Relationship of holo-free and transthyretin-bound plasma retinol-binding protein levels with liver vitamin A in rats

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Vitamin A containing (holo) free and transthyretin-bound (TTR) retinol binding protein (RBP) concentrations in plasma from rats with variable vitamin A status were determined by high performance liquid chromatography. Two different methods were used: (1) molecular exclusion with a TSK 2000 column and (2) reverse phase using a Protesil Octyl 300 column. Holo TTR-RBP peak areas were positively correlated to liver vitamin A concentrations (r = 0.79 for molecular exclusion, 0.81 for reverse phase) in rats with marginal and normal vitamin A status. This correlation was higher than the correlation of serum retinol to liver vitamin A in these rats (r = 0.58). The correlation of holo–TTR-RBP to liver vitamin A was also higher than its correlations to plasma vitamin A. Therefore, plasma concentrations of holo–TTR-RBP may be influenced by marginal vitamin A liver stores to a greater extent than plasma retinol is. This suggests that holo–TTR-RBP protein concentrations may be the better indicator of marginal vitamin A nutritional status in rats. The correlations of both holo–TTR-RBP and serum retinol decreased sharply at high liver vitamin A concentrations. Neither method is suitable for measuring sub-toxicity in rats.

Keywords: retinol-binding protein; vitamin A; rats; liver

Introduction

Retinol binding protein (RBP) is the major retinol-transporting protein in blood serum.[1,2] Usually RBP circulates in blood in a 1:1 complex with transthyretin, (TTR) but a significant fraction of free RBP (not complexed to transthyretin) is present in the blood. Immunologically active (apo + holo) RBP concentration has been used to detect vitamin A deficiencies in animals and man because it is influenced by the amount of vitamin A stored in the liver.[3-6]

Unfortunately, other factors such as protein-calorie malnutrition also influence apo + holo RBP, making it unreliable as a mass screening test for vitamin A deficiency.[7-12] Holo-free RBP is reportedly a good indicator of vitamin A status that is not influenced by alcoholism.[13] That study did not actually measure holo-free RBP, but calculated it from the relative amounts of RBP and TTR in the serum. We have developed two independent high performance liquid chromotography (HPLC) methods that quantitate holo-free and holo-TTR-RBP separately. We used these techniques to investigate whether holo-free, holo–TTR-RBP concentrations, or plasma vitamin A concentration is a better indicator of liver vitamin A status. We find that plasma holo–TTR-RBP concentrations are a good indicator of vitamin A status in marginally deficient and normal rats, with a high positive correlation to liver vitamin A concentrations. This correlation is higher than the correlation of plasma retinol to liver vitamin A or to apo + holo-RBP.

Materials and methods

Instrumentation

Holo-free and holo–TTR-RBP concentrations were assayed on a Model 1084B liquid chromatograph (Hewlett Packard, Avon-

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dale, PA, USA) equipped with an autosampler. Detection was by a Kratos FS 970 fluorometer (ABI Analytical, Ramsey, NJ, USA) using a 330 nm excitation wavelength and a 418 nm emission filter. Molecular exclusion HPLC was done on a 7.5 mm × 30 cm Altex Spherogel TSK 2000 analytical column with a 7.5 mm × 7.5 cm Altex Spherogel TSK guard column (Beckman Inst., Fullerton, CA, USA). Reverse phase HPLC was done with a 4.5 mm × 25 cm Whatman Protesil Octyl 300 column, equipped with a 4.5 mm × 25 cm Whatman Solvecon precolumn (Whatman, Clifton, NJ, USA) and a 3.2 mm × 1.5 cm Newguard column (Pierce, Rockford, IL, USA). Fluorescent and UV peak areas were compared on a Perkin Elmer Series 400 chromatograph equipped with two LCI 100 integrators, one for fluorescent detection and the other for UV detection with an LC 90 UV Vis spectrophotometric detector.

Serum and liver vitamin A were assayed on a Series 400 liquid chromatograph equipped with an ISS-100 autosampler and an LCI 100 computing integrator (Perkin Elmer, San Jose, CA, USA). Detection was by a Perkin Elmer LC 90 UV-Vis Variable Wavelength Spectrophotometer at 330 nm. Reverse phase HPLC was done with a 4.5 mm × 8.0 cm Perkin Elmer C18 column.

