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Fast minimicroassay of serum retinol (vitamin A) by capillary zone electrophoresis with laser-excited fluoresence detection

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ABSTRACT

In this paper, we present a fast minimicroassay of serum vitamin A by capillary zone electrophoresis with laser-excited fluorescence detection. A 60 cm × 50 μm I.D. fused-silica capillary was used for the separation, and the polymer coating was burned off 20 cm from the cathodic end to form a detection window. The buffer system consisted of 50 m*M* sodium phosphate plus 10 m*M* sodium chloride at pH 7.8. A helium–cadmium laser set at 325 nm was used for excitation, and the fluorescence of the vitamin A–retinol-binding protein complex was monitored at 465 nm using a photodiode. The stray and scattered radiation were removed by two special filters. Using this system, about 8 nl of serum sample were injected for direct analysis without any sample preparation. The analysis time for each sample was less than 6 min, and subfemtomole levels of vitamin A in human or animal blood could be easily detected. Therefore, the method is potentially useful for finger-prick vitamin A analysis, especially for babies and young children.

INTRODUCTION

As is well known, retinol (vitamin A) is extremely important for the health of humans and animals. Xerophthalmia, keratomalacia, night blindness and irreversible blindness are caused by vitamin A deficiency [1–5]. Low serum retanol (vitamin A alcohol) levels are diagnostic of extreme vitamin A deficiency, and serum retanol response to oral vitamin A doses (the relative dose response) is useful in assessing human vitamin A status [6]. Presently, in Third World nations, many young children are plagued by night blindness and vitamin A-related diseases. It has been estimated that more than one million children become permanantly blind each year because of vitamin A deficiency [7]. Therefore, development of a fast minimicroassay method of serum retinol is of international importance.

Retinol, which is poorly soluble in an aqueous medium, is normally transported as a retinol–retinol-binding protein (RBP) complex (holo-RBP)

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in plasma. Plasma RBP is a single polypeptide chain with a molecular mass of about 21 000 and has a single binding site for one molecule of retinol [8]. Holo-RBP further interacts strongly with another protein, plasma transthyretin (TTR, previously called prealbumin) [9] and normally circulates in plasma as a 1:1 complex (molar ratio) with TTR. The molecular mass of the holo-RBP–TTR complex is approximately 76 000 [10]. Retinol itself has a maximum absorbance at 325 nm and fluoresces at 425 nm. However, the intensity of the fluorescence is enhanced ten- to fourteen-fold when retinol is bound to RBP [11], and the fluorescence shifts to 465 nm [12].

Conventional determinations of serum retinol require initial precipitation of serum proteins followed by organic solvent extraction of retinol. The extracted retinol is resolved from other compounds by chromatography and then estimated by UV absorbance or fluorescence [13–15]. The methods are time-consuming and results can be significantly affected by analyte losses during sample preparation.

Retinol is labile to light, oxygen and heat, making it difficult to handle, especially when it is removed from the protection of its biological matrices. Extracted retinol decomposes rapidly even at subambient temperature exposed to normal light [16]. In contrast, retinol is stable in frozen serum at −70°C for at least eight years [17–19]. Based on these characteristics of retinol, direct determination of retinol in serum would be an ideal form of analysis.

A micromethod involving gel electrophoretic separation of serum, with subsequent estimation of the retinol–RBP complex by fluorimetric scanning of the gel has been reported [11]. This method avoids solvent extraction, but is still limited by large sample requirements, long separation times and gel scanning, which make it of limited use for surveys of vitamin A status.

Recently, size-exclusion high-performance liquid chromatography (SE-HPLC) with fluorescence detection has been used to measure retinol–RBP in animal and human serum [20,21]. Compared to previous methods, less serum is required and preliminary sample treatment is avoided. However, a 0.05-ml blood sample is still needed for analysis and 20–30 min of HPLC separation time are required. In addition, at least 1 ng of retinol–RBP is required for detection. These factors make it difficult to do minimicroassay, especially for babies and young children, where only small blood samples are available.

