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Journal of Chromatography B, 716 (1998) 129–137

JOURNAL OF CHROMATOGRAPHY B

Application of cloud-point extraction–reversed-phase high-performance liquid chromatography A preliminary study of the extraction and quantification of vitamins A and E in human serum and whole blood

Sarath R. Sirimanne[a,*], Donald G. Patterson Jr. [a], Li Ma[b], Joseph B. Justice Jr. [b]

[a]*Division of Environmental Health Laboratory Sciences, National Centers for Environmental Health, Centers for Disease Control and Prevention (CDC), Public Health Service, US Department of Health and Human Services, 4770 Buford Highway NE, Atlanta, GA 30341-3724, USA*

[b]*Department of Chemistry, Emory University, Atlanta, GA 30322, USA*

Received 26 June 1997; received in revised form 9 June 1998; accepted 9 June 1998

Abstract

Methods available for quantification of vitamins A and E in serum or blood requires preconcentration and clean-up by liquid–liquid extraction, evaporation of the extract, and reconstitution of the extract in a solvent of choice before analysis. This process not only involves the use of toxic organic solvents but also requires a long sample preparation time. The lipids and other non-polar coextractants often require additional steps for sample clean-up and evaporation, which may cause sample losses. The use of cloud-point extraction eliminates most of these sample clean-up problems. We recently demonstrated that cloud-point extraction (CPE) can be used for extraction and quantification of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) from human serum. We now demonstrate how CPE can be used with human serum and blood, at volumes as low as 50 μl, and report a methodology for extracting and quantifying two clinically important vitamins, (A and E) from human serum and blood. Vitamins A and E were extracted from human serum and blood by using Genapol X-80 as the cloud-point extractant under salting out conditions. Serum and blood samples were diluted in organic-free water to get sufficiently large sample volumes for CPE. The surfactant-rich phases were separated by centrifugation, and the samples were analyzed by HPLC–UV after deleterious coextractants were removed by precipitating them with acetonitrile. The recoveries of spiked vitamins A and E were found to be 85.6±0.4% and 82.6±5.2%, respectively. The average concentration of vitamins A and E in a serum pool after correction for recoveries were found to be 43.4±1.8 μg/dl (1.5±0.1 μmol/l) and 564.3±65.3 μg/dl (13.1±1.5 μmol/l), respectively. Vitamin A and E concentrations in whole blood were found to be 26.3±0.4 μg/dl (0.92±0.01 μmol/l) and 457.5±15.6 μg/dl (10.6±0.4 μmol/l), respectively. These values are comparable with those obtained by the reference method used at the Centers for Disease Control and Prevention. The success of the preliminary study will lead to a comprehensive validation of this method for vitamins A and E in serum and blood.

