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Assay of human transthyretin-bound holo-retinol-binding protein with reversed-phase high-performance liquid chromatography

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ABSTRACT

We describe a reversed-phase high-performance liquid chromatographic method for the determination of vitamin A-transporting (holo) transthyretin-bound (TTR) retinol-binding protein (RBP) concentrations in serum or plasma. Holo-TTR–RBP and free retinol derived primarily from free RBP are consistently observed with this chromatographic method. Holo-TTR–RBP concentrations determined by this method are highly correlated to holo-TTR–RBP concentrations measured by chromatography. This method has the advantage of using less expensive columns and having peak areas which are more proportional to their true concentrations in plasma, as determined by comparison to purified protein spectrophotometry and radial immunodiffusion. The percentage of RBP circulating as holo-TTR–RBP decreased significantly as the total concentration of RBP or retinol increased. Because purified holo-TTR–RBP did not dissociate under these chromatographic conditions, this suggests that more vitamin A circulates as holo-free RBP or free retinol in the blood of people with high serum RBP.

INTRODUCTION

Retinol-binding protein (RBP) is the major carrier protein for retinol from its storage site in liver to the tissues [1,2]. RBP is attached to the protein transthyretin (TTR) in the liver before it is secreted into blood [1,2]. Most RBP in the blood is bound to transthyretin, although a significant amount circulates free in solution. Retinol-bound (holo) TTR-RBP concentrations were highly correlated to liver vitamin A stores in marginally deficient and normal rats [3]. This correlation was higher than that of total plasma retinol or holo-free RBP to liver vitamin A in these rats. This suggests that determining holo-TTR-RBP concentrations could be an excellent test of vitamin A status if an inexpensive, simple assay is available.

All immunological methods for RBP displace TTR from the TTR–RBP complex [4]. There are only two known methods that separate and measure TTR–RBP specifically: (1) electrophoresis with detection by fluorimetric densitometry [5,6]; and (2) high-performance size-exclusion chromatography with fluorimetric detection [7–9]. Both methods have drawbacks. The electrophoretic method is

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difficult to use, requires expensive equipment not available in most laboratories and is insensitive. Size-exclusion chromatography does not have these problems, but results in a highly fluorescent hole-free RBP peak that suggests that the holo-free RBP measured has a different molecular configuration than it normally takes in plasma. The reversed-phase high-performance liquid chromatographic (HPLC) method described shows both holo-TTR–RBP and retinol from free holo-RBP more in proportion to their relative concentrations in plasma than does size-exclusion chromatography.

EXPERIMENTAL

Samples

We collected blood from 42 healthy adult human volunteers, aged 19 to 46 years. Eighteen were women, 24 were men. None of the women were taking oral contraceptives. Serum or plasma was harvested by centrifugation at 1500 *g* and stored in the dark at −20°C until use.

Reagents and chemicals

Purified RBP and TTR were from Calbiochem (San Diego, CA, U.S.A.), and radial immunodiffusion (RID) plates to RBP and TTR were from Behring Diagnostic (La Jolla, CA, U.S.A.). All other chemicals were HPLC or reagent grade. The mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

We determined concentrations of retinol in plasma or in holo-RBP chromatography peaks by a standard reversed-phase chromatographic technique [10,11]. Plasma total RBP (apo + holo, free and TTR-bound) was determined by RID following the manufacturer's instructions.

Reversed-phase HPLC of holo-RBP

RBP was initially assayed on a Model 1084B liquid chromatograph (Hewlett Packard, Palo Alto, CA, U.S.A.). Detection was with a Kratos FS 970 fluorimeter (ABI Analytical, Ramsey, NJ, U.S.A.) using a 330-nm excitation wavelength and a 418-nm emission filter. Later, the assay was done on a Series 400 liquid chromatograph (Perkin-Elmer, Mountain View, CA, U.S.A.) equipped with a Kratos FS 980 fluorometer and analyzed with a System Gold chromatography workstation (Beckman, Fullerton CA, U.S.A.). Reversed-phase HPLC of RBP was done with a 250 mm × 4.6 mm I.D. Whatman Protesil Octyl 300 column equipped with a 250 mm × 4.6 mm I.D. Solvecon precolumn (Whatman, Clifton, NJ, U.S.A.) and a 15 mm × 3.2. mm I.D. Newguard column (Pierce, Rockford, IL, U.S.A.). Serum or plasma was diluted 1:3 with 0.13% (w/v) trifluoroacetic acid (TFA) and 13.3% (v/v) isopropanol in distilled water, vortex-mixed for

approximately 10 s, then centrifuged at 4000 *g* for 10 min to remove particulate matter.