Capillary zone electrophoresis (CZE) has been proven to be a modern separation technique which offers higher resolution and shorter analysis time than HPLC. It requires only a very small amount of sample (nano- or picoliter) and has a very low detection limit (attomoles) [22]. However, no prior research has been done on the separation of holo-RBP by CZE. Therefore, in this paper, we have developed a fast, easy and very sensitive method to separate and detect retinol in human serum. Subfemtomole levels of retinol can be easily detected, and the response is linear over the range 0.1–0.6 μg/ml, which is the physiological range in human serum.

EXPERIMENTAL[a]

Equipment set-up

The equipment set-up, illustrated in Fig. 1, is similar to that described previously [23] with some modifications. A Model CZE 1000R high-voltage power supply was bought from Spellman (Plainview, NY, USA). A 60 cm × 50 μm I.D. capillary tubing (Polymicro Techniques, Phoenix, AZ, USA) was used for the separation. The polymer coating was burned off 20 cm from the cathodic end of the capillary to form the detection window. The anodic high-voltage end of the capillary was isolated in a plexiglass box for safety while the cathodic end was held at ground potential.

A Model 4240NB helium–cadmium (He–Cd)

[a] Disclaimer Statement. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendations or endorsement by the National Instititute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

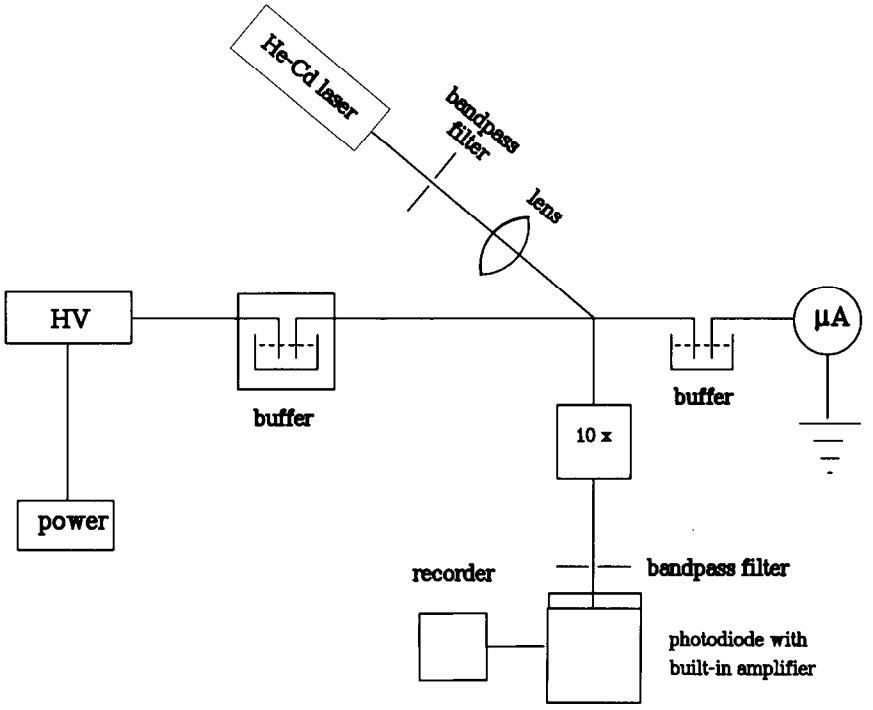

Fig. 1. Instrumental set-up for the CZE analysis of retinol in human serum with laser-excited fluorescence detection.

laser (Liconix, Santa Clara, CA, USA) operating at 325 nm was used for excitation. A Model UG-11 bandpass filter (250–400 nm) (Ealing, Holliston, MA, USA) was used to reject stray and scattered radiation from the laser head. The laser was focused onto the capillary with a 1 cm focal length lens, and the fluorescence was collected with a 10× microscope objective at a 90° angle to the incident light. The fluorescent image was focused onto a silicon photodiode combined with a Model HC220-01 built-in amplifier (Hamamatsu, Bridgewater, NJ, USA). Another bandpass filter (400–539 nm) (Ealing) was used to isolate the fluorescence (465 nm) from the vitamin A–RBP complex. The voltage from the photodiode was monitored with a Model 197A autoranging microvolt DMM (Keithley, Cleveland, OH, USA) and a Model 1201-0000 chart recorder (Cole-Parmer, Chicago, IL, USA).

