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Extensive Binding of the Bioflavonoid Quercetin to Human Plasma Proteins

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Abstract

Although the bioflavonoids, a large group of polyphenolic natural products, exert chemopreventive effects in cardiovascular disease and cancer, there is little information about the disposition of these dietary components in man. The objective of this study was to investigate the plasma-protein binding of the most abundant bioflavonoid, quercetin, using ^{14}C-labelled quercetin.

An ultracentrifugation assay (170 000 *g* for 16 h at 20°C) was shown to sediment plasma proteins. Binding of quercetin to normal plasma was extensive (99·1 ± 0·5%, mean ± s.d., n = 5). The unbound fraction varied as much as 6-fold, 0·3–1·8%, between subjects. This high binding was independent of quercetin concentration over the range 1·5–15 μM (0·5–5 μg mL^{-1}). Human serum albumin was the primary protein responsible for the binding of quercetin in plasma (99·4 ± 0·1%). Binding by α_1-acid glycoprotein (39·2 ± 0·5%) and very-low-density lipoproteins (< 0·5% of total quercetin) did not make substantial contributions to overall plasma binding. The equilibrium association constant for the binding of quercetin to serum albumin was $267 \pm 33 \times 10^3$ M^{-1} (n = 15). Thermodynamic data for the binding of quercetin to serum albumin indicated spontaneous, endothermic association. Displacement studies suggested that in man the 'IIA' subdomain binding site of human serum albumin was the primary binding site for quercetin. Association of quercetin with erythrocytes was significantly ($P < 0.001$) reduced by plasma protein binding.

These data indicate poor cellular availability of quercetin because of its extensive binding to plasma proteins.

The bioflavonoids are a large group of polyphenolic natural products widely distributed in fruits and vegetables and in beverages produced from these sources. They are thus normal constituents of our daily diet (Hertog et al 1992, 1993; Cook & Samman 1996) and the total average daily intake of bioflavonoids has been estimated to be approximately 1 g (Smith & Yang 1994). Dietary bioflavonoids are increasingly being recognized as having a protective role in coronary heart disease and stroke through several mechanisms, including antioxidant effects and inhibition of platelet aggregation (Formica & Regelson 1995; Pace-Asciak et al 1995; Keli et al 1996; Knekt et al 1996). Similarly, bioflavonoid intake has been shown to have a chemoprotective role in cancer (Wang et al 1994; Ames et al 1995) through complex effects on signal transduction involved in cell proliferation (De Azevedo et al 1996; Weber et al 1996; Lepley & Pelling 1997) and angiogenesis (Fotsis et al 1997). Bioflavonoids have also been shown to have effects on many enzymes (Tsyrlov et al 1994; Walle et al 1995) and on a wide variety of other biological processes which are also likely to be of importance in human health (Formica & Regelson 1995).

Although bioflavonoid intake has been associated with many beneficial effects, the extent to which these natural products are bioavailable in man is unclear; it is also unclear whether the effects of bioflavonoids observed in-vitro can occur in-vivo. It is therefore necessary to enhance our understanding of the fate of bioflavonoids in man. One important factor to consider in the disposition of these agents is their transport in blood by plasma proteins.

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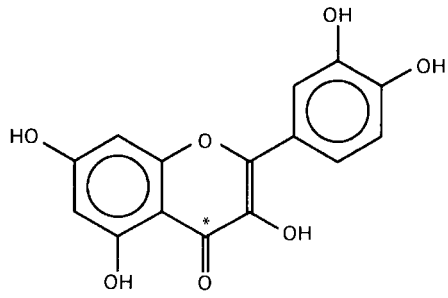

Figure 1. The chemical structure of quercetin (*indicates the position of the ^{14}C radiolabel).

Quercetin, found in relatively large amounts in many fruits and vegetables, is one of the most abundant bioflavonoids in our diet (Hertog et al 1993). The chemical structure of quercetin is shown in Figure 1. Preliminary studies indicate that quercetin is tightly bound to plasma proteins but the actual amount in the unbound form remains unknown (Gugler et al 1975; Manach et al 1995). In the current study, the extent and nature of the binding of quercetin to plasma proteins in man was examined by use of radiolabelled quercetin. Erythrocytes were used as a model to investigate the effect of plasma protein binding on the cellular association of quercetin.

