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Development of a simple HPLC method for simultaneous determination of medimin E and α-tocopherol in swine serum

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HPLC를 이용한 돼지혈청중 medimin E와 α-tocopherol의 동시정량분석법의 확립

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ABSTRACT : A rapid and simple HPLC method has been developed for simultaneous determination of medimin E, a new α-tocopherol derivate and α-tocopherol in swine serum. The analytes were extracted with ethanol from 0.2 ml of swine serum and separated by HPLC on a C_{18} reversed-phase column using methanol as a mobile phase and UV detection at 285 nm. The method was validated over a concentration range of 2-100 μg/ml with a correlation coefficient of 0.998 for medimin E and 0.2-10 μg/ml with 0.996 for α-tocopherol. The lower limit of quantification (LOQ) in serum were 2 μg/ml for medimin and 0.2 μg/ml for α-tocopherol: intra- and inter-day coefficients of variation were ≤13.0% except for LOQ. Advantages of this validated assay include small samples volume, simplified serum extraction, excellent extraction recovery and fast run time. This method can be applied to analysis of medimin E and α-tocopherol in clinical pharmacokinetics and biological activities studies.

Key words: Medimin E; α-Tocopherol; Reversed-phase HPLC; Validation

INTRODUCTION

Vitamin E functions as a chain-breaking antioxidant, inhibiting the propagation of lipid peroxidation, and thus preventing membranes or lipoproteins from oxidative damage. This constitutes an important biological function of vitamin E, since the deterioration of cellular membranes is associated with cellular dysfunction and because oxidative modification of lipoproteins plays a role in the formation of the atherosclerotic plaque (Diaz et al., 1997). In addition to antioxidant and anti-atherosclerotic activities (Carr et al., 2000), vitamin E exhibits a number of other biological activities, including impact on cellular signaling and prevention of infertility in animals (Brigelius-Flohé and Traber, 1999).

Because of the increasing interest in the biological activities of vitamin E, measurement of their serum concentrations has attracted considerable attention. Also, accurate analytical data are essential for studies relating antioxidants vitamin E status in serum to health and disease, for establishing appropriate vitamin E

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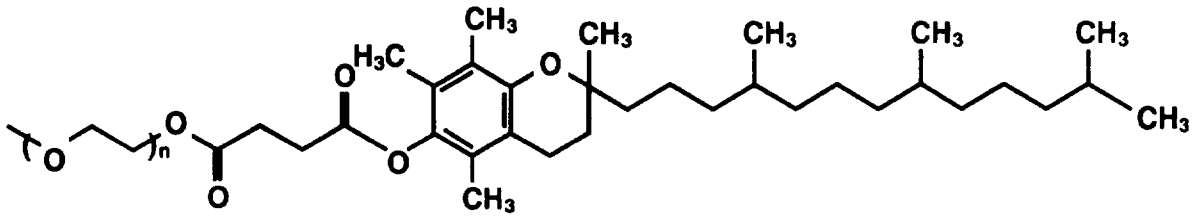
intake and supplementation guide lines. High performance liquid chromatography (HPLC) with reversed-phase is the most analytical technique to determinate vitamin E in biological samples (Caye-Vaugien et al., 1990; Barua et al., 1993; Yakushina and Taranova, 1995).

