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Analytical Validation of Mass Spectrometric Plasma Oxysterol Measurement

Kütle Spektrometrik Plazma Oksisterol Ölçümünün Analitik Validasyonu

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ABSTRACT

Aim: Oxysterols are derivatives of cholesterol which are formed by oxidation through numerous chemical reactions and play an important role in many physiological processes and in various degenerative and metabolic diseases, lipid metabolism disorders. In this study, analytical validation of liquid chromatography-tandem mass spectrometric method was evaluated by using various extraction and derivation steps in the measurement of Cholestan-3β,5α,6β-triol and 7-ketocholesterol which are important oxysterols.

Material and Methods: Optimization studies were performed in liquid chromatography-tandem mass spectrometry analyser with positive electrospray ionization in multiple-reaction monitoring mode. The analytical validation of cholestan-3β,5α,6β-triol and 7-ketocholesterol multiplex measurements with dimethylglycine derivatization was evaluated in human plasma samples. For this purpose, linearity, accuracy, repeatability, detection and quantitation limits, recovery and carry-over analysis were studied, and the results were evaluated statistically.

Results: The time of the analysis was 10 minutes for both parameters (cholestan-3β,5α,6β-triol: 3.1 minutes, 7-ketocholesterol: 7.26 minutes). The r2 value of the calibration curves for cholestan-3β,5α,6β-triol was 0.999, for 7-ketocholesterol was 0.994. The intra- and inter-assay variation coefficients assessed at three concentrations were below 15%. The assay accuracies of analytes ranged from 93.76 - 102.25 %. Plasma Cholestan-3β,5α,6β-triol levels were determined in a control group of 25 individuals (min: 15.7, max: 38.61; mean±SD: 25.61±9.2 ng/mL). Although valid results were obtained in calibration and quality control samples for 7-ketocholesterol, reproducible and reliable results were not obtained for plasma samples of healthy controls.

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Conclusion: A rapid, sensitive and specific oxysterol analytical method has been tried and made ready for implementation in clinical laboratory by method validation for the diagnosis and/or monitoring of Niemann Pick-C disease and some neurodegenerative disorders including Alzheimer's disease, Multiple Sclerosis and spastic paraplegias in this study.

Key Words: *Oxysterols, Cholestan-3β,5α,6β-triol, 7-ketocholesterol, LC-MS/MS, analytical validation*

ÖZET

Amaç: Oksisteroller, kolesterolün çok sayıda kimyasal tepkime yoluyla oksidasyona uğramasıyla oluşan ve birçok fizyolojik süreçte ve çeşitli dejeneratif ve metabolik hastalıklarda, lipid metabolizması bozukluklarında önemli rol oynayan türevlerdir. Bu çalışmada, önemli oksisteroller arasında yer alan kolestan-3β,5α,6β-triol ve 7-ketokolesterol ölçümünde çeşitli ekstraksiyon ve türevlendirme basamakları denenerek, sıvı kromatografisi-ardışık kütle spektrometrik yöntemin analitik validasyonunun yapılması amaçlanmıştır.

Materyal ve Metod: Optimizasyon çalışmaları, çoklu reaksiyon izlem modunda, pozitif elektrosprey iyonizasyon ile sıvı kromatografisi-ardışık kütle spektrofotometresi cihazında yapılmıştır. İnsan plazma örneklerinde dimetilglisin türevlendirmesiyle kolestan-3β,5α,6β-triol ve 7-ketokolesterol çoklu ölçümünün analitik validasyonu değerlendirilmiştir. Bu amaçla; linearite, doğruluk analizi, tekrarlanabilirlik, saptama ve ölçüm limitleri, geri kazanım, carry-over analizi gibi parametreler çalışılarak sonuçlar istatistiksel olarak değerlendirilmiştir.

Bulgular: Analiz süresi her iki parametre için 10 dakika olarak belirlendi (kolestan-3β,5α,6β-triol: 3.1 dakika, 7-ketokolesterol: 7.26 dakika). Oluşturulan kalibrasyon eğrilerinde; kolestan-3β,5α,6β-triol için r2:0.999, 7-ketokolesterol için r2:0,994 olarak bulundu. Üç farklı kalite kontrol örneğinde yapılan çalışma içi ve çalışma arası kesinlik sonuçlarında varyasyon katsayısı %15'in altında, doğruluk % 93.76-102.25 arasında bulundu. Sağlıklı kontrollerde (n=25) plazma kolestan-3β,5α,6β-triol düzeyleri ortalama 25.61±9.2 ng/mL (min: 15.7, maks: 38.61) olarak bulundu. 7-ketokolesterol ölçümlerinde kalibrasyon ve kalite kontrol sonuçları geçerli olmasına rağmen kontrol plazma örneklerinde tekrarlanabilir sonuçlar alınamadı.

