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(54) Title: A METHOD FOR PREPARING LIPOPROTEIN FROM A BLOOD SOURCE

(57) Abstract: There is provided a method for the precipitation of lipoprotein from a blood source such as serum or plasma using a divalent metal cation in combination with a polyanion precipitating agent such as dextran sulfate. The divalent metal cation will generally be a Group IIA divalent metal cation such as calcium or magnesium ion.



A Method for Preparing Lipoprotein from a Blood Source

Field of the Invention

The present invention relates to the preparation of lipoprotein from a blood source involving the precipitation of the lipoprotein from the blood source utilising a divalent
5 metal cation in combination with a polyanion precipitating agent.

Background to the Invention

Serum cholesterol levels are commonly monitored for the purpose of indicating general health and well being as elevated levels are associated with a range of conditions including artherosclerosis, coronary artery diseases, metabolic dysfunction, thyroid related
10 diseases, liver disease and diabetes mellitus. Cholesterol levels are typically determined by comparison to standard values obtained using known amounts of cholesterol. Cholesterol is also commonly used as a component in culture medium for the growth and maintenance of micro-organisms such as bacterial and mycoplasma species.

In order to meet the demand for cholesterol a number of processes for the
15 preparation of cholesterol-rich lipoprotein fractions have been developed. For instance, US Patent No. 4,290,774 describes a method for purification of lipoprotein cholesterol that involves absorbing the lipoprotein from blood plasma or serum onto silica, eluting the absorbed lipoprotein and further processing the eluted material by adjusting pH and salt concentration prior to heat treatment. An alkaline carbonate and alkaline earth salt is then
20 added to the heat treated material to obtain a precipitate containing denatured proteins which is discarded during the recovery of the remaining cholesterol. Similar processes are described in US Patent No. 5,409,840 and US Patent No. 4,762,792.

Another method which utilises dextran sulfate and calcium to precipitate a cholesterol-rich lipoprotein extract from bovine serum has also been described (Proksch,
25 G.J. and Bonderman, D.P. *Clinical Chemistry*, Vol 22.8, 1302-1305 (1976)). This method involves obtaining an initial precipitate from the serum by adding dextran sulfate in an amount of 0.25 g/litre of serum and CaCl_2 in an amount of 5.55g/litre while maintaining the pH of the serum at 7.4 using NaOH.

A precipitate (lipoprotein I) forms immediately and is removed after being allowed to settle for two hours at 25°C. An additional 2.6g/litre of dextran sulfate and a further 27.75 g/litre of CaCl₂ is then added to the remaining supernatant immediately producing another precipitate (lipoprotein II). Both the lipoprotein I and II precipitates are
5 subsequently washed several times and then resolubilised for the purpose of removing the dextran sulfate. While the lipoprotein I fraction was found to contain only 10% of the bovine serum cholesterol, when added to human serum and lyophilised, the reconstituted product was extremely turbid.

The lipoprotein II precipitate obtained using the higher dextran sulfate and CaCl₂
10 concentrations contained 66% of the bovine serum cholesterol. However, when heat treated for prolonging shelf life involving heating to 80°C for three hours and then maintaining at a temperature of 60°C for a total of 10 hours, the lipoprotein II fraction has been found to become turbid making it unsuitable for subsequent use.

On the basis of analysis data reported for the lipoprotein II fraction in the Proksch
15 and Bonderman (1976) article, the albumin content of the fraction at a dilution of 1 in 5 equates to 3 g/litre while total protein and cholesterol in the fraction at the same dilution was found to be 8.5g/litre and 2.7 g/litre, respectively. This converts to a total protein to cholesterol ratio in the lipoprotein II fraction of 3.15:1 which is exceedingly high. Of the
20 total protein in the lipoprotein II precipitate, 67% was reported to be associated with high density lipoprotein (HDL), 10% was associated with pre-B-lipoprotein and 23% was non lipoprotein binding. Similar processes are disclosed in US Patent No's 4,216,116, US 4,045,176 and US 3,955,925 by the same authors.