Keywords: Cloud-point extraction; Vitamins; Retinol; α-Tocopherol

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00287-4

1. Introduction

Vitamins A (retinol) and E (α-tocopherol) have been implicated as chemopreventive agents for some cancers [1–3]. The possible protective effect of vitamin E against environmental and drug toxicity has also attracted the attention of many investigators [4,5]. Measurements of these vitamins are, therefore, important in clinical trials related to cancer therapy and general nutrition studies. Most of the sample preparation methods for these vitamins involve multi step procedures that require the use of toxic organic solvents [6–9]. As an alternative to solvent extraction methods, cloud-point extraction (CPE) is being used by analytical chemists because of its efficiency, cost effectiveness and environmental friendliness [10]. Micellar aqueous solutions prepared with many non-ionic surfactants undergo phase separation above a certain temperature also known as the cloud-point temperature [10]. During the cloud-point phase-separation process, these micellar vesicles, which attract non-polar analytes because of hydrophobic interactions, aggregate into a surfactant-rich phase. The analyte/surfactant-rich phase permits analysis and quantification of the analytes by techniques such as reversed-phase high-performance liquid chromatography (HPLC) without further sample clean-up. The CPE process is attractive to analytical chemists because of features such as its capacity to concentrate a variety of analytes with high concentration factors; its safety and cost benefits (it is an excellent alternative to conventional sample concentration methods that require the use of large amounts of toxic and flammable organic solvents); the ease with which surfactant used in the process can be incinerated in the presence of waste acetone or ethanol; its capacity to enhance detection by reconcentrating the analyte; the compatibility of the surfactant rich-phase with micellar liquid chromatographic techniques; the preclusion of analyte losses during the evaporation of solvents used in traditional liquid–liquid extraction techniques; and the inhibition of adsorption of non-polar analytes to glass surfaces. The capacity of this process to concentrate and separate non-polar target species from aqueous matrices has been demonstrated in analytical chemistry and separation science [10,11]. The cloud-point methodology has recently been applied to the extraction of a wide range of analytes from water, including polycyclic aromatic hydrocarbons (PAHs), porphyrins, metallo-porphyrins, vitamin A, vitamin E, β-estradiol, estriol, estrone, progesterone and proteins [2–18]. We recently demonstrated that CPE can be used to extract PAHs and polychlorinated dibenzo-*p*-dioxins (PCDDs) from human serum with excellent recoveries by using Triton X-100 as the surfactant [19]. In this study, we extend the application of CPE to the quantification of two fat-soluble vitamins (A and E) in human serum and whole blood. The surfactant-rich phase obtained from CPE of these vitamins can be analyzed by HPLC without further clean-up or evaporation steps. HPLC has been shown to be a powerful technique for separating of these compounds [7–9,20–22]. In addition, photodiode array detection allowed simultaneous detection of these vitamins at different wavelengths. In this paper, we report the quantification of vitamins A and E in human-serum and whole-blood samples by CPE using Genapol X-080 as the surfactant and HPLC with UV photodiode array detection.

2. Experimental

2.1. Instrumentation

The HPLC system used for the analysis consisted of a Waters (Waters Associates, Milford, MA, USA) fluid handling unit with Model 600E system controller, a Model 994 programmable photo diode array detector, and a Model 717 plus autosampler. The data acquisition and processing were performed on the HPLC system equipped with Millennium chromatography manager software. Incubation and centrifugation of samples were performed by using a Precision Scientific Model 25 reciprocal shaking bath (Precision Scientific, Chicago, IL, USA) and a Model HN-S centrifuge (International Equipment, Needham Heights, MA, USA).

2.2. Reagents

The non-ionic surfactant Genapol X-080 was obtained from Fluka (Ronkonkoma, NY, USA) and used without further purification. HPLC-grade acetonitrile was obtained from Burdick and Jackson

(Baxter Healthcare, Muskegon, MI, USA). Spectrophotometric grade ethanol was purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade water was obtained from J.T. Baker (Phillipsburgh, NJ, USA). The standards of vitamins A and E were from the Nutritional Biochemistry Branch of the Centers for Disease Control and Prevention, and retinyl acetate and retinyl butyrate were synthesized in our laboratory. Serum and blood samples were obtained from National Health and Nutrition Examination Survey (NHANES) pools of the Nutritional Biochemistry Branch of the Centers for Disease Control and Prevention.

2.3. CPE procedure

Fifty μl of human serum or blood and 10 μl of internal standard retinyl acetate (3 ng/μl) were added to a 15 ml tapered centrifuge tube. To this were added 400 μl of an aqueous solution of Genapol X-80 at the desired concentration and 0.125 g crystalline sodium chloride. The contents were mixed well with a Vortex Genie Mixer until the sodium chloride completely dissolved and then incubated in a thermostatted shaking water bath for the desired time at the desired temperature (40–60°C). The micellar solution containing serum or blood was then centrifuged at 3500 rpm (1500 *g*) for 5 min. The water phase was then removed to obtain a surfactant-rich phase stuck to the wall of the tube. The greasy surfactant-rich phase was then diluted with 100 μl water after which 5 μl of retinyl butyrate was added as the external standard (6 ng/μl). Coextractants such as hydrophobic proteins and most of the surfactant were then removed from the surfactant-rich phase by precipitation with 300 μl of acetonitrile and filtration through silanized glass wool packed into a disposable glass pipet. The analytical samples were immediately analyzed by HPLC or stored at −20°C until analyzed.