Sonuç: Bu çalışma ile, Niemann Pick-C hastalığı ve Alzheimer hastalığı, Multiple Skleroz, spastik parapleji gibi bazı nörodejeneratif bozuklukların tanı ve/veya izlemi için hızlı, duyarlı ve spesifik bir oksisterol analitik yöntemi denenmiş ve metod validasyonu yapılarak klinik laboratuvarda kullanıma hazır hale getirilmiştir.

INTRODUCTION

Cholesterol molecules located in the cell membrane are highly susceptible to oxidation. Oxysterols are structurally close related to cholesterol and can be defined as oxidition products of cholesterol. They are formed mostly by cytochrome P450 (CYP) enzymes. Oxysterols can also be formed via nonenzymatic reactions in oxidative stress. They are bioactive molecules, and their manifold and complex functions are not completely known or understood. They have a very wide range of properties including acting as ligands to nuclear receptors and to G protein-coupled receptors and modulators of *N*-methyl-D-aspartate receptors (1). Lipid-protein interactions are very important for the cellular activities of oxysterols particularly in the regulation of key proteins in the cholesterol metabolism (2).

Under normal conditions, oxysterol concentration is observed at low levels even in the presence of high cholesterol. However, in some pathophysiological conditions, the oxysterol concentration can be seen at very high levels (total sterol concentration >20%), in these cases the effect of oxysterols on membrane properties is important (3,4,5). Oxysterol concentrations increase in pathologic conditions such as atherosclerosis, inflammation, cancer and neurodegenerative diseases.

On the other hand, some biomarker studies of oxysterols were initiated in patients with Niemann Pick type-C disease (NP-C) because of the notion that oxidative stress plays an important role in the pathogenesis of NP-C disease. Two of the oxysterols cholestane-3β-5α-6β-triol (C-triol) and 7-ketocholesterol (7-KC) were found to be highly elevated in the plasma of NP-C patients and have been proposed to be the biomarkers for NP-C (6,7). It is more sensitive than invasive methods such as the Philippine staining test in skin fibroblasts.

Studies on cellular oxysterol content and location of subcellular oxysterols have reported that the identification of these oxysterols is complex. Oxysterol analysis is not easy as it is found in biological fluids at very low concentration. The pre-treatment and derivatization processes should be done very carefully. Blood samples must be free of hemolysis and should be taken into the freezer in a short time. These limitations can create false high results, especially for 7-KC, which has fast autooxidation property. Therefore, accurate analysis is very important to evaluate oxysterol levels in various pathological conditions (8,9).

Gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are used for the analysis of plasma oxysterols for rapid diagnosis of NP-C. Different ionization-based mass spectrometric methods such as atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) with LC-MS/MS are continued to develop (10,11, 12,13,14,15). Although GC/MS method is sensitive to oxysterols in complex structure, its use has been limited because its require intensive labor, long sample preparation procedures, and relatively larger plasma volumes (16,17). The speed and cost-effectiveness of LC-MS/MS analysis is better than GC/MS (18.19). Different derivatization types have been tried using Girard hydrazone, picolinyl ester and dimethylglycine ester (DMG) since the most important step in oxysterol analysis is derivatization. Girard derivatization process is not preferred today because it is a very laborious and time-consuming method due to the presence of enzymatic steps and an overnight incubation. Due to the saponification step and incubation at high temperature (80 ^{o}C) in the derivatization process with picolinyl ester, quantitation problems related to the autoxidation of cholesterol were experienced. The derivatization process with dimethylglycine (DMG) is preferred for C-Triol and 7-KC analysis due to the efficiency of the method, its ability to detect free non-esterified oxysterols, and the need for derivatization at lower temperatures (limiting the formation of autoxidation products) (10,20).

In this study, it was aimed to perform analytical validation of the ESI LC-MS/MS method by various extraction and derivatization steps in the measurement of C-Triol and 7-KC oxysterols.

MATERIALS AND METHODS

This study was carried out in the Mass Spectrometry Laboratory of the Medical Biochemistry Department of Akdeniz University Faculty of Medicine. Ethics committee approval was received from Akdeniz University Faculty of Medicine Ethics Committee (Decision no: 332).

