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Analytical method for determination of allylic isoprenols in rat tissues by liquid chromatography/tandem mass spectrometry following chemical derivatization with 3-nitrophtalic anhydride

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Abstract

A liquid chromatography-tandem mass spectrometric (LC–MS/MS) method following chemical derivatization with 3-nitrophtalic anhydride was developed for the simultaneous determination of farnesol and geranylgeraniol in rat liver and testis. One analogue compound of the analytes, *n*-pentadecanol, was used as an internal standard (IS) for both analytes in this method. Rat tissues were disintegrated with 8% KOH ethanol solution, and then farnesol, geranylgeraniol and IS were extracted with a mixture of *n*-hexane–ethanol (98.5:1.5, v/v) in twice. Farnesol, geranylgeraniol and IS were then converted to 3-nitrophtalic derivatives of each analyte, and extracted with *n*-hexane. A turbo ion spray interface was used as the ionization source of LC–MS/MS and the analysis was performed in the multiple reaction monitoring (MRM) mode. The calibration curve at the spiked concentrations of 0.15–15 μg/g for both analytes showed good linearity. The method was precise; the relative standard deviations of the method for rat liver were not more than 13.4 and 5.4% for farnesol and geranylgeraniol, respectively, and those for rat testis were not more than 8.4 and 8.6% for farnesol and geranylgeraniol, respectively. The accuracies of the method for both rat liver and testis were good, with the deviations between the nominal concentration and calculated concentration of farnesol and geranylgeraniol typically being within 12.3 and 10.2%, respectively. This method provided reliable concentration levels for farnesol and geranylgeraniol in rat liver and testis.

Keywords: Farnesol; Geranylgeraniol; Derivatization; Quantitation; LC–MS/MS

1. Introduction

The allylic isoprenols, farnesol and geranylgeraniol (Fig. 1), could be produced in mammalian cells by the action of rat liver microsomal phosphatase [1], and are utilized for sterol biosynthesis and protein isoprenylation [2]. The isoprenols play a physiological role in the regulated proteolysis of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [3–5]. HMG-CoA reductase inhibitors, a group of the major lipid lowering agents, show some side effects such as skeletal myopathy [6], and this toxicity is thought to be due to the inhibition of mevalonate synthesis [7]. Since mevalonate is metabolized to farnesyl pyrophosphate and then branches to several different biosynthetic pathways [8,9], the inhibition of mevalonate synthesis results in the reduction of isoprenoids such as farnesol, geranylgeraniol and ubiquinone. Thus the concentration changes of these isoprenoids indicate the pharmacological or toxicological effects of lipid lowering agents [10], and it is considered that the analytical methods for these isoprenoids are important.

For the analytical method of ubiquinone, we have developed a liquid chromatography/tandem mass spectrometric method applying the 1-alkylamine adduct ion to enhance detection sensitivity, and reported in previous paper [11]. The analytical techniques for farnesol have been reported using high-performance liquid chromatography [12] and capillary gas chromatography/mass spectrometry [13], however, these methods have been developed for analysis of healthcare products and liquors. Additionally, to our knowledge, no quantitative analytical method for geranylgeraniol has been reported. Because of this, our interests were the development of a highly sensitive and selective method for simultaneous determination of farnesol and geranylgeraniol in biological matrices. Firstly,

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we investigated the analytical sensitivity for authentic samples of farnesol by a conventional LC–MS/MS method using a turbo ion spray interface. However, the analytical sensitivity for farnesol was relatively low, possibly due to low ionization efficiency. To enhance the sensitivity in LC–MS analysis, some articles show the benefits of an analyte derivatization method in which the introduction of permanently changed moieties or readily ionized species may dramatically improve the ionization efficiency [14,15]. Therefore, we applied a chemical derivatization method to LC–MS/MS analysis for farnesol and geranylgeraniol. In this paper, we described the establishment of an analytical method for farnesol and geranylgeraniol in rat liver and testis by LC–MS/MS following chemical derivatization with 3-nitrophtalic anhydride.

