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Determination of retinoids in human serum, tocopherol and retinyl acetate in pharmaceuticals by RP-LC with electrochemical detection

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Abstract

A liquid chromatography with a electrochemical detector method has been developed of the quantitative measurement for the three retinoids in human serum (13-*cis* and all-*trans* retinoic acid and retinol), as well as tocopherol acetate, retinyl acetate and retinol in pharmaceuticals. The detection cell consisted of a glassy carbon electrode held at 1.0 V versus an Ag/AgCl reference electrode. The maximum electrochemical signal was obtained with a supporting electrolyte containing 92% methanol 0.1 M acetate buffer (pH 4.72) as the mobile phase. The quantification limits are 0.5, 0.2, 0.4, 0.5, 0.8 and 0.8 ng for tocopherol acetate, all-*trans*-RA, 13-*cis*-RA, retinol, retinal and retinyl acetate, respectively. The electrooxidation process is applied for the simultaneous quantitative determination of retinoids in human serum. Comparison with results obtained from HPLC-UV shows agreement.
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Keywords: Retinoids; Tocopherol acetate; Human serum; Pharmaceuticals; Electrochemical detection

1. Introduction

All-*trans*-retinoic acid (all-*trans*-RA) is a natural oxidative metabolite of retinol in blood. Both retinoids are biologically active forms of vitamin A. *Trans*-retinoic acid is further metabolized to 13-*cis*-retinoic acid (13-*cis*-RA) in a reversible manner. Many liquid chromatographic methods have been proposed for the single retinol [1–5], as well as the simultaneous determination of the all-*trans*-RA and 13-*cis*-RA [6–16]. Although the simultaneous measurement of these three interrelated endogenous retinoids is relevant, very few method exist to do this on a routine basis [10,12]. Liquid chromatography can provide the selectivity required to isolate most forms of the retinoids, but UV absorbance detection provides only low picomole detection limits. Fluorescence detection has been investigated as a means of enhancing detection sensitivity for retinoids. Unfortunately, the fluorescence signal was very dependent on pH, and other retinoids did not demonstrate the same level of fluorescence. β-Carotene is important as a

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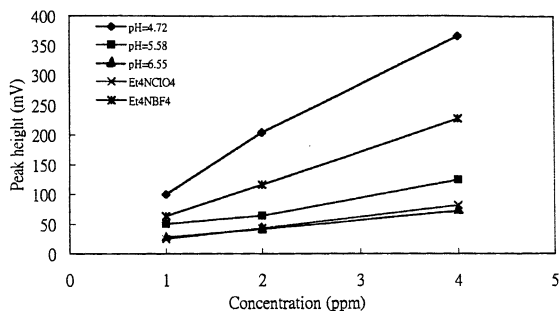

Fig. 1. ECD response with increasing all-*trans*-RA concentration in various supporting electrolytes. Potential, + 1000mV versus Ag/AgCl.

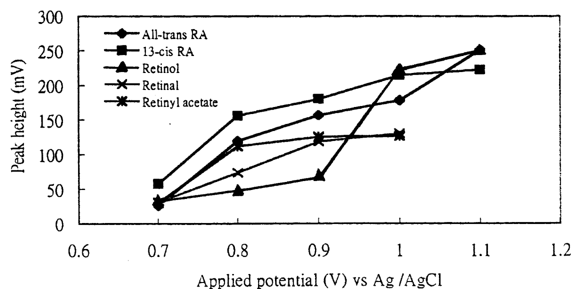

Fig. 2. Hydrodynamic voltammograms of retinoids. Conditions: electrode, 3 mm diameter glassy carbon; mobile phase, methanol–water (92:8, v/v) containing 0.1 M acetate buffer pH 4.72; flow rate, 1.0 ml/min.