Chemicals and Reagents

3β, 5α, 6β-Trihydroxycholestane (T795100), 3β, 5α, 6β-Trihydroxycholestane-d7 (T795102), 7-ketocholesterol (K185050) and 7-ketocholesterol-d7 (K185052) were purchased from Toronto Research Chemicals (Canada, US). N, N-dimethylglycine hydrochloride (DMG) (2491-06-7), N-(3-dimethylaminopropyl)-N′-ethylcarboimide hydrochloride (EDC) (E1769), 4-(dimethylamino) pyridine (DMAP) (1122-58-3), hexane (650552), ammonium formate (540-69-2) and chloroform (650471) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Formic acid (64-18-6) and acetonitrile (75-05-8) were purchased from

Merck (Darmstadt, Germany). Charcoal stripped human plasma pool (4×charcoal, Ethylene Diamine Tetra Acetic Acid (EDTA) pooled gender: product code HMPLEDTA2-STRPD-HEV-53432) was purchased from Seralab (Haywards Heath, UK).

Sample Collection

Blood samples from 25 healthy subjects, 15 males and 10 females respectively, were drawn into 5 mL K^2 EDTA tubes. The age (mean ± SD) for males was 28.6±10.7 years, while for females 29.7±11.7 years. Blood samples were centrifuged at 4000 rpm for 5 minutes (min) and the resulting plasma was aliquoted and stored at -80 °C for 30 days until analysis time. Plasma C-triol and 7-KC levels were determined.

Liquid Chromatography-Tandem Mass Spectrometry Conditions

A triple quadrupole mass spectrometry LCMS-8040 (Shimadzu Corporation, Japan) combined with electrospray ionization welded ultra fast liquid chromatography (LC-20 AD UFLC XR, Shimadzu Corporation, Japan) was used. An optimized multiple reaction monitoring (MRM) method was utilized. The device performed in ESI (+) positive mode. The measurements were multiplexed. The precursor to product transitions for multiple reaction monitoring (MRM) analysis were *m/z* 591.50□>104.20 Da for the C-triol standard and *m/z* 597.50□>□104.10 Da for the C-triol-d7 internal standard; *m/z* 485.50□>□104.10 Da for 7-KC standard and *m/z* 492.50□>104.00 Da for the 7-KC-d7 internal standard.

Raptor™ ARC-18 (100x2.1 mm; 2.7 ⌠m particle size) column was used for chromatographic separation. Device flow rate was 0.5 mL / min, column temperature 45 °C and injection volume 20 µL, gradient program solvent B, 80% (0.01-4.0 min), 100% (4.01-8.0 min), 40% (8.01-10.0) min). The total time of the analysis was 10 min for both parameters (C-Triol: 3.1 min, 7-KC: 7.26 min).

Preparation of Reagents

Preparation of Internal Standards

1 mg d7 labeled C-triol and 7-KC internal standards were dissolved with 1 mL methanol and prepared as a stock solution in 1 mg / mL construction. Mixed internal standards were prepared from these stocks as intermediate stock at a concentration of 1000 ng / mL. A mixed internal standard of 100 ng / mL was used for the sample analyzes. Stock solutions were stored at -20 °C.

Preparation of Standards

1 mg / mL stock solution was prepared by dissolving 10 mg of C-triol and 7-KC with 10 mL of methanol. Mixed intermediate stock solution at a concentration of 1000 ng / mL was prepared from this stock solution. Stock solutions were stored at -20 °C.

Preparation of Quality Control (QC) Samples

The standards at known concentrations were added to the charcoal stripped human plasma pool to determine the reliability of the method for C-triol and 7-KC. These concentrations were 25 ng/mL, 100 ng/mL, 200 ng / mL respectively.

Preparation of Samples and Derivatization Solutions

Different derivatization and extraction protocols were applicated for method. These protocols are enlisted in Table 1 and 2.

Preparation of Mobile Phases

The mobile phase A consisted of 1 mM ammonium formate in water and the mobile phase B consisted of 1 mM ammonium formate in acetonitrile-water (95:5). Both of the mobile phases adjusted with formic acid to pH 3.

Preparation of Stock Solutions for Optimization

For optimization, stock standards were prepared by dissolving C-triol and 7-KC and

their labeled standards with methanol to 1 mg / mL. 10 µL of 1 mg / mL stocks were put into eppendorf tubes and evaporated for about 30 min under nitrogen gas. Then derivatization solutions were prepared as in Table 1 and 2. 40 µL of the freshly prepared derivative solution was added to the eppendorf tubes after the evaporation process was completed. It was vortexed and re-evaporated in the nitrogen gas unit. After the evaporation process was completed, the samples were thawed with acetonitrile-water solution (70:30, v/v). Then, 200 µL of solved samples were transferred to glass insert vials prior to injection.