2.4. Analysis of vitamins A and E in deproteinized blood

An aliquot of whole blood (50 μl) was treated with 300 μl of acetonitrile and filtered through glass wool packed in a disposable glass pipet. The filtrate was then analyzed by HPLC under the same conditions described in Section 2.5.

2.5. HPLC analysis

Aliquots of cloud-point extracts of vitamins A and E from human serum (75 or 100 μl) were analyzed with an isocratic HPLC system equipped with an octadecyl silica column (15×0.46 cm, 5 μm OD5, Burdick and Jackson) equilibrated with ethanol–acetonitrile–water (45:45:10) at a flow-rate of 1 ml/min. UV absorbance at 300 and 325 nm were monitored with the photodiode array detector. Data processing and quantification were performed by using the Millennium Chromatography Manager software. Retinol, retinyl butyrate and retinyl acetate were monitored at 325 nm; vitamin E (α-tocopherol) was monitored at 300 nm.

3. Results and discussion

Genapol X-080 was chosen as the CPE surfactant because of its low UV absorbance and low cost. Genapol X-080 is a polyoxyethylene glycol monoether-type surfactant that has eight oxyethylene units and tridecyl alkyl moieties [critical micellar concentration (CMC)=0.05 mmol/l (0.028%, w/v), cloud-point=42°C (in pure water)]. The phase separation at cloud-point of an aqueous micellar solution proceeds normally, and the phases can be separated after centrifugation. We have found that, unlike simple aqueous micellar solutions, micellar serum–water or blood–water samples proceed with no temperature-induced phase separation unless the solution properties are altered. The addition of a modifier such as salt was necessary to induce phase separation of these micellar serum–water or blood–water samples. The separation of the surfactant-rich phase from micellar serum or blood samples at the desired temperature was achieved by centrifugation.

Modifiers that have been used in CPE include urea, sodium chloride, sodium azide and potassium chloride [10]. These modifiers may either lower or elevate the normal cloud-point temperature. Because of the relatively high levels of vitamins A and E, the quantification can be carried out with serum samples as small as 50 μl. However, we adjusted the volume

of the working solution to 0.5 ml with water in order to overcome practical problems associated with the sample handling in the CPE. Sodium chloride was the modifier of choice because it is both cost-effective and environmentally friendly. The surfactant-rich phase from the serum and blood samples obtained through salting-out phase separation contained unwanted coextractants such as non-polar serum proteins. Moreover, these coextractants created problems in the HPLC analysis because of the precipitation in the HPLC injector. These deleterious substances were removed from the surfactant-rich phase by precipitating with acetonitrile and filtration through silanized glass wool. Silanized glass wool is not necessary since the surfactant reportedly inhibits adsorption onto glass [23]. During this process, most of the dissolved salt, proteins and the surfactant in the surfactant-rich phase precipitate from the extract. This step was included in order to avoid injector clogging that we experienced due to precipitation occurring in the injector. The nature of the precipitate was not investigated in detail to find out what was removed from the extract. Our comparison studies indicate that the precipitation step does not affect the recovery significantly. It is not known whether serum lipids are extracted during this process. However, even if the lipids have been extracted with the analytes, there was no significant adverse effect or interference that created any problem with the HPLC analysis. The CPE at salt concentration of 25% (w/v) gave the optimum recovery of vitamins A and E. No significant improvement of recovery was observed above this salt concentration.

