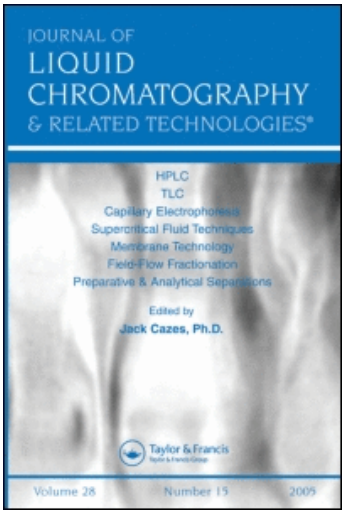

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Simultaneous Determination of Retinoic Acid, Retinol, and Retinyl Palmitate in Ram Plasma by Liquid Chromatography

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ABSTRACT

A liquid chromatographic method is described for the simultaneous determination of retinoic acid, retinol, and retinyl palmitate in ram plasma. Samples of 0.2 mL are extracted with 2-propanol-dichloromethane, the extracts are centrifuged, the supernatants are collected, evaporated, reconstituted in mobile phase, and analyzed on a C_8 column using two consecutive isocratic elutions with methanol tetrahydrofuran-acetate buffer. Detection is performed at 350 and

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325 nm using wavelength change during the run. The method exhibits analytical characteristics well within acceptable limits. Overall recoveries were 73.7% for retinoic acid, 90.2% for retinol, and 87.7% for retinyl palmitate. Precision values, expressed as % relative standard deviation, were in the range of 1.16–6.18%, while limits of detection were in the range of 0.3–10.0 ng/mL for all analytes.

Key Words: Retinoic acid; Retinol; Retinyl palmitate; Ram plasma; Liquid chromatography.

INTRODUCTION

Vitamin A, or retinol, is an essential micronutrient for animals and humans that plays a major role in a variety of physiological processes including vision, reproduction, cell growth and differentiation, and immune modulation.[1] Its potential role as a potent antioxidant in modifying the risk for conditions that may result from oxidative stress has recently stimulated further research efforts, increased interest in micronutrient supplements, and heightened consumer concern in vitamin A and its metabolites.[2]

Although, over 90% of the total body reserve of vitamin A is stored in the liver,[3] retinol occurs in blood plasma in high concentrations, whereas retinyl palmitate, the predominant esterified form of retinol, and retinoic acid, the major product of retinol metabolism, are both present in plasma in lower concentrations.[4] Thus, measuring retinol concentrations in plasma is a common method to assess vitamin A status. However, retinol is subject to homeostatic control and even in slightly deficient conditions, the concentrations of retinol are not significantly altered. As the interpretation of retinol concentrations alone can be difficult under such conditions, simultaneous determination of retinol, retinyl palmitate, and retinoic acid in plasma can be used as a better approach.

Several liquid chromatographic methods have been published for the determination of retinol,[5–9] retinol and retinyl palmitate,[10–13] retinoic acid,[14] and retinol and retinoic acid[15–17] in plasma or serum. Very few methods, however, allow simultaneous determination of retinoic acid, retinol, and retinyl palmitate[18] in plasma or serum. This is probably due to the difficulty in extracting and analyzing compounds of different polarity. Methods that are available for the analysis of retinoic acid do not usually allow simultaneous analysis of the less polar retinyl palmitate, whereas methods that can analyze retinol and retinyl palmitate cannot be used to separate the more polar retinoic acid. The huge difference in polarity between retinoic acid and retinyl palmitate makes it difficult to obtain satisfactory

chromatographic separation in a reasonable amount of time. Moreover, extraction has to be equally efficient for the removal from the sample of both very polar and hydrophobic compounds. Apart from these, analysis of vitamin A and its metabolites in biological samples constitutes, generally, a challenging analytical problem, due to low endogenous concentrations, sensitivity of the compounds in light and heat, and strong protein binding.