Table 1. Derivatization and Sample Preparation Protocol-1
Tablo 1. Türevlendirme ve Numune Hazırlama Protokolü-1

Derivatization Solution-1

Volumes were calculated according to 20 sample analyzes

- 1 M N, N-Dimethylglycine hydrochloride (DMG): Weighed to prepare 0.5 mL from derivatizing agent with a molecular weight of 139.58 g/mol.
- 2 M 4-Dimethylamino pyridine (DMAP) Preparation: Weighed to prepare 0.5 mL from derivatizing agent with a molecular weight of 122.17 g/mol.
- DMG and DMAP agents weighed were transferred to the same eppendorf, dissolved with 0.5mL chloroform, and vortexed for 30 seconds.
- 1 M N-3-Dimethylaminopropyl-N′-ethylcarboimide hydrochloride (EDC) Preparation: Weighed to prepare 0.5 mL from derivatizing agent with a molecular weight of 191.70 g/mol. The weighed EDC was transferred to the eppendorf and dissolved with 0.5 mL of chloroform and vortexed for 30 seconds.

The prepared DMG and DMAP and EDC solutions were transferred to the new eppendorf in equal volumes, vortexed and derivatization solution was prepared. As the derivatization solution influenced the derivatization, freshly prepared for each analysis.

Sample Preparation-1

Samples, calibrators and quality control samples were subjected to the following extraction procedures prior to LC-MS/MS analysis.

- All samples and solutions were brought to room temperature prior to analysis.
- Prepared calibrators, quality controls and samples were vortexed for 30 seconds.
- 50 µL samples were placed in eppendorfs.
- 300 µL of the mix internal standard at a concentration of 100 ng / mL was added to the samples.
- Eppendorfs were closed and vortexed for 30 seconds.
- Vortexed samples were centrifuged at 3000 rpm for 10 minutes.
- The supernatant was transferred to a new eppendorf tube. Plasma proteins were removed by precipitation because internal standards were prepared with methanol at this stage.
- Supernatants were subjected to nitrogen drying. At this stage, attention was paid to dry the samples completely.
- 40 µL of derivatization solution-1 was added to the dried samples.
- Samples were vortexed for 30 seconds.
- Eppendorf tubes were incubated for 1 hour at 45 °C. After this step, it is important to see a dense yellow color for successful derivatization.
- The reaction was stopped by adding 20 µL of methanol to the Eppendorf tubes.
- Samples were dried under nitrogen gas.
- 200 µL of acetonitrile-water solution (70:30, v/v) was added to the dried samples.
- Each eppendorf tube was carefully vortexed to dissolve the samples.
- 200 µL samples were transferred to glass insert vials and given to LC-MS / MS for injection.
- The injection volume was adjusted to 20 µL.

Table 2. Derivatization and Sample Preparation Protocol-2
Tablo 2. Türevlendirme ve Numune Hazırlama Protokolü-2

Derivatization Solution-2
Volumes were calculated according to 20 sample analyzes
- 0.5 M DMG Preparation: Weighed to prepare 2.5 mL from derivatizing agent with a molecular weight of 139.58 g/mol.
- 2 M DMAP Preparation: Weighed to prepare 2.5 mL from derivatizing agent with a molecular weight of 122.17 g/mol.
- DMG and DMAP agents weighed were transferred to the same eppendorf, dissolved with 2.5 mL of chloroform and vortexed for 30 seconds.
- 1 M EDC Preparation: Weighed to prepare 2.5 mL from derivatizing agent with a molecular weight of 191.70 g/mol. The weighed EDC was transferred to the eppendorf and dissolved with 2.5 mL of chloroform and vortexed for 30 seconds.
The prepared DMG and DMAP and EDC solutions were transferred to the new eppendorf in equal volumes, vortexed and derivatization solution was prepared. As the derivatization solution influenced the derivatization, freshly prepared for each analysis and it was used the solution without yellowing.
Sample Preparation-2
Samples, calibrators and quality control samples were subjected to the following extraction procedures prior to LC-MS/MS analysis.
- All samples and solutions were brought to room temperature prior to analysis.
- Prepared calibrators, quality controls and samples were vortexed for 30 seconds.
- 50 µL samples were placed in eppendorfs.
- 300 µL of the mix internal standard at a concentration of 100 ng / mL was added to the samples.
- Eppendorfs were closed and vortexed for 30 seconds.
- Vortexed samples were centrifuged at 3000 rpm for 10 minutes.
- The supernatant was transferred to a new eppendorf tube. At this stage, plasma proteins were precipitated and removed because internal standards were prepared with methanol.
- The supernatants were evaporated under nitrogen gas. At this stage, attention was paid to dry the samples completely.
- 200 µL of derivatization solution-2 was added to the dried samples.
- Samples were vortexed for 30 seconds.
- Eppendorf tubes were incubated for 1 hour at 45 °C. After this step, it is important to see a dense yellow color for successful derivatization.
- The reaction was stopped by adding 100 µL of methanol to the Eppendorf tubes.
- Samples were dried under nitrogen gas.
- 200 µL of acetonitrile-water solution (70:30, v/v) was added to the dried samples.
- Each eppendorf tube was carefully vortexed to dissolve the samples.
- 200 µL samples were transferred to glass insert vials and given to LC-MS / MS for injection.
- The injection volume was adjusted to 20 µL.