source of vitamin A, since it is absorbed in the animal systems and undergoes an oxidative cleavage at the 15,15′ double bond in the liver to all-*trans*-retinal. Detecting retinoids of bovine serum by capillary liquid chromatography with amperometric electrochemical detection was reported by J.J. Hagen [11]. In spite of the high sensitivity and selectivity of this method, the sample throughput was very low and not suitable for large studies. Most of these liquid chromatographic procedures require additional instrumentation such as an automated device for solid-phase extraction and a binary gradient elution system. Electrochemistry of β-carotene, retinal and retinol was reported by Su-Moon Park [17], in which oxidation and reduction electrode mechanisms were measured with chronoamperometry, chronopotentiometry, cyclic voltammetry and controlled potential coulometry methods. Based on the UV spectra of vitamins in the HPLC mobile phase, the wavelengths selected for absorbance determination were 285 nm for tocopherol acetate and 340 nm for retinoids, respectively; thus, that UV spectra could not perform the simultaneous determination of both vitamins in a mixture. In this paper, a simple and rapid HPLC with ECD allow the simultaneous separation of 13-*cis*-RA, all-*trans*-RA, and retinol in human serum, retinyl acetate and tocopherol acetate in pharmaceuticals.

Table 1
Comparison of ECD versus UV of quantification (LOQ), equations for the linear regression lines and the coefficients of correlation (R) for tocopherol acetate and each retinoid

Vitamins	LOQ (ng)		Linear regression line		R	
	ECD	UV	ECD (Y)	UV (Y)	ECD	UV
Tocopherol acetate	0.5	- - -[a]	54.4 × −67.3	- - -[a]	0.9990	- - -[a]
All-*trans*-RA	0.2	4.2	205 × −8.93	19.8 × −177.7	0.9999	0.9985
13-*cis*-RA	0.4	4.8	298 × −4.84	24.6 × −215.5	0.9995	0.9982
Retinol	0.5	4.3	192 × −37.2	13.0 × −116.9	0.9999	0.9995
Retinal	0.8	51	72.9 × −30.7	9.97 × −67.8	0.9970	0.9982
Retinyl acetate	0.8	7.8	138 × −21.1	5.641 × −0.026	0.9991	0.9983

[a] - - - , not determined.

Table 2
Recovery of retinoids from fortified human serum by LCEC[a]

Fortified concentration found recovery								
Serum in μg/ml (%)								
All *trans*-RA			13-*cis* RA			Retinol		
Control								
0.400	0.396	99.1(2.4)	0.200	0.198	99.1(5)[b]	0.666	0.656	98.4 (4.5)
Volunteer								
0.200	0.164	82 (6.5)	0.200	0.185	92.5 (4.5)	0.200	0.144	72 (5.5)

[a] Number of determination ($n = 3$).
[b] R.S.D., relative standard deviation.

