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DETERMINATION OF SERUM AND PLASMA CONCENTRATIONS OF RETINOL USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic high-performance liquid chromatographic method specifically developed to allow simple and rapid determination of retinol concentrations in serum and plasma is reported. Retinol and retinol acetate (the internal standard) are extracted into butanol—ethyl acetate, with no subsequent evaporation step. Separation is achieved on a reversed-phase C-18 column, with a mobile phase consisting of acetonitrile—1% ammonium acetate (89:11), and UV detection at 313 nm. Recoveries of both retinol and the internal standard were 100%, and both compounds were stable in the extraction solvent for at least 2.5 h. Three anticoagulants (oxalate, citrate, EDTA) and perchloric acid (used in some methods to denature protein) all caused losses of retinol. Each run required 9 min; same-day coefficient of variation (C.V.) for identical samples averaged 2.5%; between-day C.V. was 6.4%; sensitivity was better than 10 ng/ml, while clinical concentrations were 400—1200 ng/ml. This method permits simple, rapid, sensitive, precise, and accurate determination of retinol using 0.5 ml serum or heparinized plasma.

INTRODUCTION

Compounds with "vitamin A activity" such as retinol and β-carotene may be clinically important anti-cancer agents [1—4]. This family of compounds has been used in experimental animals to prevent or delay the development of a wide range of cancers. While the mechanism(s) of this chemopreventive effect has not been firmly established, epidemiologic data in human populations have suggested a relationship between dietary intake or blood levels of these compounds, and lower risk of cancer. Our institution has recently begun a prospective, randomized, cooperative study to evaluate the chemopreventive effect of β-carotene upon the recurrence rate of non-melanoma skin cancers. This study requires the yearly determination of plasma concentrations of both

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β-carotene and retinol in all study patients (approximately 2000 determinations of each compound each year). A simple, rapid, precise, accurate, sensitive, and inexpensive assay was required for each compound.

Until the late 1970's, retinol (R) blood concentrations were determined by spectrophotometric methods, colorimetric assays or fluorescence assays. However, all three of these methods were relatively time-consuming and non-selective, even though they did achieve adequate sensitivity [5]. High-performance liquid chromatography (HPLC) seemed to be an ideal method, since sample preparation and the analysis itself allowed rapid, sensitive, and selective quantitation of R in blood based upon retinol's high intrinsic UV absorption. Indeed, several HPLC methods for determination of serum or plasma R levels have been recently published. However, none of them has been ideal.

Many of the methods required extraction of R from serum or plasma into an organic solvent, which was then evaporated to dryness [6—14]. This step would make the analysis of 2000 samples per year considerably more time-consuming; in addition, the evaporation step, even if performed under nitrogen, can lead to excessive loss of retinol [15]. Thus, extraction methods which do not require this evaporation step would be preferable. One of the earliest HPLC methods reported met this requirement [16], but the internal standard employed was not commercially available, the peaks of R and internal standard were not totally separated, and the type of HPLC column used did not have sufficient longevity. More recently, a method was published which also avoided an evaporation step [17]. However, this report did not mention percent recovery of R. In addition, the method as reported did not include the use of an internal standard, and gave non-reproducible results in our hands. Most recently, a method has been reported which avoided solvent evaporation, included the use of a commercially available internal standard, and had excellent precision [18]. However, 1 ml of serum was required, standard curves were not performed, each run required 16 min, and recovery rates of both R and the internal standard in different plasma samples were not determined. In addition, there was no information about the equivalence of R levels in serum and heparinized plasma.

Therefore, we decided to attempt to develop an optimal HPLC method specifically designed to determine serum or plasma R concentrations simply and rapidly, incorporating the positve features outlined above. This paper concerns a method which has the required ease, speed, sensitivity, selectivity, precision, and accuracy for routine high-volume use.

MATERIALS AND METHODS

Chemicals

Retinol (R) and retinol acetate (RA) were the highest grade available, and were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium heparin (10,000 units/ml) was purchased from Elkins-Sinn (Cherry Hill, NJ, U.S.A.). All solvents were HPLC grade, manufactured by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium acetate HPLC grade, 70% perchloric acid, and potassium dihydrogen phosphate were obtained from Fisher Scientific

(Fairlawn, NJ, U.S.A.). Water used for HPLC mobile phase preparation was house distilled, then passed through a Milli-Q Purification System (Millipore, Bedford, MA, U.S.A.).

Blood samples

Vacutainer glass tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.) were used to collect serum and four types of plasma (anticoagulated with lithium heparin, oxalate, citrate, or EDTA). Serum and plasma samples were protected from direct sunlight and fluorescent light by wrapping the tubes in aluminum foil, and were stored frozen at −35°C in Nunc polypropylene tubes (AH Thomas Company, Philadelphia, PA, U.S.A.) until analysis.

