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Rapid Measurement of Retinol, Retinal, 13-*Cis*-Retinoic Acid and All-*Trans*-Retinoic Acid by High Performance Liquid Chromatography

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ABSTRACT

An isocratic liquid chromatographic method has been developed for rapid measurements of retinol, retinal, all-*trans*-retinoic acid and 13-*cis*-retinoic acid. Using 85% methanol and 15% 0.01 M sodium acetate buffer, pH 5.2, as the mobile phase and at a flow rate of 1.5 mL/min, retinol, retinal, all-*trans*-retinoic acid and 13-*cis*-retinoic acid were eluted from a 5 μm C18 reversed-phase column (4.6 mm I.D., 15 cm) in less than 14 min. Retinyl acetate, when desired, was eluted in 25.5 min under the above conditions. Each compound was detected at the most sensitive wavelength by coupling fluorescence and UV detection.

Key words: retinol, retinal, retinoic acid, HPLC

INTRODUCTION

Vitamin A is essential for normal vision, epithelial tissue growth and differentiation, bone growth, immune function, reproduction and embryonic development. Three different forms of vitamin A (retinol, retinal and retinoic acid) are active in the body with their own physiological roles[1-5]. Studies of the pharmacokinetics and metabolism of retinol and various natural and synthetic retinoids are essential to elucidate the mechanisms by which they exert their chemotherapeutic and chemopreventive effects, as well as their various cellular functions[5-8]. These studies require analytical techniques capable of distinguishing between closely related compounds. The separation, identification and quantitation of retinol and its metabolites, retinal and retinoic acid, are most commonly done using reversed phase high performance liquid chromatography (HPLC) because these compounds have a wide range of polarity[9-11]. Among the methods suggested, some involve the use of gradient elution, which requires additional time for column re-equilibration. Available isocratic methods require relatively long retention times (up to 40 min) in order to separate retinol and its metabolites. Attempts to shorten the retention times often result in incomplete separation of the compounds.

We have developed a rapid reverse phase HPLC method for the simultaneous measurement of retinol, retinal, all-*trans*-retinoic acid and 13-*cis*-retinoic acid. The method employs an acidic buffered mobile phase with isocratic elution and allows separation and detection of these compounds at the most sensitive wavelength in less than 14 min.

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MATERIALS AND METHODS

I. *Reagents*

HPLC grade hexane and methanol were purchased from EM Science (Gibbstown, NJ, USA). Ethanol (95%) was obtained from AAPER Alcohol and Chemical (Shelbyville, KY, USA). All-*trans*-retinal, all-*trans* and 13-*cis*-retinoic acids, retinol acetate and sodium acetate were purchased from Sigma Chemical (St. Louis, MO, USA). All-*trans*-retinol was a gift from Hoffmann-La Roche (Nutley, NJ, USA). Glacial acetic acid was purchased from Fisher Scientific (Fairlawn, NJ, USA).

II. *Preparation of Standards*

Stock solution (10 μg/mL) was prepared in methanol and stored at -20°C. Fresh stock solutions were prepared weekly and protected from light. Working standards of 0.1-5.0 μg/mL (or 2-100 ng per measurement) were prepared daily.

III. *High Performance Liquid Chromatography*

Samples, continuously protected from light, were injected into the HPLC system by a TosoHaas TSK-6080 sample processor (TosoHaas, Philadelphia, PA, USA) equipped with a 20-μL sample loop. A Beckman 112 solvent delivery module (Beckman Instruments, Fullerton, CA, USA) was used to deliver the mobile phase, which was 85% HPLC grade methanol and 15% 0.01 M sodium acetate buffer, pH 5.2, at a flow rate of 1.5 mL/min at room temperature. A Waters 490 programmable multi-wavelength detec-

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tor (Waters Chromatography Div., Milford, MA, USA), and a Kratos FS 970 LC fluorometer (Kratos Analytical Instruments, Ramsey, NJ, USA) were connected in series. The Waters detector was programmed to monitor 343 nm for 8 min in order to detect retinoic acids, then shifted to 400 nm for the remainder of the run to detect retinal. Retinol and retinyl acetate were detected with the fluorometer using excitation wavelength of 348 nm and emission wavelength of 470 nm. An Alltech 5 μm C18 column (Alltech Assoc., Deerfield, IL, USA; 15 cm × 4.6 mm I.D.) was used along with a 4 cm C18 guard column. Peaks were recorded using Shimadzu CR3A and CR601 Chromatopacs (Shimadzu Scientific Instruments, Columbia, MD, USA).