2. Experimental

2.1. Materials and reagents

Authentic samples of farnesol and geranylgeraniol were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The preparation of *n*-pentadecanol as internal standard (IS) was also made by Tokyo Chemical Industry Co. Ltd. The chemical structures of these compounds are shown in Fig. 1. HPLC-grade methanol, ethanol, *n*-hexane and acetonitrile, as well as analytical-grade 3-nitrophtalic anhydride, ammonium acetate, potassium hydroxide (KOH), magnesium chloride ($MgCl_2$), Tris–hydroxymethyl aminomethane, 2 mol/L hydrochloric acid, sodium dihydrogenphosphate dihydrate, phosphoric acid and acetic acid, were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Animals

Tissues from male Crj:IGS rats weighing approximately 250 g ($N = 10$, Charles River Japan, Yokohama, Japan) were used to develop this analytical method. The rats were anesthetized with ether and the liver and testis were excised. The tissues were immediately frozen and stored at −80 °C.

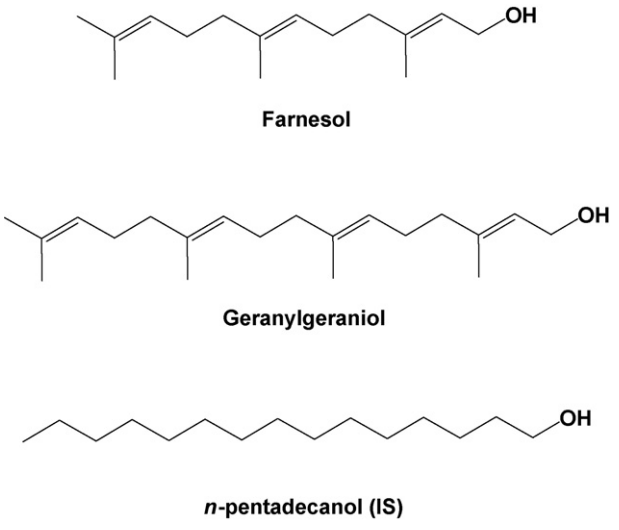


Fig. 1. Chemical structures of farnesol, geranylgeraniol and *n*-pentadecanol (IS).

2.3. Instrumentation and conditions for LC–MS/MS and ^1H NMR analysis

The HPLC system used was an Agilent 1100 Series (Agilent Technologies Inc., CA, USA) coupled with API 300 triple-stage quadrupole mass spectrometer (Applied Biosystems, CA, USA). Turbo ion spray interface was used for analyte ionization. The HPLC column used was Symmetry Shield RP8 (150 mm × 2.1 mm ID, Waters, MA, USA). A mixture of acetonitrile–10 mmol/L ammonium acetate–acetic acid (90:10:0.1, v/v/v) was used as a mobile phase at a flow rate of 0.2 mL/min. The column temperature was maintained at 40 °C. MS/MS detection was performed by the MRM from the deprotonated molecule ($[M-H]^-$) of the analyte derivatives to its product ion under the negative ion mode. The turbo ion spray voltage and turbo probe temperature were set to 3900 V and 425 °C, respectively. The flow rate of the curtain gas, nebulizer gas and heater gas were 1.25 L/min (nitrogen), 1.46 L/min (air) and 7 L/min (nitrogen), respectively. The orifice, ring and multiplier voltages were set at 50, 300 and 2200 V, respectively. The collision induced dissociation of the $[M-H]^-$ of the analytes were performed using a nitrogen gas set rate of 2.8×10^{-5} Torr and a collision energy at 14 eV for both analytes and the IS derivative. The monitoring ions (precursor → product ion) for MRM analysis were set to m/z 414 → 166 for the farnesol derivative, m/z 482 → 166 for the geranylgeraniol derivative and m/z 420 → 166 for the IS derivative.

^1H NMR (500 MHz) spectra of derivatives were recorded on a JEOL ECA500 spectrometer (JEOL Ltd., Tokyo, Japan) equipped with a 5-mm ID probe. The solvent used was deuterated chloroform ($CDCl_3$) containing 0.03% of tetramethylsilane for chemical shift reference. Sixteen scans were acquired with a spectral width of 9384 Hz, an acquisition time of 1.75 s and a recycle delay of 5 s.

2.4. Standard solutions

Approximately 50 mg of farnesol and geranylgeraniol were weighed separately and dissolved in 50 mL of methanol to prepare the stock solution at a concentration of 1000 μg/mL. Equal volumes of each stock solution of farnesol and geranylgeraniol were mixed together and serially diluted with methanol to provide working solutions with concentrations of 10, 8, 4, 2, 1, 0.2 and 0.1 μg/mL for each compound. In addition, the solution of the IS was prepared by dissolving 2.5 mg of *n*-pentadecanol with 50 mL of methanol resulting in a concentration of 50 μg/mL.