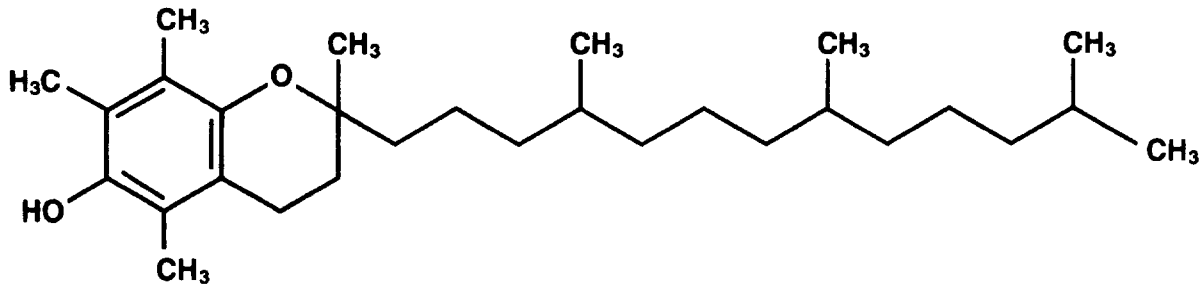
Vitamin E is the collective name for the eight naturally occurring forms α, β, γ and δ-tocopherols and α, β, γ and δ-tocotrienols. Historically, α-tocopherol was reported to have the highest biological activity (Andrikopoulos et al., 2002; Stocker et al., 2003).

Medimin E is a new α-tocopherol derivate agent and potential prodrugs for antioxidant and an increase of fertility in animal (Fig. 1). The analytical technique to determinate medimin E is essential for establishing the pharmacokinetic and bioavailability evaluation necessary for drug development. Furthermore, procedures capable of simultaneously detecting medimin E and its metabolite in body fluids are of considerable interest for pharmacokinetic and clinical studies. It has been suggested that medimin E is primarily metabolized to α-tocopherol in the animal body following administration. So we established a rapid and simple HPLC method for the simultaneous determination of medimin E and α-tocopherol.

This report describes a validated HPLC method using isocratic elution with UV detection for the simultaneous measurement of medimin E and α-tocopherol in swine serum using a α-tocopherol acetate as internal standard. The method has potential for using to evaluate the pharmacokinetics of medimin E and α-tocopherol.



Medimin E



α-Tocopherol

Fig. 1. Structures of medimin E and α-tocopherol.

MATERIALS and METHODS

Reagents and chemicals

Medimin E (Lot no. ME010411) was synthesized and characterized by LG Life Sciences Ltd., Seoul, Korea. α-tocopherol and α-tocopherol acetate were obtained from Sigma (St. Louis, MO, USA). Ethanol and methanol were purchased as HPLC grade reagents from Merck (Darmstadt, Germany) and HPLC columns from Shiseido Co., Ltd (Tokyo, Japan). Swine serum was obtained from the Cheju Regional Office of National Verterinary Research & Quarantine Service (Cheju, Korea).

Intruments and HPLC conditions

The HPLC system consisted of two isocratic pumps (LC-10AT) and a UV/VIS detector (SPD-10A), controlled by software data acquisition (Class-LC-10), all from Shimadzu (Tokyo, Japan). Temperature was controlled by Waters temperature control module (Waters Ltd., Watford, UK). The analytical column used was a Capcell Pak C18 (250 X 4.6 mm I.D., 5-μm) (Shiseido, Tokyo, Japan). Mobile phase consisted of methanol (100%) filtered through 0.45-μm membranes (Millipore, Bedford, USA) and degassed ultrasonically before use. Analyses were run at a flow rate of 1.0 ml/min at 25℃. Detection was carried out at 285 nm and peak areas were measured.

UV-Vis spectra of medimin E (1㎎/㎖), α-tocopherol (0.1㎎/㎖) and α-tocopherol acetate as internal standard (0.1㎎/㎖) in mobile phase were recorded using a UVIKON 922 spectrophotometer (Kontron Instruments, Milano, Italy). All measurements were performed in triplicate in a 1cm×1cm optical length quartz cell.

Preparation of stock solutions and spiked standards

Stock solutions were prepared at 1㎎/㎖ for medimin E, α-tocopherol and α-tocopherol acetate (internal standard) in methanol. Solutions were stored at -60℃ and were used to spike serum samples. Standards and quality control (QC) samples were made by addition of the determined quantity of stock solution to drug free serum. Calibration standards sample concentrations were 2, 6, 10, 20, 60 and 100 μg/㎖ for medimin E and 0.2, 0.6, 1, 2, 6 and 10 μg/㎖ for α-tocopherol and QC sample concentrations were 6, 10, 20 and 100 μg/㎖ for medimin E and 0.6, 1, 2 and 10 μg/㎖ for α-tocopherol. Standards and QC samples were stored at -60℃. The working solution of internal standard was prepared at 40 μg/㎖ in methanol.

