) for 30 min once, then centrifuged and filtered. We also tried repeated extraction of 10 min for three times using sample 4 as a representative sample. Comparison of the second and the third extraction to the first one using MRM for quantitation showed about 90% of recovery obtained from the first extraction, so one extraction of 30 min was used for screening purpose in this study. For an accurate quantification and a better absolute recovery, combination of more extractions with longer extraction time may be desired.

We use all the four product ion ratios to determine if a sample is positive for a qualitative screening. The raw MRM data are processed using Agilent quantitation software, which automatically calculates the ion ratios (Table 2) using one or more levels of calibration standards and extracts MRM chromatograms of each transition (Fig. 2). Most of the calculated concentrations in the samples were out of the calibration range without dilution under the extraction condition because of significant levels of lovastatin in the samples. The criteria to judge a positive sample are that the ion ratios of the analyte found in the sample should be within 20% matching with that in the standard and also with signal to noise (S/N) ratios > 10 for the quantifier ion transition and >3 for the others as qualifier transitions. The estimated LOD is 200 ng/g based on the weight of red yeast rice sample.

There are 12 samples that were tested in this work including 8 capsules, 2 tablets, 2 raw red yeast rice samples. Ten of the samples (1–5 and 7–11) were found to be positive, which showed matching of all the four product ion transitions within 20%. No ion ratio matching was found for the raw rice sample 6 and sample 12 although two product ions were found for sample 6 and sample 12 with S/N ratios < 3. So, both samples are negative or the levels

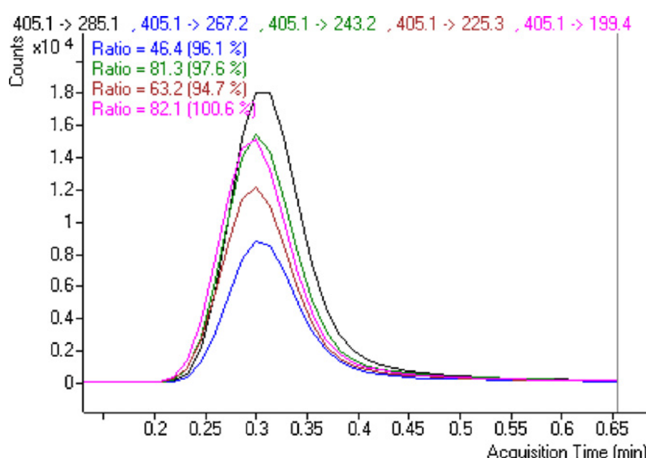

Fig. 2. Extracted MRM transition chromatograms of lovastatin of sample 1.

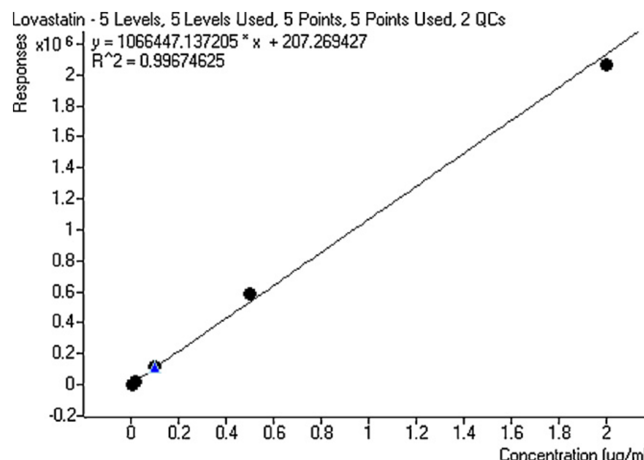

Fig. 3. Calibration curve of lovastatin standards from flow injection of tandem mass spectrometry.

of lovastatin in these samples are below LOD. Although matching one or two transition ratios seems to be adequate with LC–MS/MS, monitoring more product ion transitions is recommended for this type of screening since no retention time is used to confirm the identity of the analyte without column separation.

