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Determination of vitamin A in dried human blood spots by high-performance capillary electrophoresis with laser-excited fluorescence detection

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Abstract

We have developed a high-performance capillary electrophoresis (HPCE) method to analyze the retinol (vitamin A) concentration as retinol–retinol binding protein (holo-RBP) from microvolumes of serum (5–10 μl) or one to two drops ($\sim$20 μl) of blood collected and air-dried on blood collection filter paper. A 0.64-cm diameter disk was cut from the dried whole blood specimens and the samples were dissolved in a pretreatment buffer and filtered. Filtrate was injected onto the HPCE column for analysis. The separation was carried out in a 60 cm $\times$ 50 μm I.D. fused-silica capillary and the running voltage was 20 kV. A He–Cd laser with a wavelength of 325 nm was used for excitation, and the fluorescence of the holo-RBP complex was monitored at 465 nm by a photodiode. A virtual linear relationship was obtained for the retinol concentrations between HPCE and HPLC for 28 serum samples, 19 dried venous blood samples and 9 capillary dried blood spot samples, indicating that valid measures of serum retinol can be obtained from one to two drops of capillary blood collected on filter paper. The absolute detection limit for retinol by HPCE is below 3 μg/l. The method is very useful for vitamin A level screening, especially for children and premature new-born babies.

1. Introduction

Severe vitamin A deficiency manifested as xerophthalmia, keratomalacia, is the leading cause of childhood blindness in the world [1]. Even subclinical vitamin A deficiency is associated with an increased risk of childhood morbidity [2–5] and mortality [6]. A meta-analysis of eight intervention trials in Asia and Africa indicates that vitamin A suppletion to deficient children can be expected to decrease mortality rates by 23% among children aged from 6 months to 5 years [7]. These findings demonstrated the importance of the vitamin A level in the human body to human health. Therefore, development of a quantitative method for determining the vitamin A level in small amounts of serum or blood will be very important for nutritional studies and human health screening. It will be important to identify vitamin A de-

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ficient populations and monitor the impact of interventions.

The serum retinol concentration is the most commonly used indicator of vitamin A status. The preferred method for analysis of retinol is high-performance liquid chromatography (HPLC) [8–11]. The method involves collection of at least 200 μl of whole blood, centrifugation within hours of collection, and keeping the samples in the cold during transport and storage. In the laboratory, the serum proteins are precipitated and retinol is extracted with organic solvents. The extracts are separated by HPLC and retinol is detected by UV absorbance or fluorescence. The limitations of this method are: (1) a large amount of serum sample (100 μl or more) is needed, which is often difficult to obtain by capillary sampling and represents a large volume from infants, especially for neonates and low-birth-weight infants; (2) once separated from RBP, retinol is light-, oxygen- and heat-sensitive, increasing the likelihood of error during analysis; (3) processing time is relatively long.

Recently, we have reported a minimicroassay method for the quantitative analysis of retinol as holo-RBP by high-performance capillary electrophoresis (HPCE) [12]. The method is sensitive, fast, requires a sample volume of only 10 μl, and does not require solvent extraction. However, adsorption of the serum proteins and other matrix components on the capillary wall limited the reproducibility of the method. In addition, the method can not be used to analyze retinol in dried blood spots due to capillary blocking by the blood cell membranes.

In this paper, we present a modification of our original method, which optimizes separation conditions and improves reliability. Microvolume serum samples are mixed with a urea-based buffer and filtered prior to injection. Secondly, we present a further modification for the analysis of retinol concentration from whole blood collected and dried on blood collection paper. HPCE results were validated by comparing to values obtained by the conventional HPLC technique. Results from venous blood and capillary blood are also compared.

2. Experimental

2.1. Equipment

All of the experiments were performed under yellow light [19] except when otherwise indicated.

The equipment set-up is similar to that described previously [12]. A Model CZE 1000R high-voltage power supply (Spellman, Plainview, NY, USA) was used to supply the electromotive force across the capillary. 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 60 cm × 50 μm I.D. fused-silica capillary tubing (Polymicro Techniques, Phoenix, AZ, USA) was used for the separation. The polymer coating was burned off at 25 cm from the cathodic end of the capillary to form the detection window. 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 with running buffer. The capillary was ready for use thereafter.