Chemicals and reagents

Vitamin A deficient rat diet was from US Biochemical Cleveland, OH, USA; diet 23345). Diet composition is shown in *Table 1*. Normal rat chow was from Ralston Purina (Richmond, IN, USA; Basal diet 5755).

The vitamin A-deficient diet was nutritionally adequate in all nutrients, except vitamin A. Vitamin A concentration in 20 g aliquots of diet was extracted and chromatographed as described[14,15] with the exception that the extraction procedure was scaled up by a factor of 20. No measurable vitamin A was detected. Ketamine hydrochloride (Vetalar) was from Parke-Davis (Morris Plains, NJ, USA). Human RBP standards and purified TTR (prealbumin) were from Behring Diagnostics (San Diego, CA, USA) Retinyl acetate was from Sigma (St. Louis, MO, USA). Purity, as determined by HPLC, was 98%. All other reagents and chemicals were HPLC or reagent grade.

Animals

In the first of two experiments, 23 weanling male Sprague Dawley rats (initial weight about 70 g) were housed separately in hanging wire cages in a temperature- and humidity-controlled room with normal 12-hr light:dark cycles. Food and water were available ad libitum throughout the study. Rat weights and food consumption (adjusted for spillage) were measured twice a week throughout the study. Rats were fed vitamin A deficient diet for 6 weeks, then divided into four groups (of 5, 6, 6, and 6 animals, respectively), so that the average weight of each group was similar. Rats in each group were then fed vitamin A deficient diet supplemented with the addition of either 0.5, 1.0, 3.0, or 10.0 mg retinyl palmitate/kg diet for 2 weeks. (The National Research Council recommends a minimal daily allowance of 4000 IU, or 2.2 mg retinyl palmitate/kg diet.[16] Rats were anesthetized with ketamine hydrochloride, then decapitated. Rat livers were excised, weighed, then quick frozen in liquid nitrogen. Trunk blood was collected (EDTA anticoagulant), then centrifuged at 2000*g* for 15 min at 4° C.

The livers, red blood cells, and serum were stored at −20° C in the dark until use. In the second experiment, male weanling rats were fed rat chow supplemented with 10 mg retinyl palmitate/kg diet for 72 days. All other procedures were as described for the first experiment.

All procedures using rats conformed to the National Research Council's guides for care and use of laboratory animals and were approved by the animal use committees of the USDA and of the Letterman Army Institute of Research.

Humans

Blood samples from humans with suspected vitamin A deficiencies and toxicities were kindly donated by Dr. Robert Russell, Tufts University, Boston, MA, USA; Harold Zwick, Letterman Army Institute of Research, San Francisco, CA, USA; and Daniel Bankson, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

HPLC of retinol esters from liver and serum

Plasma and liver retinol and retinol esters were extracted and chromatographed by previously described methods.[14,15]

Molecular exclusion HPLC of retinol binding protein

Plasma was diluted 1:3 with 0.15 M saline filtered through a 0.20 μ Acro LC 13 filter (Gelman Security, Ann Arbor, MI, USA) and injected directly onto the TSK column. Isocratic elution of 50 μL diluted serum was with 0.15 M sodium phosphate, 0.001 M EDTA, 0.002 M β-mercaptoethanol, pH 7.0 at a flow rate of 0.7 mL/min.[17]

Peak identities were confirmed by comparison of retention times to purified TTR, holo-free RBP, and molecular weight standards, by extraction of retinol from HPLC peaks, by repeated chromatography on the reverse phase column, and by comparison to human RBP.[17] An aliquot from a human plasma pool of known RBP concentration was chromatographed after every 10 rats to correct for possible changes in the chromatographic column.

Table 1 Vitamin A-deficient rat diet

Component	g/100 g Diet	g/100 g Supplement	
Dried yeast (vitamin D)	8	Calcium carbonate	30.985
Corn starch	65	Cobalt chloride, $6H_2O$	0.260
Vegetable oil	5	Cupric sulfate	0.021
Vitamin free casein	18	Ferric citrate, $5H_2$	2.840
Vitamin D_2	224 units	Magnesium sulfate	5.112
Salt mixture #2	4	Manganese sulfate	0.413
		Potassium iodide	0.083
Salt mixture #2	g/100 g supplement	Sodium chloride	17.300
Aluminum potassium sulfate	0.021	Sodium fluoride	0.026
Calcium diphosphate, $2H_2$	9.812	Sodium tetraborate	0.026
Potassium diphosphate	33.309	Zinc chloride	0.026