Method Validation

The method was validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), recovery and carry-over according to Eurachem and CLSI-C62-A guidelines (21,22). Linearity was calculated by creating a linear regression curve with standards prepared in seven different concentrations with methanol. Accuracy was evaluated with quality control samples prepared at three different concentrations. The intra-assay precision was examined in three quality control samples in independent replicates on the same day. The inter-assay precision was evaluated by the same analyst different days under the same measurement conditions. Accuracy was expressed as percent deviation from the nominal concentration and precision was expressed in terms of percent coefficient of variation (CV%). LOD and LOQ were determined by calculating the standard deviation of the method. Recovery analysis was performed in charcoal stripped human plasma pool samples.

RESULTS

Analytical Validation Parameters

Linearity

C-triol and 7-KC standards were prepared by diluted with methanol from 1 mg / mL stock standards at 0, 3.125, 6.25, 12.5, 25, 50, 200 ng / mL concentrations. The calibrators prepared for linearity analysis at 7 different concentrations analyzed injecting 3 times in LC-MS / MS. The chromatograms of the 200 ng / mL C-triol and 7-KC standards were shown in Figure 1 and 2.

The linear regression curves were plotted with the obtained areas versus known concentration. The correlation coefficients of these curves were calculated and the r^2 value obtained for each calibration curve was found above 0.99 (0.999 for C-triol, 0.994 for 7-KC) in Figure 3A and B.

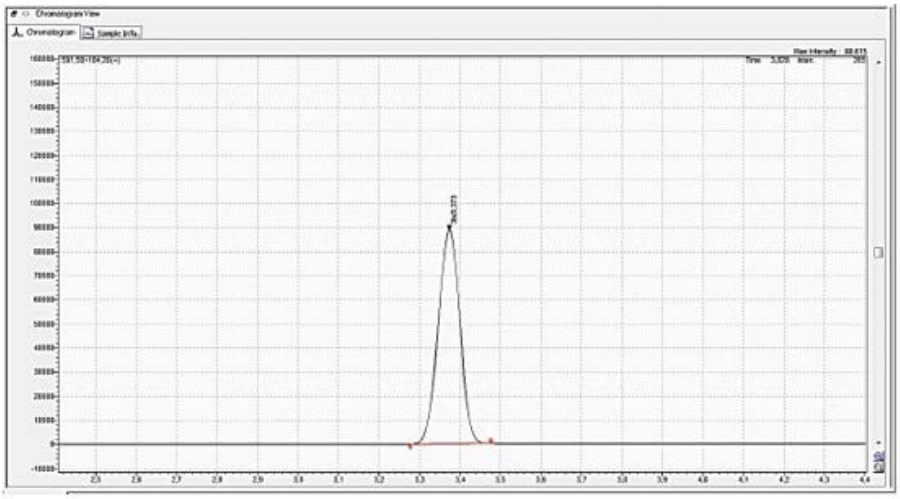

Figure 1. 200 ng/mL of Cholestane-3β,5α,6β-triol standard peak in UFLC-MS/MS chromatogram.
Şekil 1. UFLC-MS/MS kromatogramında 200 ng/mL Kolestan-3β,5α,6β-triol standart piki
UFLC-MS/MS: ultra fast liquid chromatography tandem mass spectrometry

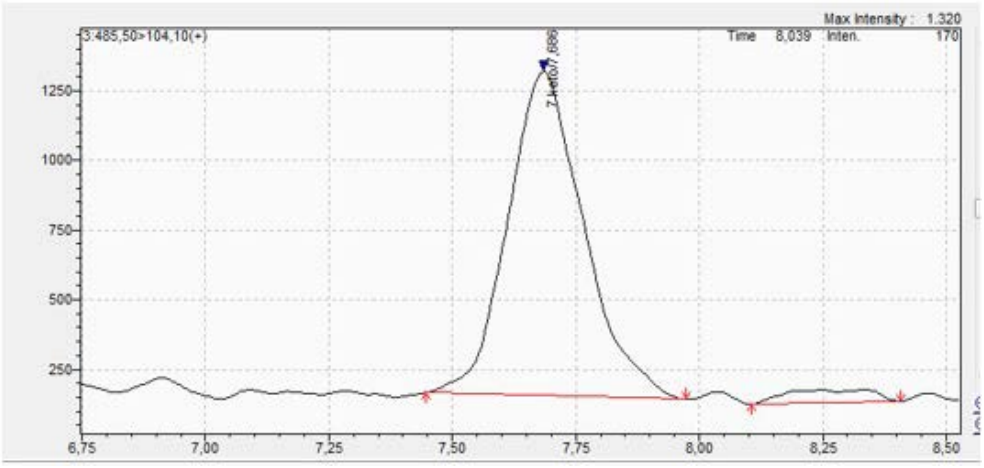

Figure 2. 200 ng/mL 7-ketocholesterol standard peak in LC-MS/MS chromatogram.
Şekil 2. LC-MS/MS kromatogramında 200 ng/mL 7-ketokolesterol standart piki