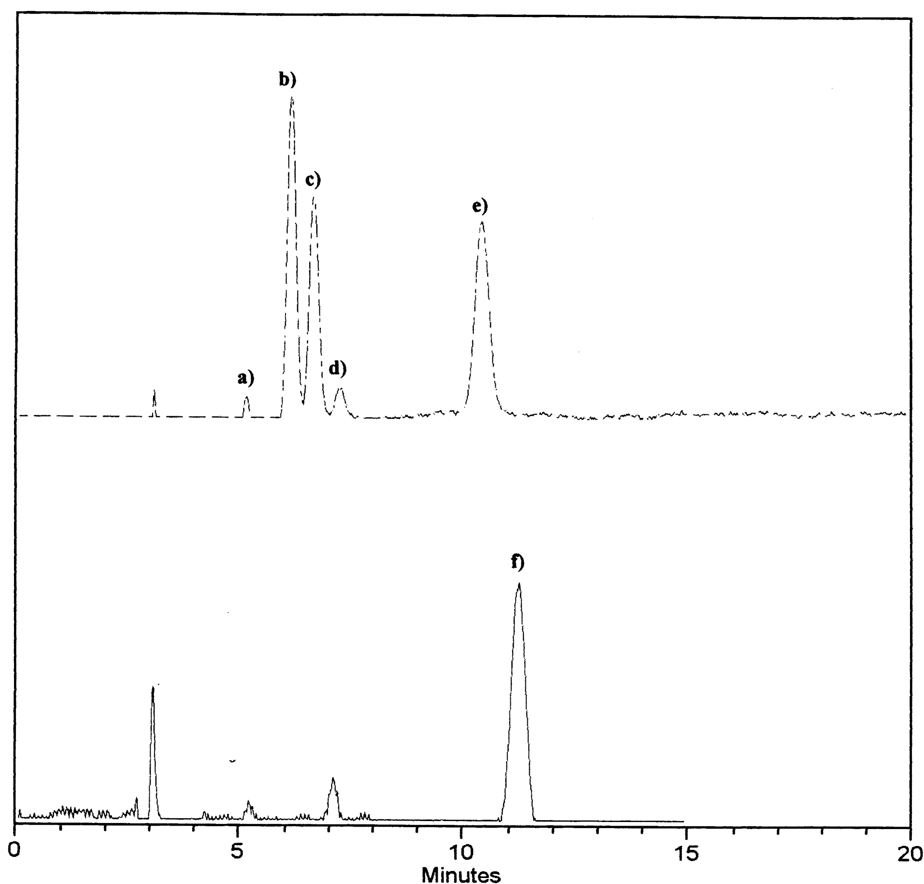

Fig. 3. Chromatograms obtained by LCEC of (a) tocopherol acetate; (b) 13-*cis* RA; (c) all-*trans*-RA; (d) retinol; (e) all-*trans*-retinal; (f) retinyl acetate. Stationary phase, μ Bondapack C_{18} (3.9 mm × 30 cm); mobile phase, methanol–water (92:8 v/v) containing 0.1 M acetate buffer pH 4.72; flow rate, 1.0 ml/min. Detection was at 1.0 V versus the Ag/AgCl reference electrode.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Model 576 pump (Gasuknro Kogyo, Japan) a Rheodine 7125 injection valve with 20 μl sample loop, an EG& G PARC 400 electrochemical detector and a Model 502 U spectrodetector. Chromatograms and peak areas were obtained with a SISC chromatogram Data Integrator. Absorbance measurements were recorded with Cary UV-VIS spectrophotometer (Varian Australia Pty Led). The electrodes were as follows: Ag/AgCl/0.1M KCl reference electrode, steel auxiliary electrode, and glassy carbon electrode for the detection of retionids. All solvents and analytes were filtrated through 0.45 μm cellulose acetate membrane filters (Millipore) and ultra-high-quality water obtained from a Watermaster WD 1106 Ultra purification system was used.

2.2. Materials

All-*trans*-RA and tocopherol acetate were purchased from TCI (Tokyo Kasei Co., JP) and all-*trans* retinol and retinyl acetate from Acros Organics (Geel Belgium, NJ), respectively. 13-*cis*-RA and 13-*cis*-retinal were purchased from Aldrich (Milwaukee, WI). Control human serum

