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DETERMINATION OF VITAMINS A AND E IN SERUM AND PLASMA USING A SIMPLIFIED CLARIFICATION METHOD AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method of sample clarification and high-performance liquid chromatography specifically developed to permit precise and rapid determination of vitamin A (retinol) and vitamin E (α-tocopherol) in serum and plasma is reported. Serum proteins were denatured by the addition of acetonitrile containing α-tocopherol acetate, the internal standard; the vitamins were subsequently extracted into an organic matrix consisting of ethyl acetate—butanol (1:1); no solvent evaporation step was required. The three vitamins of interest were eluted from a reversed-phase C_{18} column with an isocratic mobile phase methanol—water (95:5); detection was accomplished by measuring ultraviolet absorption at 280 nm. Recoveries of retinol, α-tocopherol and α-tocopherol acetate from spiked aqueous samples averaged 100.0, 100.0 and 98.8%, respectively. Recoveries of retinol, α-tocopherol and α-tocopherol acetate from plasma and serum relative to water were 102.6, 96.9 and 96.5%, respectively. Retinol and α-tocopherol were stable in the extraction matrix for up to 3.5 h, and were stable in heparinized plasma stored at room temperature for two days. Oxalate, citrate and EDTA caused significant losses of retinol and α-tocopherol, while vitamin levels in serum and heparinized plasma were similar. Limits of detection for retinol and α-tocopherol were 60 ng/ml and 0.9 μg/ml, respectively. Each run required 12 min. Same-day coefficients of variation were 3.5 and 3.6% for retinol and α-tocopherol, respectively (n = 11). Between-day coefficients of variation for retinol and α-tocopherol were 4.8 and 5.5%, respectively (n = 5). This method permits simple, rapid, sensitive, selective and precise determination of retinol and α-tocopherol using 0.5 ml of serum or heparinized plasma.

INTRODUCTION

There has recently been increased interest in measuring retinol (R) and α-tocopherol (E) in serum or plasma. This interest has been stimulated by the

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possibility that both R [1—3] and E [4—6] may act as cancer-chemopreventive agents. Our medical center now has underway a prospective clinical study to ascertain whether E is effective in preventing recurrent colonic polyps; the study requires the determination of serum concentrations of R, E and β-carotene.

Excellent analytical methods for measuring serum concentrations of these vitamins already exist, although each has certain disadvantages. All three compounds may be measured during the same high-performance liquid chromatographic (HPLC) run using either gradient elution [7] or isocratic elution [8]. While these methods are useful in overall nutritional assessment, they require solvent evaporation steps and may not have the required sensitivity or precision for prospective clinical drug intervention trials. Retinol [9] and β-carotene [10] can be readily determined using methods which do not require solvent evaporation steps, and which provide sufficient precision and sensitivity. In addition, several methods already published allow the measurement of R and E during the same run [11—15]. However, all of these methods require extraction into hexane or heptane, with subsequent solvent evaporation and resuspension of the residue. These steps are time-consuming, and are associated with variable losses of R and E. Also, we noted difficulty resuspending the lipid extract residue after solvent evaporation when we used these methods.

Prior reports did not address such issues as whether protective antioxidants needed to be used during the extraction procedure; whether different anticoagulants affected R and E recoveries; which compound would make the best internal standard; and whether recoveries of R and E from sera from different patients were constant.

We therefore sought to develop a method of plasma/serum extraction and HPLC analysis which would address the above issues, and allow the simultaneous measurement of R and E during the same run using UV detection at one wavelength. In addition, we sought a method which had optimal selectivity, sensitivity and precision, while avoiding the use of a solvent evaporation step. The extraction and chromatographic method described in this paper meets the requirements outlined above.