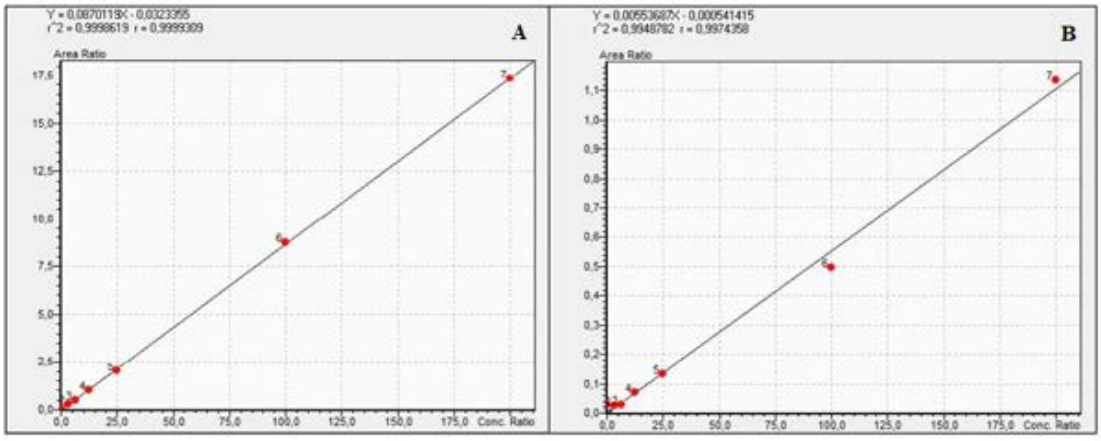

Figure 3. (A) Calibration curve of Cholestan-3β,5α,6β-triol. (B) Calibration curve of 7-Ketocholesterol.
Şekil 3. (A) Kolestan-3β,5α,6β-triol'ün kalibrasyon eğrisi. (B) 7-Ketokolesterolün kalibrasyon eğrisi.

Accuracy and Precision

A charcoal stripped human plasma pool containing K^2 EDTA, a matrix suitable for both analytes, was used when preparing the quality control samples. Control samples were prepared at three different concentrations (25, 100 and 200 ng/mL).

Accuracy is expressed as percent deviation from the nominal concentration. Our minimal criteria for accuracy were ≤ 15% at all values above the LOQ. Our acceptance criteria for precision were that at each concentration level, % CV should not exceed 15%. The intra-assay and inter-assay precisions (CV%) were 0.36-6.65 % and 4.05-8.97 % for C-triol; 2.20-4.48 % and 4.25-13.22 % for 7-KC, respectively. Precision and accuracy data for both parameters is shown in Table 3.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Table 3. Intra- and inter-assay precision and accuracy of C-Triol and 7-KC analysis in LC–MS/MS.
Tablo 3. LC-MS/MS'de C-Triol ve 7-KC ölçümünün analiz içi ve analizler arası kesinliği ve doğruluğu.

C-Triol			
Nominal concentration (ng/mL)	**Precisions (CV%)**		**Accuracy (%)**
	Intra-assay	**Inter-assay**	
25	6.65	8.97	93.76
100	1.85	4.05	101.89
200	0.36	4.72	99.64
7-KC			
Nominal concentration (ng/mL)	**Precisions (CV%)**		**Accuracy (%)**
	Intra-assay	**Inter-assay**	
25	2.84	13.22	97.24
100	4.48	9.62	95.71
200	2.20	4.25	102.25

Accuracies are expressed as percent deviation from the nominal concentration. The intra- and inter-assay variation coefficients assessed at three concentrations were below 15%.

Doğruluklar, nominal konsantrasyondan yüzde sapma olarak ifade edilir. Üç konsantrasyonda değerlendirilen analiz içi ve analizler arası varyasyon katsayıları %15'in altındaydı.

The charcoal stripped human plasma pool containing K2 EDTA as the blank sample was used without any analyte added. The LOD values were calculated from the standard deviations of the blank samples. Blank samples were calculated using the formula "$X_{LOD} = X_{BLANK} + 3 \times SD$" by giving five injections for C-Triol and 7-KC analytes. In the formula, X_{LOD} refers to the mean of the limit of detections, X_{BLANK} refers to the mean of the blank samples concentrations measured, and SD refers to the standard deviations of the results of the blank samples.