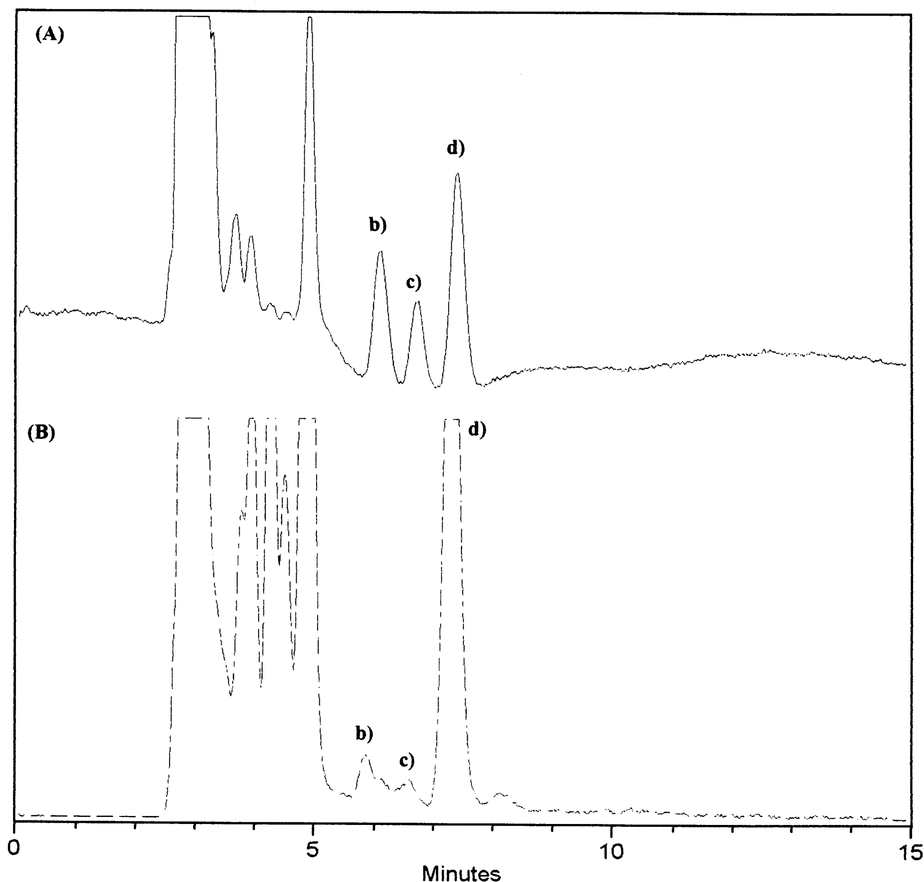

Fig. 4. Chromatograms obtained by LCEC from the (A) one week after administration of β-carotene capsule (30000 IU) female volunteer serum (B) human serum (from Sigma). The peaks are identified as follows: (b) 13-*cis* RA;(c) all-*trans* RA; (d) retinol. Analysis conditions are identical to those listed in Fig. 3.

Table 3
Within-day and between-day of all-*trans*-RA, 13-*cis*-RA and retinol in serum from healthy adult and control (Sigma) subjects

Mean concentration (ng/ml $n = 6$)[a]							
Subjects		All-*trans*-RA		13-*cis*-RA		Retinol	
Number	sex	ECD (%)	UV (%)	ECD (%)	UV(%)	ECD (%)	UV (%)
1	F	12.7 (7.1%)[b]	- - -[c]	- - -[c]	- - -[c]	289 (8.8)	210(5.0)
2	F	- - -[c]	- - -[c]	14.6(8.4)	- - -[c]	230 (6.5)	243(3.4)
3	F	- - -[c]	- - -[c]	- - -[c]	- - -[c]	331 (9.2)	322(6.4)
4	M	44.5(9.3)	35.1(20)	- - -[c]	- - -[c]	459(1.2)	423(5.5)
5	M	15.6(23.1)	7.17(15)	23.7 (3.6)	18.8(10)	497(13)	486(10)
6	M	14.7(10.1)	6.55(15)	- - -[c]	- - -[c]	302(2.7)	264(10)
7	Control	7.87(28)	- - -[c]	6.58 (48)	- - -[c]	194(2.2)	161(2.3)

[a] Number of determination ($n = 6$).
[b] Relative standard deviation.
[c] - - - , not determtined.