EXPERIMENTAL

Reagents

All chemicals were the highest grade commercially available; all solvents were HPLC grade. Water was house-distilled, then passed through a Milli-Q purification system (Millipore, Milford, MA, U.S.A.). Butanol-1 and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). D-α-Tocopherol (oil) was obtained from Kodak (Rochester, NY, U.S.A.). D-α-Tocopherol acetate (EA), all-*trans*-retinol and BHT (butylated hydroxytoluene) were obtained from Sigma (St. Louis, MO, U.S.A.). Absolute ethanol was purchased from U.S. Industrial Chemicals (Tuscola, IL, U.S.A.). Dipotassium monohydrogen phosphate was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Blood samples

All blood samples were drawn directly into Vacutainer sample tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.). Tubes contained either no anticoagulant (red top No. 6490 or royal blue top No. 6526) or contained lithium heparin (No. 6484), oxalate (No. 6445), citrate (No. 6389), or EDTA (No. 6385). All tubes were handled under 25-W incandescent bulbs only and were protected from sunlight and fluorescent light. After 60 min to allow for clot retraction, all tubes were centrifuged at 1500 *g* for 10 min, and the serum or plasma was transferred to polypropylene freezer tubes using a glass disposable pipet. All samples were kept frozen at −35°C until used. When needed, samples were thawed to room temperature, vortexed for 10 s, then centrifuged at 1500 *g* for 10 min to remove any cryoprecipitate. Large amounts of single-donor heparinized plasma were obtained from the blood bank in plastic bags (Transfer Pack Unit, Fenwal Labs., Deerfield, IL, U.S.A.), mixed thoroughly and stored in polypropylene freezer tubes.

Stock solutions

A stock solution of D-α-tocopherol was prepared by dissolving 100 mg of D-α-tocopherol oil in 20 ml of acetonitrile. This stock solution demonstrated a 4% loss over a period of three months when stored at 4°C under nitrogen, and had only one peak when subjected to HPLC analysis. D-α-Tocopherol acetate stock solution was prepared by dissolving approximately 400 mg of crystralline EA in 20 ml of acetonitrile. The retinol stock solution was prepared by dissolving 25 mg crystalline R in 20 ml acetonitrile.

Serial dilutions of these stock solutions into acetonitrile were made daily. After dilutions of R (to 25, 15 and 5 μg/ml), E (to 500, 300 and 100 μg/ml) and EA (to 500 μg/ml) were made, actual concentrations were determined by measuring the UV absorption of the most dilute solutions with a spectrophotometer. Extinction coefficients (E 1%, 1 cm in ethanol) and wavelengths used were 1850/325 nm for R [9], 75.8/292 nm for E [13], and 43.6/285 nm for EA [13]. We had previously determined that UV absorption for all three compounds was equal (to within 0.3%) in ethanol and acetonitrile.

Sample clarification

Our serum/plasma clarification procedure was a modification of our previously described method for R alone [9]. Human serum or plasma (500 μl) was transferred into a 1.5-ml polypropylene microcentrifuge tube; 50 μl acetonitrile and 50 μl of an EA solution in acetonitrile (approximately 500 μg/ml) were added to provide the internal standard and to initiate protein precipitation. After vortexing for 15 s, 250 μl of butanol—ethyl acetate (1:1) were added, with vortexing for 60 s. Finally 150 μl of an aqueous solution of dipotassium monohydrogen phosphate (1.2 g/ml) were added, the solution was vortexed for 30 s, then centrifuged at 13 000 *g* for 1 min (Fisher microcentrifuge Model 235B). The organic upper layer was transferred by glass pipet to a 0.5-ml polypropylene microcentrifuge tube, and again centrifuged for 1 min at 13 000 *g*. A 50-μl aliquot of this organic matrix was then injected directly onto the HPLC apparatus.

Daily standard solutions

To 500 μl water were added known amounts of R, E and EA contained in the diluted stock solutions described above. One of the R solutions (25 μl), one of the E solutions (25 μl) and 50 μl of the EA solution were used. The final concentrations in these three aqueous solutions (ng of each analyte added per 1 ml water) were: solution A—R 250 ng/ml, E 5 μg/ml, EA 50 μg/ml; solution B—R 750 ng/ml, E 15 μg/ml, EA 50 μg/ml; and solution C—R 1250 ng/ml, E 25 μg/ml, EA 50 μg/ml. These three aqueous solutions were extracted and used to generate the two daily standard curves.

