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Simultaneous quantitative determination of urinary cholesterol, desmosterol and lanosterol in pravastatin treated rats by gas chromatography/mass spectrometry

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Gas Chromatography/Mass Spectrometry를 이용한 Pravastatin 투여 쥐의 뇨 중 Cholesterol, Desmosterol, Lanosterol의 동시분석법

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Abstract: A simultaneous determination method for cholesterol, lanosterol and desmosterol was developed using gas chromatography/mass spectrometry. Urine was enzymatically hydrolyzed with β-glucuronidase/arylsulfatase. Samples were prepared using extractions with a mixture of ethyl acetate-hexane (2:3, v/v), followed by derivatization with a mixture of MSTFA/TMSI/TMCS (100:2:5 v/v/v). All analyses were performed using GC/MS in selective ion monitoring mode. Good linearities (r^2=0.998~0.999) in calibration curve and a satisfactory recovery (80.0%~113%) were achieved. Accuracy and precision values within ±15% in the concentration range of 5 to 200 ng/mL were also observed for all compounds. The developed method was applied to pravastatin-treated (70 and 250 mg/kg/day for 7 days, oral) hyperlipidemia rats. Those sterols were significantly lower in drug-treated rats compared to the controls, which justifies the drug efficacy. Therefore, these results indicate that the developed method was successfully applied to examine statin drug efficacy with urine sample.

요 약: 가스크로마토그래피-질량분석기 (GC/MS)를 이용하여 뇨 중의 cholesterol, lanosterol, desmosterol의 동시분석법을 개발하였다. 뇨 시료는 β-glucuronidase/arylsulfatase를 첨가하여 효소가수분해한 후 에틸아세테이트-헥산 (2:3, v/v)으로 추출하였으며 추출한 잔사를 MSTFA/TMSI/TMCS (100:2:5 v/v/v)로 유도

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체화한 후 GC/MS의 selective ion monitoring mode에서 분석하였다. 개발된 분석방법은 검량선에서 좋은 직선성 (r^2=0.998~0.999)과 회수율 (80.0%~113%) 을 보였으며 5-200 ng/mL의 농도범위에서 15% 이내의 정확도와 정밀도를 보였다. 개발된 분석법은 고지혈증 유도 쥐에 pravastatin을 7일동안 70과 250 mg/kg/day로 경구투여한 후 채취한 뇨 시료를 분석하는데 적용하였다. 측정 결과 뇨 중 스테롤의 농도가 약물 투여한 쥐에서 대조군에서 보다 유의성 있게 낮아 약물효과를 관찰할 수 있었다. 이 결과로부터 개발된 방법을 뇨 중 스테롤의 농도를 정량 함으로서 스타틴약물의 효과를 측정하는데 적용할 수 있음을 확인하였다.

Key words : cholesterol, desmosterol, lanosterol, pravastatin, GC/MS

1. Introduction

Hypercholesterolemia is a major coronary risk factor, and extensive epidemiological data have shown that there is a strong positive correlation between serum cholesterol levels and the incidence of coronary heart disease (CHD).[1-4] The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase converts HMG-CoA to mevalonate, and inhibition of this enzyme results in decreased synthesis of cholesterol and other cholesterol precursors, downstream products of mevalonate (*Fig.* 1). The rate of cholesterol synthesis can be determined indirectly by measuring cholesterol precursors, as these are positively correlated with cholesterol synthesis rate.[5-10] In the cholesterol biosynthetic pathway, lanosterol is the first cyclic sterol and desmosterol is the ultimate cholesterol precursor. These molecules have both been reported as markers of the cholesterol synthesis rate.[11] Therefore, these two compounds with cholesterol can be used as biomarkers for drug effect of statin in hyperlipidemia patients. Cholesterol metabolites can be analyzed by various methods and there have been many reports of quantitative analyses of cholesterol, desmosterol and lanosterol. Concentrations of cholesterol precursors at biological fluids are very low; therefore, the sensitivity of their analysis is a crucial factor.