In investigating the effect of vitamin A on the fertilizing ability of rams, we developed a liquid chromatographic method that allows simultaneous determination of retinoic acid, retinol, and retinyl palmitate in the plasma of rams. The primary target in developing this method was to minimize the time, labor, glassware, and cost of the materials required. The performance of the developed method was evaluated in terms of precision, accuracy, linearity, and sensitivity.

EXPERIMENTAL

Instrumentation

Liquid chromatography was carried out on a Hewlett-Packard system (HP1100, Agilent Technologies, Palo Alto, CA) consisting of a G1311A quaternary pump, a G1322A vacuum degasser, a Rheodyne, Model 7725, an injection valve (Cotati, CA) equipped with a 100 μL loop, and a G 1314A variable wavelength detector.

Reagents

LC grade methanol and tetrahydrofuran were purchased from Malinckrodt Baker BV (Deventer, Holland). LC grade dichloromethane was purchased from Labscan Ltd (Dublin, Ireland), whereas 2-propanol from Merck (Darmstadt, Germany). LC grade water was produced from tap water using a USF ELGA Purelab Plus purification system (Ransbach-Baumbach, Germany).

All trans-retinoic acid and all trans-retinyl palmitate were purchased from Sigma (St. Louis, MO), all trans-retinol from Fluka (Buchs, Switzerland).

Chromatographic Conditions

LC analysis was performed at ambient temperature on a reverse phase MZ column (Mainz, Germany), 250×4.0 mm, packed with Hypersil MOS, C_8, 5 μm. The mobile phase consisted of methanol containing 0.1 M sodium

acetate and 0.01 M acetic acid (solvent A), water containing 0.1 M sodium acetate and 0.01 M acetic acid (solvent B), and tetrahydrofuran (solvent C). At the beginning of each run, the composition of the mobile phase was set at 87% solvent A, 12.9% solvent B, and 0.1% solvent C, and the elution was maintained isocratic for 9 min. A short linear gradient from the initial mobile phase composition to 94% solvent A, 1% solvent B, and 5% solvent C, was applied between 9 and 9.5 min, followed by isocratic elution for an additional 10 min. The flow rate of the mobile phase was set at 1 mL/min.

At the end of each run, a 3 min column washing was performed using a solvent system consisting of 69% solvent A, 1% solvent B, and 30% solvent C. Following column washing, the composition of the solvent system was reversed to the initial conditions and the column was allowed to equilibrate for 10 min before next injection.

Detection of all three analytes was made at their absorbance maximum, which for retinol and retinyl palmitate was at 325 nm, and for retinoic acid at 350 nm. Thus, the detector wavelength was programmed at 350 nm from 0 to 8.5 min of run, and at 325 nm thereafter.

Laboratory Precautions

To avoid isomerization and degradation of the analytes,[19,20] all handling and experimental procedures including preparation of standard solutions, sample treatment, and chromatography were carried out under fluorescent lighting of moderate intensity, eliminating all sources of daylight from the laboratory, while vitamin A and its metabolites were being analyzed.

Preparation of Standard Solutions

Stock standard solutions of retinoic acid, retinol, and retinyl palmitate were prepared by individually weighing 5 mg of each compound in 10-mL volumetric flasks and dissolving to volume with methanol. Intermediate standard solutions were prepared by transferring an aliquot from each stock solution in 25-mL volumetric flasks and diluting to volume with methanol. Stock and intermediate standard solutions were flushed with nitrogen and stored at -20°C.

A concentrated mixed working standard solution containing 30 ng/mL retinoic acid, 3000 ng/mL retinol, and 300 ng/mL retinyl palmitate was prepared by transferring aliquots from intermediate solutions in a 100-mL volumetric flask and diluting to volume with the mobile phase (methanol–water–tetrahydrofuran, 94 : 1 : 5, v/v/v). Serial dilutions of the concentrated mixed working standard

solution with the mobile phase resulted in six mixed working solutions containing retinoic acid in the range 2–30 ng/mL, retinol in the range 200–3000 ng/mL, and retinyl palmitate in the range 20–300 ng/mL. Working standard solutions were stored in the dark at 4°C and prepared fresh weekly.