2.5. Preparation, extraction and chemical derivatization with 3-nitrophtalic anhydride

Rat liver and testis (1.0 g each) were each mixed with 8% KOH in ethanol solution (2 mL) and the samples were incubated at 80 °C for 2 h. The samples were agitated during the incubation to disintegrate the liver and testis efficiently. The

obtained solutions were cooled to room temperature, centrifuged at 1660 × *g* for 5 min and the supernatants (20 μL) were transferred to glass tubes. The working solutions (10 μL) were added to these extracts to prepare the calibration standard samples with the spiked concentrations of 15, 6, 3, 1.5, 0.3 and 0.15 μg/g for each analyte. Similarly, quality control (QC) samples with the three spiked concentrations of 12, 1.5 and 0.15 μg/g for each analyte were prepared from the working solutions described above. To these samples, 10 μL of the IS solution and 2 mL of 1.5 mol/L Tris–HCl buffer (pH 8.6) containing 0.25 mol/L $MgCl_2$ were added. Farnesol, geranylgeraniol and the IS in the solutions were extracted with 5 mL of a mixture of *n*-hexane–ethanol (98.5:1.5, v/v, 2 times), and the supernatants obtained by centrifugation at 1660 × *g* for 5 min were evaporated at 40 °C under a stream of nitrogen gas. The residues were dissolved with 250 μL of 3-nitrophtalic anhydride pyridine solution (10 mg/mL), and incubated for 30 min at 70 °C. After the incubation, the solutions were evaporated at 40 °C under a stream of nitrogen gas, and the residues were dissolved in methanol (200 μL), then 500 μL of 50 mmol/L phosphate buffer, which was prepared by dissolving sodium dihydrogenphosphate dihydrate with water and adjusting to pH 3 by the addition of phosphoric acid, was added. The 3-nitrophtalic acid derivatives of farnesol, geranylgeraniol and IS were extracted with *n*-hexane (5 mL), and the *n*-hexane layers were evaporated at 40 °C under a nitrogen gas stream and the residues were dissolved in 500 μL of a mixture of methanol–water–acetic acid (75:25:0.1, v/v/v). Fifty microliters aliquots of the solutions were injected into the LC–MS/MS system.

2.6. Recovery

The extraction recoveries of the analytes from rat liver and testis were evaluated at the spiked concentration of 12 μg/g. The extraction recovery was calculated by comparing the peak area of extracted analyte to that of non-extracted analyte. Extracted analytes were prepared by the procedure described in Section 2.5. For the non-extracted sample, analytes were spiked after the extraction with the mixture of *n*-hexane–ethanol in the procedure.

2.7. Calculation

Farnesol and geranylgeraniol are endogenous compounds and it is hard to obtain the analyte-free matrix. In this case, there are two approaches to construct the calibration curve. First approach is the standard addition method that the standard samples are spiked to the control tissue and provide the calibration curve using the tissue samples. As second approach, if the slope of the calibration curves provided from standard (matrix-free) samples are identical to that from the spiked tissue samples, the standard (matrix-free) samples can be used as calibration curves. It is considered that both approaches are acceptable to quantify the endogenous compounds, and the first approach (standard addition method) was used to construct the calibration curve for farnesol and geranylgeraniol in this analytical method.

The standard curve was obtained by a $1/C$ weighted least-squares linear regression on the peak area ratio of each analyte to the IS versus the concentrations of each analyte in the spiked samples:

$$y = a + b \times C_{\text{theor}},$$

where y, C_{theor}, a and b are the peak area ratio, spiked concentration, y-intercept and slope, respectively. The concentration of endogenous farnesol and geranylgeraniol were calculated by dividing a by b. The nominal concentrations of the spiked samples and the QC samples were expressed as the sum of the spiked and endogenous concentrations of the analytes.