Reverse phase HPLC of retinol binding protein

Rat plasma was diluted 1:3 with 13.3% isopropanol and 0.133% trifluoroacetic acid (TFA) in distilled water, vortexed for about 10 sec, then centrifuged at 4000*g* for 10 min. The precipitate was discarded and the supernatant filtered through a 0.20 μ Acro LC 13 filter. A 20 μL aliquot was injected onto a Protesil 300 Octyl Reverse Phase Column (Whatman, Inc., Clifton, NJ, USA) to quantify peak 1 (holo–TTR-RBP).To quantify peak 2b (vitamin A derived from holo-free RBP and from an unidentified high molecular weight lipoprotein associated peak), 120 μL of the diluted sample preparation was reinjected. Elution of both peaks was with a gradient of buffer A (0.1% TFA in distilled water) and buffer B (0.1% TFA in isopropanol). The gradient was started with 10% buffer B for 1 min, then increased linearly to 70% buffer B over 19 min. The gradient stabilized at 70% buffer B for 1 min, then decreased linearly to 10% buffer B for 9 min. The gradient was maintained at 10% buffer B over the last 10 min of the run (total run time 40 min). The flow rate was a constant 1.0 mL/min, with pH constant at 2.2. The identity of peak 1 was confirmed by repeated chromatography on the TSK molecular exclusion column, by extraction of retinol from the HPLC peaks, and by comparison with human RBP. An aliquot from a human plasma pool of known RBP concentration was run after approximately every tenth rat sample, to correct for possible changes in the column.

Comparison of TSK and reverse phase HPLC

The areas of peak 1 (holo–TTR-RBP) and peak 2a (holo-free RBP) from the TSK 2000 molecular exclusion column were correlated to the areas of peak 1 and peak 2b derived from the reverse phase Protesil Octyl 300 column. Linear correlation coefficients were derived from SAS (Statistical Analysis System, Cary, NC, USA), using the best least squares fit lines.

Quantitation of holo-RBP

Holo-free and holo–TTR-RBP concentrations were estimated by three previously described[17] independent methods. These methods were: (1) extraction and quantitation of retinol from the HPLC peaks; (2) comparison to purified human vitamin A-saturated free RBP; and (3) comparison of UV detection at 330 nm with fluorescent detection at 330 nm excitation, 418 emission. The estimates from each method were averaged to get the reported RBP concentration.

Estimation of apo RBP concentrations

A small amount of a concentrated solution of retinol in 95% ethanol was added to samples of rat plasma so that the retinol was present in a 4 to 10 times excess molar concentration relative to RBP. These vitamin A-saturated preparations were incubated overnight at 4° C, then run on the TSK HPLC column using standard conditions. Apo RBP concentrations were estimated by subtracting the peak areas of rat plasma incubated with 95% ethanol alone from the peak areas of rat plasma incubated with retinol.

Peak areas for the unknown samples were compared to HPLC peak areas of known standards. First order linear regression coefficients were derived with Sigmaplot (Jandel Scientific, Sausalito, CA, USA) and with SAS and were based on the best least squares fit of the data.

Results

The rats used in the first study had vitamin A liver stores ranging from marginally deficient to adequate. The rats in the second study were adequate or subtoxic. Most rats had adequate liver vitamin A stores at the end of the experiments. At no time did any of these rats show symptoms of gross vitamin A deficiency or toxicity. All rats continued to gain weight during the study, though the rate of growth was slowed during the last week the rats were fed the unsupplemented vitamin A-deficient diet. Coats, eyes, and other physical symptoms remained normal. Rat weights, liver weights, and hematocrits were not significantly different in any group of rats at the beginning or end of the times they were on different vitamin A supplements. Liver and plasma vitamin A concentrations, however, did differ among groups. The rats given the lowest vitamin A supplement had the lowest liver vitamin A concentrations (*Table 2*).

Rat holo-RBP has very similar properties to human holo-RBP on molecular exclusion HPLC[17] (*Figures 1a and 1b*). Three peaks are present in molecular exclusion chromatograms of RBP. The 17-minute peak (peak 1) has been identified as holo–TTR-RBP.[17] The 24-minute peak (peak 2a) has been identified as holo-free RBP.[17] The third peak is identifiable in all of our chromatograms from rat plasma but only some of our chromatograms from human plasma. This peak (peak 0) migrates at 12 minutes and appears to be a high molecular weight complex that contains vitamin A and lipoprotein.