Materials and Methods

Materials

4-[^{14}C]Quercetin dihydrate ([^{14}C]quercetin) was custom-synthesized by Chemsyn Science Laboratories (Lenexa, KS) for the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Its specific activity was 52·9 mCi mM^{-1} by ultraviolet spectrophotometric assay and it was shown to be $\geq$98% pure by high-performance liquid chromatography (HPLC). [^{14}C]Quercetin powder (200 μCi) was dissolved in dimethylsulphoxide and stored under argon at −20°C; it was thawed immediately before use. Crystalline human serum albumin (fraction V, high purity) and crystalline α_1-acid glycoprotein (fraction VI, ~99%) were purchased from Calbiochem-Novabiochem (La Jolla, CA). Crystalline bovine serum albumin was purchased from Sigma (St Louis, MO). Distilled, deionized water was used in all experiments and other chemicals were of analytical grade or better.

Collection of blood samples

Informed consent was obtained in writing from five healthy volunteers (3 male, 2 female) before collection of blood. The collection protocol was approved by the local Institutional Review Board. Blood (50 mL) was obtained by venepuncture of a forearm vein and collected into heparinized glass vacuum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood was centrifuged (2000 *g* for 15 min) and the plasma aspirated and used immediately for plasma-binding experiments. Excess plasma was stored at −20°C until required for binding experiments with diluted plasma. Blood collected for erythrocyte association experiments was used immediately.

Protein binding assay

Pilot studies with ultrafiltration units (Ultrafree-MC, Millipore, Bedford, MA) found that approximately 60% of [^{14}C]quercetin was bound to the low-binding cellulose membranes after centrifugation of 3 μM solutions in isotonic phosphate buffer (pH 7·4). This high membrane binding made ultrafiltration unsuitable for these studies. Extensive membrane binding also precluded equilibrium dialysis as a suitable method for plasma-protein binding studies. Ultracentrifugation was therefore considered the most appropriate method for examining the binding of [^{14}C]quercetin to plasma proteins.

A pilot study designed to determine the amount of ultracentrifugation required to sediment plasma proteins was performed using bovine serum albumin as model protein. [^{14}C]Quercetin was added to solutions of bovine serum albumin (40 mg mL^{-1}) in isotonic phosphate buffer (pH 7·4) to give a final [^{14}C]quercetin concentration of 3 μM in 4·1 mL. The solutions were ultracentrifuged at 170 000 *g* (37 500 rev min^{-1}) at 20 ± 1°C (Sorvall RC 80 ultracentrifuge; Sorvall Instruments, du Pont, Wilmington, DE) in 5-mL polycarbonate tubes (Sorvall Instruments) in a six-place swinging bucket rotor (AH60, Sorvall Instruments). The spin was stopped after 1, 2, 3, 4, 8, 12, 16 and 20 h without braking. Two 100-μL samples were withdrawn from the top of the solution at each time point, one for determination of radioactivity, the other for determination of protein concentration. To determine the radioactivity of a sample, scintillation fluid (Scintisafe Econo 2, Fischer Scientific, Fair Lawn, NJ; 10 mL) was added to the sample and the disintegrations min^{-1} counted by liquid scintillation (LS 6000SC, Beckman Instruments, Fullerton, NJ). Quench correction was by the external standard ratio method. The total protein concentration of samples was determined by the method of Lowry et al (1951); the limit of detection was 1·5 μg.

The potential for adsorptive loss of [^{14}C]quercetin (3 μM in isotonic phosphate buffer (pH 7·4)) to various types of container (polycarbonate, polyethylene, glass and silanized glass) was examined

in the dark at room temperature. Samples of the isotonic phosphate buffer (pH 7·4) containing [^{14}C]quercetin were withdrawn from the airtight containers at various times over a period of 24 h and subjected to liquid scintillation counting.

Protein-binding studies

Before ultracentrifugation of all samples, 100 μL was withdrawn for liquid scintillation counting. After ultracentrifugation, two 100-μL samples were withdrawn from the water layer for liquid scintillation counting and determination of protein concentration. Samples with detectable protein in the water layer were not used for data analysis. [^{14}C]Quercetin was added to plasma from each individual to give final [^{14}C]quercetin concentrations of 1·5, 3, 7·5, 12 and 15 μM (0·5–5 μg mL^{-1}) in a final volume of 4·1 mL; the solution was then subjected to ultracentrifugation (n = 5 sets of concentrations). Human serum albumin (40 mg mL^{-1}) dissolved in isotonic phosphate buffer (pH 7·4) was treated identically. The binding of quercetin to α_1-acid glycoprotein (0·66 g L^{-1}) was examined in the same buffer to which 1·5 μM [^{14}C]quercetin had been added (n = 3).

