Limitations of protein-coated charcoal in the separation of free from bound vitamin B₁₂ in serum

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SUMMARY The effect of haemoglobin and albumin-coated charcoal on the concentration of vitamin B₁₂ binding proteins in serum has been investigated. As commonly employed, coated charcoal removes a significant amount of transcobalamin II (TCII) from serum, but does not affect transcobalamin I and III (TCI and III). Increasing the protein coat up to about 10 mg of protein per 25 mg of charcoal reduces the adsorption of TCII, but increasing the protein concentration beyond this has little added effect. The amount of adsorption is proportional to the amount of coated charcoal employed, but even small amounts adsorb some TCII. These results indicate that protein-coated charcoal is not the ideal way of separating free from bound vitamin B₁₂ in serum; it cannot reliably be used for the measurement of the concentration of apo-TCII but can be employed for the measurement of apo-TCI and III.

There are two major vitamin B₁₂ binding proteins in human serum, transcobalamins I and III and transcobalamin II. Transcobalamins I and III (TCI and III), also known as cobalophilin, are mainly saturated with vitamin B₁₂ and probably serve a storage function. Transcobalamin II (TCII) is mainly unsaturated and is the major transport protein for the vitamin. The unsaturated (apo) TCI and III and TCII together constitute the unsaturated vitamin B₁₂ binding capacity of serum. To measure this, it is the practice to add excess radioactive labelled vitamin B₁₂ which saturates all the binding sites and leaves excess free radioactive vitamin B₁₂. The next step involves separating the bound from the free vitamin B₁₂ for which a number of methods have been described including microbiological assay,¹ electrophoresis,¹ dialysis,² gel filtration,³ ultrafiltration,⁴ and adsorption with haemoglobin-coated⁵ or albumin-coated⁶ charcoal. Because of its simplicity, the coated charcoal method has achieved wide popularity.

In measuring the concentration of apo-TCII in serum, it was noted that treatment with coated charcoal appeared to decrease the amount of measurable TCII. We report the results of a systematic study of this phenomenon.

Material and methods

Sera were obtained from normal volunteers and from patients undergoing therapeutic phlebotomy. The blood was allowed to clot at room temperature, the serum separated and frozen in aliquots at -20°C until used.

RADIOACTIVE VITAMIN B₁₂
Vitamin B₁₂ labelled with ⁵⁷Co (⁵⁷CoB₁₂) with a specific activity of approximately 15.0 μCi/μg was obtained from Amersham Corporation.

ZIRCONYL PHOSPHATE GEL
Zirconyl chloride (ZrOCl₂·8H₂O) was obtained from BDH Chemicals Ltd. Zirconyl phosphate gel pH 6.25 (Z-gel) was prepared according to the method of Hansen *et al.*⁷ This was used to measure the apo-TCII concentration of serum by the method of Tan and Hansen⁸ as modified by Sonneborn *et al.*⁹ with minor changes. Five hundred picograms ⁵⁷CoB₁₂ in 1.0 ml saline was added to duplicate aliquots of 0.1 ml serum and the mixtures incubated at room temperature for 30 min; 2.5 ml of Z-gel and 5.0 ml of 0.1 mol/l ammonium acetate pH 6.25 were added. The tubes were mixed, centrifuged at 1600 g for 5 min, the supernatant decanted, the sediment washed with another 5 ml ammonium acetate solution and the radioactivity of the pellet measured in a well scintillation counter.

Limitations of protein-coated charcoal

COATED CHARCOAL

The following charcoal preparations were investigated:

1 Norit "A", pharmaceutical grade, neutral charcoal, from Amend Drug and Chemical Company, lot no B10311H18.

2 The same as above but lot no D11718B01.

3 DARCO G-60 activated carbon from Fisher Scientific Company, lot no 746274.

4 Norit A, neutral, pharmaceutical charcoal powder for decolorising from BDH Chemicals, lot no 83116/3417.

5 Norit SG activated charcoal powder from MCB Manufacturing Chemists.

Haemoglobin-coated charcoal was prepared according to the method of Lau *et al.*⁵ using one part of haemoglobin (prepared from human red cells) to 10 parts of charcoal. Albumin-coated charcoal was prepared as described by Gottlieb *et al.*⁶, using one part of albumin to five of charcoal.