HPLC system

We used a Waters 510 dual-piston pump (Waters Assoc., Milford, MA, U.S.A.), an SSI 0.5-μm in-line filter (Rainin Instruments, Woburn, MA, U.S.A.), a Rheodyne 7125 injector with a 100-μl loop (Rainin), a Brownlee precolumn (30 × 4.6 mm) packed with 5-μm RP-18 material (Rainin), a Waters μBondapak column (300 × 3.9 mm) packed with 10-μm irregular RP-18 material, a Beckman 160 UV detector equipped with a 280-nm filter (Beckman Instruments, Wakefield, MA, U.S.A.), and a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A reporter—integrator. For two days, a Waters 490 detector with variable-wavelength, multi-channel capabilities was used to confirm peak identities.

The detector sensitivity was set at 0.017 a.u.f.s. The mobile phase consisted of methanol—water (95:5) and was vacuum-filtered through a 0.45-μm Nylon filter (Rainin) prior to use. Flow-rate was 2.5 ml/min at ambient temperature, generating a back-pressure of 129 bar (1900 p.s.i.). Peak identification was made by retention times being identical with those of known standards.

Calculations

Peak areas under the R, E and EA peaks were measured using routine integration parameters, and the R/EA and E/EA peak area ratios were calculated. Two standard curves were generated every day from the three extracted aqueous solutions. The ordinate represented the R/EA (or E/EA) peak area ratio, while the abscissa represented the R (or E) concentrations of the solutions used in generating the standard curve. Best-fit linear regression lines were calculated using the method of least squares. The correlation coefficient (r) was calculated for each standard curve; if the r value was less than 0.99, the standard curve was repeated.

In experiments in which recoveries were tested under different conditions, the significance of differences between group means was examined using one-way analysis of variance. If significant differences were found, they were further analyzed using the Student—Neuman—Keuls test [16]. Results of multiple determinations (n) were expressed as mean ± standard deviation (S.D.). Precision was reported as the coefficient of variation (C.V.) of multiple determinations (C.V. = S.D./mean).

RESULTS AND DISCUSSION

Chromatographic and clarification conditions

The column and mobile phase were selected in order to allow the latest peak

(EA) to elute in less than 12 min, and still allow adequate separation of the R peak from earlier peaks on the chromatogram (see Fig. 1). The organic matrix used for the extraction (ethyl acetate—butanol, 1:1) gave superior recoveries and peak shapes when compared to organic matrices consisting of *n*-butyl acetate, methyl-*tert*.-butyl ether and butyl chloride, alone or in combination.

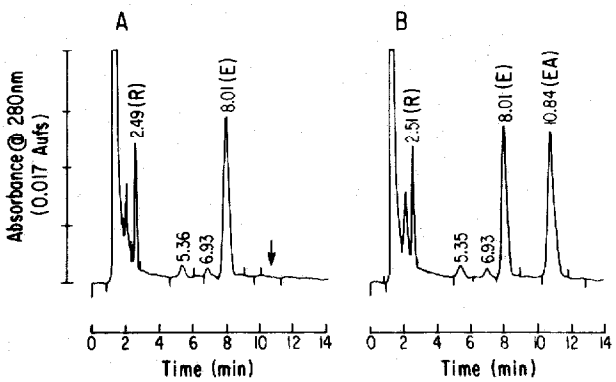

Fig. 1. Representative chromatograms of serum from a healthy volunteer. Panel A demonstrates peaks for retinol (R) and α-tocopherol (E), and no intrinsic peak at 10.84 min (arrow), the retention time for EA; Panel B demonstrates the same intrinsic peaks, plus the peak of the internal standard (EA) added during the clarification process. In this subject, concentrations of R and E were 700 ng/ml and 18.51 μg/ml, respectively (both within the normal range).

Tocol has been used by other investigators as an internal standard [7, 12], but is not commercially available. EA, used by other authors [7, 11, 13], elutes after E on the chromatogram, thus prolonging each run by 3 min (see Fig. 1). In about 10% of our plasma samples, there was a small peak which eluted 0.6 min prior to the EA internal standard peak. The peak area of this uknown peak was always less than 4% of the peak area of the EA peak. Therefore, although baseline separation was not achieved, this unknown peak did not interfere with the use of EA as the internal standard. In addition, we studied serum samples from ten subjects each taking 400 mg of EA orally each day. In no sample was endogenous EA detected, although E levels were increased significantly above baseline values. Thus, EA may be used as an internal standard, even if EA is the form of vitamin E given orally.