A spiking solution for the precision and accuracy studies was prepared by transferring an aliquot from each intermediate standard solution into a 10-mL volumetric flask and diluting to volume with methanol.

Analytical Procedure

A measured volume of plasma sample (200–1000 μL), the volume depending on the expected level of the analytes in the examined sample, was placed in a 5-mL centrifuge tube where three volumes of an extraction solvent mixture consisted of 2-propanol and dichloromethane (2 : 1, v/v) were also added. The content of the tube was vortexed for 30 s and centrifuged at 3000 g for 5 min. Following centrifugation, the supernatant solution was transferred into another tube, evaporated to near dryness under nitrogen stream. The residue was dissolved in 100 μL of tetrahydrofuran and methanol (15 : 85, v/v), made up to 300 μL with methanol, and an aliquot of 100 μL was injected into the LC.

Determination

Calibration curves were generated by running working standard solutions, plotting recorded peak heights vs. the corresponding concentrations of the analytes injected, and computing slope, intercept, and least squared fit of standard curves. The HP Chemstation software was used for data analysis. Calibration curve slopes and intercept data were used to determine the concentration of the analytes in plasma extracts.

RESULTS AND DISCUSSION

Extraction

The classical procedure for extracting retinoids from serum or plasma is by use of a water immiscible solvent, usually hexane, after protein precipitation with ethanol. Application of this procedure in our samples gave very good recoveries for retinol, good for retinyl palmitate, and poor for retinoic acid. The results did not change when ethanol was substituted with acetonitrile and when hexane was substituted with petroleum ether or chloroform.

A possible reason for this poor recovery could be the fact that retinoic acid, in contrast to retinol and retinyl palmitate that are insoluble in water, is rather water

soluble.[21] Therefore, retinoic acid would not be extracted efficiently when water-immiscible solvents were used. As far as the lower recovery of retinyl palmitate compared to that of retinol is concerned, this is not in agreement with some previous results, suggesting that hexane extracted retinol with recoveries in the range 94–101% from human serum[12,22–23] and retinyl esters with 100% recovery.[3] It lends support, however, to other reports[24] suggesting that hexane was most efficient in the extraction of retinol from rat serum, while ethyl acetate gave seven times higher recovery of retinyl palmitate, compared to hexane.

Since water immiscible solvents did not work well with our analytes, extraction with water miscible solvents was investigated as an alternative. Extraction with water miscible solvents could precipitate proteins, liberate the analytes, and give quantitative recoveries if composition and volume were properly adjusted. Using a mixture of 2-propanol and tetrahydrofuran (2 : 1, v/v), we found that all three analytes could be acceptably extracted from plasma samples. However, the extraction efficiency of this mixture was found to be lower than that of the mixture of 2-propanol and dichloromethane (2 : 1, v/v) used by Barua and Olson[18] for extracting retinol, retinyl palmitate, and retinoic acid from human or rat serum. Thus, the extraction solvent suggested by Barua and Olson[18] was finally adopted. Adding 10 μL of glacial acetic acid per 100 mL of plasma, as suggested by Barua and Olson,[18] did not improve the recovery of retinoic acid in our system, so this step was not applied.

A complication arose when reconstituting the residue after the extract evaporation, since small volumes of mobile phase (methanol–water–tetrahydrofuran, 94 : 1 : 5, v/v/v) could not redissolve the residue completely, affecting the recovery, particularly, of retinyl palmitate. A solvent mixture of tetrahydrofuran and methanol (15 : 85, v/v) gave complete redissolution but distorted the peak shapes. As a successful compromise, we carefully dissolved the residue in 100 μL of a solvent mixture of tetrahydrofuran and methanol (15 : 85, v/v) and made up the solution to 300 mL with methanol.