Preparation of serum samples

Serum (200 μl) was combinded with 50 μl of internal standard (α-tocopherol acetate, 40 μg/㎖) in a 1.5㎖ polypropylene tube. The sample was vortexed briefly, followed by addition of 0.75㎖ ethanol. The tubes were capped and shaken at low speed (120 cycles/min) for 10 min and then centrifuged for 10 min at 5000×g. The upper organic layer was transferred to a clean glass tube and evaporated to dryness at 40℃ under a stream of nitrogen. Following reconstitution of the residue in 160 μl of mobile phase, 50 μl of the mixture was injected onto the column.

Linearity

Linearity of calibration was tested by extraction and assayed in duplicate daily for five days. The peak area of medimin E or α-tocopherol to the internal standard (medimin E/α-tocopherol acetate, α-tocopherol/α-tocopherol acetate) was used as the assay parameter. α-Tocopherol was calculated with the values obtained, after subtracting the blank run from peak area of the concentration of each

standard. The peak area ratios were plotted against nominal concentrations. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data ($y=ax+b$), where y is the peak area ratio and x the nominal concentrations.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD (signal-to-noise ratio=3) and LOQ (signal-to-noise=10) (International Conference on Harmonization, Draft Guideline on Validation of Analytical Procedures: Methodology, ICH-Q2B, November, 1996) were determined from the signal to noise ratios of standard solutions of medimin E and α-tocopherol at low concentrations (0.1-6 μg/ml for medimin E and 0.01-0.6 μg/ml for α-tocopherol).

Precision and accuracy

Intra-day accuracy and precision (each, n=5) were evaluated by analysis of QC samples for medimin E and α-tocopherol. Inter-day accuracy and precision were determined by repeated analysis over five consecutive days (n=2 series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Precision was expressed as the coefficient of variation (CV%), and accuracy as the mean relative error (RE%). A precision (CV%) $\leq$15% and an accuracy (RE%) $\leq$15% are acceptable (Shah et al., 1992).

Stability and recovery

Stability in serum was also tested by assaying frozen QC samples after storage at -60℃ for 1, 7 and 30 days. QC samples for serum were tested in triplicate for stability over three freeze/thaw cycles. Stability was expressed as a percentage of nominal concentration.

Absolute recoveries of QC samples in serum were determined by comparing the peak areas of medimin E, α-tocopherol and internal standard with those obtained from direct injection of the compounds dissolved in aqueous supernatant of processed blank serum.

RESULTS and DISCUSSION

UV spectrum analysis

UV-Vis spectra were recorded for determination of the optimum wavelength for simultaneous measurement of medimin E and α-tocopherol (Fig. 2). Medimin E and α-tocopherol acetate were showed a absorption maximum at UV 285 nm while for α-tocopherol the maximum sensitivity was obtained at UV 292 nm. Although α-tocopherol was showed a absorption maximum at UV 292 nm, UV at 285 nm was chosen for the simultaneous quantitation of medimin E and α-tocopherol, increasing the sensitivity of the medimin E.

Chromatography

Representative chromatograms of serum are shown in Fig. 3. α-Tocopherol acetate was chosen as internal standard because it used as the internal standard for HPLC assay of α-tocopherol (Talwar et al., 1998; Su et al., 1999) and its peak was sufficiently separated from those of medimin E and α-tocopherol. The retention times for medimin E, α-tocopherol and internal standard were 8.62, 9.36 and 11.34 min, respectively and no endogenous peaks that would interfere with the detection of medimin E and α-tocopherol acetate was observed.