Retinyl acetate (RA) has also been used as an internal standard [11, 13—15]. However, in many of our plasma samples, there was an intrinsic peak which nearly co-eluted with RA, despite various modifications of the mobile phase. This peak was also seen in papers by other authors, but was not commented upon [11, 13]. In other papers, no chromatogram was supplied, so the presence or absence of this interfering peak could not be ascertained [12, 14, 15]. In addition, when plasma was stored in blood collection bags, a very large peak nearly co-eluted with RA. This substance did not extract well from the bag using water, but extracted from the bag easily using methanol. Given its solubility properties, UV absorbance and elution properties, we believe this substance is most likely di(2-ethylhexyl)phthalate, commonly used as a plasticizer [17]. These observations led us to use EA as our internal standard.

Sensitivity and precision

The limits of detection for R and E were 60 ng/ml and 0.9 μg/ml, respectively (peak height > 5× background noise). While this sensitivity could have been considerably improved either by increasing the injection size or by increasing detector sensitivity (to as low as 0.001 a.u.f.s. on our detector), this was not necessary, since the usual serum levels of R and E encountered were at least five times greater than these levels of detection.

Same-day precision was determined by determining R and E levels in eleven aliquots of the same serum sample. The values of the C.V. for R, E and EA were 3.5, 3.6 and 2.0%, respectively. When a serum sample was analyzed in duplicate each day for five days, the between-day precision for R and E was 4.8 and 5.5%, respectively. These values of sensitivity, same-day precision and between-day precision compare favorably with values obtained in earlier studies [11—15].

Recovery and stability

We first ascertained net recoveries of R, E and EA from spiked water samples. To 500 μl of water were added 100 μl of an acetonitrile solution of either R (5 μg/ml), E (125 μg/ml) or EA (250 μg/ml). The UV absorbance at the appropriate wavelength was measured in the aqueous phase before extraction, and again after the full sample extraction procedure. Blanks were prepared from water to which pure acetonitrile had been added. As seen in Table IA, all three analytes of interest were essentially completely extracted from the aqueous into the organic matrix.

Second, we investigated the possibility that these substances might adsorb

TABLE I

RECOVERY AND STABILITY OF R, E AND EA UNDER VARIOUS CONDITIONS

All data are normalized to percentage recovery obtained under control conditions defined in the text. Values are expressed as mean ± S.D. of n samples, aliquots or patients. No difference between group means was significant at the $p = 0.05$ level.

Condition	Recovery (%)		
	R	E	EA
A. Recovery from water	100.0 ± 0.0 (n = 3)	100.0 ± 0.0 (n = 3)	98.8 ± 0.0 (n = 3)
B. Recovery from water after one tube transfer	97.9 ± 1.1 (n = 2)	101.1 ± 0.6 (n = 2)	102.1 ± 1.1 (n = 2)
C. Recovery from plasma relative to water	102.6 ± 3.4 (n = 4)	96.9 ± 6.2 (n = 20)	96.5 ± 3.5 (n = 20)
D. Recovery from plasma after 1 h incubation	95.1 ± 5.9 (n = 2)	95.7 ± 3.7 (n = 2)	94.9 ± 0.4 (n = 2)
E. Stability in plasma over 48 h	101.0 ± 0.0 (n = 2)	102.4 ± 0.0 (n = 2)	—
F. Stability in organic matrix over 2.5—3.5 h	99.3 ± 0.9 (n = 4)	104.4 ± 4.6 (n = 5)	100.8 ± 1.7 (n = 5)
G. Recovery using BHT during clarification process	99.5 ± 2.6 (n = 9)	101.3 ± 5.9 (n = 10)	99.5 ± 5.2 (n = 10)

to the walls of the polypropylene tubes. A 3-ml volume of an aqueous solution containing R, E and EA was prepared in a polypropylene tube. Two aliquots were removed and analyzed (defined as 100% for each analyte). The remaining liquid was transferred to a second polypropylene tube, vortexed, and two more aliquots were removed and analyzed. Recoveries of R, E and EA after the transfer were unchanged (see Table IB), evidence that these substances do not adsorb to the walls of the polypropylene tubes.