3. Result and discussion

3.1. Chemical derivatization and mass spectrometry

Firstly, the detection sensitivity of farnesol in the LC–MS/MS analysis was checked using a farnesol solution (1 mg/mL). The mass transition for detection of farnesol was set based on the autotune program so that the precursor ion and product ion were optimized at *m/z* 222 and *m/z* 123, respectively. Unfortunately, the peak intensity of farnesol in the MRM chromatogram was only 1300 cps and the signal to noise (S/N) ratio was 6 when 100 ng of farnesol was applied onto the column (Fig. 2). This brief survey result suggested that the ionization efficiency of farnesol was too poor to develop a highly sensitive quantification method for farnesol. Therefore, chemical derivatization of farnesol was investigated to enhance the detection sensitiv-

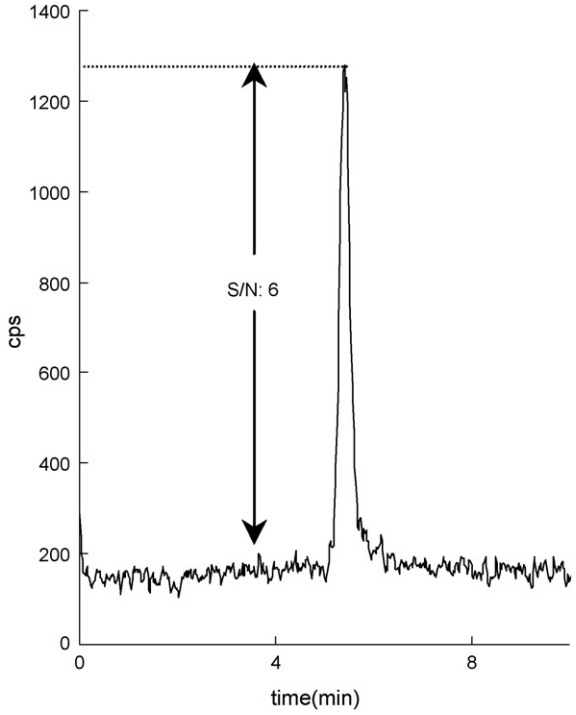


Fig. 2. MRM chromatogram of farnesol after applying 100 ng onto column. Column: Develosil C8-UG-5 (150 mm × 2.0 mm ID; Nomura chemical), mobile phase: methanol–10 mmol/L ammonium acetate–acetic acid (80/20/0.1, v/v/v), ionization: turbo ion spray (positive), mass transition: *m/z* 222 → 123.

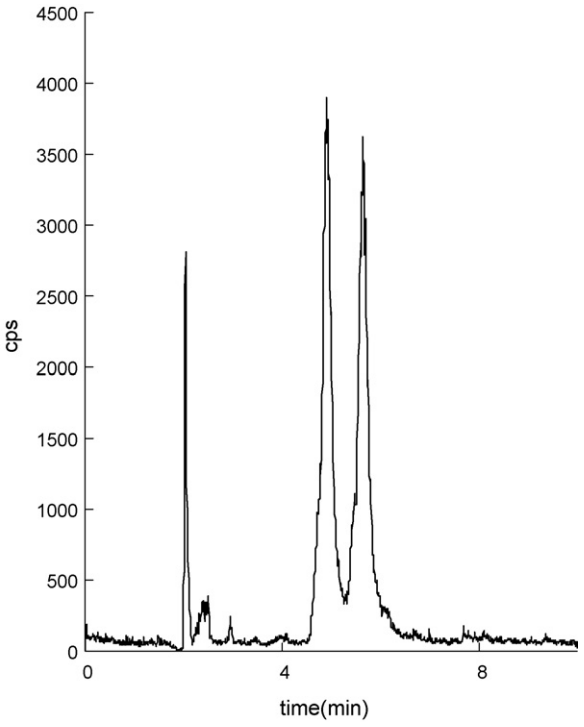


Fig. 3. MRM chromatogram of the trimellitic acid derivative of farnesol after applying 50 ng onto column. Column: Xterra RP18 (150 mm × 2.1 mm ID; Waters), mobile phase: methanol–5 mmol/L ammonium formate–formic acid (85/15/0.05, v/v/v), ionization: turbo ion spray (negative), mass transition: *m/z* 413 → 369.