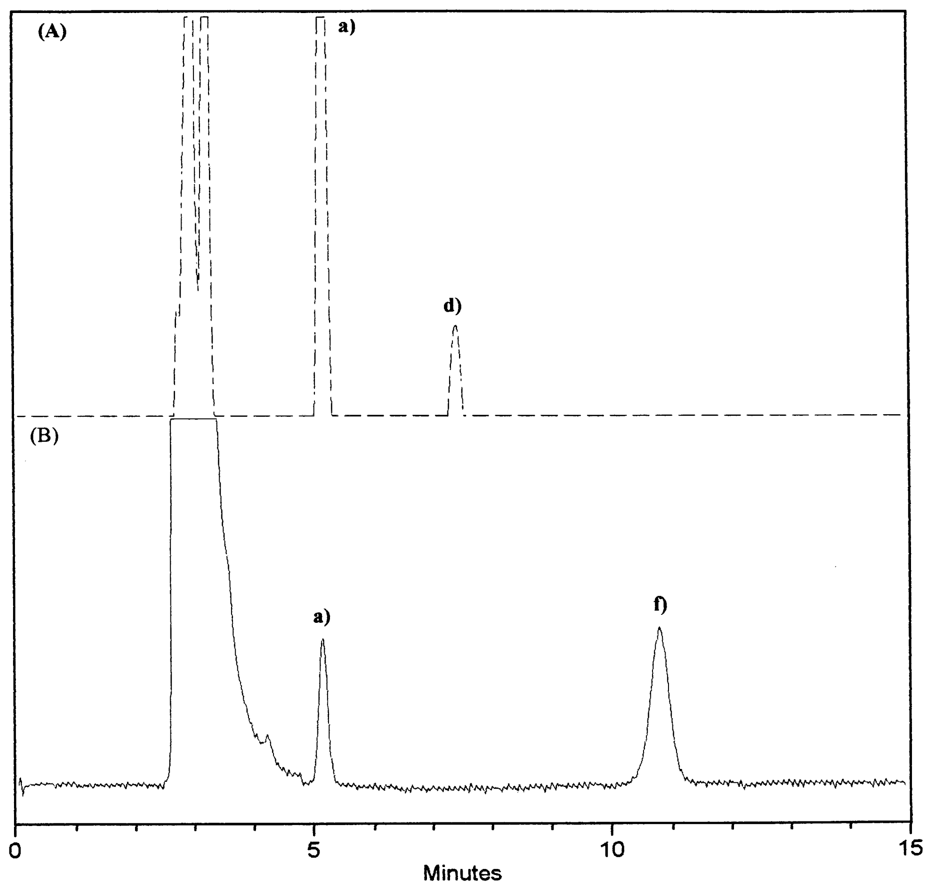

Fig. 5. Chromatograms obtained by LCEC from commercial (A) anti-wrinkle cream; (B) multi vitamin capsule. The peaks are identified as follows: (a) tocopherol acetate; (d) retinol; (f) retinyl acetate. Analysis conditions are identical to those listed in Fig. 3.

Table 4
Recovery of tocopherol acetate, retinol and retinyl acetate from cosmetics and pharmaceuticals

Sample	Tocopherol acetate			Retinol			Retinyl acetate		
	Added (μg/ml)	Found (μg/ml)	Recovery (%) $n = 3$[a]	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Added (μg/ml)	Found (μg/ml)	Recovery (%)
Anti-wrinkle cream	2.00	1.999	99.9 (5.5%)[b]	2.00	2.10	105 (5%)	- - -	- - -	- - -
Vitamins capsule	4.00	4.10	103 (6.5%)	- - -	- - -	- - -	4.00	4.55	113 (5%)

[a] Number of determination.
[b] R.S.D.

was purchased from Sigma Chemical Co. (St. Louis. MO). All other chemicals were of analyti-cal reagent grade. Samples of anti-wrinkle cream and vitamins capsule were bought from a number of retail outlets in the south of Taiwan.