The supernatant was filtered with a 0.20-μl Acro LC 13 filter (Gelman Scientific, Ann Arbor, MI, U.S.A.), then a 50-μl aliquot was injected onto the HPLC column. Elution was with a gradient of buffer A (0.1% TFA in distilled water) and buffer B (0.1% TFA in isopropanol). In a typical run, the gradient was initialized with 10% buffer B for 1 min, then increased linearly to 70% B over 19 min. The gradient was stabilized at 70% buffer B for 1 min, then decreased linearly to 10% buffer B over 10 min, then stabilized at 10% buffer B for 8 min (total run time 39 min). The flow-rate was constant at 1.0 ml/min, with pH constant at approximately 2.2. Sample processing time could be decreased either by increasing the gradient slope or by increasing the flow-rate without compromising peak resolution. Peak areas of samples were compared to peak areas of a standard plasma pool of known RBP concentrations. We confirmed peak identities with three methods. We compared sample peaks to purified holo-free RBP, TTR, holo-TTR–RBP and free retinol standards, collected peaks and rechromatographed them on a TSK 2000 size-exclusion column and checked immunological activities by RID against antibodies to total-RBP and TTR. Holo-TTR–RBP was quantitated by RID and by comparison of the HPLC peak areas to purified, vitamin A-saturated TTR–RBP of known concentration. Areas of peaks derived from the TSK 2000 and Protesil Octyl 300 columns were correlated with SAS version 6.03 for the personal computer [12]. Correlation coefficients were determined using the best least-squares fit of the data.

Molecular exclusion HPLC of holo-RBP

Size-exclusion HPLC of holo-RBP was done on a TSK 2000 column (Beckman Instruments, Fullerton, CA, U.S.A.) as previously described [8]. Standard chromatographic conditions were as follows: plasma was diluted 1:3 (v/v) with normal saline, samples were eluted isocratically with 0.15 *M* sodium phosphate–0.002 *M* β-mercaptoethanol–0.001 *M* ethylenediaminetetraacetatic acid, pH 7.0 at 0.5 ml/min.

Rechromatography of reversed-phase peaks on TSK 2000 column

Six 200-μl injections of human plasma were chromatographed on the reversed-phase Protesil Octyl 300 column under standard conditions. The areas of highest fluorescent activity from all three peaks were collected and concentrated by centrifugation at 15 000 *g* for 45 min. A 200-μl aliquot of each peak was chromatographed on a TSK 2000 size-exclusion chromatographic column under standard conditions [8]. A typical chromatogram is shown in Fig. 1. Similarly, six 200-μl injections of human plasma were chromatographed on a TSK 2000 size-exclusion column, and the areas of highest fluorescence from each peak were collected, pooled and concentrated. A 200-μl aliquot of each peak was chromatographed on the Protesil Octyl 300 reversed-phase column under the standard reversed-phase conditions previously described.

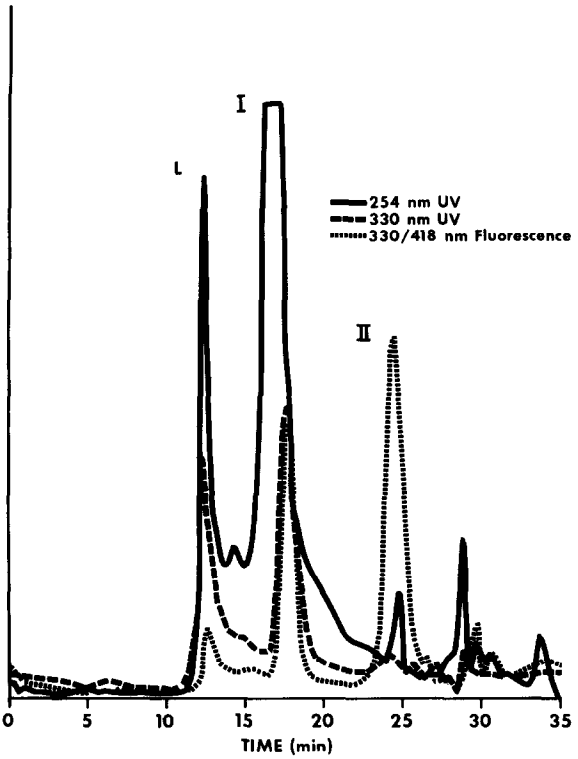

Fig. 1. Typical chromatogram of normal human plasma (50-μl aliquot of 1:3 diluted plasma) on the TSK 2000 size-exclusion column. Procedures are as described in text. Peaks: L = vitamin A associated with lipid and protein; I = holo-TTR-RBP; II = holo-free RBP. From ref. 8.