Liquid Chromatography

Despite continuous development and improvement of analytical equipment and LC columns, the chromatographic determination of vitamin A and its metabolites in biological samples still represents a considerable challenge. This is mainly due to the low concentrations of the analytes combined with their wide polarity range and their instability towards daylight, temperature, and oxygen.

Several reversed-phase chromatographic procedures have been described for the separation of vitamin A and its metabolites,[25] which are mostly based on gradient elution[10,12,16,18,26,27] and only a very few allow isocratic separation of the analytes.[3,13,17] Since the majority of these are based on the

use of C_{18} stationary phases with acetonitrile–water or methanol-water mobile phases, we initially tested such LC systems for setting up an isocratic system suitable for the analysis of retinoic acid, retinol, and retinyl palmitate.

Using a mobile phase containing 93% methanol, a solvent found to be more effective than acetonitrile for eluting the analytes, retinoic acid was eluted at 2.9 min, retinol at 3.8 min, and retinyl palmitate at 26.2 min. In an attempt to increase the retention time of retinoic acid, which was eluted quite near to the solvent front, we decreased the elution strength of the mobile phase by lowering its methanol content. With this change, the peak of retinoic acid was removed from the solvent front but the retention time of retinyl palmitate increased unacceptably. Since there was no mobile phase to isocratically elute all analytes in a reasonable amount of time, we tested various gradient elution systems. Barua and Olson[18] have described a linear gradient from methanol–water (3 : 1, v/v) to methanol–dichloromethane (4 : 1, v/v) over a period of 15 min followed by isocratic elution for an additional 15 min to elute retinoic acid at 10.5 min, retinol at 12.9 min, and retinyl palmitate at 24.1 min.

By applying a linear gradient starting with 82% methanol and ending in 99% methanol in water within 25 min, retinoic acid was eluted at 6.9 min, retinol at 8.4 min, and retinyl palmitate at 21.5 min. In order to achieve a similar retention profile without applying any gradient elution, we added in the mobile phase tetrahydrofuran, a non polar but water miscible solvent, that could greatly increase the mobile phase strength. By this addition, retinoic acid could be eluted isocratically at 6.8 min with a mobile phase containing 87% methanol, 12.9% water, and 0.1% tetrahydrofuran, whereas retinol and retinyl palmitate could also be eluted isocratically at 11.2 and 17.2 min, respectively, with a mobile phase containing 94% methanol, 1% water, and 5% tetrahydrofuran (Fig. 1). With this system, however, the mobile phase had to change linearly between 9.00 and 9.50 min from the starting to the ending composition.

To suppress the ionization of retinoic acid, an acetate buffer was used. Without the acetate buffer, large variations in the retention time of retinoic acid was observed, obviously due to the different degree of ionization of the analyte depending on the pH of each individual sample. Considering that the pK_a of retinoic acid is between 6 and 8, which is dependent on the medium,[28] buffering the mobile phases at pH 5 with a mixture of 0.1 M sodium acetate and 0.01 M acetic acid was, therefore, sufficient to suppress ionization.

Detection

Retinol and retinyl palmitate exhibit a maximum of absorbance at 325 nm, whereas retinoic acid has a maximum at 350 nm.[25] In a method for the determination of retinoic acid and retinol in human plasma,[15] a detection