The LOQ values, the lowest acceptable and actual measured analyte concentration, were calculated as three times the LOD values using the formula "$X_{LOQ} = X_{LOD} \times 3$". The data were shown in the Table 4.

Recovery

Standards of 100 ng / mL were prepared for C-Triol and 7-KC in a charcoal stripped human plasma pool containing K^2 EDTA. Recoveries were calculated according to the formula "% Recovery = Concentration measured / Reference concentration x 100" in the six repeated measurement results obtained. Recovery results were 94% and 99% for C-Triol and 7-KC, respectively.

Carry over

Carry-over was evaluated by performing several blank injections after a high concentration of standard measurement. We did not observe any finding of carry over in our experiments.

Comparison of Analysis with Different Derivatization Protocols

Table 4. Limit of detection and limit of quantitation values for C-Triol and 7-KC.

Tablo 4. C-Triol ve 7-KC için saptama sınırı ve ölçüm sınırı değerleri

	C-Triol (ng/mL)	7-KC (ng/mL)
1. injection	1.02	1.72
2. injection	1.10	1.88
3. injection	0.95	1.18
4. injection	1.10	1.01
5. injection	1.09	1.60
Mean	1.05	1.48
SD	0.06	0.33
LOD	**1.23**	**2.47**
LOQ	**3.69**	**7.40**

The LOD values were calculated from the standard deviations of five replicate analysis of the blank samples. The LOQ values were calculated by using a formula derived from LOD.

LOD değerleri, boş numunelerin beş tekrarlı analizinin standart sapmalarından hesaplandı. LOQ değerleri LOD'den türetilen bir formül kullanılarak hesaplandı.

The results obtained from the studies performed by changing the concentration and volume of the derivatization solution were given in Table 5. The analysis results obtained were found to be similar to the Derivatization Solution-1.

Table 5. Comparison of analyzes with derivatization solutions 1 and 2

Tablo 5. Analizlerin türevlendirme çözümü 1 ve 2 ile karşılaştırılması.

	Derivatization Solution-1				Derivatization Solution-2			
C-Triol (ng/mL)	**1.Injection**	**2.Injection**	**3.Injection**	**Mean**	**1.Injection**	**2.Injection**	**3.Injection**	**Mean**
0	1.01	1.01	0.95	0.99	0.08	0.02	0.04	0.05
100	104.22	100.50	100.96	101.89	94.22	89.43	85.59	89.75
200	192.97	202.61	202.26	199.28	180.67	209.68	182.58	190.98
7-KC (ng/mL)	**1.Injection**	**2.Injection**	**3.Injection**	**Mean**	**1.Injection**	**2.Injection**	**3.Injection**	**Mean**
0	1.72	1.01	1.18	1.3	0.1	0.6	0.1	0.26
100	95.71	83.25	94.71	91.22	99.88	100.02	94.55	98.15
200	213.61	191.84	208.05	204.5	208.56	202.64	201.4	204.2

Plasma Oxysterol Levels

Plasma C-Triol levels in healthy controls (mean 25.61 ± 9.2 ng / mL) ranged from 15.7 ng / mL and to 38.61 ng / mL. These ranges were found to be 15.7-38.61 ng / mL in women and 18.63-33.3 ng / mL in men. There was no statistically significant difference between men and women in terms of plasma C-Triol levels. Although valid results were obtained in calibration and quality control samples for 7-KC, reproducible and reliable results were not obtained in the measurements made on plasma samples of healthy controls.

DISCUSSION

The concentrations of oxysterols, which play a role in physiological processes such as modulation of cholesterol-lipid metabolism and membrane structure, increases in some pathophysiological and pathological conditions including atherosclerosis, inflammation, cancer, Niemann Pick-C (NP-C) disease and some neurodegenerative disorders (3,4,5,6,7). Among these oxysterols, especially C-Triol and 7-KC, have recently been shown as diagnostic biomarkers of NP-C Disease (6).

C-Triol and 7-KC analysis performed by mass spectrometric methods offer much higher sensitivity and specificity. However, in these methods, chemical derivatization is required to increase the ionization efficiency of oxysterols that degrade very rapidly, and many different derivatization agents have been tried until today (23). One of these methods, derivatization with picolinyl ester, is a method developed to detect oxysterols (24, 25). Honda et al. reported in their study that the procedure they applied for the conversion of oxysterols to picolinyl ester included 1 hour incubation at 80°C, which could cause cholesterol autooxidation and could create false positive results (24). On the other hand, Griffiths et al., analyzed the Girard hydrazone derivatives of oxysterols by LC-MS / MS method, but could not provide quantitative data (26). ESI and APCI ionization sources have been tested in other mass spectrometric methods involving derivatization with N, N-dimethylglycine (DMG) esters, and both are considered to be sensitive and reliable (10,12,20,27). Measurements for C-Triol with ESI ionization using different derivatizing agents such as trimethylglycine (TMG) and mono-dimethylaminoethyl-succinyl esters (MDMAES) were found to be sensitive compared to DMG, but they were not as successful as DMG in reflecting concentration increases (27). In this study, we observed that C-Triol can be successfully measured by mass spectrometric method using DMG, DMAP and EDC derivatization agents and ESI ionization source. However, measurements made with the same derivatization agents were not found sensitive and reproducible for 7-KC.