Total protein in bovine serum including protein in lipoprotein itself, is typically in a range of from 72 to 79 g/l while the amount of albumin in the serum typically ranges from
25 24g/litre to 38 g/litre. As a comparison, and taking dilutions into account, a human serum sample analysed in the Proksch and Bonderman (1976) article contained 36.75 g/litre of albumin and a total protein content of 56 g/litre.

Summary of the Invention

The present invention stems from the finding that the amount of albumin and other
30 contaminating proteins that precipitate with lipoprotein from a source such as plasma or serum using a divalent metal cation and polyanion precipitating agent can be substantially

decreased by increasing the amount of the divalent cation with respect to the concentration of the polyanion used. This is highly surprising as it would be expected that by increasing the concentration of the divalent metal cation more contaminating protein and in particular, more albumin would be precipitated resulting in a less pure lipoprotein
5 fraction. Accordingly, by manipulating the amount of the divalent metal cation used relative to the selected polyanion precipitating agent, the amount of the albumin and other proteins in the precipitate can be manipulated and it is this observation which has led to the present invention.

In one aspect of the present invention there is provided a method for preparing
10 lipoprotein from a liquid blood component containing albumin, the method comprising:
adding a divalent metal cation and a polyanion precipitating agent to the blood component;
mixing the divalent metal cation and the precipitating agent with the blood
component to obtain a precipitate containing lipoprotein; and
15 collecting the precipitate from the blood component for further purification of the lipoprotein;
wherein the divalent metal cation is added to the blood component in an amount relative to the precipitating agent such that less than 10% of the albumin in the blood component is precipitated with the lipoprotein, and the precipitate obtained has a ratio of
20 albumin to total protein below 1:3 by weight and contains greater than 20% of the lipoprotein associated cholesterol present in the blood component.

Preferably, the collecting of the precipitate will comprise adding a filter aid to the blood component following the precipitation of the precipitate to provide a solution of the filter aid and the precipitate, and recovering the precipitate and the filter aid from the
25 solution. The filter aid will generally comprise diatomaceous earth.

Preferably, the purification of the lipoprotein from the precipitate collected from the blood component will comprise:

- (i) preparing a dissolving solution;
- (ii) dissolving the lipoprotein in the dissolving solution from the precipitate;
- 30 and
- (iii) separating the dissolved lipoprotein from undissolved matter.

The dissolving solution will usually comprise one or more agents for inhibiting the undissolved matter dissolving into the solution with the lipoprotein.

The cholesterol may also be separated from the precipitated lipoprotein and collected. Hence, the invention further extends to a method of preparing cholesterol from
5 the precipitated lipoprotein.

Accordingly, in another aspect of the present invention there is provided a method for preparing cholesterol from a liquid blood component containing albumin, the method comprising:

- 10 (a) adding a divalent metal cation and a polyanion precipitating agent to the blood component;
- (b) mixing the divalent metal cation and the precipitating agent with the blood component to obtain a precipitate containing lipoprotein;
- (c) collecting the precipitate from the blood component; and
- (d) purifying the cholesterol from the precipitated lipoprotein;
- 15 wherein the divalent metal cation is added to the blood component in an amount relative to the precipitating agent such that less than 10% of the albumin in the blood component is precipitated with the lipoprotein, and the precipitate obtained has a ratio of albumin to total protein in the precipitate below 1:3 by weight and contains greater than 20% of the lipoprotein associated cholesterol present in the blood component.

20 Typically, the lipoprotein precipitated in a method of the invention will be obtained in a single stage precipitation process. By "single stage precipitation process" is meant that the lipoprotein is obtained from the blood component without preparing the blood component for the precipitation of the lipoprotein by firstly precipitating one or more initial fractions from the blood component.

25 The amount of the divalent metal cation needed to be added to the liquid blood component in order to attain a reduced albumin or other conterminating protein level in the precipitate in combination with the polyanion precipitating agent, may be determined by varying the amount of the divalent metal cation relative to the amount of the polyanion precipitating agent and evaluating the albumin and/or total protein content in the
30 resulting precipitate.