ity for the method and trimellitic anhydride was used as the derivatization reagent. The introduction of a carboxylic group is a commonly used strategy to improve detection sensitivity in the negative ion mode, because these acidic groups are readily deprotonated and are able to obtain the high ionization efficiency [14]. Although the detection sensitivity was enhanced by derivatization using trimellitic anhydride, there were two peaks in the MRM chromatogram for the product after the reaction of trimellitic anhydride and farnesol in pyridine solution at 70 °C for 20 min (Fig. 3). It was considered that structural isomers were formed in the reaction due to the lack of the selectivity for the binding site (Fig. 4). To avoid the derivatization to isomers, 3-nitrophtalic anhydride was selected as a derivatization reagent [16]. Nitro functionality in 3-nitrophtalic anhydride has an electron-withdrawing inductive effect leading the selectivity of the reaction between 3-nitrophtalic acid and farnesol. 3-Nitorophtalic anhydride were reacted in pyridine solution at 70 °C as same condition for trimellitic anhydride. The duration of the derivatization was set for 30 min, since the peak areas of 3-nitrophtalic acid reached plateau within 30 min in determination of peak areas with the reaction time for 15, 30 and 60 min. When farnesol and 3-nitrophtalic anhydride were reacted in pyridine solution at 70 °C for 30 min, one 3-nitrophtalic acid derivative of farnesol was generated by esterification between the hydroxy group of farnesol and the carboxyl group at the 2-position of 3-nitrophtalic acid (Fig. 5). Chemical structures of 3-nitorophtalic acid derivatives for farnesol were identified based on ^{1}H NMR spectral data (Table 1). Proton signals from 3-nitrophtalic acid moiety were observed at 7.7–8.4 ppm and those from farnesol

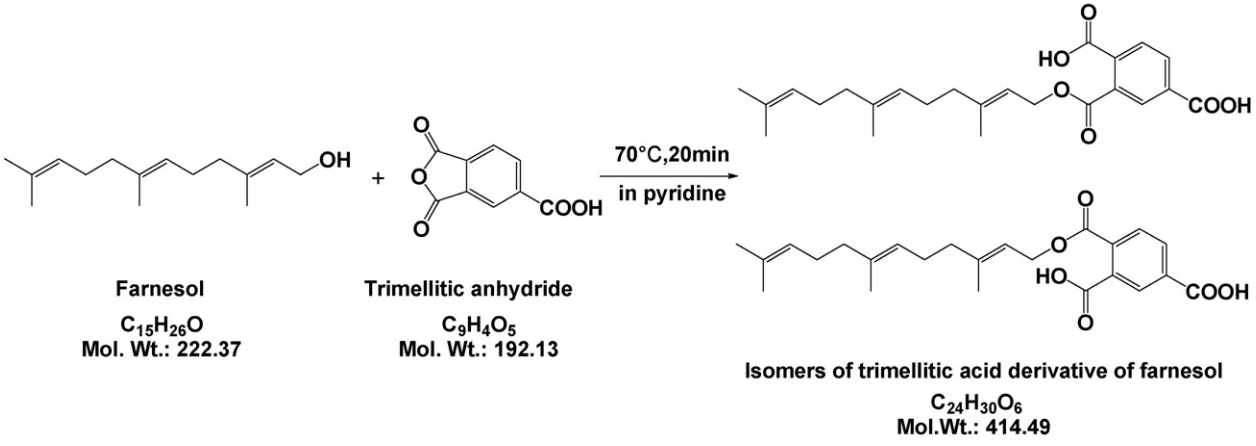


Fig. 4. Chemical derivatization scheme of farnesol and trimellitic anhydride.

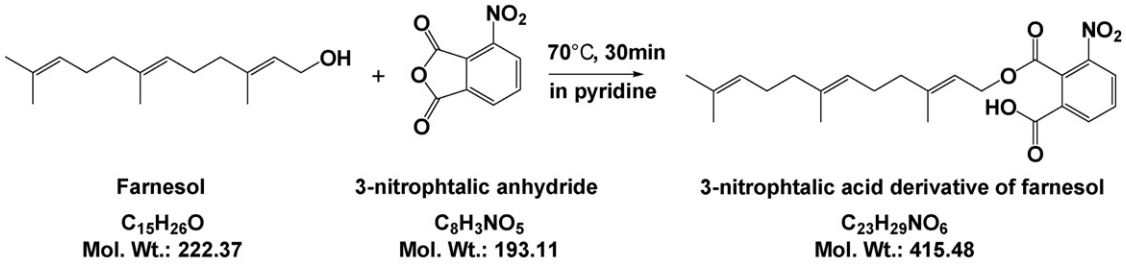
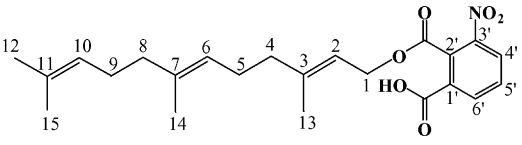
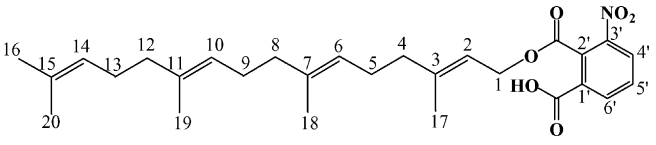


Fig. 5. Chemical derivatization scheme of farnesol and 3-nitrophtalic anhydride.