VITAMIN B₁₂-SATURATED CHARCOAL TREATED SERUM

This was prepared by the method of Scott *et al.*¹⁰ Five hundred picograms of ⁵⁷CoB₁₂ in 1 ml of saline was added to 0.1 ml of serum. This amount of ⁵⁷CoB₁₂ was in excess of the vitamin B₁₂ binding capacity of the serum. The mixture was incubated at room temperature for 30 min. An aliquot (1.0 ml) of coated charcoal, containing 25 mg charcoal, was then added to adsorb the free ⁵⁷CoB₁₂, the tube mixed on a vortex mixer, centrifuged, and the supernatant serum decanted; this is hereafter termed charcoal-treated serum.

SEPHADEX GEL FILTRATION

Sephadex gel filtration experiments were carried out using Sephadex G-150 in a 2.5 × 100 cm column. Serum (500 μl) was applied to the column and eluted with 0.04 mol/l phosphate buffer containing 0.5 mol/l NaCl, pH 7.38. Fractions (4 ml) were collected, the optical density measured at 280 nm and the radioactivity of the fractions determined using a well scintillation counter. The radioactivity of the fractions included in the TCI and III and the TCII peaks were summed to give the respective amounts of these vitamin B₁₂ binding apoproteins. It was found that the charcoal-treated serum, in spite of extensive centrifugation, contained a small amount of charcoal particles which tended to block the column. Experiments showed that aliquots of sera directly applied to the column, or passed through an 0.45 μm millipore filter, contained identical amounts of vitamin B₁₂ binders. The charcoal-treated serum was therefore passed through such a filter and 500 μl of the filtrate applied to the column.

PURIFICATION OF TCII

TCII was purified from Cohn Fraction III* prepared from human plasma as described by Savage *et al.*¹¹ This purified material was shown to be free of cobalophilin by sodium dodecyl sulphate-polyacrylamide gel electrophoresis*.¹² The purified TCII was saturated with ⁵⁷CoB₁₂ and 340 μg of this protein combined with 1.0 ml of Freund's adjuvant was injected into the foot pads of each of three New Zealand white rabbits. Twenty-three days later a repeat injection containing 85 μg of the same material with 0.5 ml Freund's adjuvant was given. The rise in TCII antibody titre as measured by PEG precipitation,¹³ was followed weekly and serum collected when the antibody titre was 1200 ng/ml or more. This antibody was used to confirm the identity of the TCII peak in Sephadex column chromatography experiments. The addition of the antibody caused the peak to be shifted to the void volume.

*See acknowledgements.

Results

Effect of coated charcoal on apo-TCII concentration
⁵⁷CoB₁₂ was added to seven sera in more than ade-

Fig. 1 Representative Sephadex G-150 column chromatography experiment. ⁵⁷CoB₁₂ was added to a serum in excess of its binding capacity. One aliquot was applied directly to the column (.....○) and one was pretreated with haemoglobin-coated charcoal (●—●). It will be seen that treatment with charcoal removed the free ⁵⁷CoB₁₂ and a significant amount of TCII.

Table 1 *Apo-TCII concentration (pg/ml)*

	Before haemoglobin-coated charcoal		After haemoglobin-coated charcoal	
	Sephadex	Z gel	Sephadex	Z gel
1	998	986	646	577
2	862	819	569	509
3	554	558	212	264
4	1560	1528	911	946
5	870	868	625	600
6	714	704	373	449
7	1038	946	611	685

quate amounts to saturate all the vitamin B₁₂ binders. Each serum was then divided into two aliquots, one of which was treated with haemoglobin-coated charcoal using charcoal No 1 (25 mg in 1 ml) as described above. The TCII content of each aliquot was then determined both by the Z-gel assay and by Sephadex G-150 column chromatography. A representative column chromatography experiment is shown in Fig. 1 and the results for the seven sera are shown in Table 1. It will be seen that there is good agreement between the two methods for measurement of apo-TCII, but in each case there is a substantial reduction in the apparent TCII concentration in the charcoal treated sera which ranged from 28 to 62%.

On five different occasions, three aliquots of one serum were taken. Excess ⁵⁷CoB₁₂ was added to each aliquot. On the first aliquot the apo-TCII content was estimated by the Z-gel assay. The mean $\pm$ SD TCII content was 1080 ± 54 pg/ml. The free ⁵⁷CoB₁₂ in the second and third aliquots was removed with albumin-coated charcoal or haemoglobin-coated charcoal. The apo-TCII concentration of the second aliquot was then estimated by the Z-gel assay. The mean $\pm$ SD was 674 ± 63 pg/ml. To the third aliquot further ⁵⁷CoB₁₂ was added and a Z-gel assay for apo-TCII performed. The mean $\pm$ SD was 733 ± 35 pg/ml. The failure of

the addition of further ⁵⁷CoB₁₂ to restore the measurable apo-TCII to the value in the first aliquot (1080 ± 54 pg/ml) indicates that the treatment with protein-coated charcoal has removed TCII and not simply removed ⁵⁷CoB₁₂ from its binding with TCII.