The similarity of rat and human holo-RBP on reverse phase chromatography is equally striking, as shown by comparisons of *Figures 2a and 2b*. Two peaks are present in the reversed phase chromatogram of either rat or human serum, and both are in similar locations. The major peak, (peak 1), reacts with antibodies to RBP and to TTR in human serum and has been identified as holo–TTR-RBP because of its fluorescent properties, its physiochemical properties, and its vitamin A content (*Table 3*). The small peak (peak 2b) has been identified as vitamin A derived from holo-free RBP and from the high molecular weight lipoprotein-associated complex, based on its fluorescent and physiochemical properties, its vitamin A content, and its lack of reactivity to either RBP or transthyretin antibodies in human serum. There usually is only a small amount of lipoprotein-associated vitamin A in human plasma, so peak 2b corresponds closely to peak 2a of *Figure 1a and 1b* (free-holo RBP on the molecular exclusion column) in humans. Peak 2b in rats has high correlations with the high molecular weight lipoprotein associated vitamin A complex (peak 0, *Figure 1a*) and a lesser correlation to free-holo-RBP (peak 2a, *Figure 1a*), because it consists of vitamin A derived from both complexes.

Holo–TTR-RBP does not dissociate in either the molecular exclusion[17] or the reverse phase method when the holo–TTR-RBP peak is collected and rechromatographed three times.

Molecular exclusion HPLC and reverse phase HPLC peak areas are highly correlated to each other (*Figure 3*). The peak areas of peak 1 (identified as holo–TTR-RBP) showed the highest correlations between these two methods ($r = 0.96$). The peak areas of peak 2a (free-holo RBP, molecular exclusion) and

Table 2 Physiochemical properties of rats fed different amounts of vitamin A

Mg RP Kg diet	n	Rat weight (final)	Rat liver weight (final) g	Liver vitamin A μg RE g liver
0.5	5	433 ± 58	14.2 ± 2.7	26.8 ± 16.3
1.0	6	422 ± 63	16.9 ± 3.8	32.8 ± 7.4
3.0	6	418 ± 22	16.3 ± 1.8	29.4 ± 7.4
10.0	6	448 ± 30	16.9 ± 1.3	67.5 ± 10.7[a]
Study 2	6	514 ± 17	16.7 ± 0.6	352 ± 149[a]

RP, retinyl palmitate; RE, retinol and retinyl esters.
[a]Significant difference, $P < 0.05$.

2b (free-holo RBP and lipoprotein associated vitamin A, reverse phase) are not as highly correlated in these two methods ($r = 0.64$). There is a higher correlation between peaks 0 (lipoprotein associated vitamin A, molecular exclusion) and 2b (reverse phase), but only in the rat. This is because reverse phase chromatography disassociates retinol from both holo-free RBP and from the unidentified high molecular weight complex of lipoprotein associated with vitamin A. Apo-free and apo–TTR-RBP are not present in significant concentrations in these rats. The difference in fluorescence between plasma incubated with ethanol or with saturating amounts of vitamin A ranged from −6% to +14%, and were not dependent on the amount of vitamin A fed to the rats.

The effects of liver vitamin A concentration on holo-free and holo–TTR-RBP in the first experiment, as measured by molecular exclusion chromatography, is shown in *Figure 4*. Holo–TTR-RBP (peak 1) was positively correlated to liver vitamin A stores ($r = 0.79$) in marginal or adequate rats. The high molecular weight complex containing lipoprotein and vitamin A is also positively correlated to liver vitamin A ($r = 0.79$). Holo-free RBP, however, has no significant correlation to liver vitamin A ($r = 0.14$). These correlations are similar to those derived independently from reverse phase HPLC (*Figure 5*). Holo–TTR-RBP has an almost identical correlation with liver vitamin A ($r = 0.81$). The correlation of reverse phase peak 2b (holo-free RBP and the high molecular weight vitamin