Table 1
^{1}H NMR of 3-nitrophtalic acid derivatives of farnesol and geranylgeraniol

Farnesol derivative				Geranylgeraniol derivative			
Position	*d* (ppm)	Multiplicity	*J* (Hz)	Position	*d* (ppm)	Multiplicity	*J* (Hz)
4′, 6′	8.41	d	8.1	4′, 6′	8.41	d	8.0
5′	7.70	t	8.1	5′	7.69	t	8.0
1	4.95	d	7.5	1	4.95	d	7.4
2	5.49	t	7.5	2	5.49	t	7.4
6, 10	5.05–5.11	m	–	6, 10, 14	5.08–5.10	m	–
4, 5, 8, 9	1.94–2.14	m	–	4, 5, 8, 9, 12, 13	1.96–2.16	m	–
12, 15	1.57	s	–	16, 20	1.58	s	–
13	1.77	s	–	17	1.77	s	–
14	1.67	s	–	18	1.67	s	–
				19	1.59	s	–





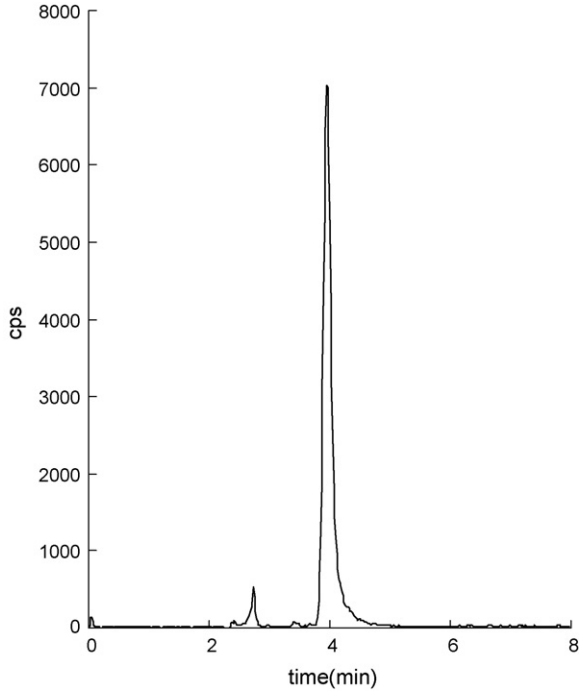


Fig. 6. MRM chromatogram of the 3-nitrophtalic derivative of farnesol after applying 5 ng onto the column. Column: Symmetry Shield RP8 (150 mm × 2.1 mm ID; Waters), mobile phase: methanol–10 mmol/L ammonium acetate–acetic acid (90/10/0.1, v/v/v), ionization: turbo ion spray (negative), mass transition: *m*/*z* 414 → 166.

moiety were appeared at 1.6–5.5 ppm. For negative MRM analysis of this derivative, the precursor and product ion was set at *m*/*z* 414 and *m*/*z* 166, respectively. The precursor ion at *m*/*z* 414 corresponds to $[M-H]^-$ of the 3-nitrophtalic acid derivative of farnesol, and the product ion at *m*/*z* 166 was derived from nitrobenzoic acid. Under these conditions, the peak intensity of the farnesol derivative in the MRM chromatogram was observed with 7000 cps when 5 ng of farnesol derivative was injected into column (Fig. 6). From these results, it was clear that the sensitivity of 3-nitrophtalic acid derivative of farnesol is over 100-fold higher than that of farnesol. This sensitivity enhancement effect could be also applied to geranylgeraniol due to its similar chemical structure. Thus, 3-nitrophtalic anhydride was used as the derivatization reagent for the analytical method of farnesol and geranylgeraniol.