Effect of coated charcoal on apo-TCI and III

The apo-TCI and III concentrations in the above seven sera before and after treatment with haemoglobin-coated charcoal were measured by Sephadex G-150 column chromatography (Fig. 1). The results for the seven sera are shown in Table 2.

Effect of amount of coated charcoal

⁵⁷CoB₁₂ was added to a serum in more than adequate amounts to saturate all the vitamin B₁₂ binders.

Table 2 *Apo-TCI and III concentrations (pg/ml) as measured by Sephadex gel filtration*

	Before haemoglobin-coated charcoal	After haemoglobin-coated charcoal
1	593	577
2	132	150
3	197	217
4	563	529
5	184	149
6	283	278
7	223	238

This was divided into five aliquots and amounts of haemoglobin-coated charcoal (charcoal No 1) ranging from 6.25 to 25 mg added to four of them. The TCII content of the untreated and the charcoal-treated aliquots were then measured by the Z-gel assay in duplicate. This was done on five different occasions on the same serum and the mean and standard deviation of the TCII concentrations for each of the aliquots estimated. The results are shown in Fig. 2. It will be seen that there is a linear reduction in measurable apo-TCII concentration

Fig. 2 The apo-TCII content of a serum as measured by the Z-gel assay directly and after adsorption with different amounts of haemoglobin-coated charcoal using one part of Hb to ten of charcoal. Each point represents the mean of duplicate observations made on five different occasions and the vertical lines, the standard deviations of the means.

Table 3 Apo-TCII concentration (pg/ml) as measured by Z gel assay

Serum	Without charcoal	Haemoglobin-coated charcoal				
		Charcoal 1	Charcoal 2	Charcoal 3	Charcoal 4	Charcoal 5
1	687	503	502	367	498	573
2	854	591	608	432	633	722
3	539	331	356	235	380	430
		Albumin-coated charcoal				
4	869	509	529	577	609	678
5	710	445	458	510	557	560
6	605	264	214	332	338	402

with the addition of increasing amounts of haemoglobin-coated charcoal.

Effect of the amount of protein used to coat the charcoal

Haemoglobin-coated charcoal using charcoal No 1 was prepared with amounts of haemoglobin (Hb) ranging from 1.25 to 25 mg of Hb per 25 mg of charcoal. Similarly albumin-coated charcoal using charcoal No 1 was prepared with amounts of albumin ranging from 2.5 to 25 mg per 25 mg of charcoal. The effect of 25 mg of these different coated charcoals on the apo-TCII content of 0.1 ml aliquots of a serum was measured in duplicate by the Z-gel assay. The results are shown in Fig. 3. As the ratio of protein to charcoal is increased, the amounts of apo-TCII remaining in the serum increases, but from 10 mg of protein to 25 mg of protein per 25 mg of charcoal there is little change.

Effect of different charcoal preparations

Three sera were divided into aliquots and the apo-TCII content measured by the Z-gel assay, both directly and after treatment with haemoglobin-

coated charcoal (25 mg charcoal) prepared from the five different lots of charcoal listed under material and methods, using one part of haemoglobin to ten of charcoal. Three sera were similarly investigated using albumin-coated charcoal (25 mg charcoal) prepared from the five different lots of charcoal using one part of albumin to five of charcoal. The results are shown in Table 3. In each case, the various coated charcoals reduce the amount of measurable apo-TCII.