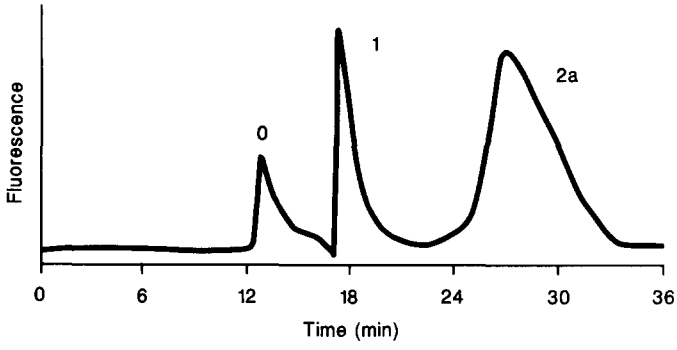


Figure 1a Typical molecular exclusion (TSK) chromatogram of rat holo-RBP. Chromatogram is of 25 μL 1:3 diluted rat plasma. HPLC method is as described in text.

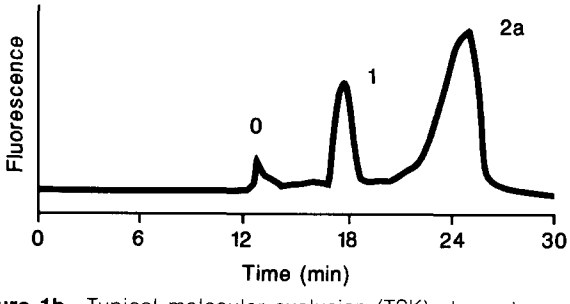


Figure 1b Typical molecular exclusion (TSK) chromatogram of human holo-RBP. Chromatogram is of 25 μL of 1:3 diluted human plasma. HPLC method is as described in text.

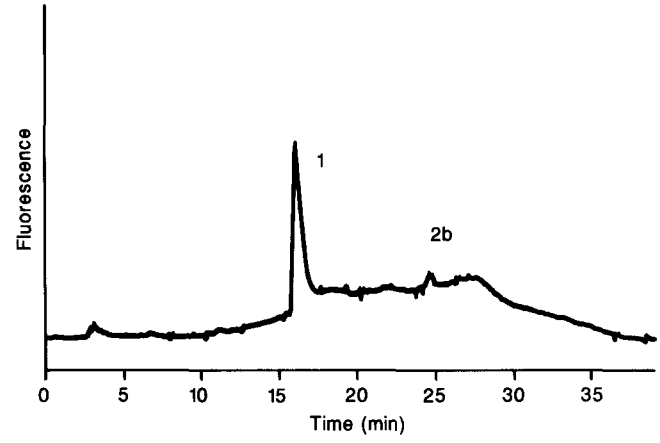


Figure 2a Typical reverse phase chromatogram of rat holo-RBP. Chromatogram is of 20 μL 1:3 diluted rat plasma. HPLC method is as described in the text.

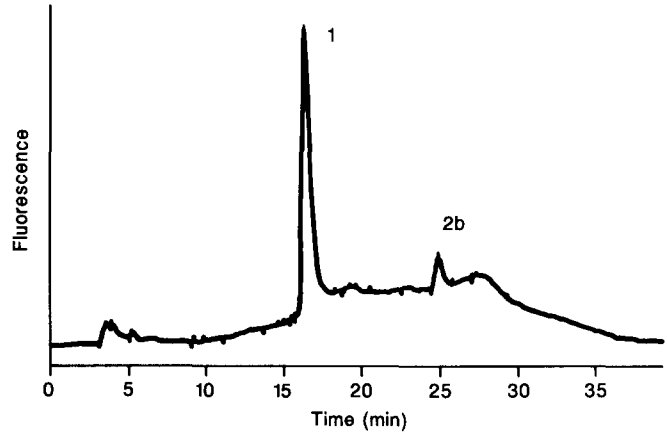


Figure 2b Typical reverse phase chromatogram of human holo-RBP. Chromatogram is of 100 μL 1:3 diluted human plasma. HPLC method is as described in the text.