3.2. Chromatography

As a brief summary of checking the detection sensitivity for farnesol, a Develosil C8-UG-5 (150 mm × 2.0 mm ID) column was used and the mobile phase was a mixture of methanol–10 mmol/L ammonium acetate–acetic acid (80/20/0.1, v/v/v). These chromatographic conditions were changed to a Symmetry Shield RP8 (150 mm × 2.1 mm ID) column with the mobile phase of methanol–10 mmol/L ammonium acetate–acetic acid (90/10/0.1, v/v/v) to adjust for the physicochemical properties of the 3-nitrophtalic acid derivative of farnesol. Then the organic solvent was changed to acetonitrile from methanol to shorten the total analytical time for simul-

Table 2
Linearity regression parameters for the determination of farnesol and geranylgeraniol in rat liver and testis

Tissue	Analyte	Spiked concentration (ng/g)	Slope	*y*-Intercept	Correlation coefficient
Liver	Farnesol	0.15–15	7.460E−02	1.466E−02	0.9975
	Geranylgeraniol	0.15–15	3.852E−02	7.745E−02	0.9927
Testis	Farnesol	0.15–15	4.964E−02	9.232E−03	0.9955
	Geranylgeraniol	0.15–15	4.565E−02	2.205E−02	0.9991

Table 3
Precision and accuracy of the method

Tissue	Analyte	Concentration (μg/g)			R.S.D. (%)	RE (%)
		Spiked	Nominal	Measured[a]		
Liver	Farnesol	0.148	0.345	0.313	13.4	−9.1
		1.48	1.68	1.66	7.7	−1.2
		11.8	12.0	10.6	5.5	−12.3
	Geranylgeraniol	0.150	2.21	2.01	5.4	−9.0
		1.50	3.56	3.57	4.8	0.4
		12.0	14.0	13.0	1.4	−7.0
Testis	Farnesol	0.148	0.334	0.326	7.7	−2.4
		1.48	1.67	1.63	8.4	−2.2
		11.8	12.0	10.7	6.8	−11.1
	Geranylgeraniol	0.150	0.633	0.685	8.6	8.3
		1.50	1.98	2.08	6.8	5.1
		12.0	12.4	11.2	4.8	−10.2

[a] Mean values of five determination.

taneous determination of farnesol and geranylgeraniol. As a result, the Symmetry Shield RP8 (150 mm × 2.1 mm ID) column with the mobile phase of acetonitrile–10 mmol/L ammonium acetate–acetic acid (90/10/0.1, v/v/v) was found to provide appropriate chromatographic conditions.

3.3. Linearity, precision, accuracy and recovery

Linearity, precision and accuracy were evaluated using the method established. For linearity, the least square regression fit showed good linearity with correlation coefficients of greater