The analysis of sterols have been performed by gas chromatography/mass spectrometry (GC/MS)[12-16] and liquid chromatography/mass spectrometry (LC/MS),[10] liquid chromatography/ultraviolet detection (LC/UV).[17,18] However, UV detection has poor sensitivity and selectivity for

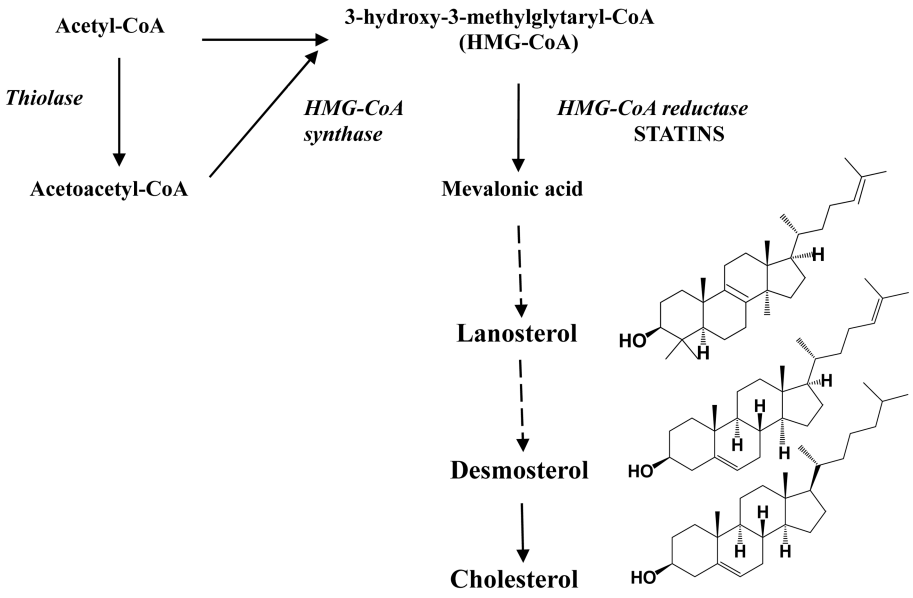

Fig. 1. Biosynthetic pathway of cholesterol.

sterols and electrospray ionized MS detection showed ion suppression in LC analysis.[19] Additional methods have been developed using nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and fluorescence spectroscopy.[20] But these techniques require large volume samples and are not also suitable for quantitative analysis.[21] On the other hand, GC/MS is reported to be suited better for the quantitative determination of sterols presented at low concentrations.[22] To improve selectivity and sensitivity for sterols in GC/MS analysis, adequate extraction procedures and derivatization steps are necessary since sterols have been analyzed from various biological samples such as serum,[1,23] microsomes,[24] plasma,[10,12] yeast cell,[13] tissue,[23,14] amniotic fluid[15] and feces.[16] However, published extraction procedures which include saponification, neutralization, solid phase extraction and prolonged derivatization were so complex to analyze a large number of biological samples.[14] Another reported method, a solid phase micro-extraction (SPME) microfiber in headspace mode also showed less sensitivity for sterols due to large variation and low recovery.[25-26]

In this study, we developed a simultaneous quantitative method for lanosterol, desmosterol and cholesterol in urine samples. Urine is a non-invasive and convenient biological fluid to test for many clinical effects. If simple and effective analytical method for urinary cholesterol, desmosterol and lanosterol is available, the effect of statin drug could be checked in urine, instead of blood. We are particularly interested in simplifying the extraction procedure and reducing time for sample preparation. Furthermore, we report that sterols in urine can be extracted with improved efficiency and determined simultaneously in a simple, straightforward manner, with favorable accuracy and precision. Pravastatin (*Fig.* 2), a representative competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, has been demonstrated to effectively reduce plasma cholesterol in animal studies[27] and in clinical trials.[28,29] Therefore, we also examined the feasibility of using this method to evaluate the effect of statin drugs.

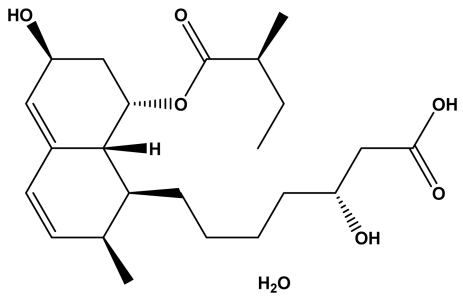

Fig. 2. Chemical structure of pravastatin.