After ultracentrifugation of plasma samples for 16 h at 20 ± 1°C, several layers were observed: a top layer of very-low-density lipoproteins (Legg & Rowland 1987); a second 'protein-free' water layer; and a number of protein layers. The entire very-low-density lipoprotein layer was carefully aspirated and subjected to liquid scintillation counting. After ultracentrifugation of buffered protein solutions only two layers were observed: an upper 'protein-free' water layer and a lower protein layer.

Because of its poor solubility in water the range of workable quercetin concentrations was limited. Therefore, to examine binding at different quercetin-to-protein ratios plasma from each individual was diluted with isotonic phosphate buffer (pH 7·4) to 0·01, 0·05, 0·1, 1, 10 and 50% plasma content, [^{14}C]quercetin was added to a final concentration of 1·5 μM and the mixture was ultracentrifuged for 16 h at 20 ± 1°C (n = 5 sets of dilutions). A 40 mg mL^{-1} solution of human serum albumin was treated identically (n = 3 sets of dilutions).

The effect of temperature on the binding of quercetin to human serum albumin was examined in 40 mg mL^{-1} solutions in isotonic phosphate buffer (pH 7·4) to which [^{14}C]quercetin had been added to give final concentrations of 1·5, 3, 7·5, 12 and 15 μM. The solutions were ultracentrifuged at 170 000 *g* for 16 h at 5 ± 1, 12 ± 1 or 20 ± 1°C (n = 1 set of 5 concentrations at each temperature).

Displacement studies

The identity of the quercetin binding site on human serum albumin was examined by determining the extent to which it was displaced by either (±)-warfarin or (±)-ibuprofen, which bind primarily to the 'IIA' or 'IIIA' subdomain binding sites, respectively (He & Carter 1992). Various amounts of (±)-warfarin or (±)-ibuprofen were added to centrifuge tubes to give final concentrations of 0, 10, 100, 200 and 1000 μM in 4·1 mL. Plasma diluted to a concentration of 1% with isotonic phosphate buffer (pH 7·4) was added to the tubes and [^{14}C]quercetin was added to a final concentration of 1·5 μM. The solutions were thoroughly mixed and a 100-μL sample taken for liquid scintillation counting. Samples were prepared in triplicate and ultracentrifuged and analysed as described above for protein binding studies.

Erythrocyte association

The effect of plasma proteins on the association of quercetin with erythrocytes was examined both in whole blood and in a protein-free erythrocyte suspension. [^{14}C]Quercetin was added to freshly collected heparinized blood from five volunteers to give final concentrations of 1·5, 3, 7·5 and 15 μM in a final volume of 3 mL. After thorough mixing, 0·5 mL of blood from each concentration and volunteer was placed in six sealed polypropylene tubes. A second sample of blood from each volunteer was centrifuged (2000 *g* for 15 min), the plasma was aspirated and the erythrocytes washed three times with normal saline. Erythrocytes were then re-suspended in isotonic phosphate buffer (pH 7·4) to give an identical haematocrit to that of to the original blood samples. As with blood samples, [^{14}C]quercetin was added to erythrocyte suspensions to give final concentrations of 1·5, 3, 7·5 and 15 μM in final volumes of 3 mL; 0·5 mL of each concentration was then placed in each of six sealed polypropylene tubes.

The whole-blood and erythrocyte suspensions with added [^{14}C]quercetin were incubated in a shaking water bath at 37°C. One tube of each concentration from each group was removed 5, 15, 30, 60, 120 and 240 min after the initiation of incubation and centrifuged at 11 400 *g* for 2 min. Samples (100 μL) of the supernatant were subjected to liquid scintillation counting.