Discussion

Miller¹⁴ appears to have been the first to describe the use of charcoal adsorption to separate free from protein-bound vitamin B₁₂ in serum. However, Gregory and Holdsworth¹⁵ and Ardeman and Chanarin¹⁶ showed that activated charcoal would also adsorb some vitamin B₁₂ binding proteins. Gottlieb *et al*⁶ suggested that the charcoal should first be coated with another protein to prevent this. They added excess radioactive vitamin B₁₂ to 0.5 ml of serum and added 50 mg of albumin-coated charcoal (one part of albumin to five of charcoal by weight) to

Fig. 3 The measurable apo-TCII concentrations of 0.1 ml aliquots of a serum treated with 25 mg of charcoal coated with different amounts of Hb and albumin. The left most point on the former represents a concentration of one part of Hb to 20 of charcoal as advocated by Lau *et al*.⁵ The second point from the left on the latter corresponds with the concentration of one part of albumin to five of charcoal as recommended by Gottlieb *et al*.⁶ The horizontal dotted line is the "true" apo-TCII concentration as measured by the Z-gel assay without exposure to coated charcoal.

remove the free radioactive vitamin B₁₂ and assumed that the radioactivity remaining in the supernatant was a measure of the unsaturated vitamin B₁₂ binding capacity of the serum. The same workers⁵ later also recommended haemoglobin as a coating agent (one part of haemoglobin solution to 20 of charcoal by weight). Subsequently Scott *et al*¹⁰ from the same laboratory, used this haemoglobin-coated charcoal in the ratio of 25 mg of charcoal to 0.1 ml of serum to remove excess free radioactive vitamin B₁₂.

Adams and McEwan¹⁷ made a systematic study of several methods for separating bound from free vitamin B₁₂. They noted that the results using charcoal could be significantly affected by a number of factors including the type of binder, the form of vitamin B₁₂, the type and amount of charcoal and the amount of coating. However, they did not study the vitamin B₁₂ binding proteins of serum. Stenman¹⁸ also found that coated charcoal adsorbed some vitamin B₁₂ binding proteins, especially from samples with a low protein content. Begley and Hall¹⁹ reported that the use of albumin-coated charcoal as recommended by Gottlieb *et al*⁶ reduced the apparent unsaturated vitamin B₁₂ binding capacity of serum by about 10%, but did not determine which binding proteins were adsorbed.

Our accidental finding that the apparent apo-TCII concentration of a serum as measured by the Z-gel assay was considerably reduced by prior treatment with haemoglobin-coated charcoal prompted this study. The results of the Z-gel assay on other sera before and after treatment with coated charcoal (Table 1) indicated that this reduction was a constant phenomenon. We have further shown that this reduction is due to adsorption of TCII and not to the splitting off of ⁵⁷CoB₁₂ from its binding with TCII. Sephadex G-150 column chromatography confirmed that the reduction of vitamin B₁₂ binding protein was confined to apo-TCII and that there was no reduction in the concentration of apo-TCI and III (Fig. 1 and Table 2). The experiments illustrated in Fig. 2 show that the amount of apo-TCII adsorbed increases stoichiometrically with the amount of coated charcoal added. If 50 mg of coated charcoal is used with 0.5 ml of serum as recommended by Gottlieb *et al*⁶ (or 10 mg/0.1 ml serum), then the adsorption of TCII will be proportionately reduced. However, even in these proportions, there will still be a loss of up to 25% of apo-TCII in some sera. Furthermore, if the amount of coated charcoal used is reduced too much, all the free vitamin B₁₂ added to the serum may not be adsorbed.¹⁷

To see whether increasing the amount of protein coating the charcoal would prevent the adsorption

of apo-TCII, experiments were carried out with increasing amounts of haemoglobin and albumin coating (Fig. 3). The amount of apo-TCII adsorbed decreased with increasing protein concentration up to about 10 mg of protein per 25 mg of charcoal, but increasing the concentration beyond this point had little effect. Even when equal amounts of protein and charcoal were employed, there was still significant reduction in the amount of measurable apo-TCII.

Herbert *et al*²⁰ stressed the importance of the type of activated charcoal used and stressed that it must be "Norit 'A' pharmaceutical grade neutral charcoal, purchased from Amend Drug and Chemical Company, New York". Two different batches of this particular charcoal gave similar results, as did three other commercially available brands of charcoal. The adsorption of TCII by protein-coated charcoal therefore seems to be a generalised property, independent of the type of charcoal.

It is apparent from these studies that the use of haemoglobin or albumin-coated charcoal is not a reliable technique for quantitatively separating bound from free vitamin B₁₂ in serum. When employed in the measurement of unsaturated vitamin B₁₂ binding capacity or apo-TCII concentration in serum, falsely low values will result. However, since it does not adsorb TCI and III its use is valid for the measurement of these serum binders. Finally, this study suggests that when serum is used as the binder in radioisotope dilution assays for vitamin B₁₂, the use of protein-coated charcoal to separate bound from free vitamin B₁₂ needs to be reassessed.

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