2. Experimental

2.1. Reagents

Lanosterol, desmosterol, cholesterol and d_6 (2, 2, 3, 4, 4, 6)-cholesterol (d_6-cholesterol) were purchased from Steraloids Inc (Newport R.I., USA). Pravastatin was obtained from SynFine Research Inc (Ontario, Canada). β-Glucuronidase/arylsulfatase (β-glucuronidase activity: 400-600 U/mL, arylsulfatase activity: 15-40 U/mL) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Methanol, methylene chloride, ethyl acetate and n-hexane were obtained from SK Chemicals (Ulsan, Republic of Korea, HPLC grade). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TMSI) and trimethylchlorosiline (TMCS) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ) was obtained from a MilliQ apparatus from Millipore (Milford, USA).

2.2. Animal studies

Six- to eight-week-old male Sprague-Dawley rats (weighing 280-325 g) were purchased from Orientbio Korea (Seoul, Republic of Korea) and were housed in a room with an ambient temperature of 20-23 °C, a 12 hour light (7:00-19:00) and dark (19:00-7:00) cycle, and a relative humidity of 50±5%. Rats were given a diet of standard rodent chow (Samtaco Inc., Seoul, Republic of Korea) and tap water. Hyperlipidemia rats were produced by administration of a saline solution of poloxamer-407 (P-407) through intraperitoneal (i.p.) injection at a dose of 1 g/kg/every 3 days, to maintain consistent level of cholesterol. Hyperlipidemia was checked via the serum cholesterol level. Rats were then divided into pravastatin-treated and hyperlipidemia control groups (*Fig.* 3). Drugs were administrated orally to rats for 7 days at the dose of 70 and 250 mg/kg/day (*Fig.* 4). Following drug administration, rats were given access to standard rodent chow and tap water ad libitum. Urine was collected for the 24 hours immediately following the last drug administration, using a metabolic cage under an ice bath to avoid any metabolite degradation. Blood was withdrawn from the carotid artery after cannulation when all urine collections were finished and then centrifuged at 15000 rpm for 2

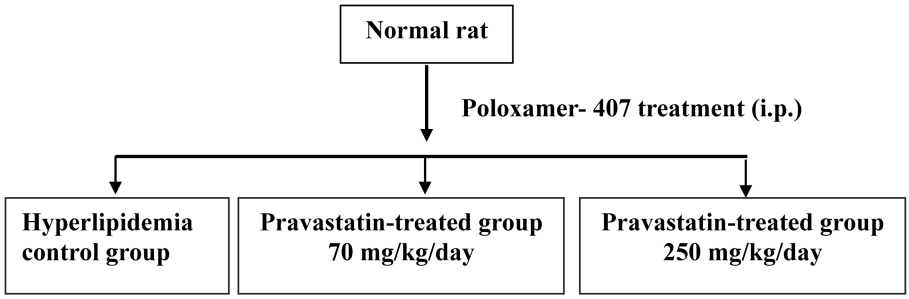

Fig. 3. Grouping of experimental animals for the oral administration of pravastatin in rats.

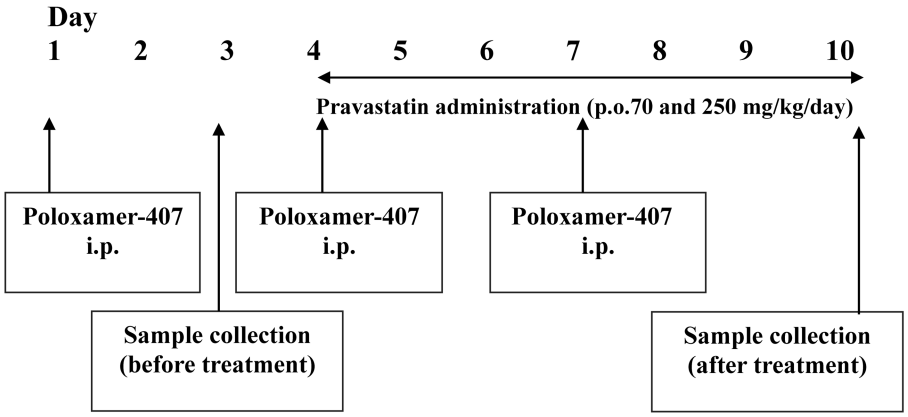

Fig. 4. Schedules for the administration of poloxamer-407 (i.p.) and pravastatin (oral).

minutes to separate the serum. All urine and serum samples were stored at -20 °C before analysis.