Standard solutions

Retinol and retinol acetate were dissolved in acetonitrile. Serial dilutions of each were made until R solutions were obtained with approximate concentrations of 10, 6.7, and 3.3 μg/ml (stock solutions), and RA solutions were obtained with approximate concentrations of 10 (stock solution) and 3 μg/ml. Exact concentrations were calculated by measuring the UV absorption of the most dilute solutions at 325 nm with a Varian Techtron Model 635 spectrophotometer (Springfield, NJ, U.S.A.). Extinction coefficients for 1% (w/v) solutions of R (1850) and RA (1565) in ethanol were used, once it was demonstrated that both compounds had equal absorption in pure acetonitrile and pure ethanol. Standard solutions for extraction were prepared fresh daily by adding 50 μl of the R and RA stock solutions to 500 μl water.

Extraction procedures

All extractions were performed in a dark room, illuminated with a 25-W incandescent bulb. In method A, 500 μl of serum, plasma, or water were placed in a 1.5-ml polypropylene microcentrifuge tube (Fisher Scientific). To this were added 50 μl of the RA stock solution (approximately 10 μg/ml) (or 50 μl the R stock solution), and 50 μl of acetonitrile. After vortexing for 15 sec, 250 μl of butanol—ethyl acetate (1:1) were added, with further vortexing for 60 sec. Finally, 150 μl of an aqueous solution of potassium dihydrogen phosphate (1.2 g/ml) were added, the solution vortexed for 30 sec, and then centrifuged at 13,000 *g* for 1 min (Fisher Micro-Centrifuge Model 235B). The organic upper layer was transferred by pipet to a 0.5-ml polypropylene microcentrifuge tube (Fisher Scientific) and centrifuged at 13,000 *g* for 1 min. Fifty μl of this organic solution were then injected directly onto the HPLC system.

Method B required the addition of 100 μl of 5% perchloric acid to 500 μl of plasma in a 1.5-ml microcentrifuge tube. After vortexing for 45 sec, 500 μl of butanol—ethyl acetate (1:1) or 500 μl of ethyl acetate were added, with subsequent vortexing for 1 min. Centrifugation was performed, as in method A. An extraction solvent consisting of butanol—ethyl acetate (1:1) was finally chosen because it produced the best peak shapes, greatest peak heights, optimal viscosity, and optimal eluotropic value on alumina, when compared to either pure ethyl acetate or pure butanol.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 dual-piston pump (Waters Assoc., Milford, MA, U.S.A.), an SSI 0.5-μm in-line filter (Rainin Instruments, Woburn, MA, U.S.A.), a Rheodyne Model 7125 injector with a 100-μl loop (Rainin), a Brownlee pre-column (30 × 4.6 mm) packed with 5 μm diameter RP-18 material (Rainin), an Altex Ultrasphere-ODS column (250 × 4.6 mm, Beckman Instruments, Wakefield, MA, U.S.A.), a Beckman Model 160 UV detector equipped with a 313-nm filter, and a one-channel strip-chart recorder (Model D-5119-1, Houston Instruments, Austin, TX, U.S.A.). Detector sensitivity was set at 0.030 absorbance units full scale (aufs). The mobile phase was acetonitrile—1% ammonium acetate (89:11), flowing at 2.5 ml/min (pressure 166 bars).

Calculations

R and RA peak heights were measured, and the R/RA ratio calculated. A standard curve was generated each day from the three extracted standard solutions, by plotting the R/RA ratio on the ordinate versus R concentration (μg/ml) on the abscissa. The best fit linear regression line was calculated using the method of least squares. In experiments in which recoveries were tested under different conditions, the significance of differences between means was explored using one-way analysis of variance. When significant differences occurred, they were further investigated using the Student—Newman—Keuls test [19].

RESULTS

Extractions using perchloric acid

Using extraction method B with 100% ethyl acetate as originally reported [17], heparinized plasma samples from six subjects were analyzed. The RA peak heights should have been constant, since the same amount was added to each sample (approximately 1000 ng/ml). However, the measured RA peak heights showed a coefficient of variation (C.V.) of 16.3%. Comparison of R peak heights could not be made, since the samples were from six different subjects. However, extraction methods A and B (each using 500 μl of butanol—ethyl acetate) were then applied to triplicate plasma samples from the same subject. R peak heights by method B showed a C.V. = 15.8%, compared to a C.V. = 4.9% by method A. Similarly, RA peak heights showed a C.V. = 14.6% by method B, compared to a C.V. = 5.0% by method A. Since method A gave more precise results, the effect of perchloric acid upon R and RA stability was measured. An aqueous solution of 0.83% perchloric acid had R and RA added (each approximately 1000 ng/ml), and was then extracted with butanol—ethyl acetate as in method A after incubation at 22°C for 1, 4 or 15 min. The results are summarized in Table I. There was a pronounced deterioration in R peak height over 15 min (reduction to 13.9% of control), while the reduction in RA peak height was smaller (reduction to 83.5% of control). Because the R height declined more than the RA height, the R/RA ratio declined as well (reduced to 16.7% of control).