RESULTS

Typical chromatograms of a mixture of 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol, retinal and retinyl acetate are shown in Figure 1. Under the conditions specified, retention times were 4.5, 5.7, 11.0, 12.5 and 25.5 min for 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol, retinal and retinyl acetate, respectively.

Standard curves for retinol and retinyl acetate (0.1 to 5.0 μg/mL, or 2 to 100 ng/measurement) are shown in Figure 2, and those for 13-*cis* and all-*trans*-retinoic acid and retinal (0.2 to 10.0 μg/mL, or 4 to 200 ng/measurement) are shown in Figure 3. Standard curves are linear for the concentrations employed for each compound. The coefficients of variation for these 5 compounds ranged between 1.2% to 8.3% (n = 6; increasing values with decreasing quantities). Lower limits of accurate measurement were approximately 2.5 ng for retinol and 13-*cis* and *trans*-retinoic acid and 5.0 ng for retinal and retinyl acetate under the experimental conditions using a 20-μL sample loop.

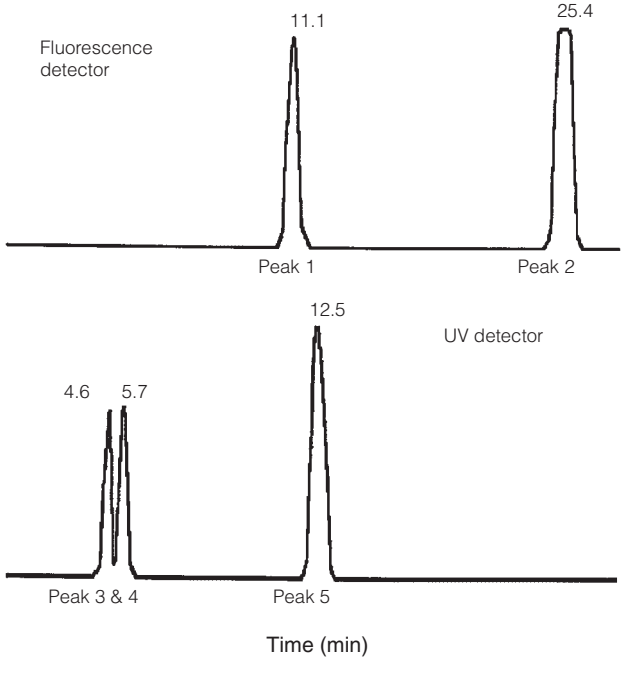

Figure 1. Typical chromatograms of 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol, retinal and retinol acetate mixture. The retention time is 11 min for retinol (peak 1), 25.5 min for retinyl acetate (peak 2), 4.5 min for 13-*cis*-retinoic acid (peak 3), 5.7 min for all-*trans*-retinoic acid (peak 4) and 12.5 min for retinal (peak 5). Experimental conditions: 15 cm C18 reversed-phase column; flow rate: 1.5 mL/min; mobile phase: 85% methanol/15% 0.01 M Na acetate buffer, pH 5.2; UV detector monitored at 343 nm for first 8 min, then 400 nm; fluorescence detector excitation at 348 nm and emission at 470 nm.

DISCUSSION

There are a variety of HPLC methods for measuring

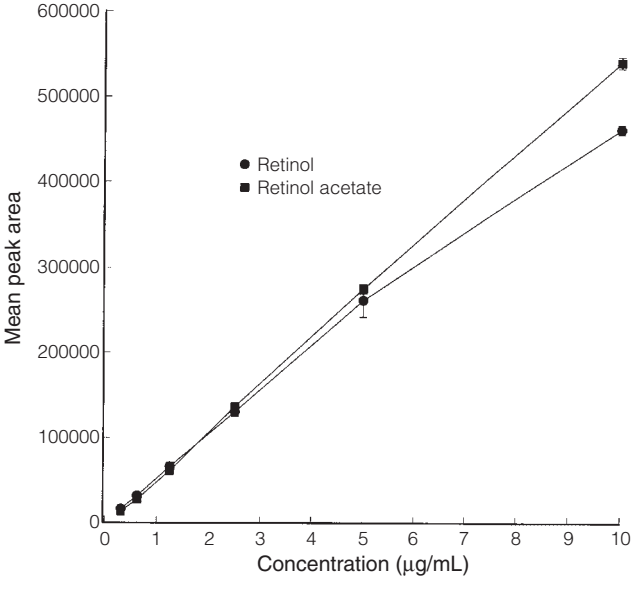

Figure 2. Standard curves for retinol and retinol acetate. See Figure 1 for conditions.