Data analysis

The unbound fractions of [^{14}C]quercetin in plasma and protein solutions were calculated as the ratio of the number of disintegrations min^{-1} in the water layer to that of the sample before ultracentrifugation. Unbound fractions of quercetin in plasma

were analysed for the effect of quercetin concentration and for differences between individuals by two-way analysis of variance (Instat 2.00; GraphPad Software, San Diego, CA). Differences between unbound fractions in plasma and human serum albumin were analysed by means of the Mann-Whitney *U*-test (Instat 2.00). The apparent equilibrium association constant (K_a) for the binding of quercetin to human serum albumin was determined by use of equation 1 derived from the law of mass action:

$$K_a = C_b/[C_f(P_t - C_b)] \quad (1)$$

where C_b is the concentration of quercetin bound to human serum albumin, C_f is the unbound concentration of quercetin and P_t is the total concentration of serum albumin.

The enthalpy change (ΔH) for the association between quercetin and human serum albumin was determined from the slope of best fit obtained by linear least-squares regression (Minim 2.0.8; Dr R. Purves, University of Otago, Dunedin, New Zealand) of the dependence of ln K_a against the reciprocal of temperature for the three temperatures studied. Entropy (ΔS) and Gibb's free energy (ΔG) changes were then calculated from standard thermodynamic equations.

The fraction of quercetin distributed into erythrocytes was determined by subtracting the radioactivity in the supernatant (after correction for haematocrit) from the total radioactivity added. The significance of differences between the amount of quercetin in plasma and buffer in erythrocyte association experiments were determined by means of the Mann-Whitney *U*-test (Instat 2.00). Two-way analysis of variance was used to examine erythrocyte association data for effect of time and quercetin concentration (Instat 2.00). Data are reported as mean ± standard deviation. $P \leq 0.05$ was taken as indicative of significance.

Results

Ultracentrifugation

In the pilot study of bovine serum albumin sedimentation, protein concentrations in the top layer declined sigmoidally with ultracentrifugation time, with no protein detectable in this layer after 16 h. Radioactivity in the water layer also declined sigmoidally with ultracentrifugation time and remained constant (0·66%) after 12 h. Sixteen hours was therefore chosen as the standard spin time for subsequent experiments; this was found to be sufficient to sediment plasma proteins to below the limit of detection of the protein assay in subsequent experiments. Radioactivity in samples taken from the top of 15 μM [^{14}C]quercetin buffer solution were unchanged after centrifugation for 16 h under these conditions, indicating quercetin was not sedimented during the spin at this concentration. [^{14}C]Quercetin in buffer alone did not bind significantly to polycarbonate, polyethylene, glass or silanized glass over a 24-h period (linear regression, Instat 2.00).

Binding of quercetin to plasma and to human serum albumin

The binding of quercetin to plasma proteins was extensive, with 99·1 ± 0·5 and 99·4 ± 0·1% of quercetin being bound to plasma and human serum albumin (40 mg mL^{-1}), respectively (see Table 1). There were significant differences between the extent of binding of quercetin to plasma from different individuals ($P < 0.001$) which did not appear to be related to age or sex. There was no significant difference between the fraction of quercetin bound to plasma and to the physiological concentration of human serum albumin employed, suggesting that this protein is responsible for the majority of the binding of quercetin to plasma. Quercetin concentration had no significant effect on the fraction of the compound bound either to plasma or to human serum albumin over the range studied (1·5–15 μM). Saturation of the quercetin binding site(s) is thus unlikely to occur at physiological or

Table 1. Fractions (%) of quercetin bound to plasma, to human serum albumin and to α_1-acid glycoprotein at 20 ± 1°C.

Sample	Fraction of bound quercetin	Range
Plasma		
1	99·6 ± 0·1	99·6–99·7
2	98·7 ± 0·3	98·2–99·0
3	99·2 ± 0·2	99·1–99·5
4	98·6 ± 0·3	98·4–99·0
5	99·5 ± 0·1	99·5–99·6
Mean	99·1 ± 0·5	
Human serum albumin (40 mg mL^{-1})		
1	99·3 ± 0·1	99·2–99·5
2	99·4 ± 0·1	99·3–99·4
3	99·4 ± 0·1	99·4–99·5
Mean	99·4 ± 0·1	
α_1-Acid glycoprotein (0·66 mg mL^{-1})		
Mean	39·2 ± 0·5	38·8–39·8

Results are means ± s.d. (%). For experiments with plasma and human serum albumin the quercetin concentration was 1·5–15 μM (five concentrations in each set). For experiments with α_1-acid glycoprotein the quercetin concentration was 1·5 μM (n = 3 replicates).

potentially therapeutic concentrations of quercetin. The mean K_a value for the binding of quercetin to human serum albumin at 20°C was $267 \pm 33 \times 10^3$ M^{-1} (n = 15). Similar sigmoidal relationships between the fraction of bound quercetin and the total protein concentration were obtained for both diluted plasma and human serum albumin solutions (see Figure 2). This similar association also suggests that in man serum albumin is the primary protein responsible for the plasma protein binding of quercetin.