We also tried to measure the amount of lovastatin semi-quantitatively and the results were shown in Table 3. Five levels of standard solutions of lovastatin were prepared at 5 ng/ml, 20 ng/ml, 100 ng/ml, 500 ng/ml and 2.0 μg/ml in 75:25 methanol/water (v/v). The correlation coefficient using linear fitting with weighting of $1/x$ was 0.9967, as shown in Fig. 3. For the semi-quantification, the sample extracts were diluted 100 fold to lower the concentrations of lovastatin into the calibration range. The standards and samples were also analyzed by the liquid chromatography tandem mass spectrometry for comparison. The m/z of 285.2 was used as quantifier and the other two product ions of m/z 225.2, 199.2 were used as qualifier ions. The retention times and ion ratios (20% matching with the standard) were used as confirmation of lovastatin (t_R, 10.70 min). The results from the two methods are comparable, and the concentrations obtained from flow injection are consistently lower than these obtained from LC/MS–MS. It is probably due to more matrix suppression by FI–MS/MS than LC–MS/MS, which is expected with more interference in the ionization process from the compounds in the matrices that could not be removed without column separation. The matrix effect of flow injection was evaluated by spiking the standard in the matrix of sample 6 at 100 ng/ml, 250 ng/ml and 1 μg/ml. Complete evaluation of matrix suppression for all of the different samples is difficult since blank samples from each type of the sample matrices with different formulations for the capsule and tablet samples are not available. With the 100-fold dilutions of sample extracts, the result showed that the matrix suppression in sample 6 is less than 20%. The matrix suppression in the flow injection is higher for samples 2, 3 and 5 than samples 1, 4, 7, 8, 9, 10, 11 based on the ratios of the calculated concentrations from the two methods in Table 3. The correlation of the results from the two methods showed the effectiveness of the flow injection as a semi-quantitative method in this application. More accurate quantification with the use of isotope labeled internal standards or the matrix-matched internal, external standards could be achieved.

We also briefly explored the possibility of detection of multiple drug adulteration in dietary supplement materials by adding simvastatin in the method. With a dwell time of 20 ms between transitions for each transition including pause time, 25 transitions can be scanned within 0.5 s when using five transitions for each analyte for a group of five analytes. For a peak width of 10 s from flow injection tandem mass spectrometry analysis, twenty data points for quantitation can be obtained. So the method should be able to analyze multiple drug adulteration. Product ion transitions from simvastatin were observed a few of these samples (1–5, 7–11) from qualitative screening of simvastatin together with lovastatin using the same product ions for both. The results showed that for sample 8 the four ion ratios were matched with the reference standard of simvastatin within 20% tolerance. Further experiment on liquid chromatography tandem mass spectrometry showed a possibility of existence of monacolin X [16], a compound also with the m/z of 418 as simvastatin, but eluted 1.38 min earlier than simvastatin under experimental condition (9.34 min verse 11.12 min), and no presence of simvastatin in the samples. The major fragments from monacolin X as described in Fig. 1 are probably the same as these from lovastatin and simvastatin.

Quantification of monacolin K in red yeast rice by HPLC with photo-diode array detector with a total run time of 30 min has been published [17]. Li et al. [16] also reported their results of chromatographic chemical profiling of 14 monocolins for the qualitative evaluation of red yeast rice products. The problem with these methods is that possible co-eluting interferences could cause overestimation of quantities of the analytes. Accurate quantification of multiple monocolins by a LC–MS/MS method using a triple quadrupole mass spectrometer is difficult given that most of the reference standards are not commercially available. The flow injection tandem mass spectrometry method presented in this work offers a cost effective way for fast screening of lovastatin in red yeast rice products by reducing the consumption of solvents and elimination of HPLC column.

The method here could be adapted to detect other types of adulterated drugs in other dietary supplement materials although in some cases there might be some limitations when potential interference peaks from the matrices or solvent arose. However, the ion ratios from the interferences will not likely match to that of the standards closely when we used five or more transition ion ratios except analogue compounds such as the ones mentioned above. Significant amounts of drugs are typically detected in these adulterated samples, which are probably intended for the desired therapeutic effect [11]. Most of the naturally occurred interfering compounds in the samples are presented at much lower concentration in general, therefore may not generate significant interference on the detection of the analyte after 100 or 500 fold of dilution of the sample extract as demonstrated in this work. When a match is met with one or two of the product ion transitions with signal over noise ratios better than 3, a run with chromatographic separation may be necessary to find out if it is a positive or a negative. A positive sample may need to be confirmed further with LC–MS/MS for regulatory samples.

In conclusion, this method could be used as a fast qualitative screening of lovastatin in red yeast rice products. Semi-quantification of lovastatin with this method seems also to be effective.

This work should not be taken as reflecting FDA policy or regulations.

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