A Model 4240 NB helium–cadmium (He–Cd) laser (Liconix, Santa Clara, CA, USA) operating at 325 nm was used for excitation. A band-pass filter (250–400 nm) (Model UG-11, 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 built-in amplifier (Model HC220-01, Hamamatsu, Bridgewater, NJ, USA). Another band-pass filter (400–539 nm) (Model 35-532, Ealing) was used to isolate the fluorescence (465 nm) from the retinol–RBP complex. The voltage from the photodiode was monitored with an autoranging microvolt DMM (Model 197A, Keithley, Cleveland, OH, USA) and the signal was recorded with a Model C-R3A integrator (Shimadzu, Columbia, MD, USA).

2.2. Reagents and buffers

All chemicals and solvents were of analytical reagent grade or HPLC grade unless stated otherwise. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). All-*trans* retinol standard for HPLC was purchased from Sigma (St. Louis, MO, USA) and was purified by HPLC whenever necessary before use. Retinyl acetate was purchased from Aldrich (Milwaukee, WI, USA)

The electrophoresis running buffer was 50 m*M* Na_2HPO_4, the pH being adjusted to 7.8 with 1.5 *M* H_3PO_4. The running buffer was filtered with a 0.45-μm membrane and degassed before use.

The sample pretreatment buffer was 50 m*M* Na_2HPO_4 with 6 *M* urea and the pH was adjusted to 7.8 with 1.5 *M* H_3PO_4.

2.3. Serum and blood spot preparation

Fasting blood samples were obtained by venipuncture from 19 student volunteers at Northeast Missouri State University and Kirksville College of Osteopathic Medical Center. All procedures were approved by Northeast Missouri State University Internal Review Board.

Venous blood was immediately spotted onto blood collection filter paper (grade 903, Schleicher and Schuell, Keene, NH, USA). This paper absorbs a fixed volume of blood in a given diameter of paper. Specimens were allowed to air dry at room temperature in the dark. Remaining blood samples were allowed to clot at room temperature in the dark prior to preparation of serum by centrifugation. Nine additional serum samples were obtained from a blood bank. From 9 volunteers, capillary blood samples were obtained at the same time that the venous samples were collected. Capillary specimen samples were collected directly onto the filter paper following recommended procedures published by the manufacturer and the National Committee for Clinical Standards [13]. In brief, following finger-prick, the unprinted side of the filter paper was gently touched against the puncture wound, and in one step a sufficient quantity of blood was allowed to soak through to fill the printed circle of the filter paper. We avoided lifting the paper on and off the wound which may have resulted in a layer of blood on the paper, rather than uniform filling of a standardized volume. Each dried blood sample was placed in a separate paper envelope. The envelopes were then sealed in a Ziploc storage bag with desiccant beads and stored at −70°C until analysis.

2.4. Pretreatment of serum and blood spot samples

All samples were pretreated with a 6 *M* urea in phosphate buffer solution. This step results in dissociating transthyretin (TTR) from the TTR–retinol–RBP complex without dissociating the retinol–RBP complex [14,15].

To 10 μl of serum, 200–500 μl (generally 300 μl) of ice-cold sample pretreatment buffer was added. The mixture was mixed well on a vortex-mixer (Fisher, St. Louis, MO, USA). The solution was allowed to stand for ca. 5 min, then a 100-μl volume was taken out and put into a Microcon-30 filter unit (Amicon, Beverly, MA, USA) (this filter unit allows molecules with a molecular mass < 30 kDa to pass through), and filtered by centrifugation. The filtrate was then ready for injection onto the HPCE column for analysis.

For dried blood spot samples, a 0.64-cm diameter disk was cut from the center of the blood spot using an ordinary paper hole punch, and put into a 1.5-ml microcentrifuge tube. Following addition of 200–500 μl (usually 300 μl) of ice-cold sample pretreatment buffer, the mixture was stirred on a vortex-mixer intermittently for about 5 min until the blood spot was completely dissolved in the buffer. Finally 100 μl of the blood spot solution was taken and filtered in a Microcon-30 filter unit by centrifugation in the same way as for serum samples. The filtrates were then ready for HPCE analysis. Pilot work demonstrated that the holo-RBP complex is stable in filtrate for at least 24 h when stored on ice or at 4°C.