Binding of quercetin to α_1-acid glycoprotein (39·6 ± 0·5%, n = 3) was significantly less ($P < 0.01$) than to serum albumin (n = 5 concentration sets) (Table 1). Given the relatively lower physiological concentration of α_1-acid glycoprotein (0·5–1 g L^{-1}) compared with human serum albumin (Kremer et al 1988) and its lower binding of quercetin, α_1-acid glycoprotein does not seem to make a substantial contribution to the overall binding of quercetin in plasma. Similarly, only small amounts of quercetin (< 0·5% of total radioactivity added) were associated with the very-low-density lipoprotein layer, suggesting this plasma fraction also does not contribute substantially to the overall plasma binding of quercetin.

Binding of quercetin to physiological concentrations of serum albumin increased with decreasing temperature from $267 \pm 33 \times 10^3$ to $330 \pm 18 \times 10^3$ and $413 \pm 20 \times 10^3$ M^{-1} at 20, 12 and 4°C, respectively, giving a ΔH value of 2·05 ± 0·18 kJ M^{-1} (r = 0·95, n = 25), indicating endothermic association. Extrapolation of these data to 37°C gave a K_a value of 177×10^3 M^{-1}, corresponding to approximately 97·9% binding of quercetin at body temperature. The Gibb's free energy change (ΔG) and the entropy change (ΔS) for quercetin–serum albumin association were calculated to be −28·7 ± 0·3 kJ M^{-1} at 20°C (n = 15) and 111 ± 1 J M^{-1} K^{-1} (n = 15), respectively. The negative value calculated for ΔG suggests that in man association between quercetin and serum albumin was spontaneous. The positive value calculated for ΔS of the quercetin–human serum albumin association indicates a loss of order on binding. This loss of order is probably because of a loss of structured water at the binding site.

The fraction of quercetin bound to 1% plasma generally decreased on addition of up to 1000 μM (±)-warfarin, suggesting that quercetin was displaced from the 'IIA' subdomain site of human serum albumin (Figure 3). Displacement of quercetin by (±)-ibuprofen did not occur up to 1000 μM under these conditions, indicating that in man the 'IIIA' subdomain site is not a major site for the binding of quercetin to serum albumin. The displacement of quercetin by (±)-warfarin was only significantly different from that by (±)-ibuprofen at the 1000 μM concentration ($P < 0.01$).

Erythrocyte association

Whole-blood incubation experiments revealed that 83·8 ± 4·8% of quercetin remained in plasma. In contrast, when quercetin was incubated with erythrocytes in buffer in the absence of plasma proteins, only 4·1 ± 1·6% of quercetin remained in the buffer. The difference between erythrocyte asso-

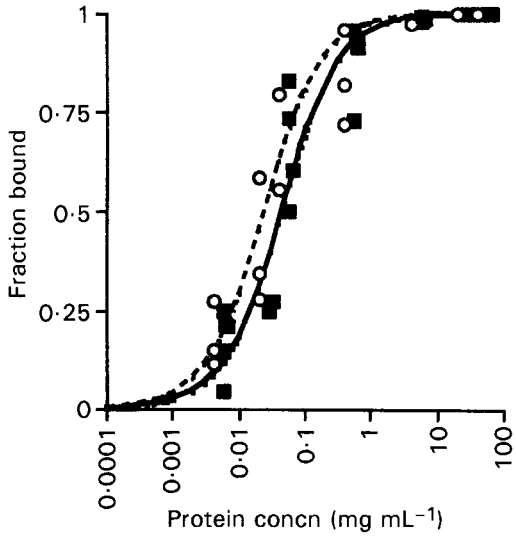

Figure 2. Plot of the fraction of bound quercetin against protein concentration for diluted human plasma (■) and for human serum albumin solution (○); the lines of best fit (solid and dashed lines, respectively) were determined by non-linear regression analysis.