2.3. Solutions of standards and internal standards

Stock solutions of lanosterol, desmosterol and cholesterol were prepared at a concentration of 1 mg/mL in a methylene choloride:methanol (1:1 v/v) solution and diluted in the same solvent to varied concentrations (0.1 to 100 μg/mL) for calibration curves and method validation. The solutions were then stored at 4 °C. All the quantitative calculations were based on the peak area ratios relatives to the internal standard (ISTD, d_6-cholesterol, 10 μg/mL).

2.4. Sample preparation

Ten microlitres of d_6-cholesterol (ISTD, 10 μg/mL) was added to 500 μL of rat urine during sample preparation. Following the addition of 1.5 mL of acetate buffer (0.2 M, pH 5.2) and 50 μL of β-glucuronidase/arylsulfatase, enzyme hydrolysis was carried out at 55 °C for three hours. Liquid-liquid extraction was then performed twice with a 5 mL of ethyl acetate: n-hexane (2:3) solvent mixture. The organic phase was separated by centrifugation at 2500 rpm for 5 minutes, and the supernatant was taken after freezing (-25 °C) for 5 minutes. The removed supernatant was then dried in a gentle stream of nitrogen at 37 °C and kept in a vacuum desiccator over P_2O_5/KOH for at least 30 minutes. For derivatization, a 40 μL mixture of MSTFA/TMSI/TMCS (100:2:5, v/v/v) was added to the dried residue and allowed to react at 60 °C for 20 minutes.

2.5. GC-MS conditions

An Agilent 6890 series gas chromatography system directly connected with an Agilent 5975 series mass selective detector were used. Samples were injected into a fused-silica capillary column coated with cross-linked methyl silicone (Ultra-1, 25 m×0.2 mm i.d., 0.33 μm film thickness) by an Agilent 7683 B series auto-sampler. Helium was used as the carrier gas at a constant flow rate 0.9 mL/min. The inlet temperature was 280 °C, and the split ratio was 10:1. The oven temperature was controlled

as follows: the initial temperature was 240 °C, which was raised to 290 °C at a rate of 40 °C/min and it was maintained for 9 min. Finally, the temperature was increased to 320 °C at a rate 30 °C/min and it was maintained for 2 min. The ion source and detector temperatures were 150 °C and 230 °C, respectively. The auxiliary temperature was 300 °C. The electron impact (EI) ionization mode was used, and all of the ions were monitored in the selected ion monitoring (SIM) mode.

2.6. Calibration curves and validation

Nine calibration samples (over a concentration range of 5 ng/mL to 1000 ng/mL) including a blank were prepared with sterol free urine and then extracted as the same procedure for sample extraction. Sterol free urine used as matrices for calibration curves and validation study was prepared in house from normal urine. It was prepared as described Moon *et al.*[30] by percolating urine sample through Serdolit PAD-1 (0.1-0.2 mm analytical grade; Serva, Heidelberg, Germany). In this way, urinary sterols and other potential interfering compounds are retained in the cartridges and the eluates were collected as sterol free urine. The calibration factors were calculated according to a least-squares linear regression. The analytical recovery was determined by comparing the response before and after extraction. To test the recovery efficiency, samples were prepared at low (15 ng/mL), medium (75 ng/mL) and high (200 ng/mL) concentrations for each compound by spiking an appropriate amount in sterol free urine and then extracted and derivatized. The response was compared with the response of each derivatized standard without extraction. The precision was expressed as coefficient of variation (% CV) and the accuracy of the method was determined based on quality control (QC) samples at low (15 ng/mL), medium (75 ng/mL) and high (200 ng/mL) concentrations in triplicate for each compound. For the intraday validations, samples were examined three times on the same day, and for the interday validations, calibration samples were analyzed on three different days.

The LOD was defined as the lowest concentration with a signal-to-noise (S/N) ratio more than 3. The LOQ was defined as the concentration at which both the relative standard deviation (RSD) and the percentage deviation from the nominal concentration were less than 20%.