Pretreatment of the capillary column

Each new capillary column was filled with 1.0 *M* sodium hydroxide solution for about 30 min to clean the column. The column was then washed with 0.1 *M* sodium hydroxide followed by deionized water and finally running buffer. The capillary was ready for use thereafter.

Reagents

All chemicals were of analytical reagent grade unless stated otherwise. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). The absolute ethanol (200 proof) was purchased from Midwest Grain Products (Pekin, IL, USA). RBP was purchased from Calbiochem (San Diego, CA, USA). Standard retinol samples in human serum (from low to high levels) and pure retinol standard were obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA).

Running buffer preparation

The running buffer was composed of 50 m*M* sodium phosphate plus 10 m*M* sodium chloride with a pH range from 2.0 to 11.5. It was prepared by mixing appropriate amounts of mono- and di-

sodium phosphate solution, and the pH was adjusted by using sodium hydroxide or phosphoric acid.

Standard retinol–RBP preparation

Standard retinol–RBP was prepared using a modification of the method of Moffa and Krause [24]. First, 10 mg of pure retinol standard were dissolved in 10 ml of absolute ethanol (concentration was 1 g/l); 10 μg of standard RBP were dissolved in 5 ml of 0.1 *M* sodium phosphate buffer (pH 7.7). Then, 20 μl of retinol standard solution were slowly transferred into 5 ml of standard RBP solution with appropriate stirring. The retinol–RBP mixture was allowed to stand for 3 h at 25°C and was then ready for injection.

Serum samples

Frozen human serum was obtained from the Department of Nutritional Science in the University of Connecticut (Storrs, CT, USA). Fresh human blood samples were obtained from volunteers in Grim-Smith Hospital (Kirksville, MO, USA). All the blood samples were centrifuged for 7–8 min at 3700 rpm (approximately 1500 *g*) to separate red blood cells from the serum. Serum was then directly injected into the high-performance capillary zone electrophoresis (HPCZE) system for analysis. Unused serum was kept at −20°C until analysis.

Conventional extraction and HPLC analysis of retinol from serum

To 100 μl freshly thawed serum was added an equal volume of methanol containing an internal standard (retinyl hexanoate); each sample was extracted three times with an equal volume of hexane. Hexane from combined hexane extracts was evaporated under a gentle stream of argon, and the residue was dissolved in 50 μl 2-propanol–dichloromethane; 20 μl were injected for HPLC analysis. Samples were analyzed by reversed-phase HPLC on a 5-μm Resolve C_{18} column (Waters Assoc., Milford, MA, USA) using a mobile phase of acetonitrile–dichloromethane–methanol–*n*-butanol (90:15:10:0.1), containing 0.1% ammonium acetate, at a flow-rate of 1.0 ml/min, with detection at 300 nm [25].

HPCZE analysis

Serum samples were injected electrokinetically at 10 kV for 10 s (approximately 8–10 nl were injected), and the separation was carried out at 24 kV for 5 min. The electrophoretic current was monitored with a multimeter throughout the separation to ensure the reproducibility. After five to seven injections, the capillary required cleaning due to the adsorption of serum proteins on the capillary wall. Cleaning was accomplished by flushing the capillary for 4 min with 1 *M* sodium hydroxide, then 2 min with deionized water and finally for 2 min with running buffer.