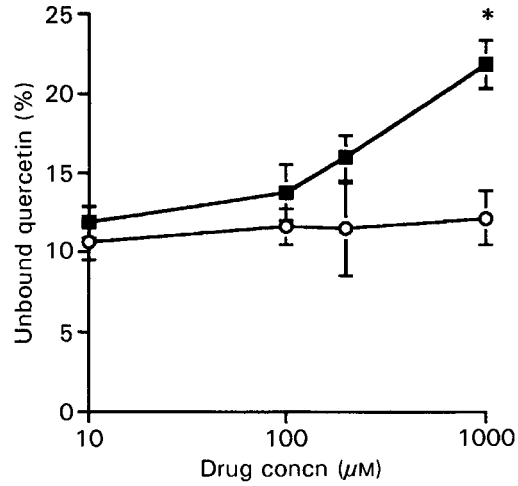

Figure 3. Plot of percentage unbound quercetin in 1% human plasma in isotonic phosphate buffer (pH 7·4) against concentration of (±)-warfarin (■) or (±)-ibuprofen (○). Data are means ± s.d. *$P < 0.01$, significant difference between results for the two drugs at 1000 μM.

ciation of quercetin in whole blood and in the erythrocyte suspension was highly significant ($P < 0.001$). This suggests plasma proteins significantly limit the association of quercetin with the cells. There were no significant effects of time (5–240 min) or concentration (1·5–5 μM) on the association of quercetin with erythrocytes under the conditions employed.

Discussion

The principal finding in this study is that quercetin is highly bound to plasma proteins, resulting in a small and highly variable unbound fraction, 0·3–1·8%, in five healthy normal volunteers.

Two previous studies have examined the plasma binding of quercetin by gel filtration (Gugler et al 1975) and ultrafiltration (Manach et al 1995). These studies suggested very high binding because no unbound quercetin could be detected with the fluorimetric or ultraviolet detection methodologies employed. The use of radioactively labelled quercetin in our study was an important improvement in sensitivity, enabling quantification of the small amounts of unbound compound. In addition, the use of an ultracentrifugation binding assay was necessary to avoid unacceptable losses of quercetin as a result of adsorption to filters and membranes.

In man, the main plasma-binding protein for quercetin was serum albumin with only minor contributions from α_1-acid glycoprotein and very-low-density lipoproteins. Thermodynamic experiments predicted less binding of quercetin to human serum albumin at 37°C (97·9%) than at 20°C (99·4%), the temperature at which these ultracentrifugation experiments were conducted. The estimated K_a value for the binding of quercetin and serum albumin in man at 37°C, 177×10^3 M^{-1}, is of similar magnitude to that for other drugs (e.g. warfarin (231×10^3 M^{-1}) and phenylbutazone (230×10^3 M^{-1})) which are extensively (i.e. > 98%) bound to human serum albumin (Tillement et al 1974).

In an attempt to characterize the binding site(s) for quercetin on human serum albumin, inhibition experiments were conducted with drugs which bind predominantly to the major hydrophobic binding sites on the IIA ((±)-warfarin) and IIIA ((±)-ibuprofen) subdomains of the protein (He & Carter 1992). Only the highest (±)-warfarin concentration showed some inhibition of the binding of quercetin to serum albumin. Of further interest is whether other flavonoids bind as strongly to human serum albumin and plasma.

The 6-fold variability of the unbound fraction of quercetin in plasma (0·3–1·8%) for the five normal healthy subjects studied was of interest. This variability did not seem to be related to the age or sex of the subjects but might be a result of competition with other dietary flavonoids or endogenous compounds. This question warrants further investigation.

Of considerable pharmacological significance is the extent to which plasma binding inhibits the cellular association of quercetin. It is clear from this study that this binding reduced the extensive ability of the red blood cells to accumulate quercetin in the absence of plasma proteins. This observation is also in accord with data recently reported on the intravenous dose kinetics of quercetin in a phase I trial in cancer patients (Ferry et al 1996). In that study, a steady-state volume of distribution of only 4·7 L was observed, indicating that quercetin is contained largely within the plasma compartment.

In summary, we have shown that in man quercetin is extensively bound to plasma proteins, mainly serum albumin, with 2% or less present in the unbound form in-vivo. This small fraction implies that the cellular availability of quercetin from the systemic circulation is poor. Pharmacological studies with quercetin using protein-free buffers in-vitro should be interpreted with this in mind when extrapolating to the in-vivo situation.

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