2.3. Procedure

Stock solution of standards were prepared by dissolving 10 mg of retinoids and tocopherol acetate in 10 ml methanol, respectively. Appropriate amounts of each stock solution were diluted with methanol to give working solutions in the range 0.02–40.0 μg/ml. Working standards were prepared daily by adding 100 μl of the spiking standards to 0.5 ml of control serum. 0.04% Tretinoin (all-*trans*-RA), 0.05% isotretinoin (13-*cis*-RA) cream and 30000 IUβ-carotene capsule were given skin and orally to three male and three female (aged 18–22 year). Human venous blood samples were taken from adult male and female volunteers and collected in tubes (protected from light with aluminium foil to minimize light-induced degration of retinoids). The human serum sample were prepared by mixing 100 μl of ethanol and 200 μl of *n*-hexane-ethylacetate (1:1 v/v) with 200 μl of serum, and the resulting solution was centrifuged at 4000 × *g* for 10 min to sediment protein aggregates. The deproteinized serum was filtered and evaporated to dryness with a stream of nitrogen. Methanol (300 μl) was immediately added to the residue and, after mixing for 5 s, as much as possible of the methanol was transferred to an injection vial. Taking into account about the retinoids content of the anti-wrinkle creams and vitamins capsules, samples (approximately 10–20 mg) of the latter were weighed accurately in a 5 μl beaker, diluted to about 5 μl with methanol and deionized water, dissolved and centrifuged. The supernatant was transferred into a 10 μl amberized calibrated flasks. An aliquot of the solution was filtered through a 0.45 μm membrane filter prior to HPLC analysis. A μ Bondapack C_{18} analytical column (particle size 5 μm, 3.9 × 300 mm i.d.) (purchased from Waters Corporation, USA) eluted with methanol-water (92:8, v/v) containing 0.1 M acetate buffer (pH 4.72) at 1 ml/min. The EC detector was operated at + 1.0 V. By means of the injection value, 20 μl of the prepared sample solution and standard solution was chromatographed under the operating conditions described above. Quantitation was based on the peak area of the sample.

Table 5
Analytical results for the determination of tocopherol acetate, retinol and retinyl acetate in commercial anti-wrinkle creams and vitamins capsules

Concentration (w/w, %)[a]						
Samples	Tocopherol acetate (%)	Expected content	Retinol (%)	Expected content (%)	Retinyl acetate	E content (%)
Anti-wrinkle cream 1	0.466 (2.4)[b]	[c]	0.069 (2.6)	- - -[d]	- - -[d]	[c]
2	0.298 (2.6)	[c]	0.081 (2.1)	0.089		[c]
3	0.136 (0.5)	[c]	0.083 (1.2%)	- - -[d]	- - -[d]	[c]
Vitamins capsule 1	1.68 (0.7)	[c]	- - -[d]	- - -[d]	0.140 (0.7)	0.192
2	0.305 (5.5)	- - -[d]	- - -[d]	- - -[d]	0.173 (3.5)	0.174
3	1.04 (5.6)	–	- - -[d]	- - -[d]	- - -[d]	0.117
Vitamin tablet	- - -[d]	- - -[d]	- - -[d]	- - -[d]	- - -[d]	0.089
	0.0878 (6.0)	- - -[d]	- - -[d]	- - -[d]	0.184 (0.8)	0.214

[a] Number of determination ($n = 3$).
[b] Relative standard deviation.
[c] Not indicate.
[d] - - - , not determined.

3. Results and discussion

3.1. Optimization of LCEC conditions

Retinoids are pentaene and may under oxidation at glassy carbon electrode (GCE) by a similar mechanism to other conjugated double –bond system [2]. The oxidation was found to occur in two steps and to be irreversible; the reactant was found to undergo adsorption at the GCE [3]. In earlier study [18], retinoids are readily oxidized with GCE, thin-film copper modified GCE and thin-film lead modified GCE in methanol containing sodium perchlorate at potentials greater than 0.9 V versus SCE. Electrodeposited metals are extremely pure and highly active. However, the uniform thickness of deposit is greatly influenced by the electrochemical methods. Hence the voltammetric cell designs and systems, which have not been developed for use with HPLC, and the GCE is used in the presented work. To optimize the supporting electrolyte solution, the effects of electrolyte pH and composition in the mobile phase were investigated. Fig. 1 shows the increase in EC response (peak height) as electrolyte pH decrease. A acetate buffer (pH 4.72) was chosen for the experiments since the EC response of the all-*trans*-RA was found to be much higher than in the other supporting electrolytes. In order to determine the optimum applied potential for electrochemical detection, we constructed hydrodynamic voltammograms for 13-*cis*-RA, all-*trans* RA, retinol, retinal and retinyl acetate (Fig. 2.). The maximum current, measured as peak height, was achieved at a potential of $+1.0$ V or greater; however, less interference occurred in the serum sample when a potential of $+1.0$ V was used. The optimum potential for this was used as the working potential for all retinoids LCEC studies Table 1.