RESULTS AND DISCUSSION

Effect of pH on separation and signal

Adsorption of proteins onto the capillary wall is a serious problem when separating proteins by CZE using uncoated silica columns. Performing the separation at a pH above the isoelectric point (p*I*) of the proteins under investigation is one [26] of the most active ways to minimize protein build up. In this way the coulombic repulsion between negatively charged proteins and the capillary wall will minimize the protein adsorption. However, the retinol–RBP complex is sensitive to the pH of the buffer, whereby higher pH may cause the retinol–RBP complex to decompose [27]. In order to optimize the separation and detection of the retinol–RBP complex, a wide range of buffer pH (3–11.5) was investigated. At lower pH (2.0–6.0), no retinol–RBP signal was observed. Two phenomena contribute to this observation. First, since the p*I* of RBP is between 4.4 to 4.8 [12], the retinol–RBP complex was heavily adsorbed onto the capillary when the pH of the buffer was near to or lower than the p*I* of the RBP. Secondly, the fluorescent intensity of the retinol–RBP complex at this pH is reduced. Both factors have been demonstrated experimentally. When the pH of the buffer was 6–7.2, only a single peak with a small shoulder was observed (as shown in Fig. 2A). In addition, there was no linear relationship between peak heights or peak areas and retinol–RBP levels for a series of standard samples at low or neutral pH. This may be due to incomplete

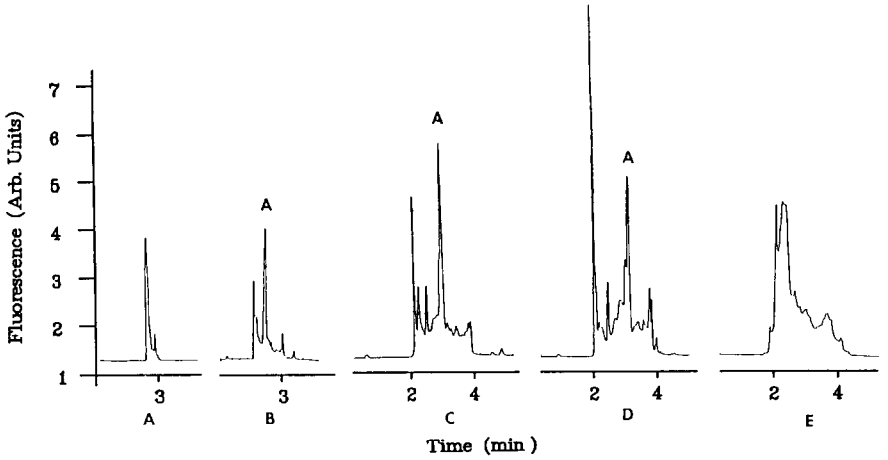

Fig. 2. Influence of buffer pH on the separation and detection of retinol–RBP. (A) pH 7.1; (B) pH 7.5; (C) pH 8.5; (D) pH 10.3; (E) pH 11.5. Buffer: 50 m*M* sodium phosphate plus 10 m*M* sodium chloride. A 10-s + 10-kV injection of the serum sample was followed by electrophoresis at +24 kV for 5 min in a 60 cm × 50 μm I.D. capillary column. Excitation wavelength: 325 nm; fluorescence was monitored at 465 nm (with a bandpass filter). Peak A = retinol–RBP complex.

separation of the serum matrix and retinol–RBP, and the partial adsorption of retinol–RBP onto the capillary wall. When the buffer pH was kept at 11.5 (as shown in Fig. 2E), the characteristic peak of retinol–RBP disappeared. This was apparently due to the decomposition of retinol–RBP complex [26]. If the buffer pH was kept at 10.3 (as shown in Fig. 2D), the retinol–RBP peak was detectable; however, the linearity of the retinol–RBP response of the standard serum samples was very poor due to partial decomposition of the retinol–RBP complex. When the buffer pH was maintained between 7.5 and 8.5 (as shown in Fig. 2B and C), both complete separation of the retinol–RBP complex from other serum components and a linear response for standard serum samples were attained. However, within this pH range, we observed that the fluorescent signal of the retinol–RBP complex was enhanced at higher pH (from 7.8 to 8.5) (as shown in Fig. 2C). Therefore, pH 7.8 was employed in this work as an optimized pH to analyze serum samples.