2.5. HPCE analysis

Pretreated samples were injected electrokinetically at 10 kV for 5 or 10 s (approximately 5 or 10 nl was injected), and the separation was carried out at 20 kV for 6 min. The mean of 3 injections is reported.

Retinol was quantitated from serum sample by two methods. First, a series of standard sera with known retinol concentration (previously determined by HPLC) were analyzed by HPCE, and a calibration curve was constructed. Second, a series of dilutions was prepared from a single serum sample following pretreatment and filtration, and a calibration curve was constructed. The retinol level of unknown serum could be found from the calibration curve.

Quantitation of the retinol concentration in the dried blood spot samples is performed similarly to that of serum samples, which can also be done in two ways. In the first method, a calibration curve was constructed with a series of standard blood spots, the serum retinol concentration of which was known (analyzed by conventional HPLC method). In the second method, a single blood spot, from blood of known serum retinol concentration (quantified by HPLC) was treated with pretreatment buffer and filtered. Prior to injection, a series of dilutions was prepared from the dried blood sample solution. Each dilution was analyzed by HPCE and a calibration curve was constructed. A blood sample with a higher retinol concentration (~ 600 μg/l) was used so that a broad range of unknown values could be compared against the resulting calibration curve. In our experiment, we used the second method for the quantitation of holo-RBP in the serum and dried blood spot samples.

2.6. Conventional extraction and HPLC analysis of serum retinol

HPLC analysis of serum retinol was carried out following a previously established method [16]. Briefly, to 100 μl of freshly thawed serum an equal volume of methanol containing an internal standard (retinyl acetate) was added; each sample was extracted three times with an equal volume of hexane. Hexane from the combined hexane extracts was evaporated under a gentle stream of argon, and the residue was dissolved in 25 μl of 2-propanol–dichloromethane (4:1, v/v); a 20-μl aliquot was injected for HPLC analysis. A Resolve 5-μm C_{18} column (150 × 3.9 mm I.D.) (Waters Associates, Milford, MA, USA) was used for separation, and the mobile phase was methanol–water (95:5, v/v) at a flow-rate of 1.0 ml/min. The UV absorbance was monitored at 325 nm.

3. Results and discussion

A typical electropherogram of a pretreated blood spot sample is shown in Fig. 1. Peak A was identified as the retinol–RBP complex peak by injecting pure holo-RBP standard. Peak 1 was identified as a component of the Microcon-30 filter unit, because the same peak resulted following injection of filtered sample pretreatment

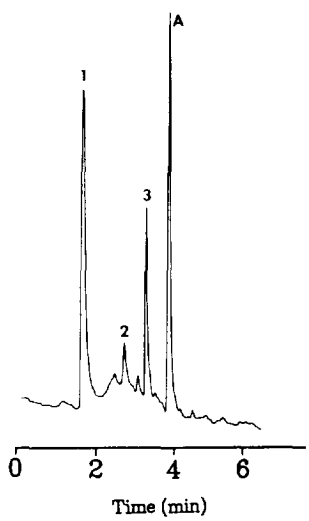

Fig. 1. Electropherogram of pretreated dried blood spot. Peak A = retinol–RBP complex. Running buffer: 50 m*M* phosphate, pH 7.8. A 10-s injection at 10 kV was followed by electrophoresis at + 20 kV for 6 min in a 60 cm × 50 μm I.D. capillary column. Excitation wavelength: 325 nm; fluorescence was monitored at 465 nm. Peak 1 originated from the microcon filter unit, peaks 2 and 3 originated from the non-serum fraction of the blood.

buffer. Compared with pretreated serum samples, all the pretreated blood spot samples showed an additional peak (peak 3) which eluted before the retinol-RBP peak (peak A). Some blood spot samples showed another small peak (peak 2). Since peaks 2 and 3 were not observed in any of the serum samples, we concluded that they originated from the non-serum fraction of the blood.