3.2. Linearity, recovery and limit of quantification of LCEC assay

Comparision of ECD versus UV of the calibration graphs of peak current ratio versus mass of all-*trans*-RA, 13-*cis*-RA and retinol injected were linear over the range 0.8–80 ng for five retinoids and tocopherol acetate. Retinoid mixtures for fortification were prepared by mixing the stock solutions and diluting with methanol. A 100 μl aliquot of the mixture was added to 0.3 ml of serum and extraction was carried out as described above. Table 2 show the LCEC traces obtained for a volunteer and control serum sample spiked with 13-*cis*-RA, all-*trans*-RA and retinol, respectively, excellent recoveries and precision were observed (recoveries ranging from 72.0 ± 5.5 to $99.1 \pm 2.4\%$). The limit of quantification 0.5, 0.2, 0.4, 0.5, 0.8 and 0.8 ng for tocopherol acetate, all-*trans*-RA, 13-*cis*-RA, retinol, retinal and retinyl acetate respectively. The chromatogram for the separation of tocopherol acetate, 13-*cis*-RA, all-*trans*-RA, retinol, retinal and retinyl acetate is shown in Fig. 3.

3.3. Application to human serum and pharmaceuticals

The proposed LCEC methods was applied to the determination of 13-*cis*-RA, all-*trans*-RA and retinol in human serum, retinyl acetate and tocopherol acetate in pharmaceuticals. A representative LCEC chromatograms for the retinoids in a volunteer serum and control serum extract are shown Fig. 4 A and B, respectively. The in Fig. 4 A and B compare with a chromatogram of pure standard (Fig. 3). Sample constituents with retention characteristics identical to those of 13-*cis*-RA, all-*trans*-RA and retinol were identified and measured. Results for the analysis of healthy adult serum was shown in Table 3. Recovery tests were carried out on cosmectic and pharmceutical for evalutation of the reproducibility and accuracy of the proposed method. Two commercial products were spiked with the amounts of the agents reported in Table 4 and subjected to full extraction procedure. As is seen, excellent recoveries and precision were observed. The mean concentrations of 13-*cis*-RA, all-*trans*-RA and retinol were 7.9, 24.9 and 419 ng/ml, respectively. The observed values were higher than earlier published values of 1.57–1.80 ng/ml for 13-*cis*-RA, and 1.07–1.77 ng/ml for all-*trans*-RA in normal subjects [2,3,10,12,14]. However, the concentrations of retinol in volunteers and control serum (from

Sigma) are in good agreement with published literature values of 324–711 ng/ml (Fig. 4B and Table 3). Since all-*trans*-RA is a natural oxidative metabolite of retinol in blood and all-*trans*-RA is further metabolized to 13-*cis*-RA. These data might be increased after vitamin A intake and administration of RA or retinol undergo oxidation. A representative LCEC chromatograms of a commercial anti-wrinkle cream and vitamin capsule is shown in Fig. 5 A and B. Analytical results are given in Table 5.

4. Conclusions

HPLC with electrochemical detection both a selective and sensitive means of monitoring retinoids. Isocratic chromatographic conditions allowed resolution for a mixture of 13-*cis*-RA, all-*trans*-RA, retinol, retinal, retinyl acetate and tocopherol acetate. In addition, our LCEC system run takes about 12 min per sample. It can be applied to a variety of clinical situations in which serum retinoids have to be measured accurately and pharmaceuticals, and provides the researcher with a tool to examine the interrelations between these endogenous substances.

Acknowledgements

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