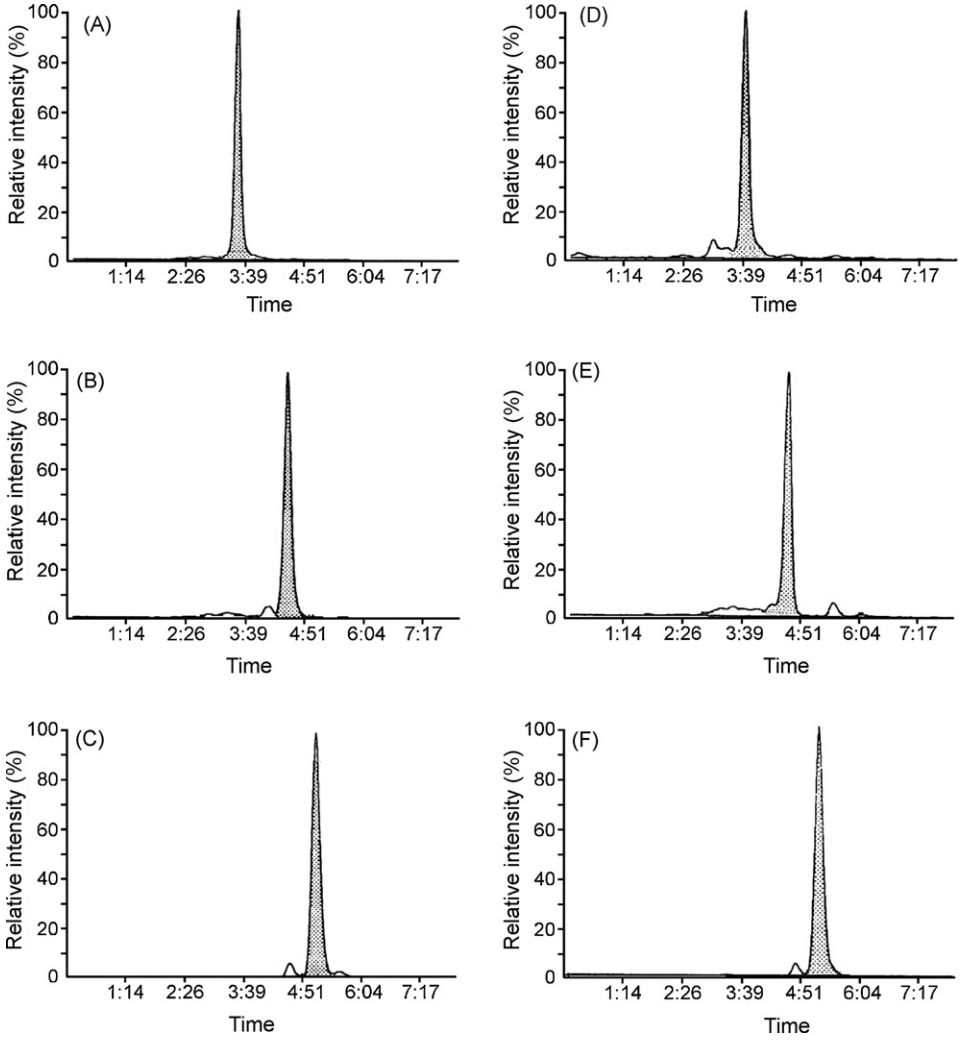


Fig. 7. MRM chromatograms for 3-nitrophtalic acid derivative of (A) farnesol in rat liver, (B) geranylgeraniol in rat liver, (C) IS in rat liver, (D) farnesol in rat testis, (E) geranylgeraniol in rat testis and (F) IS in rat testis.

Table 4
Recovery of farnesol and geranylgeraniol from rat liver and testis

Tissue	Recovery %[a]	
	Farnesol	Geranylgeraniol
Liver	111.4	98.4
Testis	108.0	107.2

[a] Mean values of two determinations.

than 0.992 for both analytes (Table 2). Table 3 presents the precision and accuracy data for the QC samples for the analytical procedure. The precision and accuracy were evaluated by the relative standard deviation (R.S.D.) and relative error (RE), respectively. The R.S.D. of the method for rat liver were not more than 13.4 and 5.4% for farnesol and geranylgeraniol, respectively, and those for rat testis were not more than 8.4 and 8.6% for farnesol and geranylgeraniol, respectively. The RE of the method for both rat liver and testis were also good, with the deviations between the nominal concentration and calculated concentration of farnesol and geranylgeraniol typically being within 12.3 and 10.2%, respectively. These results indicated that the method was reliable within the tested calibration range (0.15–15 μg/g). The extraction recovery of farnesol and geranylgeraniol from rat liver and testis were more than 98.4% (Table 4), indicating high extraction efficiency in this procedure.

3.4. Application

The LC–MS/MS method using chemical derivatization of 3-nitrophtalic anhydride can be applied to quantification of the endogenous farnesol and geranylgeraniol in rat liver and testis. The peaks of 3-nitrophtalic acid derivative of farnesol, geranylgeraniol and IS appeared with good shape and no interference peak was observed in MRM chromatograms (Fig. 7). The calculated concentrations of endogenous farnesol and geranylgeraniol were 0.197 and 2.063 μg/g, respectively, in rat liver and 0.186 and 0.483 μg/g, respectively, in rat testis. The concentration level of farnesol in rat liver was similar to that in testis. On the other hand, the geranylgeraniol concentration in rat liver was approximately 4 times higher than that in the testis.

4. Conclusion

This paper reports a quantitative analytical method for farnesol and geranylgeraniol in rat liver and testis using LC–MS/MS following chemical derivatization with 3-nitrophtalic anhydride. Farnesol and geranylgeraniol in rat tissues were extracted by liquid–liquid extraction, and then converted to the 3-nitrophtalic derivatives of each analyte. These analyte derivatives were introduced to the MS/MS system coupled with HPLC via a turbo ion spray interface. The calibration curve at the spiked concentrations of 0.15–15 μg/g for both analytes showed good linearity and the R.S.D. and RE values from the QC samples showed that this method was reliable within the tested calibration range. Using the method developed in this study the concentrations of endogenous farnesol and geranylgeraniol were determined. The calculated concentrations of farnesol and geranylgeraniol were 0.197 and 2.063 μg/g, respectively, in rat liver and 0.186 and 0.483 μg/g, respectively, in rat testis. It is considered that this method is capable of determining the concentrations of farnesol and geranylgeraniol in rat liver and testis.

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