Table 3 Properties of HPLC peaks from normal rat plasma (peak assignment)

	Lipid-protein	holo–TTR-RBP	holo-free RBP
Retention time, min			
Reverse phase	—	17.1	24.8
Molecular exclusion	12.5	17.8	25.5
Molecular mass, Da	Exclusion	68,000	20,500
% of total peak area			
Reverse phase	—	89–95	5–11
Molecular exclusion	2–8	23–28	69–75
% of total extractable vitamin A in peak			
Reverse phase	—	84–95	2–11
Molecular exclusion	3–7	82–91	3–14
Excitation max, nm			
Molecular exclusion	365	340	335
Emission max, nm			
Molecular exclusion	465	450	450

A complex) to liver vitamin A is insignificant (r = 0.21), probably reflecting the major contribution of holo-free RBP to this peak. The correlations of holo–TTR-RBP to liver vitamin A are higher than the correlation of plasma vitamin A to liver vitamin A in these rats (*Figure 6*, r = 0.58). The correlations for holo–TTR-RBP and retinol with liver vitamin A were not good in adequate-to-subtoxic rats. The correlations of liver retinyl ester concentrations with plasma retinol, holo–TTR-RBP and holo-free RBP were 0.26, 0.34, and 0.27, respectively.

Preliminary data on vitamin A-deficient humans (plasma retinol 0.2 to 0.4 μmol/L) showed that both holo–TTR-RBP and free-holo RBP were decreased significantly ($P \leq 0.01$). Serum retinyl esters increased significantly ($P \leq 0.001$) in humans with vitamin A toxicity. Serum retinol, holo–TTR-RBP and free-holo RBP did not increase significantly from the normal human range. Typical molecular exclusion chromatograms for human plasma RBP in deficiency and toxicity are shown in *Figures 7a and 7b*.

Discussion

We developed two independent HPLC methods for measuring holo-free and holo–TTR-RBP, one involving molecular exclusion chromatography on a TSK 2000 column, the other reverse phase chromatography on a Protesil Octyl 300 column. These methods give very similar results for the effect of liver and plasma vitamin A concentrations on holo–TTR-RBP. Molecular exclusion chromatography may be the superior method of the two because it does not disassociate vitamin A from holo-free RBP and from the high molecular weight vitamin A-containing complex associated with lipoprotein, and it requires less sample preparation. Neither method is species specific, and both can assay vitamin A-containing holo-free and holo–TTR-RBP.

The HPLC methods described are not strictly comparable to immunologic methods, because the HPLC methods measure holo-RBP only, while immunologic methods measure apo-RPB and any other protein with cross-reactivity to RBP antibodies. Both the HPLC and immunologic methods should usually give almost the same total RBP concentrations (within experimental error) because apo-RBP is present in very small amounts in plasma and serum. Our results showed differences of −6% to 14% between vitamin A-saturated and non-saturated plasmas in these rats,

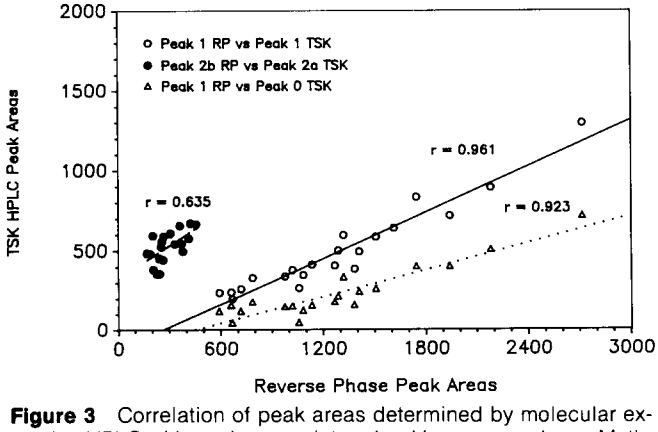


Figure 3 Correlation of peak areas determined by molecular exclusion HPLC with peak areas determined by reverse phase. Methods as described in text.

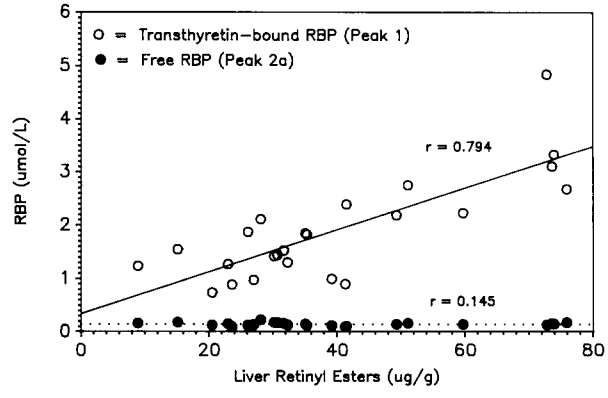


Figure 4 Correlation of liver vitamin A concentration with holo-free and holo–TTR-RBP concentrations in rat plasma, as measured by molecular exclusion HPLC. Methods are described in the text. Each point represents the mean of duplicate assays. ○ = peak 1 (holo–TTR-RBP). ● = peak 2a (holo-free RBP).