The HPLC trace in Fig. 1a corresponds to a sample obtained from CPE of vitamins A and E from human serum. The comparison of HPLC traces of a standard and that of a cloud-point extract of a human serum sample indicated that some HPLC peaks in the serum extract have the same retention times as those for vitamins A and E. Spiking experiments carried out to identify vitamin A and E revealed that the authentic A and E coelute with the two analytes extracted from human serum (see Fig. 1b). The HPLC trace b shows the chromatogram for an HPLC run from the cloud-point extract of the same human serum spiked with additional authentic vitamins A and E. It is clear that the peaks that elute at 3.84 min and 15.41 min have increased peak areas and intensities. As shown in Fig. 1c, vitamin A elutes at 3.84 min, thus confirming that the peak at 15.41 min is due to vitamin E. The UV spectra corresponding to peaks 1 and 2 were identical to those of the authentic vitamins A and E, thus providing further proof of their identities as vitamin A and vitamin E. Another compound, which has been coextracted during CPE, elutes at 12.73 min and has not been identified.

3.1. CPE and quantification of vitamins A and E in human serum

The normal background levels of vitamins A and E in human serum were sufficiently high to be detected by HPLC–UV, and hence the experiments were performed without spiking these analytes into human serum. Retinyl acetate was used as an internal standard and was spiked before CPE; retinyl butyrate was used as an external standard to correct the volume loss due to the precipitate during the precipitation of deleterious coextractants with acetonitrile. A typical HPLC trace for a mixture containing analytes, internal standard and external standard is shown in Fig. 2. The standard curves of vitamin A and vitamin E were constructed by injecting different volumes of standard solution of each analyte. Linearity of these standard curves was established with correlation coefficients averaging 0.999 for both vitamin A and vitamin E over a range of 1 to 10 ng and 10 to 75 ng, respectively. On the basis of the standard curves for vitamins A and E, the limits of detection were estimated to be 1 ng vitamin A and 10 ng for vitamin E. Normal serum contains vitamins A and E at high levels thus precluding the determination of the exact limit of detection (LOD). In order to optimize extraction, we performed a number of experiments under different experimental conditions. In Table 1 we summarize data for the effects of extraction time, surfactant concentration and extraction temperature on the CPE efficiency. When the incubation time was increased from 10 to 60 min, the concentration of vitamin A fell slightly; however, the concentration of vitamin E dropped dramatically from 399 μg/dl (9.3 μmol/l) to 289 μg/dl (6.7 μmol/l). These results imply that both compounds undergo decomposition at elevated temperatures but at different rates. A previous study [24] has also