TABLE I

EFFECT OF PERCHLORIC ACID ON RETINOL AND RETINOL ACETATE OVER TIME

R and RA were added to water or 0.86% $HClO_4$, to concentrations of approximately 1000 ng/ml. Extractions were performed using method A, after timed incubations at room temperature, in the dark.

Solution extracted	Incubation time (min)	R height	RA height	R/RA ratio
Water (control)	15	0.770 ± 0.015	0.377 ± 0.014	2.04 ± 0.05
0.83% $HClO_4$	1	0.556 ± 0.044**	0.367 ± 0.007	1.51 ± 0.10**
0.83% $HClO_4$	4	0.340 ± 0.012**	0.352 ± 0.010*	0.97 ± 0.06**
0.83% $HClO_4$	15	0.107 ± 0.015**	0.316 ± 0.011**	0.34 ± 0.04**

*$p < 0.05$.
**$p < 0.01$.

Recoveries of R and RA during extraction

Samples of 500 µl of water or pooled heparinized plasma had R (approximately 0, 200, 400 or 800 ng/ml) and RA (approximately 800 ng/ml) added, and were then extracted using method A. Net R heights were determined by subtraction of the R height of the unspiked plasma. As can be seen in Table II, the values of R, RA, and the R/RA ratio were essentially identical whether the extraction was performed on a spiked water or plasma matrix, indicating that recovery from both was equal. High recovery rates for both compounds were confirmed by demonstrating that when R and RA were added to water (both at 1000 ng/ml), exactly 100% of UV absorption in the water measured with the spectrophotometer at 325 nm was removed after the water was extracted with butanol—ethyl acetate.

RA, the internal standard, was added to a final concentration of 800—1000 ng/ml. However, RA peak heights were proportional to RA concentration when

TABLE II

STANDARD CURVES (R/RA PEAK HEIGHTS RATIO VS. R CONCENTRATION) IN SPIKED WATER AND PLASMA SAMPLES

Water and plasma were spiked with R (0, 192, 384 or 768 ng/ml) and RA (approximately 800 ng/ml).

	R (µg/ml)	R (net height)	RA (height)	R/RA ratio	Best line fit
Water	0.768	0.679	0.365	1.860	R/RA = 2.419 R + 0.003;
	0.384	0.335	0.360	0.930	$r > 0.9999$
	0.192	0.170	0.360	0.472	
	0	0.000	0.358	0.000	
Plasma	0.768	0.670	0.362	1.850	R/RA = 2.405 R + 0.003;
	0.384	0.342	0.371	0.922	$r > 0.9999$
	0.192	0.178	0.378	0.471	
	0	0.000	0.374	0.000	

RA concentrations varied from 400 to 1600 ng/ml ($r > 0.9999$ for the best fit line). Finally, it was felt necessary to demonstrate that recoveries of R and RA were equal when both were added to plasma samples from different subjects. Heparinized plasma from three subjects and water were spiked with R (800 ng/ml) and RA (800 ng/ml). In addition, the plasma samples were also analyzed with only RA added, so that the net R peak height associated with the R added could be determined. Samples for each subject were run in duplicate or triplicate. The mean RA heights (± S.D.) for the three subjects and for the water samples were 0.197 ± 0.014 (n = 6), 0.195 ± 0.008 (n = 6), 0.188 ± 0.005 (n = 5), and 0.192 ± 0.010 (n = 3), respectively. These means were not significantly different. More importantly, the R/RA ratios from the three different plasma samples and the water were 1.82 ± 0.18 (n = 2), 1.74 ± 0.23 (n = 3), 1.77 ± 0.01 (n = 2), and 1.74 ± 0.06 (n = 3). Again, these means were not significantly different, indicating that recoveries of R and RA from three different plasma samples and from water were equal.