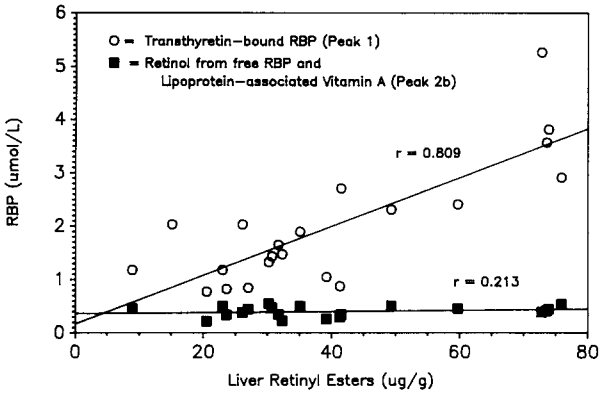


Figure 5 Correlation of liver vitamin A concentration with holo-free- and holo-TTR-RBP concentrations in rat plasma, as measured by reverse phase HPLC. Methods are described in the text. Each point represents the mean of duplicate assays. ○ = peak 1 (holo-TTR-RBP). ■ = peak 2b (vitamin A from holo-free RBP and prepeak)

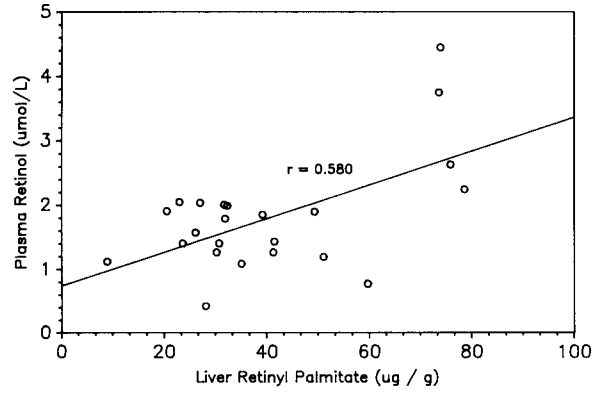


Figure 6 Correlation of liver vitamin A concentration with plasma vitamin A concentration. Liver retinol esters and plasma retinol were determined by isocratic HPLC as described in text. All assays were done in duplicate.

with no consistent effect of vitamin A concentration in the diet.

This study shows holo-TTR-RBP concentrations were better than plasma retinol concentrations as indicators of liver vitamin A stores in marginally deficient-to-normal rats. The correlations between holo-TTR-RBP and liver vitamin A concentrations were significantly better ($P = 0.01$) than the correlation of plasma vitamin A concentrations to liver vitamin A concentrations. This suggests that holo-TTR-RBP concentrations may be influenced by vitamin A liver stores to a greater extent than plasma vitamin A is in marginally deficient rats, and may be better indicators of vitamin A status in normal rats. Preliminary human data suggests that serum retinol, holo-free RBP and holo-TTR-RBP all decrease significantly in vitamin A deficiency.

Data from humans and rats suggests that these relationships break down during vitamin A subtoxicity or toxicity. In toxicity, retinyl esters were increased significantly in humans, but serum retinol, holo-TTR-RBP and holo-free RBP concentrations were not.

Our rat and human chromatograms always showed characteristic differences. Since rat plasma samples were always run with at least one human plasma control, an accurate comparison of peak areas of typical chromatograms of RBP from rats and humans was possible. This comparison shows that the unidentified high molecular weight complex associated with lipoprotein and vitamin A (peak 0, molecular exclusion) and holo-TTR-RBP (peak 1) concentrations were consistently higher in rats than in humans with similar plasma vitamin A concentrations. Holo-free-RBP concentrations were lower in rats, particularly at higher plasma vitamin A concentrations. There are two probable explanations for these differences: (1) the formation or degradation of the holo-TTR-RBP complex is regulated by different mechanisms in rats