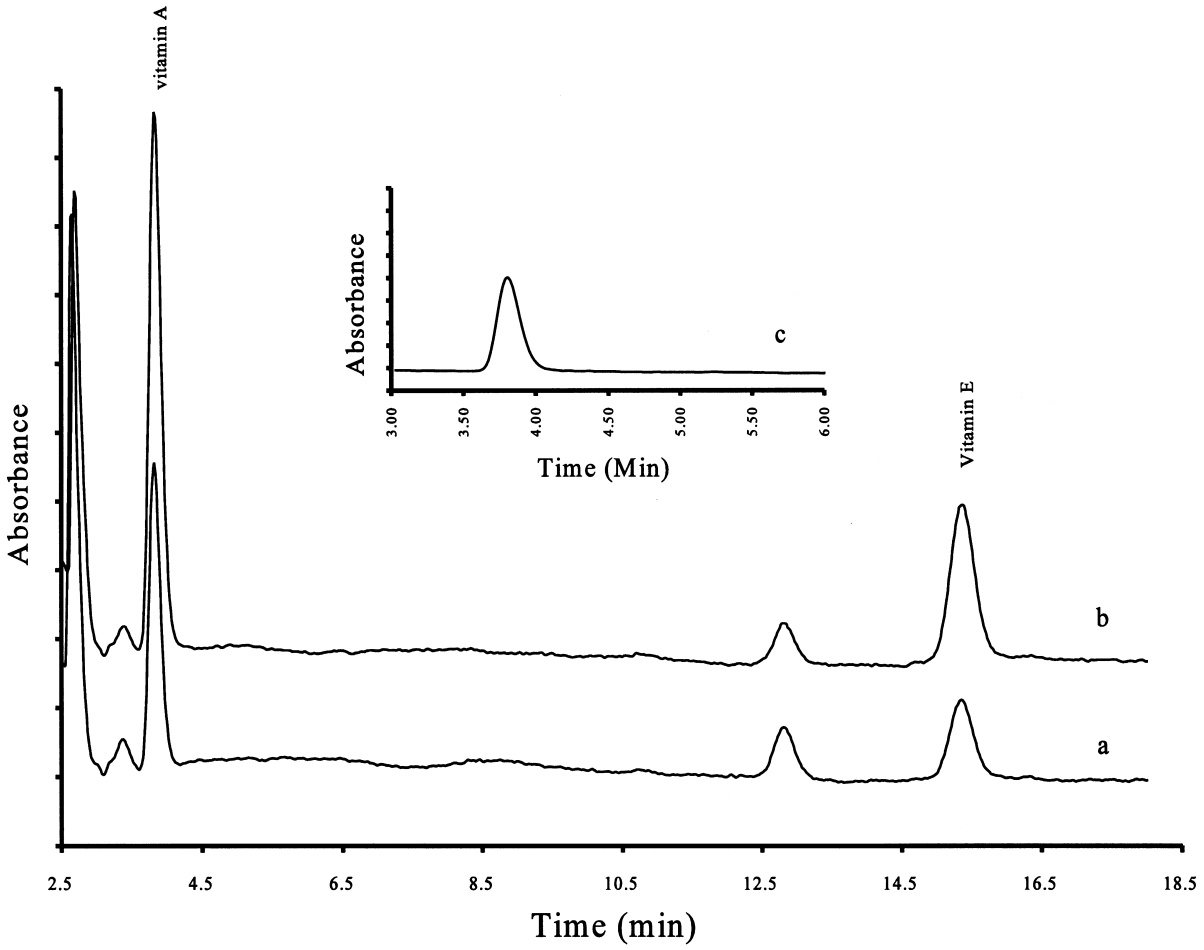

Fig. 1. Identification of HPLC peaks corresponding to vitamins A and E in cloud-point extracts of human serum. Traces: (a) a typical HPLC trace for an extract obtained from human serum. (b) A HPLC trace for same cloud-point extract spiked with authentic vitamins A and E. (c) A HPLC trace for an authentic vitamin A. Peak identification: 1=vitamin A, 2=vitamin E. HPLC conditions are described in Section 2.5.

shown that vitamin E was not as stable as vitamin A in serum during long-term storage and that the degradation of vitamin E can occur even at −20°C, whereas vitamin A is relatively stable at that temperature. Because longer extraction times are deleterious, we chose an extraction time of 10 min for the cloud-point extraction of vitamins A and E. The effect of the surfactant concentration on extraction efficiency is significant between concentrations of 2% and 16%, but the efficiency does not change appreciably above 16% (w/v). We also studied the effect of the extraction temperature on extraction efficiency over the temperature range of 40–60°C and found that decomposition at temperatures above 50°C caused lower recovery. Our preliminary studies indicated that the duration longer than 10 min leads decreased recovery. We therefore determined that the optimum extraction conditions were an extraction time of 10 min, a surfactant concentration of 16% (w/v), and a temperature of 50°C. The recovery of the internal standard was evaluated using a standard curve. Under these conditions, the recovery of the internal standard, retinyl acetate, was 94.5%. To evaluate the effectiveness of the extraction, a cloud-point extract was compared with a serum sample after protein precipitation with acetonitrile. Fig. 3 shows the HPLC (trace a) for a cloud-point extract of a serum sample and (trace b) for a serum sample