Stability of R and RA in serum and plasma

Serum and plasma anticoagulated with four different anticoagulants were obtained in duplicate from three different subjects. After standing at room temperature in the dark for 1 h, the samples were centrifuged and the serum or plasma removed. All samples were extracted using method A. The results are given in Table III. Serum and plasma anticoagulated with heparin demonstrated equivalent values of R, RA, and R/RA ratio (for each patient, the mean value from the serum samples was defined as 100%). Recovery of R was slightly less when EDTA was used as the anticoagulant, and was much less when potassium oxalate or sodium citrate were used as anticoagulants. Recoveries of RA were not affected by choice of anticoagulant.

TABLE III

RECOVERY OF R AND RA FROM SERUM AND VARIOUS PLASMA PREPARATIONS

Duplicate samples of serum and four types of plasma were obtained from three subjects. RA was added (1000 ng/ml), and peak heights of R and RA were measured. For each patient, the values of R, RA, and R/RA ratio obtained with serum samples were defined as 100%.

Treatment	R height	RA height	R/RA ratio
Serum	100.0%	100.0%	100.0%
Plasma (heparin)	98.7 ± 1.9	97.9 ± 0.3	96.6 ± 1.9
Plasma (EDTA)	92.8 ± 6.7	98.9 ± 1.9	92.1 ± 8.3
Plasma (oxalate)	82.4 ± 7.6*	97.0 ± 0.8	80.0 ± 7.4**
Plasma (citrate)	76.7 ± 8.0**	100.3 ± 2.7	76.8 ± 7.3**

*$p < 0.05$.
**$p < 0.01$.

Stability of R and RA in butanol—ethyl acetate

Because of the concern about the effect of the extraction solvent on R and RA stability [17, 18], duplicate plasma samples were extracted and injected

immediately, and injected again 2.5 h later, after having been left at room temperature in the dark. Duplicate samples were so analyzed on four different days. Relative to the sample injected immediately after extraction (defined as 100%), those injected after 2.5 h produced R heights of 99.3 ± 0.9%, RA heights of 101.3 ± 1.4% and R/RA ratios of 98.1 ± 0.9%. These differences were not significant.

Precision

Each day, 30—35 plasma samples were analyzed for R concentrations. Naturally the R concentrations varied but all specimens analyzed should have had the same RA peak height. On seven consecutive days, the RA peak heights had C.V. values of 2.8%, 2.0%, 1.8%, 1.9%, 1.5%, 4.7%, and 2.6%, with an average same-day C.V. of 2.5%. When multiple injections were made on the same day of the same plasma sample, R peak heights had similar C.V. values. Over seven consecutive days of analysis, a pooled sample of heparinized plasma was analyzed in duplicate each day. These seven daily mean values were: 460, 420, 468, 414, 421, 481 and 472 ng/ml. These seven measurements had a mean of 448 ± 29 ng/ml, with a between-day C.V. of 6.4%.

Sensitivity

Fig. 1A and B illustrate chromatograms of one subject's plasma, with and without the addition of RA (1000 ng/ml). The calculated concentration of R

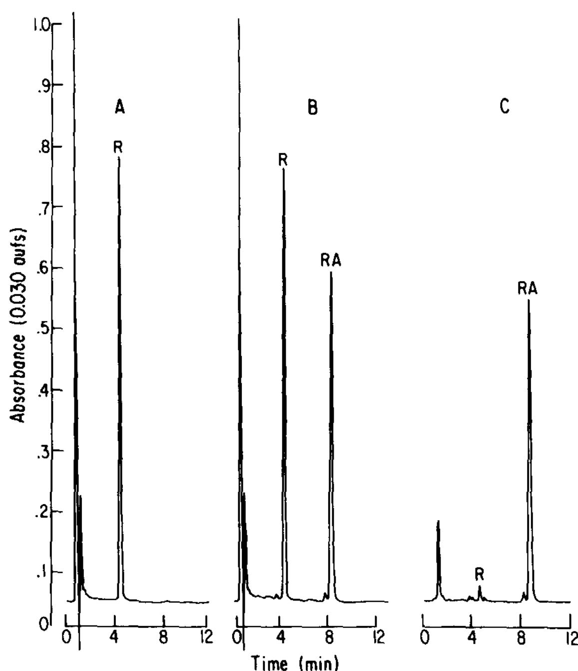

Fig. 1. Chromatograms from human plasma and aqueous samples extracted using method A, with detector sensitivity set at 0.030 a.u.f.s. (A) Human heparinized plasma with normal retinol peak (R, calculated to be 805 ng/ml); (B) the same human plasma, with retinol acetate (RA) added as the internal standard (approximately 1000 ng/ml); (C) water spiked with retinol (25 ng/ml) and retinol acetate (approximately 900 ng/ml).

was 805 ng/ml. Fig. 1C demonstrates that a standard aqueous solution containing R (25 ng/ml), carried through the extraction process, is easily quantitated. At this low detector sensitivity (0.030 aufs), solutions with R concentrations as low as 10 ng/ml are quantifiable (peak height > 3× baseline noise).