Immunological reactivity of Protesil Octyl 300 peaks

HPLC peaks were evaporated to dryness under nitrogen, resuspended in five times the original injection volume of distilled water, then spotted on RID plates with ten times the recommended volume. This was necessary because the solvents used in this chromatographic procedure would have interfered with the RID plate media.

Purification of holo-TTR–RBP

Holo-TTR–RBP was purified from human plasma by modifying the method of Grover *et al.* [13] for peak verification. Plasma (250 ml) was eluted through a 45 cm × 2.8 cm I.D. column of DEAE Sephadex, concentrated by a 65% ammonium sulfate precipitation and dialyzed extensively. The preparation was then purified further on a 90 cm × 1.5 cm I.D. column of CM Sephadex. The final concentration and purification was done by a 65% ammonium sulphate precipitation, which does not dissociate the TTR–RBP complex. The purity of holo-TTR-RBP was checked by disc gel electrophoresis and by chromatography on

reversed-phase or on size-exclusion columns, with UV detection at 330, 280 and 254 nm and fluorescent detection at 330 nm excitation, 418-nm emission cut-off. Immunological activity was measured with RID plates, as described previously.

Electrophoresis

Electrophoresis was run according to the method described by Glover and co-workers [5,6], with the following modification. Gels were stained for protein with 0.2% Coomassie Blue in 10% acetic acid, 10% isopropanol for 18 h, then destained in 10% acetic acid, 10% isopropanol for 48 h.

RESULTS

A reversed-phase chromatogram of purified holo-TTR–RBP showing UV absorbance at 280 nm and fluorescence at 330 nm excitation and 418-nm emission is shown in Fig. 2. Chromatograms of the purified preparation detected at 254 and 330 nm gave similar results, showing the presence of a single major peak. Size-

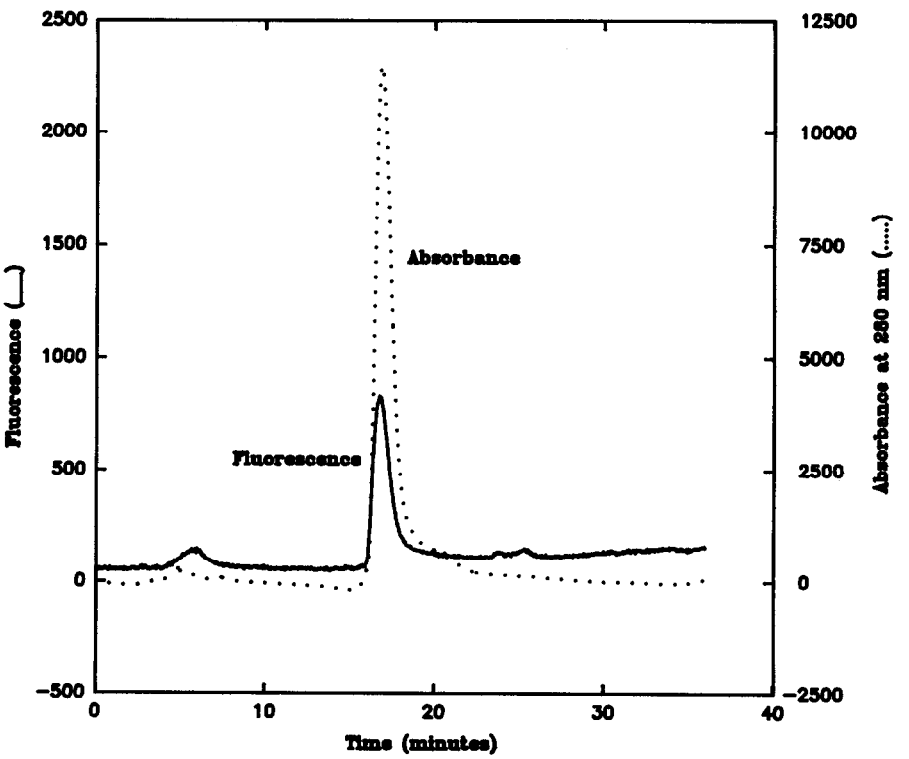

Fig. 2. Chromatogram of purified holo-TTR–RBP on a Protesil Octyl 300 reversed-phase column. Methods are described in text.