2.7. Urinary creatinine value

All concentrations calculated from the calibration curves were revised according to the respective urinary creatinine value. The urinary creatinine value of each sample was measured by the Jaffe method.[31]

3. Results and Discussion

3.1. GC/MS analysis

Peak identification was achieved by comparing the retention time and matching the area ratio of three characteristic ions for each component in SIM (Selected Ion Monitoring) mode. Among the characteristic ions, most intensive mass fragment ion was used for quantitative analysis. Characteristic ions and the ions used for the quantitative analysis were summarized at *Table* 1. The total ion chromatogram of the trimethylsilyl (TMS)-derivative of the compounds is shown in the *Fig*. 5.

3.2. Recovery and validations

Method validation was performed by evaluating linearity, LOQ, precision, accuracy and employing calibration samples made up with sterol free urine as the matrices. The calibration curve for cholesterol over the range of 10 ng/mL to 1000 ng/mL showed good linearity, with an r^2 value 0.999 (*Table* 2). For lanosterol and desmosterol, over the range of 5 to 200 ng/mL, linearity was satisfied with an r^2 value of 0.999 and 0.998,

Table 1. Characteristic ions used in the GC/MS-SIM analysis

	Characteristic ions (*m/z*)		
Cholesterol	<u>458</u>*	368	353
Desmosterol	456*	441	<u>343</u>
Lanosterol	498*	483	<u>393</u>
d_6-Cholesterol (ISTD)	<u>464</u>*	374	359

Underlined ions were used to quantify the respective sterols
*: Derivatized molecular weight of each compound

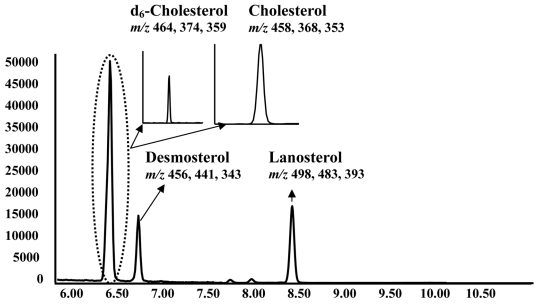

Fig. 5. Total ion chromatogram (TIC) of d_6-cholesterol, cholesterol, desmosterol and lanosterol in a 200 ng/mL of standard mixture.

Table 2. Limit of detection (LOD), limit of quantitation (LOQ), calibration range and linearity of cholesterol, desmosterol and lanosterol.

Compounds	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	Linearity
Cholesterol	1	10	10-1000	0.999
Desmosterol	0.5	5	5-200	0.998
Lanosterol	0.5	5	5-200	0.999

respectively. Limit of detection (LOD) for lanosterol and desmosterol was 0.5 ng/mL; and for cholesterol was 1 ng/mL. Limit of quantitation (LOQ) for lanosterol and desmosterol was 5 ng/mL; and for cholesterol was 10 ng/mL. The recovery of each compound was greater than 80.0% at each of the three different concentrations (15, 75 and 200 ng/mL) in the both intraday and interday validation (*Table* 3). Intraday precision and accuracy (n=3) were determined from the variability of replicate analysis of quality control (QC) samples analyzed within the same analytical run and the interday (n=3) precision and accuracy were determined from the variability of replicate analyses of QC samples analyzed on three consecutive days. The intraday and interday accuracies and precisions were within ±15% and the results were agreed with the quantitative analytical method for biological samples described by Causon.[32] The accuracies for cholesterol, desmosterol and lanosterol were within ± 15% (86.0~115%) in all quality control samples. Good precision was also observed for intraday and interday variations. The precisions for all compounds ranged 3.40 ~13.5% in intraday validation and 1.50~14.7% in interday validation. Therefore, the levels of sterols could be measured with acceptable precision and accuracy. In comparison to LOD and LOQ, our method has higher sensitivity than other gas chromatographic analysis of biological fluid.[25]