DISCUSSION

Since we must measure over 2000 samples each year we felt it would be highly advantageous to develop an extraction process which avoided a solvent evaporation step. We first attempted to use the extraction method proposed by Goodman et al. [17]. However, we noticed that this method, which precipitated serum and plasma protein with 5% perchloric acid (0.83% final concentration), produced inconsistent results. We found that R, and to some extent RA, are rapidly destroyed when exposed to 0.83% perchloric acid. Perchloric acid causes less destruction of R and RA when they are in a serum or plasma matrix, but since the precision of R and RA peak heights is suboptimal with this method, it was not used. While the original authors have used this method to quantify plasma retinol levels [20], their methods paper did not measure R recovery [17]. However, they reported that recovery of 13-*cis*-retinoic acid from plasma using this method was quite variable (83.1—94.7%), possibly reflecting similar problems with the effects of perchloric acid upon this retinoid.

Another question which our work has answered is the issue of whether serum R levels and plasma R levels are equal. This appears to depend on which anticoagulant is used. It was previously shown that recovery of R from plasma anticoagulated with EDTA was slightly less than from serum; oxalate caused an even greater loss [18]. We have confirmed this observation, and in addition demonstrated that anticoagulation with citrate should be avoided as well. Recovery of R and RA from heparinized plasma is equal to that from serum. It is possible that EDTA, oxalate, and citrate all reduce R recoveries by causing acid-induced deterioration or oxidation, similar to the destruction seen following exposure to perchloric acid. In any case, it would seem best to confine blood samples to either serum or heparinized plasma, avoiding other anticoagulants.

Concerning the stability of R and RA in the extraction solvent, we demonstrated no loss in peak heights of either compound after incubation for up to 2.5 h at room temperature in the dark in butanol—ethyl acetate. This is consistent with the results of McLean et al. [18], who found that R was stable for up to 2 h at room temperature when extracted into butanol—acetonitrile. This stability allows the lab technician to perform extractions and operate the HPLC system simultaneously, without worrying that extracted samples have to be injected onto the HPLC system immediately.

The optimal HPLC technique includes the presence of an internal standard (preferably commercially available), and the generation of a standard curve each day, made from spiked samples subjected to the extraction process. As several other groups have done [9, 14, 18], we chose RA as our internal standard, since it is commercially available, has a retention time slightly longer

than R, has similar UV absorption characteristics, and is not present in human blood. Our standard curve was prepared each day from aqueous standard solutions which contained known amounts of R and RA, and which were subsequently extracted. This has the advantage that the R height measured is the net R height, since there is no intrinsic R content of water. We demonstrated that recovery of both compounds was constant and complete from water and from plasma samples from three different subjects. Thus, the efficiency of recovery does not vary from patient to patient. While excellent (96%) recovery of R and RA has been demonstrated previously [18], other workers have experienced recoveries of R and RA as low as 70% [14]. Also, other groups have not demonstrated that recovery was equal in plasma from different patients.

It is important that any assay method has sufficient sensitivity and precision. We were easily able to quantify concentrations of R as low as 10 ng/ml with the UV detector set at 0.030 aufs. Sensitivity could be increased by either increasing the volume of organic matrix injected (we currently inject only 50 μl), or increasing the sensitivity of the UV detector (our detector is able to operate as low as 0.001 aufs, affording an approximately 30× increase in sensitivity). However, since clinically apparent R concentrations are in the range of 400—1400 ng/ml [18] it was not necessary to "push" the sensitivity of the assay further. Even at the level of 10 ng/ml, this sensitivity was better than that reported with other assays [6, 16—18]. Our same-day precision (C.V. average of 2.5%), and between-day precision (C.V. = 6.4%) were similar to those reported by other groups [6, 9, 16, 18].

One final factor of cost concerns the stability of HPLC columns under the required chromatographic conditions. We used a guard pre-column to protect the analytical column. By changing the guard column every 500 injections, the pre-column was not overloaded, pressures were constant, and the analytical column remained protected. We have made over 1100 injections on our first analytical column, and have thus far not detected any change in retention time, or any decrease in the number of theoretical plates.

In conclusion, this method should be of use to investigators who must measure serum or plasma retinol concentrations in large numbers of patients. The ease, speed, sensitivity, accuracy, and precision of the method, its established constant recoveries from different plasma samples, and its standard curve made with aqueous standard solutions utilizing a commercially available internal standard, are all features which other researchers may find useful.

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