exclusion chromatography with detection at 280, 254 or 330 nm also showed one major peak with small amounts of dissociated holo-RBP or impurities. These chromatograms, and the results of disc gel electrophoresis (Fig. 3), show that this protein preparation is approximately 95% pure holo-TTR–RBP (range 94–99%). The main peak showed immunological activity toward RBP antibodies. No appreciable dissociation of the holo-TTR–RBP peak on either chromatography column was seen. The amount of dissociation seen was not changed with different injection volumes or concentrations, but did increase gradually with storage. The amount of holo-TTR–RBP dissociated in samples stored three months at 4°C had increased from 0–3% to approximately 30% of the sample.

A typical reversed-phase chromatogram of human plasma is shown in Fig. 4. The first peak, which elutes at 16.7 min[a], was identified as holo TTR–RBP based on its retinol content, its immunological reactivity to antibodies to RBP and to TTR, and its coelution with purified, retinol-saturated TTR–RBP on reversed-phase and size-exclusion columns (Table I). When the first reversed-phase peak was chromatographed on the TSK 2000 size-exclusion column, it gave rise to a single peak of approximately 66 000 dalton, consistent with our previous finding for holo-TTR–RBP on this column. This peak showed no significant formation of breakdown products when collected and rechromatographed on the TSK 2000 column three times. The holo-TTR–RBP peak from the TSK column migrated as a single peak at 16.2 min on the Protesil Octyl reversed-phase column, and did not show significant breakdown when collected and rechromatographed three times on the reversed-phase column.

A second peak was often seen at 23.1 min. This peak was identified as an impurity in the TFA that was caused either by light or by oxidation. This impurity can be present even in freshly opened bottles of TFA if the TFA is amber in color; it is not present when clear TFA is used. The artifact peak does not interfere with either of the retinol-containing peaks.

The last (third) peak, which eluted at 25.4 min, was identified as free retinol based on its retinol content, its lack of immunological reactivity to either RBP or TTR, and its coelution with purified retinol or retinyl esters (Table I). When the holo-free RBP molecular exclusion chromatography peak from the TSK 2000 column was collected and rechromatographed on the reversed-phase column the retinol peak appeared. Purified free RBP that had been saturated with retinol also eluted as peak 3. This suggests that the third reversed-phase peak was composed of retinol derived from the breakdown of the free holo-RBP exclusion peak. There is a high correlation between the holo-TTR–RBP (peak 1) peak areas measured on the reversed-phase column and the holo-TTR–RBP (peak 1) peak

[a] Protesil Octyl 300 columns do not give exactly the same retention times for these proteins, even when the same sample is prepared identically and run on columns from the same manufacturer's lot. Reproducibility is good for within-column runs.

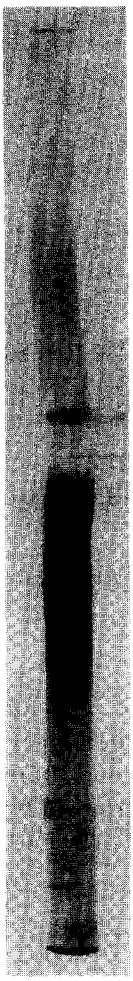

Fig. 3. Electrophoresis of purified holo-TTR–RBP. Holo-TTR–RBP is the heavy middle band. Methods are described in text.

areas measured in the same samples by size-exclusion chromatography on a TSK 2000 column ($r = 0.73$; Fig. 5). There is a higher correlation between peak 3 (called vitamin A) observed in reversed-phase chromatography and the holo-free RBP peak (peak 2) seen on the TSK 2000 column ($r = 0.87$).

Reversed-phase chromatograms for serum or plasma were almost indistinguishable, with no consistent differences in peak areas observed. The plasma anticoagulant used did not appear to be important, since duplicate samples collected in heparin, citrate or in EDTA gave identical results within experimental error [coefficient of variation (C.V.) = 5.4%, $n = 9$].