The proposed HPLC method uses an isocratic separation with only methanol as mobile phase at a flow-rate of 1 ml/min. It

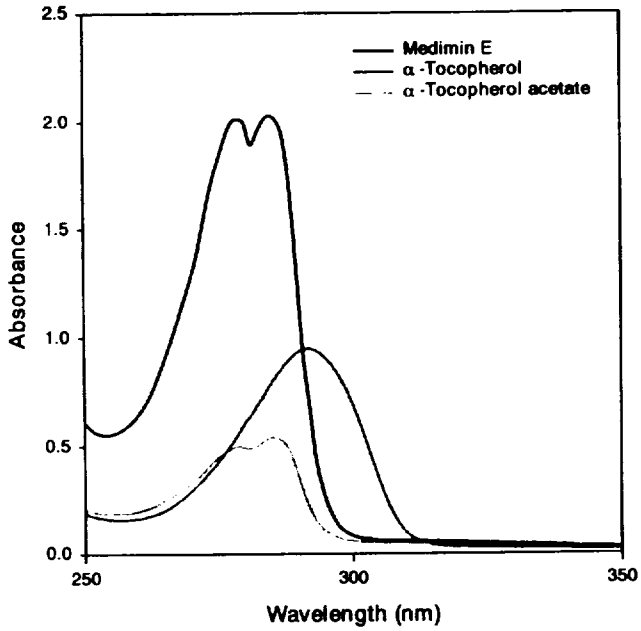


Fig. 2. Absorption spectra of medimin E, α-tocopherol and α-tocopherol acetate in methanol.

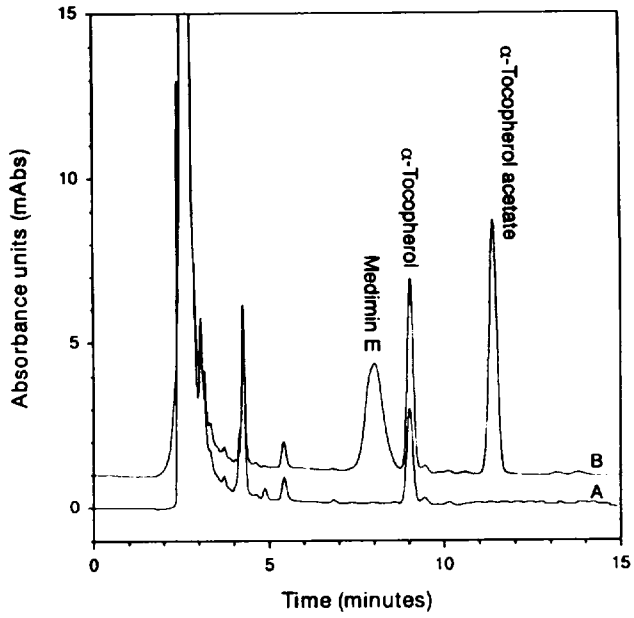


Fig. 3. HPLC chromatograms of a blank serum (A) and serum spiked with 1 mg/ml Medimin E, 0.1 mg/ml α-tocopherol and 0.5 mg/ml α-tocopherol acetate as internal standard (B).

allows a fast elution of the analytes from the column in a short time and do not require preparing complex mobile phase. The simple protein precipitation by ethanol was sufficient to isolate medimin E and α-tocopherol from serum.

Linearity of calibration curve

The calibration curve were liner within range of 2~100 μg/ml for medimin E and 0.2~10 μg/ml for α-tocopherol. The correlation coefficients were ranged in 0.99689-0.99976 for medimin E and 0.99214-0.99822 for α-tocopherol. The calibration parameters are shown in Table 1. The accuracy of back-calculated concentrations ranged in 9.3~12.6% for medimin E and in 1.3~11.3% for α-tocopherol.