Another important step in oxysterol analysis is the extraction process of the samples. It is necessary to work very carefully while performing sample extraction. Boenzi et al., increased the temperature to 65 °C and reduced the time to 15 min. in order to shorten the time reserved for derivatization in their study. When we tried this method, we observed that C-Triol and 7-KC gave false high results at this temperature. In our study, derivatization process to reduce the effects of high temperature on oxysterols, as stated in Polo's study, it was performed at 45 °C for 1 hour (20). Polo et al. preferred two-step liquid-liquid extraction with hexane for the extraction of oxysterols (20). In our study, we observed that a problem occurred in terms of reproducibility in the analysis results when we used two-step liquid-liquid extraction with hexane. This is because the liquid-liquid extraction step, which is performed to provide a clean sample before injection, is very affected by the manipulation technique and is sensitive to various external interferences. For this reason, we removed the liquid-liquid extraction step in our next trials. Then we obtained ideal results regarding repeatability and accuracy in C-Triol analysis with this change. There are several studies in the literature that have

achieved successful results by simplifying the liquid-liquid extraction stage (29). In addition, there are studies on the difference in concentration as well as the type of derivative agent in oxysterol measurements. When Klinke et al. increased the volume of DMG derivative solution five times, they observed a significant improvement in result quality and recovery values (12). In our study, all steps such as derivatization with DMG, increasing the derivative agent concentration, making or removing liquid-liquid extraction, as in the literature, were tried for both parameters. Most of the results we obtained for both parameters are consistent with the data reported in the literature. We observed that increasing the concentration of the derivatizing agent did not alter the analysis quality. Klinke et al. reported that their intra-day CV's as 2.8-7.8 % for C-Triol and 2.6-14.9 % for 7-KC, respectively. In our study, our intra-assay CVs were 0.36-6.65 % for C-Triol and 4.05-8.97 % for 7-KC. These values are below the 15 % CV accepted in analytical studies (21,22). C-Triol levels in healthy controls were reported as 7.42-21.2 ng / mL by Jiang et al. and 1.1–21.9 ng / mL by Boenzi et al. (10,30). Plasma C-Triol levels have been found between the range of 15.7-38.61 ng / mL in healthy controls of our study.

Although the accuracy, precision, recovery and calibration data were acceptable in 7-KC analysis, we did not observe acceptable results for plasma samples of our control group. These results showed that the analysis of 7-KC by using this derivatization agents was not reproducible as well as C-triol. Boenzi et al. has shown that C-Triol is the "gold standard" biomarker in the diagnosis of NP-C (30). Their ROC curves confirmed that C-Triol has better sensitivity and specificity than 7-KC. Polo et al. reported that they observed an increase of 7-KC even in the first 30 days by storing the samples at -20 ºC instead of -80 ºC (20). This report also supports that C-Triol is a more reliable biomarker than 7-KC in the diagnosis of NP-C. It can be suggested that 7-KC could be helpful in detecting sample stability and matrix effect.

There is no need for a special blood tube for oxysterol analysis. Various tubes used in routine biochemistry laboratory are sufficient for analysis. At the same time, requiring a small amount of sample volume (50 µL) is another factor that facilitates oxysterol analysis. Although EDTA plasma is preferred, samples such as serum, lithium heparined or citrated plasma can also be used for analysis (29).

Oxidative stress, which plays an important role in the oxidation of cholesterol, increases the formation of oxysterols such as C-Triol and 7-KC. Therefore, oxysterol measurements may also be useful in identifying individuals at risk in atherosclerosis and neurodegenerative diseases associated with oxidative stress (7). Although our results present a reliable and reproducible measurement method for plasma C-Triol levels, our study had some limitations. First of all, since NP-C patient samples could not be obtained, it was not possible to observe the diagnostic performance of the method. In addition, the standard deviations of plasma 7-KC concentration were found to be very high in the control group, and this was thought to be due to plasma matrix interference in 7-KC measurement. Plasma matrix interferences may affect the accuracy and precision of analytical results.

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Disclosure Statement

Coauthors report that they have no conflicts of interest.

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