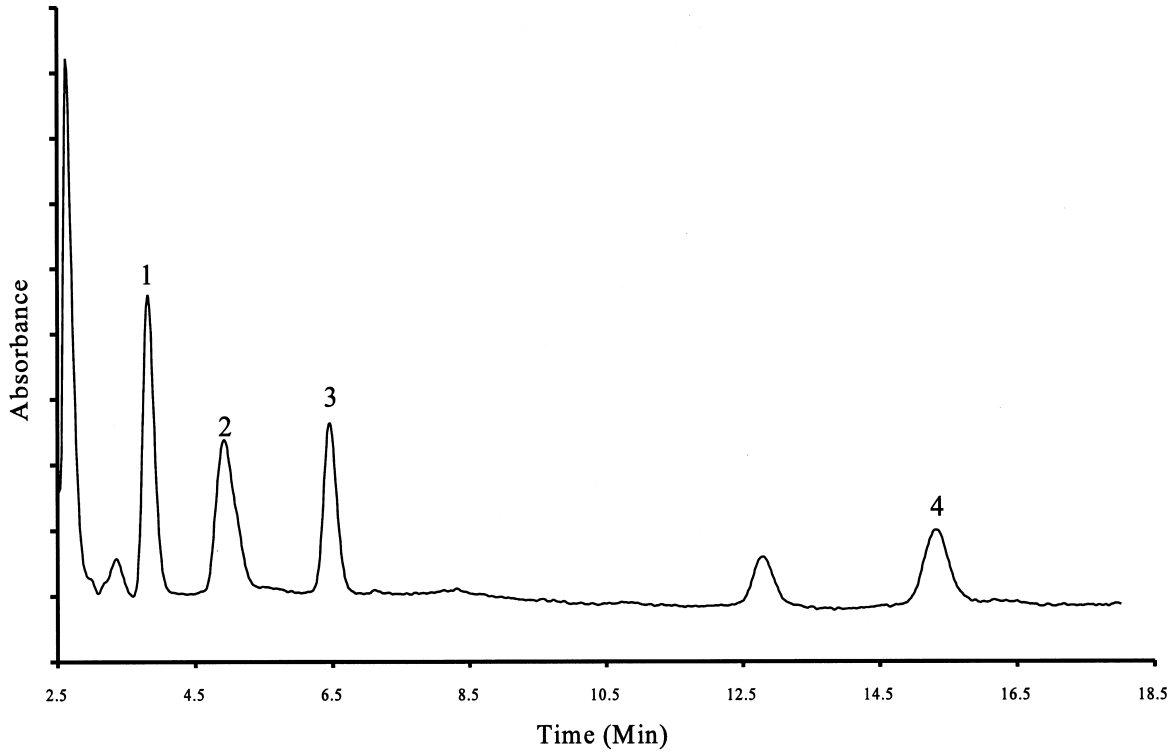

Fig. 2. A typical HPLC trace for a sample containing internal standard and the external standard. HPLC conditions are described in Section 2.5. Peak identification: 1=vitamin A, 2=retinyl acetate, 3=retinyl butyrate, 4=vitamin E.

treated with acetonitrile to remove deleterious coextractants. It is clear from Fig. 3 that the cloud-point extraction is effective in extracting analytes from

Table 1
Effects of experimental conditions on the CPE from human serum samples

Time (min)	10	35	60
Vitamin A[a] (μg/dl)	36.9	35.5	34.8
Vitamin E[a] (μg/dl)	399	340	289
X-080 concentration (%, w/v)	2	16	30
Vitamin A[b] (μg/dl)	30.7	36.9	37.9
Vitamin E[b] (μg/dl)	246	399	401
Temperature (°C)	40	50	60
Vitamin A[c] (μg/dl)	36.7	37.1	36.9
Vitamin E[c] (μg/dl)	386	466	399

[a] Temperature: 60°C, X-080: 16% (w/v)
[b] Temperature: 60°C, time: 10 min.
[c] Time: 10 min, X-080: 16% (w/v).