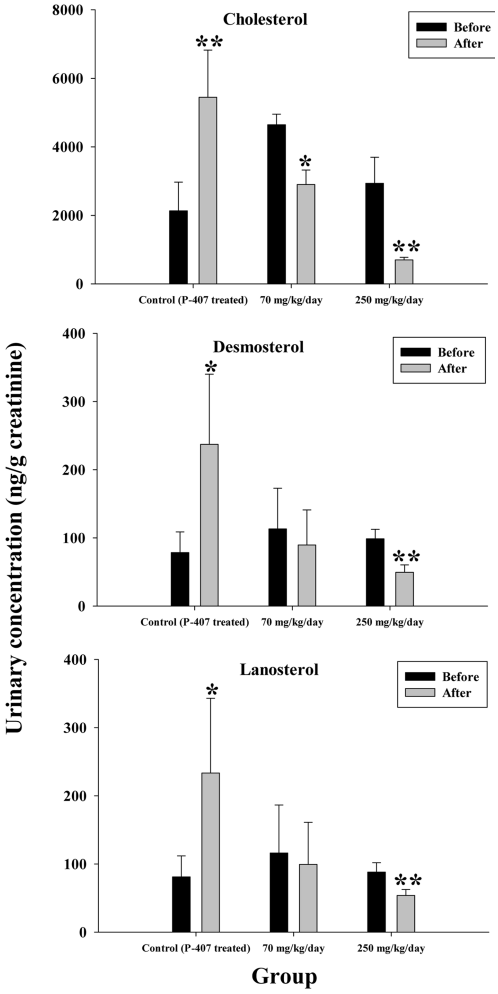

Fig. 6. Urinary concentrations (ng/g creatinine) of cholesterol, desmosterol and lanosterol in the pravastatin-treated hyperlipidemia rats. [Before: Urine collected before oral administration of pravastatin, After: Urine collected after oral administration of pravastatin] [*: p≤0.05; **: p≤0.01]

Table 3. Intraday and interday validations for the quantitative analysis of urinary cholesterol, desmosterol and lanosterol

Concentration ng/mL	Day	Recovery (%)			Accuracy (%)			Precision (%)		
		Cholesterol	Desmosterol	Lanosterol	Cholesterol	Desmosterol	Lanosterol	Cholesterol	Desmosterol	Lanosterol
15	Intraday	90.7±5.16	80.0±12.9	92.8±19.7	94.2	86.0	114	3.44	8.75	8.02
	Interday	104±11.9	81.7±2.49	95.2±4.52	93.6	90.5	114	2.34	14.7	6.10
75	Intraday	103±2.36	88.0±5.81	95.6±14.0	98.4	91.8	102	5.13	6.82	13.54
	Interday	107±4.63	98.6±15.0	92.4±4.47	91.4	114	95.5	7.03	12.0	4.40
200	Intraday	110±8.72	84.0±2.36	87.6±10.9	115	107	110	5.29	4.32	6.23
	Interday	113±4.21	82.1±2.72	84.0±5.10	106	102	107	6.71	7.10	1.50

3.3. Analysis of urinary lanosterol, desmosterol and cholesterol after oral administration of pravastatin

The urinary concentrations of lanosterol, desmosterol and cholesterol were calculated with respect to the urinary creatinine level. Notable increases in urinary sterols were observed over time, in the poloxamer-407-treated control group. The urinary concentration of cholesterol was decreased significantly and those of lanosterol and desmosterol were decreased slightly in the low-dose-treated group (70 mg/kg/day) (*Fig.* 6). Whereas, high significant decrease of cholesterol, desmosterol and lanosterol were found in high-dose-treated group (250 mg/kg/day). Compared with control rats, cholesterol decreased by 37.56% and 76.15%, desmosterol decreased by 32.33% and 50.51%, and lanosterol decreased by 14.66% and 38.64% in the low- and high-dose-treated groups, respectively. This result indicates that the developed method can be successfully applied for detecting pravastatin effects. Conventionally, serum cholesterol levels are used to verify the effects of statin drugs. The method developed here has been demonstrated to be feasible for evaluating the effects of statin drugs by measuring urinary cholesterol and its precursor's levels. Therefore, it is proposed that the urinary concentration of lanosterol and desmosterol along with cholesterol could be indicators for measuring statin drug effects.

4. Conclusion

We have developed a simultaneous quantitative method for urinary cholesterol, desmosterol and lanosterol in rats exhibiting higher sensitivity with good linearity, recovery, accuracy and precision. Moreover, we used urine sample which is non invasive and the most easy to get compare to other samples like blood (plasma and serum), tissue and microsomes. Only simple liquid-liquid extraction was performed to prepare analysis and the time for sample preparation was remarkably reduced. It was applied to evaluate pravastatin (cholesterol lowering drug) treatment effects. From this study, it was observed that lanosterol, desmosterol and cholesterol were significantly decreased as the dose of pravastatin increased. Therefore, it was demonstrated that the developed method could be successfully applied for the clinical study to evaluate statin drug effects.

Acknowledgements

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