The areas of peaks 1 and 3 varied linearly with injection volume from 5 to 100

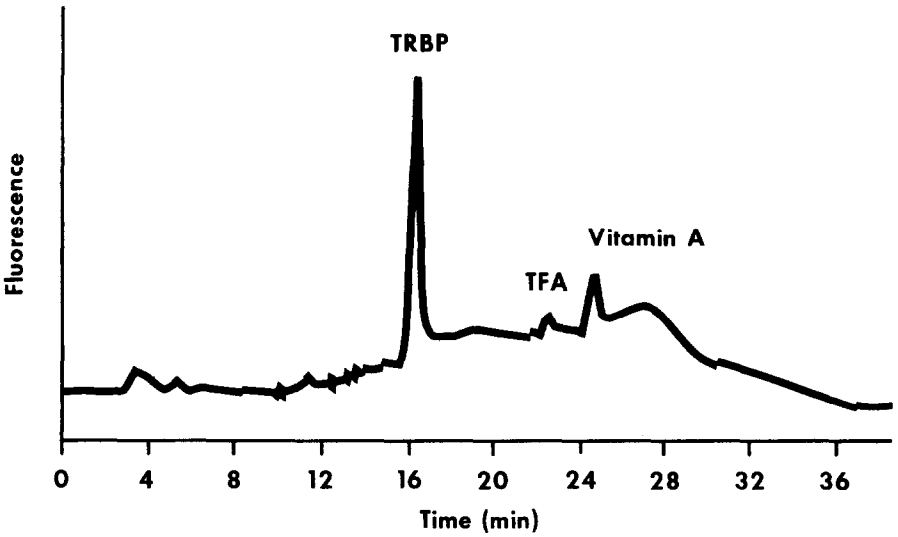

Fig. 4. Typical chromatogram of normal human plasma (50-μl aliquot of 1:3 diluted plasma) on the Protesil Octyl 300 reversed-phase HPLC column. Procedures are described in text.

μl plasma (Fig. 6). Peak-area reproducibilities were good, with a C.V. of 3.3% for holo-TTR–RBP and a C.V. of 3.8% for the retinol peak for six replicate samples. In reversed-phase chromatography of holo-RBP peak areas correspond more closely to the relative amounts of RBP contained in each peak, as determined by comparison to purified preparations of holo-free and holo-TTR–RBP of known concentrations, absorbance data and RID.

TABLE I

PROPERTIES OF HPLC PEAKS FROM NORMAL HUMAN PLASMA ELUTED FROM A PROTESIL OCTYL 300 COLUMN

Parameter	Peak 1 (transthyretin-bound RBP)	Peak 2 (trifluoroacetic acid)	Peak 3 (retinol)
Retention time (min)	16.7	23.1	25.4
Molecular mass (dalton)	66 000	–	–
Coelution with purified TTR–RBP	+	–	–
Coelution with retinol	–	–	+
Percentage of total peak area	74–89	0–15	9–18
Percentage of total extractable vitamin A in peak	83–94	0	6–17
Immunological reactivity			
Anti-RBP	+	–	–
Anti-transthyretin	+	–	–