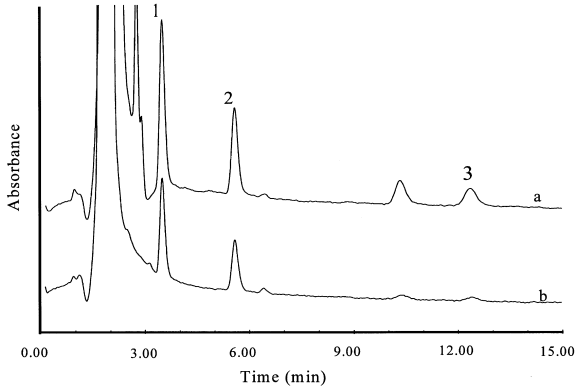

Fig. 3. A HPLC trace for a sample obtained from (A) CPE of a human whole-blood sample (trace a). (B) Filtrate from a whole-blood sample after protein precipitation with acetonitrile (50 μl of human serum was treated with 300 μl of acetonitrile and filtered through glass wool). Peak identification: 1=vitamin A, 2=retinyl acetate, 3=vitamin E.

serum more efficiently. This also indicates that during CPE analytes are extracted from the protein bound form while simple protein precipitation does not extract the protein bound analytes. Precipitation of coextractants in the CPE sample had no significant effect on the recovery. To determine whether the extraction conditions have the same effect on the extraction of vitamins A and E as on the internal standard retinyl acetate, we studied recoveries of spiked vitamin A and vitamin E from a serum sample. We divided the serum sample into two samples. One was quantified for vitamins A and E by cloud-point extraction and the other was spiked with the vitamin standards before cloud-point extraction was performed. The recoveries were calculated from the peak areas obtained by subtracting the corresponding peak areas of the unspiked serum sample from that of the spiked serum sample. The recoveries from three determinations for vitamins A and E were 85.6±0.4% and 82.6±5.2%, respectively. The difference in recoveries of the vitamins (86% and 83%) and the internal standard (94%) is probably due to a higher stability of the internal standard relative to the vitamins. The levels adjusted for recoveries of vitamin A [43.4±1.8 μg/dl (1.5±0.1 μmol/l)] and vitamin E [564.3±65.3 μg/dl (13.1±1.5 μmol/l)] in a National Health and Nutrition Examination Survey (NHANES) human serum pool are comparable with those obtained by using a reference method [24] in use at the Centers for Disease Control and Prevention [47.3±2.6 μg/dl (1.6±0.1 μmol/l) for vitamin A; 579.5±24.5 μg/dl (13.4±0.6 μmol/l) for vitamin E]. The recoveries from serum was adjusted in order to compare the values with those obtained by the CDC reference method [24]. Extraction with hexane followed by removal of hexane under vacuum and reconstitution in ethanol or acetonitrile is involved in the reference method. On the other hand, CPE uses a non-toxic surfactant and salt for the extraction and no evaporation is required. The quantification results for vitamins A and E obtained with the two methods compare very well with a