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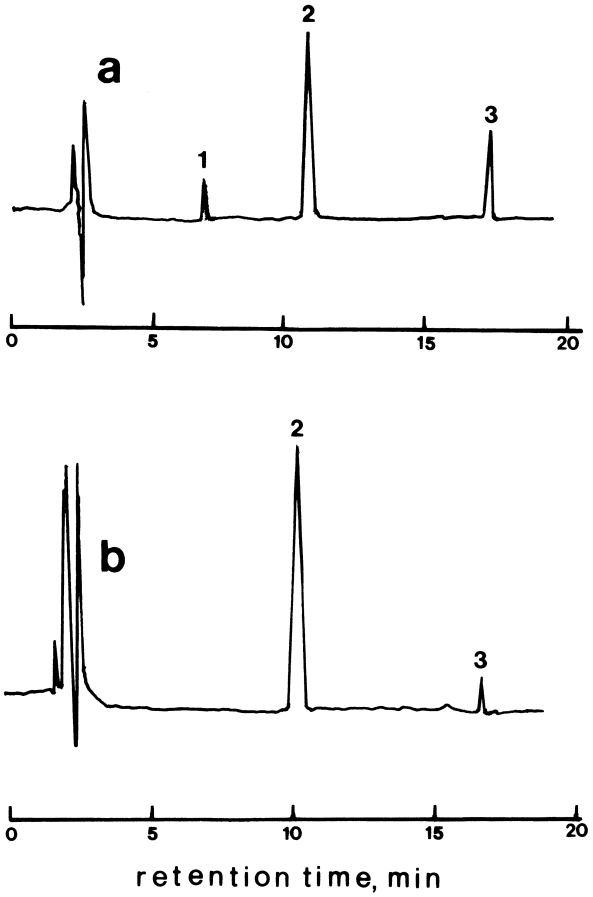

Figure 1. Typical chromatograms of a standard solution (a), and a ram plasma sample (b) containing 198.1 ng/mL retinol and 16.2 ng/mL retinyl palmitate. Peak identification: retinoic acid (1); retinol (2); and retinyl palmitate (3).

wavelength of 350 nm was selected because retinol occurs in plasma in relatively high concentrations. The very low concentrations of retinoic acid in human plasma, under physiological situations, would make its quantification difficult if 325 nm was the wavelength of choice.

In our system, peak heights of retinoic acid were found to be 13% higher when measured at 350 nm than 325 nm. Therefore, in order to increase the sensitivity of the assay, the detector was set at 350 nm from the start to 8 min of the run, and at 325 nm thereafter.

Calibration Curves and Detection Limits

The linearity of the absorbance response of the system with the amounts of the analytes injected was evaluated in the range of 0.2–3 ng for retinoic acid, 20–300 ng for retinol, and 2–30 ng for retinyl palmitate. Regression analysis of the data obtained by running 100-μL injections, in triplicate, from all six working standard solutions showed the response to be linear for all analytes in the range examined (regression equation for retinoic acid in the range 2–30 ng/mL: $y = -0.002 + 0.027x$, $r^2 = 0.9994$; regression equation for retinol in the range 200–3000 ng/mL: $y = -0.098 + 0.018x$, $r^2 = 0.9999$; regression equation for retinyl palmitate in the range 20–300 ng/mL: $y = 0.116 + 0.029x$, $r^2 = 0.9989$, where y represents peak height in mAU and x the concentration in ng/mL of the analyte).

The efficiency of the chromatographic system could allow low limits of detection to be realized in plasma samples. The detection limits that corresponded to concentrations yielding signals that could be clearly distinguished from the signals obtained from the blank (signal-to-noise ratio of at least 3 : 1) were estimated at 0.3 ng/mL for retinoic acid, 6.0 ng/mL for retinol, and 10.0 ng/mL for retinyl palmitate. These limits of detection are comparable to, or better, than those reported earlier.[25]

Accuracy, Precision, and Linearity

To study the accuracy and the precision of the method, a standard addition procedure was evaluated. Plasma samples from five rams fed vitamin A deficient diets were collected and pooled. Then, 25 subsamples of 0.2 mL were taken and divided into five groups of five samples each. Four of the groups were spiked with retinoic acid, retinol, and retinyl palmitate, each at a different fortification levels by adding 5, 10, 15, and 20 μL of a methanolic spiking solution, and all five groups were submitted to analysis by the newly developed method. The fortification range was 1.0–8.3 ng/mL for retinoic acid, 458.3–3666.7 ng/mL for retinol, and 29.8–238.1 ng/mL for retinyl palmitate. Tables 1, 2, and 3 present the analytical results and the computed precision and accuracy data of the analysis of all trans-retinoic acid, all trans-retinol, and all trans-retinyl palmitate in ram blood plasma.