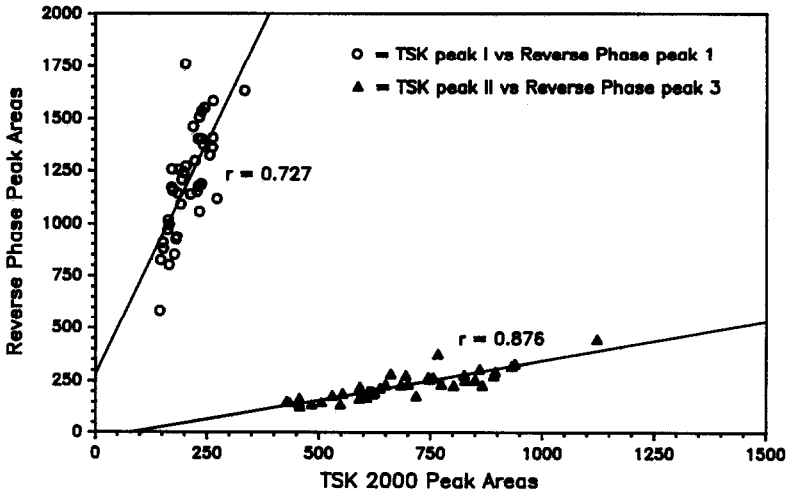

Fig. 5. Correlation of Protesil Octyl 300 reversed-phase HPLC peak areas with TSK 2000 size-exclusion peak areas. TSK 2000 samples were chromatographed under standard size-exclusion chromatographic conditions (plasma diluted 1:3 with normal saline, isocratic gradient of 0.15 *M* sodium phosphate–0.002 *M* β-mercaptoethanol–0.001 *M* ethylenediaminetetraacetic acid, pH 7.0 at 0.5 ml/min). Samples were chromatographed as described in the text. Each point represents the mean of triplicate determinations. (○) Correlation of peak 1 (holo-TTR–RBP) with exclusion peak 1 (holo-TTR–RBP; (▲) peak 3 (retinol derived from holo-free RBP) with peak 2 (holo-free RBP).

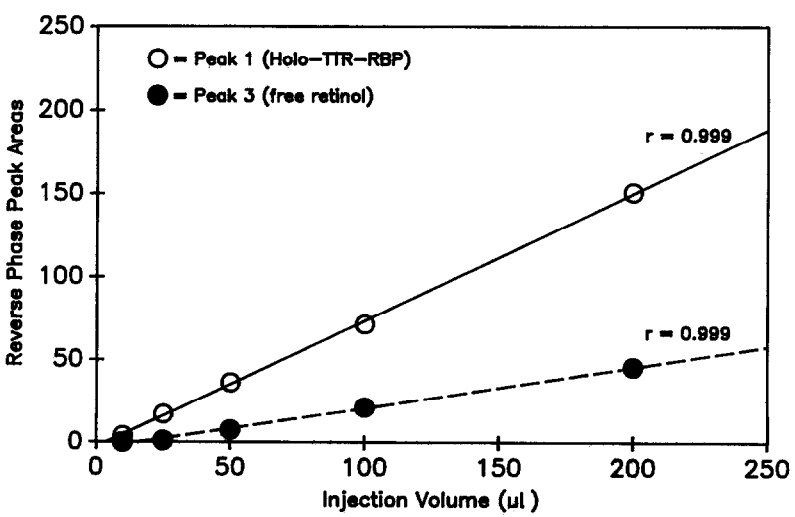

Fig. 6. Variation of Protesil Octyl 300 peak areas with injection volume. (○) Peak 1 (holo-TTR–RBP); (●) peak 3 (retinol). Each point represents the mean of triplicate assays.

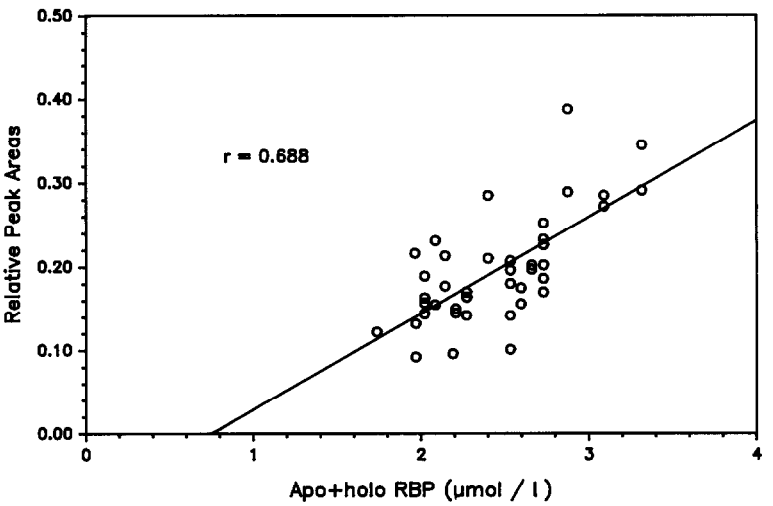

Fig. 7. Correlation of relative peak areas (dissociated vitamin A/holo-TTR-RBP) with apo- + holo-RBP in humans by RID.