Table 1. Parameters of calibration curves

	Medimin E			α-Tocopherol		
	Slope(a)	Intercept(b)	Correlation coefficient(r)	Slope(a)	Intercept(b)	Correlation coefficient(r)
Day 1	0.05169	-0.04855	0.99976	0.18414	-0.02662	0.99613
Day 2	0.04844	-0.02072	0.99790	0.16507	-0.01209	0.99822
Day 3	0.04911	-0.05717	0.99927	0.17334	-0.00531	0.99214
Day 4	0.04827	-0.02269	0.99689	0.16394	-0.01768	0.99502
Day 5	0.05061	-0.09139	0.99759	0.17941	-0.02574	0.99678
Mean	0.04962	-0.04811	0.99828	0.17318	-0.01749	0.99566
S.D.	0.00148	0.02894	0.00119	0.00880	0.00907	0.00228

$y=a\times x+b$, where y is the peak area ratio (medimin E/α-tocopherol acetate, α-tocopherol/α-tocopherol acetate) and x the nominal concentration; weighting: $1/x^2$.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for medimin were calculated to be 0.5 and 1 μg/ml, respectively, and for α-tocopherol 0.05 and 0.1 μg/ml, respectively. At the LOQ, the signal to noise ratio was greater than 10:1 for medimin E and α-tocopherol. The intra- and inter-day CV% at LOQ was 4.5~18.3% for medimin E and α-tocopherol, and the intra- and inter-day RE% was 17.9~19.8% (Table 2). These results also satisfied validation criteria (Shah et al., 1992), since the precision and accuracy were within the limit (CV% < 20% and RE% < 20%).

Table 2. Precision and accuracy of assay for determination of medimin E and α-tocopherol in serum (n=5).

	Norminal concentration(μg/㎖)	Intra-day assay			Inter-day assay		
		Measured concentration(μg/㎖) (mean±S.D.)	Precision(%)	Accuracy(%)	Measured concentration(μg/㎖) (mean±S.D.)	Precision(%)	Accuracy(%)
Medimin E	2 (LOQ)	2.49±0.11	4.5	19.8	2.39±0.11	4.8	19.5
	6	5.97±0.23	3.9	0.5	6.31±0.48	7.6	5.2
	10	10.61±0.55	5.2	5.8	10.47±0.33	3.1	4.7
	20	20.24±1.37	6.8	1.2	18.98±1.30	6.8	5.1
	100	91.12±4.72	5.2	9.3	91.08±4.60	5.1	8.9
α-Tocopherol	0.2 (LOQ)	0.24±0.04	18.3	18.2	0.24±0.03	14.7	17.9
	0.6	0.67±0.07	11.2	10.0	0.68±0.02	2.3	13.0
	1	0.92±0.08	9.1	9.2	0.94±0.11	8.5	5.9
	2	1.93±0.08	4.0	3.8	1.97±0.13	6.5	1.5
	10	9.48±0.67	6.1	5.5	9.27±0.37	4.0	7.3

Precision (%): coefficient of variation (%), accuracy (%): relative error (%), LOQ: limit of quantitation.

Precision and accuracy

The intra- and inter-day (n=5) precision and accuracy are shown in Table2. The intra- and inter-day CV% of the QC samples for medimin E was 3.1~6.8%, while that for α -tocopherol was 4.0~11.2%. The intra- and inter-day RE% of the QC samples for medimin E was 0.5~9.3%, while that for α-tocopherol was 1.5~13.0%. These results indicate that the method exhibits an adequate degree of precision (CV% 〈15%) and accuracy (RE% 〈15%) for the quantitation of medimin E and α-tocopherol (Shah et al., 1992).

Stability and recovery

All stability results are shown in Table 3. Medimin E and α-tocopherol were stable for up to 24 h at 4 °C and for at least 1 month at -60 °C in serum: the mean recoveries from the nominal concentration was 88.8~107.1% for medimin E and 85.0~110.9% for α-tocopherol. Medimin E and α-tocopherol were stable in serum samples when following three freeze-thaw cycles.

Table 4 shows the results for extraction recovery from serum. Recovery ranged between 91.1 and 106.1% for medimin and 91.6 and 111.1% for α-tocopherol.