Third, we investigated whether recovery of R, E and EA added to plasma or serum was equal to recovery from spiked aqueous samples. Our previous work with R utilized an identical extraction procedure, and demonstrated that recovery of exogenous R added to four plasma samples was 102.6 ± 3.4% relative to recovery from spiked water [9]. In this study, we measured recovery of exogenous E and EA added to plasma samples from twenty subjects, compared to their recoveries from spiked water (see Table IC). These results indicate that the recovery of exogenous R, E and EA added to plasma from different subjects is uniform and nearly as high as their recovery from spiked water.

Fourth, we investigated the possiblity that recoveries of exogenous R, E and EA added to plasma might decrease if they were allowed more time to equilibrate with and bind to plasma binding constituents. R, E and EA were added in small volumes (30 μl each) of concentrated ethanolic solutions (R 70 μg/ml, E 1500 μg/ml, EA 5000 μg/ml) to 3 ml plasma. Duplicate extractions of the vortexed plasma sample were performed 1 min after spiking, and again after 60 min incubation (see Table ID). There seemed to be minimal change in analyte recovery even after allowing 1 h incubation time to enhance binding.

Fifth, we evaluated the stability of R and E in plasma over time. We obtained fresh heparinized plasma from one normal volunteer, and performed one set of duplicate extractions and analyses immediately after venipuncture. After the plasma was kept at room temperature in subdued incandescent light for 48 h, another set of duplicate analyses was performed (see Table IE). Both R and E appeared to be stable in a plasma matrix, even when left at room temperature for up to 48 h. This finding agrees with results previously published [14].

Sixth, we investigated the stability of analytes of interest in the organic matrix used during the extraction procedure. We measured R, E and EA in plasma samples injected immediately after extraction, and again after the organic matrices had been left for 2.5—3.5 h in the dark at room temperature (see Table IF). There was no evidence that R, E or EA changed significantly if HPLC injections were not made immediately following extractions. This agreed with our earlier finding using the same extraction scheme for R [9], and with the previous observation of another group using a similar organic matrix [18].

Finally, we investigated whether the use of an antioxidant such as butylated hydroxytoluene (BHT) during the extraction process would change R, E or EA recoveries. We extracted these analytes from two spiked water samples and from eight plasma samples from normal volunteers. For each sample, duplicate aliquots were extracted without BHT (defined as 100% recovery), and in the presence of BHT (125 μg/ml final concentration, added as a concentrated acetonitrile solution in the first step of clarification). The presence of BHT in our

clarification procedure did not significantly alter recoveries of R, E or EA (see Table IG). While groups using other procedures have used BHT to protect oxidizable substances during clarification [10, 15], this has not usually been done when working with R and E [11–14]. Therefore, since BHT eluted slightly before R during the HPLC run and had significant UV absorption at 280 nm, we chose not to use BHT during the extraction procedure.

Serum versus plasma

Our prior work with R [9] and β-carotene [10], along with the work of others with R [18] indicated that serum had the highest levels of R and β-carotene, with heparinized plasma very slightly less, and progressively greater losses when EDTA, oxalate, or citrate were used as anticoagulants. We obtained fresh blood samples from three normal volunteers, allowed the samples to incubate for 1 h at room temperature, and compared recoveries of R, E and EA (as well as the R/EA and E/EA ratios) from serum and plasma matrices. Recoveries of each analyte from serum (red top Vacutainer tubes) were defined as 100% (see Table II).

As expected, the internal standard (EA) showed no difference in recovery from the various matrices. EA was added during the extraction procedure, and thus would have had little time to be in contact with the various anticoagulants. In addition, EA is more resistant to oxidation than the free alcohols R and E.

Recoveries of R and E were essentially the same from the two types of serum, and from heparinized plasma. Since plasma has higher concentrations of clotting factors than serum, we expected to see slightly higher concentrations of R and E in serum than plasma [9, 10]. Finally, the progressively decreasing recoveries of R and E in plasma anticoagulated with EDTA, citrate or oxalate

TABLE II

RECOVERY OF R, E AND EA FROM SERUM AND VARIOUS PLASMA PREPARATIONS

Two types of serum and four types of plasma were obtained from three healthy subjects. Blood collection tubes were centrifuged 1 h after venipuncture. Serum and plasma were then promptly removed, clarified (with the addition of EA) and chromatographed. For each subject, the recovery of R, E, EA, R/EA and E/EA was defined as 100% in serum obtained in red top tubes. Values represent the mean relative recoveries of each analyte found in duplicate analyses from three subjects.