The mean (± S.D.) concentration of holo-TTR–RBP in men was 2.04 ± 0.41 μmol/l. The mean (± S.D.) concentration of holo-TTR–RBP in women was 1.85 ± 0.44 μmol/l. This difference was significant ($p < 0.05$ Student's t-test). The difference for apo- + holo-RBP determined by RID was greater. The mean concentration of apo- + holo-RBP for men was 2.70 ± 0.28 μmol/l, and for women was 2.09 ± 0.17 μmol/l. This difference was significant ($p < 0.001$). The third reversed-phase peak (composed of retinol mostly dissociated from free RBP) showed a similarly significant difference between men and women ($p < 0.001$). Not surprisingly, total plasma retinol [10,11] concentrations were also higher in men than in women (1.89 ± 0.37 μmol/l for men, 1.32 ± 0.18 μmol/l for women, $p < 0.001$). The difference between the ratio of the concentrations of retinol to apo- + holo-RBP for men and women was in the same direction but not as large (0.70 for men, 0.63 for women) ($p < 0.01$).

As the concentration of apo- + holo-RBP increased in plasma, the percentage present as free retinol (peak 3) increased (Fig. 7). This increase was significant ($r = 0.688$, $p < 0.001$). There was a similar, but less significant correlation between plasma retinol and the percentage of holo-TTR–RBP (Fig. 8; $r = 0.449$, $p < 0.05$).

DISCUSSION

Holo-TTR–RBP can be assayed by reversed-phase HPLC on a Protesil Octyl 300 column. Only two vitamin A-containing peaks are present in the chromatograms from normal serum or plasma, one corresponding to retinol-transporting TTR–RBP, the other to retinol derived from holo-free RBP. Free retinol and

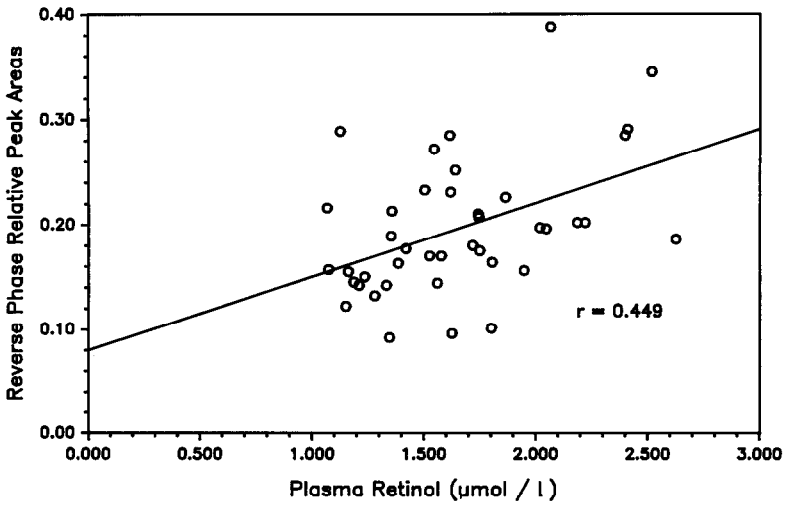

Fig. 8. Correlation of relative peak areas (dissociated vitamin A/holo-TTR–RBP with plasma retinol in humans by HPLC [10,11].

holo-TTR–RBP peak areas determined by this reversed-phase HPLC method are highly correlated to holo-free and holo-TTR–RBP peak areas measured by size-exclusion chromatography. However, peak areas derived from the reversed-phase method have areas that more accurately represent their relative concentrations in blood, determined by RID, in contrast to the peak areas observed with size-exclusion chromatography [8]. Holo-TTR–RBP appears to have a higher correlation than holo-free RBP does to liver vitamin A stores in rats [3], so this method might be an accurate method of measuring liver vitamin A stores.

We found significant differences between men and women in the concentrations of plasma retinol, apo- + holo-RBP, and holo-free and holo-TTR–RBP. Men tended to have higher concentrations of all of these species. The ratio of the concentrations of retinol to RBP were more similar. These ratios were lower than expected, but are similar to those found in several other studies of normal or repleted subjects (*e.g.* refs. 14–16).

We found that as the concentration of apo- + holo-RBP (by RID) or plasma retinol by HPLC increased, the percentage of retinol bound to holo-TTR–RBP decreased, while the percentage appearing as retinol dissociated from holo-free RBP or from free retinol increased. Purified holo-TTR–RBP does not dissociate on our reversed-phase-column. This suggests that more free retinol than had been supposed circulates in a loosely bound association to free RBP or to other molecules in normal adults. It further suggests that as the concentration of retinol or holo-RBP increases, the percentage found as free retinol, or loosely bound retinol also increases and that this increase begins well before toxic concentrations of vitamin A are present.

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