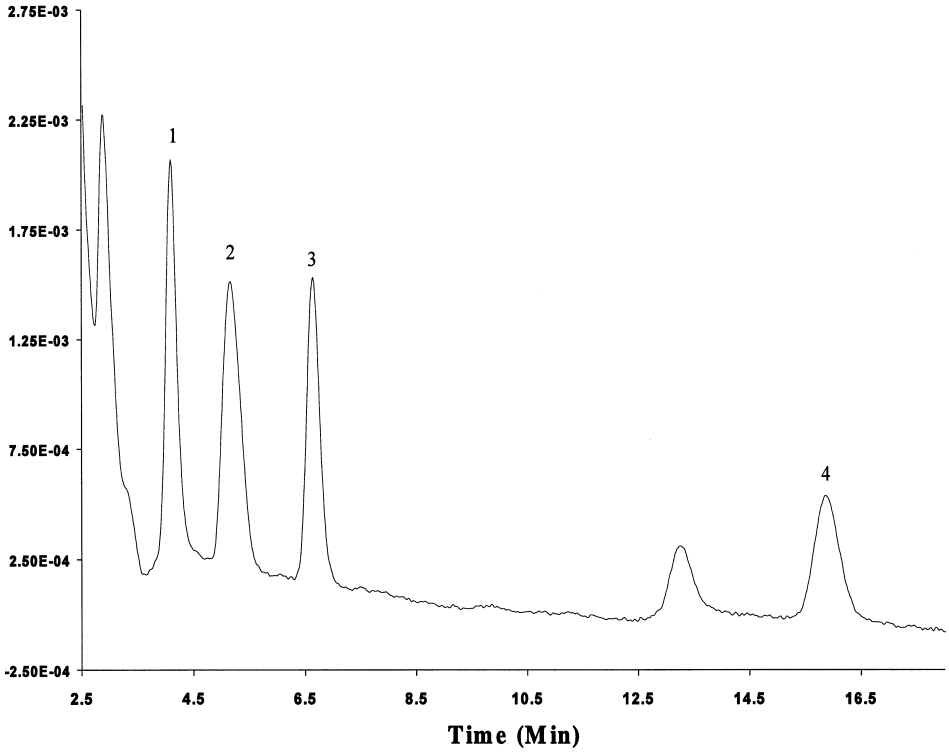

Fig. 4. A HPLC trace for a sample obtained from CPE of a human whole-blood sample. Peak identification: 1=vitamin A, 2=retinyl acetate, 3=retinyl butyrate, 4=vitamin E.

mean percent difference of 8.2% and 2.6%, respectively.

3.2. Quantification of vitamins A and E from whole blood

A typical HPLC trace for an extract obtained from CPE of a whole blood sample is shown in Fig. 4. The peaks for vitamins A and E were identified by comparing them with the peaks for authentic vitamin A and E standards; the identities of the other two peaks (3.0 and 13.5 min) in Fig. 4 are not known. It should be noted in Fig. 4 that there is a shift in the retention time which is attributed to the surface modification of the stationary phase due to the effects of residual surfactant found in the cloud point extract. Repetitive injections were found to stabilize the retention times while extended washing of the column with the mobile phase causes the retention times to shift back to the original retention time. The results of experiments to study the effect of extraction time on extraction efficiency at a temperature of 50°C and 16% Genapol X-080 and the effect of the surfactant concentration on the extraction efficiency of vitamins A and E in whole blood are shown in Table 2. A detailed study on the systematic optimization of these extraction conditions will be published elsewhere [25]. The levels do not change appreciably for surfactant concentrations above 16% (w/v). The average levels from three determinations of vitamins A and E in this whole blood sample were 26.3±0.4 μg/dl (0.9 μmol/l) for vitamin A and 457.5±15.6 μg/dl (10.6 μmol/l) vitamin E when retinyl acetate was used as the internal standard. These values are reported uncorrected for recovery since there is no reference values with which to compare these results. The external standard was used for correcting the losses due to the precipitation step in the procedure.

Table 2
Effects of extraction time and surfactant concentration on the CPE from human whole blood samples

Time (min)	10	35	60
Vitamin A[a] (μg/dl)	23.2	21.7	21.2
Vitamin E[a] (μg/dl)	433.8	404.1	388.2
X-080 concentration (%, w/v)	2	16	30
Vitamin A[b] (μg/dl)	16.3	23.2	22.2
Vitamin E[b] (μg/dl)	147.9	433.8	421.2

[a] Temperature: 50°C, X-080: 16% (w/v)
[b] Temperature: 50°C, time: 10 min.

4. Conclusions

Vitamins A and E in a very small volume of diluted serum and whole blood samples are easily and efficiently extracted by CPE. We are continuing our studies to design and test a CPE tube that can accommodate all of the steps involved in the CPE, thus shortening and simplifying the extraction procedure. Future work will involve an extensive validation study before the method can be used for accurate quantification of vitamins A and E in serum and blood.

5. Disclaimer

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services.

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