A simple and rapid HPLC method for the simultaneous determination of medimin E and α-tocopherol in swine serum has been developed and validated. This method used a simple ethanol extraction and C_{18} reversed-phase column. The results obtained exhibited good precision and accuracy. The validated assay used a 0.2㎖ serum sample, and the calibration curve range

Table 3. Stability of medimin E and α-tocopherol in serum (n=3).

		Remained (%) (mean±S.D.)							
		Medimin E, nominal concentration(μg/mℓ)				α-Tocopherol, nominal concentration(μg/mℓ)			
		6	10	20	100	0.6	1	2	10
Stability in serum									
4℃	1 day	104.2±10.5	96.9±5.2	100.6±1.9	88.8±5.4	85.3±2.2	85.6±4.8	104.6±1.6	85.0±8.1
-60℃	1 week	106.3±4.0	99.0±7.4	110.1±0.8	93.6±5.6	91.5±3.3	96.9±2.3	94.9±1.3	96.7±1.4
-60℃	1 month	103.2±7.3	90.6±9.9	107.1±2.2	90.9±8.4	98.3±1.5	110.9±2.6	96.9±8.9	94.6±7.7
Free-thaw stability									
	Cycle 1	105.0±7.2	98.6±4.1	106.5±4.9	90.3±3.4	101.3±5.6	87.6±4.7	92.9±2.1	99.8±8.5
	Cycle 2	103.9±8.6	96.1±4.2	105.8±5.1	90.2±10.3	93.6±1.8	86.8±9.3	93.0±1.5	93.9±1.2
	Cycle 2	104.7±10.1	100.8±0.0	103.6±5.3	90.4±5.2	86.8±9.8	99.0±4.8	93.9±10.2	85.8±7.6

Remained (%): measured concentration/nominal concentration x 100(%).

Table 4. Absolute recovery of medimin E and α-tocopherol in serum of (n=5).

	Nominal concentration (μg/ml)	Absolute recovery (%)		
		Mean±S.D.	CV%	Range (μg/ml)
Medimin E				
	6	99.5±3.9	3.9	5.8~6.1
	10	106.1±5.5	5.2	9.8~11.1
	20	101.2±6.9	6.8	18.6~22.4
	100	91.1±4.7	5.2	83.9~93.6
α-Tocopherol				
	0.6	111.1±7.5	11.2	0.6~0.7
	1	91.6±8.3	9.1	0.8~1.0
	2	96.3±3.9	4.0	1.8~2.0
	10	94.8±5.7	6.1	8.8~10.3

Absolute recovery (%): measured concentration/norminal concentration x 100 (%).

was 2~100μg/mℓ for medimin E and 0.2~10μg/mℓ for α-tocopherol. Additional advantages of this validated assay include a excellent extraction recovery, fast run time and a readily available internal standard. This method can be applied to analysis of medimin E and α-tocopherol in clinical pharmacokinetics studies.

요 약

본 연구에서는 α-tocopherol과 그 새로운 유도체인 medimin E의 신속 동시 정량법을 개발하였다. 0.2mℓ의 돼지 혈청을 취하여 1회 ethanol로 추출, 원심분리 후 상등액을 HPLC로 분석하였다. Column은 C_{18} (Capcell Pak)을 사용하였고, 이동상은 100% methanol 이었

으며, 검출기 조건은 285 nm 이었다. Medimin E와 α-tocopherol의 분석에 이용된 검량곡선의 농도범위는 각각 2~100 μg/ml와 0.2~10μg/ml이었으며 상관계수는 각각 0.998과 0.996 이었다. 정량한계는 medimin E가 2 μg/ml, α-tocopherol이 0.2 μg/ml이었으며 intra-day와 inter-day variation은 모든 분석에서 13.0% 이하를 나타내었다.

이런 방법에 의하면 최소한의 시료에서 1회의 간단한 추출에 의하여 신속하게 medimin E와 α-tocopherol을 동시 정량 할 수 있으며, 이는 체내동태 및 생리활성 연구에 많은 기여를 할 것으로 기대된다.

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