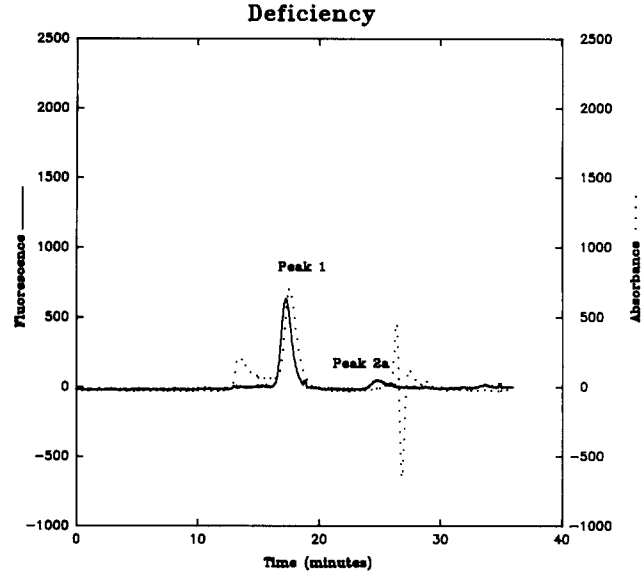
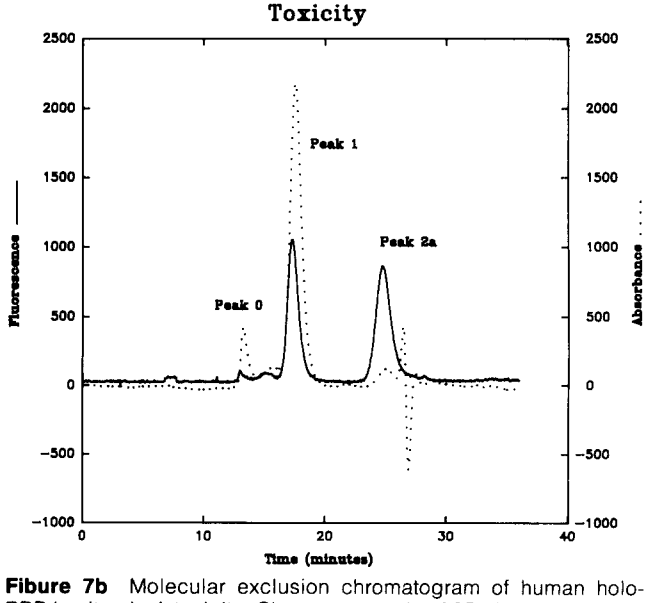


Figure 7a Molecular exclusion chromatogram of human holo-RBP in vitamin A deficiency. Chromatogram is of 25 μL of 1:3 diluted plasma. HPLC method is as described in text.



Fibure 7b Molecular exclusion chromatogram of human holo-RBP in vitamin A toxicity. Chromatogram is of 25 μL of 1:3 diluted plasma. HPLC method is as described in text.

and humans; (2) the formation of the holo–TTR-RBP complex is influenced by nutritional factors other than vitamin A status. Little is known about the factors influencing the formation of the holo–TTR-RBP complex in vivo, but there is some support for both possibilities. Rat and human RBP have different amino acid compositions,[18] and different immunologic reactivities.[19] Because RBP seems to be different in these species, it would not be surprising if the binding of RBP to TTR was different. It is also true that the typical diets of rats and humans are different. Human data suggested that free RBP is affected to a much greater extent by changes in liver function (shown in subjects with fluctuating creatinine) or by daily changes in nutrient intake (as indicated by greater day-to-day variations in the free RBP peak.[20] It is possible that RBP levels in this study may have been influenced by differences in dietary factors such as zinc,[21] protein or calories[10-12] or carbohydrate.[22]

The rats used in these studies have liver vitamin A stores which were marginally deficient to subtoxic. This narrow range of vitamin A nutriture was used in an attempt to mimic the normal range of vitamin A nutriture in the human populations of the United States, where gross vitamin A deficiency or toxicity is uncommon, but marginal status occurs fairly often.[23] It appears that holo–TTR-RBP may be a good indicator of vitamin A status in this range of vitamin A nutriture. Unfortunately, it is unclear whether the rat is a good model for humans, since it is possible that the binding of TTR to RBP is regulated by different mechanisms in these species. The mechanisms controlling the formation of the holo–TTR-RBP complex require more study than they have been given. The molecular exclusion HPLC method described should provide a useful tool for studying this complex and the mechanisms controlling its formation and degradation in animals and man.

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