Least-squares and regression analysis[29] of the data (Tables 1, 2, and 3) based on the four-level spiking, showed that the relationships between "added" (x) and "found" (y) analyte were adequately described by linear regression equations of the type $y = ax + b$, where y is the amount recovered and x the amount added. ($y = 0.107 + 0.737x$, $r^2 = 0.9985$, for retinoic acid; $y = 161.2 + 0.902x$, $r^2 = 0.9998$, for retinol; $y = 3.9 + 0.877x$, $r^2 = 0.9999$, for

Table 1. Precision and accuracy data for the determination of all trans-retinoic acid in ram plasma.

Mean[a] endogenous conc., ng/mL	Fortification level, ng/mL	Mean[a] conc. found, ng/mL	RSD, %	Mean[a] recovery, %
0	1.0	0.8 ± 0.03	3.89	77.2 ± 3.0
0	2.1	1.6 ± 0.06	3.66	78.8 ± 3.1
0	4.2	3.3 ± 0.04	1.21	79.4 ± 1.0
0	8.3	6.2 ± 0.38	6.15	74.4 ± 4.6

[a]Mean of five replicates $\pm$ SD.

retinyl palmitate). Therefore, the slopes (0.737 for retinoic acid, 0.902 for retinol, and 0.877 for retinyl palmitate) of these regression lines could be used as estimates of the overall recoveries (73.7% for retinoic acid, 90.2% for retinol, and 87.7% for retinyl palmitate) of the developed method.

Since the intercepts of the regression lines, which are actually the values in ng/mL that are predicted for the unspiked samples, were not significantly different ($p > 0.05$) from the arithmetic means of the unspiked samples (161.2 vs. 177.9 for retinol; 3.9 vs. 3.7 for retinyl palmitate) it might be concluded that interferences were not present in analyzed samples. The absence of interference permitted accuracy evaluation to be made by using the data from both the spiked and the unspiked samples.

Table 2. Precision and accuracy data for the determination of all trans-retinol in ram plasma.

Mean[a] endogenous conc., ng/mL	Fortification level, ng/mL	Mean[a] conc. found, ng/mL	RSD, %	Mean[a] recovery, %
177.9 ± 6.74	458.3	568.7 ± 24.00	4.22	84.9 ± 5.2
177.9 ± 6.74	916.7	974.7 ± 51.02	5.23	86.7 ± 5.6
177.9 ± 6.74	1833.3	1843.0 ± 27.61	1.50	90.7 ± 1.5
177.9 ± 6.74	3666.7	3456.0 ± 213.76	6.18	89.4 ± 5.8

[a]Mean of five replicates $\pm$ SD.

Table 3. Precision and accuracy data for the determination of all trans-retinyl palmitate in ram plasma.

Mean[a] endogenous conc., ng/mL	Fortification level, ng/mL	Mean[a] conc. found, ng/mL	RSD, %	Mean[a] recovery, %
3.7 ± 0.40	29.8	30.6 ± 1.73	5.70	90.4 ± 5.8
3.7 ± 0.40	59.5	56.9 ± 1.96	3.40	89.5 ± 3.3
3.7 ± 0.40	119.0	110.5 ± 1.28	1.16	89.7 ± 1.1
3.7 ± 0.40	238.1	213.3 ± 8.60	4.00	88.1 ± 3.6

[a]Mean of five replicates ± SD.

CONCLUSION

The results of the present study suggest that the developed analytical procedure is an efficient and reliable method for determining all trans-retinoic acid, all trans-retinol, and all trans-retinyl palmitate in ram blood plasma. It is a relatively simple, rapid, and inexpensive procedure that presents quite acceptable analytical characteristics with respect to recovery, precision, selectivity, and sensitivity. These advantages make the method valuable for metabolic and clinical studies where the concentrations of vitamin A and its metabolites are investigated.

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