In order to quantitate retinol in dried blood spots, we performed a recovery study from 903 specimen collection papers. A known volume of serum was spotted on the paper. After being dried, the whole spot was cut out and treated in the same way as the blood spots and analyzed by HPCE. Simultaneously, a identical volume of serum was taken and analyzed by HPCE without spotting on filter paper. The recovery was calculated based on the peak area. The recoveries of seven serum samples ranged from 89 to 113%.

Table 1 presents values for the retinol concentration obtained by HPLC from 28 venous serum samples, HPCE from 28 venous serum samples and 19 venous dried whole blood sam-

Table 1
Comparison of serum retinol (ROL) concentration (μg/l) as determined by HPLC and HPCE for serum and blood spots from the same person at the same time

Sample ID[a]	ROL (μg/l) by HPLC[b] Venous serum	ROL (μg/l) by HPCE[c]		
		Venous serum	Venous blood spot	Capillary blood spot
1M	738	678	737	
2F	352	354	390	
3F	392	379	398	
4M	494	497	551	
5F	620	356	338	
6M	780	762	793	
7F	496	527	530	
8M	594	563	588	
9M	596	600	650	
10M	578	605	607	
11M	555	550	516	530
12F	595	577	641	605
13M	674	676	715	694
14M	535	531	550	543
15F	423	438	448	437
16M	620	616	565	580
17M	968	761	745	750
18M	683	682	647	631
19M	714	695	683	680
20	259	248		
21	536	506		
22	421	416		
23	570	621		
24	435	464		
25	711	685		
26	446	442		
27	250	273		
28	637	634		

[a] M = male; F = female. Samples 20–28 were obtained from the blood bank.
[b] Average of 4 measurments; S.D. = ± 0.3– ± 0.8 (S.D. = standard deviation).
[c] Average of 3 measurements; S.D. = ± 0.7– ± 1.1.

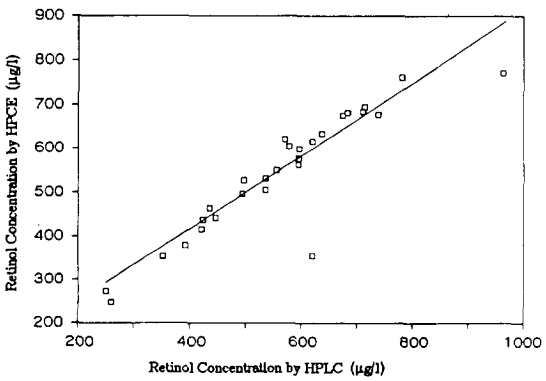

Fig. 2. Correlation of serum vitamin A level as determined by HPLC and HPCE for 28 serum samples.

ples and HPCE from 9 capillary whole blood spot samples. Fig. 2 illustrates the correlation of the values for the retinol concentrations of 28 serum samples as determined by HPLC and by HPCE. A virtual linear relationship was obtained for 28 samples ($r = 0.99$ for 26 samples, $r = 0.91$ for all 28 samples).

Fig. 3 illustrates the excellent correlation between retinol values obtained by HPCE from dried blood spot samples and by HPLC from serum for the 19 samples assessed by both methods ($r = 0.97$ for 17 samples). The two samples not falling on the regression line were from the same two individuals whose serum retinol concentrations did not correspond. The relationship between the venous blood serum

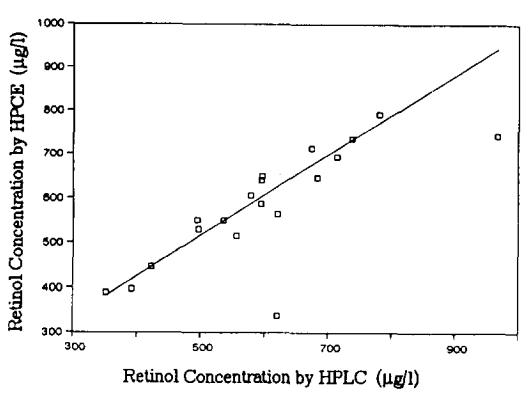

Fig. 3. Correlation between serum retinol concentration measured in venous blood by HPLC and in venous dried blood spots by HPCE for 19 blood samples.

retinol concentration as analyzed by HPLC and the retinol concentration in dried capillary blood spot as analyzed by HPCE was also virtually linear ($r = 0.92$).