Matrix	Recovery (%)				
	R	E	EA	R/EA	E/EA
Serum (red top)	100.0	100.0	100.0	100.0	100.0
Serum (blue top)	102.8	102.0	101.6	100.7	99.0
Plasma (heparin)	98.3	97.9	100.4	98.2	97.5
Plasma (EDTA)	91.7*	91.3*	98.7	95.8	94.0
Plasma (citrate)	80.3**	77.8**	97.4	82.9**	79.8**
Plasma (oxalate)	80.9**	76.6**	95.2	84.9**	80.0**

*$p < 0.05$.
**$p < 0.001$.

very closely parallels our earlier findings with retinol [9] and β-carotene [10]. We have not further explored the mechanisms for this decreased recovery, but it may be due to the action of these acidic compounds upon R and E, causing isomerization or oxidation, as noted by others [18].

Plasma E levels

This method of analysis was applied to plasma samples obtained form 41 subjects who were receiving (blindly) either placebo or β-carotene 50 mg orally once daily. DL-α-Tocopherol (6 mg, 6.6 I.U.) was present in each capsule to act as an antioxidant; this amount is less than the recommended daily allowance of 12—15 I.U. per day. It was previously shown that administration of β-carotene did not alter plasma E levels [19]. In these 41 subjects, we found that E levels ranged from 4.20 to 49.50 μg/ml (mean 15.57 ± 7.39 μg/ml). These concentrations are in the range reported by others [11, 12, 14].

Comparison to other techniques

We attempted to compare our E results with those obtained with other methods requiring ethanol—hexane extraction and solvent evaporation. However, when we used this type of method, our recoveries of R, E and EA added to plasma from twelve subjects averaged 84.9 ± 10.4%, 86.0 ± 14.3% and 67.3 ± 9.2%, respectively. In fact, it was these low and variable recoveries which stimulated us to develop an alternative method.

However, we did compare our results for R determination using the method reported in this paper, and a method which we reported previously [9]. Over two consecutive days, R was determined in plasma samples from 24 subjects and two unspiked water (blank) samples. The results obtained with the newer method (y in ng/ml) were related to the results obtained with the earlier method (x in ng/ml) by the regression $y = 0.998x + 4.09$ ($r = 0.978$). Thus the different HPLC conditions, the choice of a less sensitive UV wavelength for monitoring (280 versus 313 nm), and the use of a different internal standard (EA versus RA) did not alter R results.

Peak identification by absorbance ratios

In routine use, we identified peaks as representing R, E or EA when they had retention times identical to the known standards. In some patients, we additionally confirmed peak identities by injecting samples once, then injecting samples a second time after exogenous known analytes were added. In every case, the spiked sample still showed only one peak. However, a single peak on a chromatogram at a single wavelength of detection could conceivably represent more than one analyte.

To investigate this possibility, we used a variable-wavelength UV detector which had the capability of monitoring two different wavelengths, and plotting the ratio of UV absorbances of the two wavelengths on a third channel. We monitored our eluent at 280 and 292 nm, and plotted the ratio of UV absorbances at 292/280 nm. Using this system, we injected four extracted aqueous standards of R, E and EA, and extracted plasma samples spiked with EA from five subjects. The ratio plots for these nine samples were nearly rectangular in each case. In addition, for all nine runs, the calculated absorbance ratios for

R, E and EA were 1.74 ± 0.11, 1.40 ± 0.08 and 0.22 ± 0.01, respectively. This represents supporting evidence that peaks we identified as R, E and EA were in fact those analytes without co-eluting substances.

This method of sample extraction and HPLC analysis for R and E represents a simple, rapid, sensitive, selective and precise method for measuring these analytes of interest in human serum and heparinized plasma. We have presented supporting data concerning analyte stability in both human plasma and the organic matrix; lack of requirement for BHT during extraction; reproducible recovery during extraction of samples from different subjects; and peak identification. Problems with previously published methods such as analyte loss during solvent evaporation, and difficulties using retinyl acetate as the internal standard, have been eliminated. This method of sample clarification and analysis should prove useful to investigators who must measure R and E concentrations in serum or plasma, especially when large numbers of samples must be processed with great accuracy, precision and sensitivity.

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