Identification of retinol–RBP peak

In order to verify the retinol–RBP peak in the standard serum electropherogram (as shown in Fig. 2), pure retinol–RBP complex, which was prepared from retinol and isolated human RBP, was injected. The results was shown in Fig. 3. The pure retinol–RBP complex migrates as a single peak (A) that exactly matches the peak A in Fig. 2.

Although retinyl esters, as well as very small amounts of unesterified retinol, will be present in chylomicra, their contribution to circulating vitamin A in fasting blood is small (< 10%) [5]; retinol–RBP is the physiologically important transport form of vitamin A. Retinol–RBP is well re-

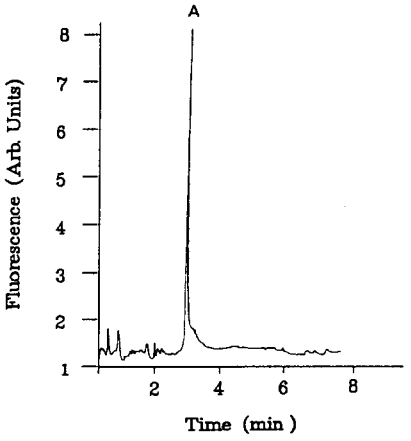

Fig. 3. Pure retinol–RBP complex electropherogram. The pH of the running buffer was 7.8. A 30-s, + 10-kV injection was used. Other conditions are the same as those in Fig. 2. Peak A = retinol–RBP complex.

TABLE I

COMPARISON OF CONCENTRATIONS OF RETINOL (VITAMIN A) IN HUMAN SERUM SAMPLES DETERMINED BY HPLC AND HPCZE

Serum sample	Concentration of retinol (ng/ml)	
	HPLC method[a]	CZE method[b]
1	463	445 ± 28
2	177	183 ± 24
3	231	268 ± 29
4	404	424 ± 27
5	216	206 ± 23
6	498	428 ± 24
7	204	270 ± 27
8	386	376 + 25

[a] The data came from Department of Nutritional Science, University of Connecticut.

[b] The data were the mean of five analyses ± standard deviation of the mean.

solved from the lipoproteins by these electrophoretic conditions.

Linearity

Linear response to graded levels of vitamin A in the serum is extremely important in quantitative analysis of vitamin A in human serum. We observed very good linearity between the fluorescence signal (peak height) and the vitamin A concentration of standard serum samples with known concentrations of vitamin A. The response is linear over the range 0.1–0.6 μg/ml, which is the physiological range in human serum. The detection limit for this technique is approximately 10 ng/ml of serum (or 10 fg of retinol) at a signal-to-noise of 5:1.

In order to make sure the separation conditions to be maintained the same, a fresh serum sample was used as a reference, which was injected after several sample injections.

Comparison of HPCZE with HPLC for vitamin A analysis

As an additional verification of the method, a series of frozen serum samples were analyzed with both conventional HPLC and HPCZE. The results from both techniques are listed in Table I. Linear regression (forced through the origin) of the correspondence between the two methods gave slope 0.981 with standard deviation 0.041 (*i.e.*, not statistically different from 1) with correlation coefficient 0.925. The average coefficient of variation (standard deviation divided by mean) for CZE analysis was 1.5%. However, the CZE method is much faster and easier than the HPLC method.

CONCLUSION

We have developed a reproducible, fast and easy method for the minimicroassay of vitamin A in human serum. The method requires less sample preparation than HPLC and is feasible for finger-prick analysis of vitamin A due to the small sample requirement. This method can easily detect subfemtomoles of vitamin A in human or animal blood, and is, therefore, better suited for vitamin A surveys in clinical chemistry and particularly appropriate in relative dose response assays of human vitamin A status.

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