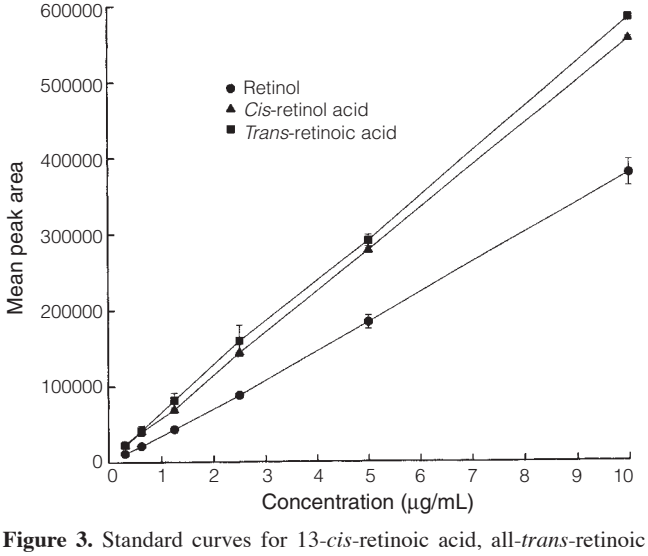

Figure 3. Standard curves for 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinal. See Figure 1 for conditions.

retinol and its metabolites[9-21]. In order to improve resolution, some investigators have adopted systems resulting in lengthy retention times, making the measurement of large numbers of samples impractical. Other researchers have resorted to measure various retinoids in multiple runs by employing both normal and reversed-phase systems, or measuring polar and apolar retinoids separatedy[13,19]. The method described here allows simultaneous measurement of retinol and its major metabolites in a relatively short run time. Satisfactory resolution of retinol and retinal is accomplished in this method by the coupling of fluorescence and UV detectors. This method allows the exploitation of the flourescence properties of retinol, which are not shared by retinal, and the absorbance at 400 nm by retinal, which is not shared by retinol. Thus, although retinol and retinal elute closely in this system, quantitation of the individual peaks is accurate at all concentrations tested. The use of dual detectors also has the advantage of allowing each compound to be detected at its optimal wavelength. Under the experimental conditions described with a 20-μL sample loop, the lower limits of quantitation for each compound were approximately 2.5-5.0 ng. The sensitivity of the measurement can be easily increased by using a larger sample loop as needed. For example, as low as 0.5 ng of retinol, and 13-*cis* and *trans*-retinoic acid, and 1.0 ng of retinal and retinyl acetate can be accurately measured using a 100-μL sample loop.

Separating isomers of retinoic acid by reversed-phase chromatography is influenced by pH and chromatographic characteristics of charged particles. In this procedure, 13-*cis* and all-*trans*-retinoic acids are satisfactorily resolved and moved away from the solvent front by employing a mobile phase buffered to a pH of 5.2. The lowered pH facilitates the separation of both *cis* and *trans*-retinoic acids and retinal. Under these conditions, 13-*cis* and all-*trans*-retinoic acid, retinal and retinol are resolved in less than 14 min. When desired, this method provides effective resolution, detection and quantitation for retinyl acetate with a total run time of approximately 26 min. To minimize undesirable changes, fresh stock solutions should be prepared weekly and protected from light as much as possible.

The method developed in this study is ideal for pharmacokinetic studies of retinoids. Accurate quantitation of retinol and retinyl esters in tissues or biological fluids can also be achieved easily using the present procedure following extraction with apolar solvents. However, retinol is not easily detectable in biological samples partly due to its conversion to retinal and retinoic acid, and the latter has a different polarity[18,22-25]. Therefore, different extraction procedure is required to measure retinal, retinal and retinoic acid in biological fluids[15-21,25,26]. Using a solid phase extraction technique, Schmidt *et al.*[18] effectively separates the apolar (retinyl esters, retinol and retinal) and polar (retinoic acid isomers) retinoids from tissues and serum samples prior to HPLC detection[26]. Therefore, following the solid phase extraction, both the polar and apolar retinoids in biological samples can be measured subsequently by this method.

In summary, a simple and rapid isocratic method has been developed for the simultaneous measurement of retinol and its metabolites. The procedure employs a buffered mobile phase and combination of fluorescence and UV detection to achieve rapid and sensitive quantitation of 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol and retinal.

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