The linearity has been studied from the standard dilution of one single blood spot with known serum retinol concentration (quantified by HPLC). The linear relationship of the holo-RBP fluorescence intensity as determined by HPCE versus retinol concentration maintains over one order of magnitude which could cover the whole range of retinol concentrations in serum ($r^2 = 0.997$). It should be noted that the absolute retinol concentration injected onto the HPCE column was much lower than that mentioned on all figures due to buffer dilution during the sample pretreatment. The absolute retinol concentration range actually detected by HPCE was 3.3–~35 µg/l. The absolute detection limit of the HPCE assay is lower than 3 µg/l.

This technique measures the holo-RBP complex which normally accounts for 90% of the total vitamin A in circulation. Under certain conditions, a greater fraction of the retinol in the circulation may be bound to other proteins [15,17], or to lipoproteins in some special case [18,19], and would not be detected by HPCE. In the present study, the retinol concentration as determined by HPCE was lower than that obtained with HPLC in two samples. In one case (sample no. 5F), the electropherogram (Fig. 4) of the sample was different from that of the other samples. An additional peak (peak X) was found at a migration time longer than that of the retinol–RBP peak (peak A). This could be a peak of retinol complexed with another protein, or a different form of RBP [15]. In the second case (sample no. 17M), the electropherogram was similar to that of the other samples, but the retinol concentration as determined by HPLC was ca. 90% of the serum retinol concentration found in adult males. It is possible that at high-normal retinol concentration, a lower fraction of the total circulating retinol is complexed with RBP compared with lower retinol concentrations.

Inherent to our method of quantitating retinol in whole blood samples is the assumption that

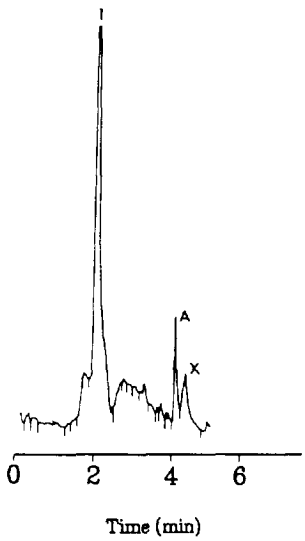

Fig. 4. Electropherogram for the serum sample (no. 5F) showing a lower level than that obtained by HPLC. The electrophoretic conditions were the same as for Fig. 1. Peak X has yet not been identified.

the red blood cell volume in unknown samples is equivalent to that of the samples used to construct the calibration curve. Since this will not be true for all specimens, this assumption is a source of error. This issue is under further investigation. However, for most samples, the magnitude of the error will be small. For example, if the retinol concentration in an unknown whole blood sample is 150 μg/l and the percent hematocrit of the sample used to construct the calibration curve is 35%, the calculated serum retinol concentration of the unknown sample would be 231 μg/l. If, in fact, the hematocrit of the unknown was 30–40%, the true serum retinol concentration would be 214–250 μg/l, respectively, and the error would be $\pm$ 7–8%. Theoretically, the error is small compared to the errors induced by sample extraction and instrumental analysis processes. Therefore, the fluctuations of the hematocrit among samples are not considered in this paper. However, as the technique is developed, we plan to choose a reference blood spot with a hematocrit concentration which is similar to that of the population being assessed.

On the other hand, since HPCE can only detect RBP-bound retinol and HPLC detects the total retinol, some vitamin A related diseases could be studied by combining the HPLC and HPCE techniques.

The stability of retinol in the dried blood spot, optimum conditions of handling and storage of blood spots are under investigation.

4. Conclusions

An HPCE technique for analyzing the retinol level in microvolumes of sera and dried whole blood spots has been developed. The main advantages of this method compared to conventional HPLC are its greater sensitivity with much lower sample volume requirements, its speed, and the ease in sample handling, transportation and preparation. From the preliminary data obtained, valid measures of the serum retinol concentration can be determined from one to two drops of capillary blood collected and dried on filter paper. It will be especially beneficial in analyzing the vitamin A level for infants and new-born babies. It could hopefully be developed into a fast routine vitamin A survey technique in the clinical laboratory.

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