Hence, in another aspect of the present invention there is provided a method for determining an amount of a divalent metal cation for preparing lipoprotein from a liquid blood component containing albumin in combination with a polyanion precipitating agent, the method comprising:

- 5 (a) adding an initial amount of the divalent metal cation and an amount of the polyanion precipitating agent to a sample of the blood component;
- (b) mixing the divalent metal cation and the polyanion precipitating agent with the blood component to obtain a precipitate from the liquid blood component containing lipoprotein comprising greater than 20% of the lipoprotein associated cholesterol present in
10 the blood component;
- (c) repeating steps (a) and (b) one or more times using a further sample of the liquid blood component and varying the amount of the divalent metal cation relative to the amount to the polyanion precipitating agent in the further blood component each time, respectively; and
- 15 (d) determining the amount of the divalent metal cation relative to the polyanion precipitating agent required to obtain a precipitate containing less than 10% of the albumin in the blood component and having a ratio of albumin to total protein in the precipitate below 1:3 by weight.

Typically, the amount of the precipitating agent used in step (c) will be held
20 substantially constant and the amount of the divalent metal cation varied each time. Generally, the amount of the divalent metal cation determined in step (d) will be greater than the initial amount of the divalent metal cation used in step (a).

The term "liquid blood component" is to be taken to mean blood plasma, serum or a fraction thereof. The plasma, serum or fraction thereof may be used neat or diluted with a
25 suitable buffer. Preferably, serum or plasma will be utilised in a method of the invention. Most preferably, serum will be used.

The polyanion precipitating agent may be any such agent that is capable of precipitating the lipoprotein from the blood component in combination with the divalent metal cation. Preferably, the polyanion precipitating agent will be selected from the group
30 consisting of sulfated polymers, sulfated polysaccharides, and mixtures thereof. Generally, the precipitating agent used will comprise a single compound as distinct from a mixture. Preferably, the precipitating agent will be dextran sulfate.

Typically the divalent metal cation used in a method of the invention will be a divalent metal cation of a salt and the method will further comprise selecting the salt for addition to the liquid blood component. Preferably, the salt will be a Group IIA metal salt.

Accordingly, in another aspect of the present invention there is provided a method
5 for preparing lipoprotein from a liquid blood component, the method comprising:

- (a) adding a divalent metal cation and dextran sulfate to the blood component;
- (b) mixing the divalent metal cation and the dextran sulfate with the blood component to obtain a precipitate containing lipoprotein; and
- (c) collecting the precipitate from the blood component for further purification
10 of the lipoprotein;

wherein the divalent metal cation and the dextran sulfate are added to the blood component in a ratio of moles of the divalent metal cation to dextran sulfate in grams per litre of the blood component of 1:9.5 or greater, and the precipitate contains less than 10% of the albumin in the blood component and greater than 20% of the lipoprotein associated
15 cholesterol present in the blood component.

In another aspect of the present invention there is provided a method for preparing cholesterol from a liquid blood component containing albumin, the method comprising:

- (a) adding a divalent metal cation and dextran sulfate to the blood component;
- (b) mixing the divalent metal cation and the dextran sulfate obtain a precipitate
20 containing lipoprotein;
- (c) collecting the precipitate from the blood component; and
- (d) purifying the cholesterol from the precipitated lipoprotein;

wherein the divalent metal cation and the dextran sulfate are added to the blood component in a ratio of moles of the divalent metal cation to dextran sulfate in grams per
25 litre of the blood component of 1:9.5 or greater, and the precipitate contains less than 10% of the albumin in the blood component and greater than 20% of the lipoprotein associated cholesterol present in the blood component.

In yet another aspect, there is provided lipoprotein prepared by a method of the invention.

In still another aspect there is provided cholesterol purified from lipoprotein prepared by a method of the invention.

Lipoprotein concentrate prepared in accordance with one or more embodiments of the present invention may contain a reduced amount of albumin and other contaminating protein(s) compared to levels obtained by prior art precipitation methods, making the concentrate suitable for subsequent heat inactivation treatment with the clarity of the concentrate being substantially unaffected by the heat treatment. Being able to heat treat the lipoprotein is highly advantageous as heat treatment is a simple and cost effective process which denatures deleterious enzymes and other proteins that may be present in the lipoprotein, and inactivates or kills any contaminating microorganisms such as viruses, without any further separation or treatment steps being required.

Throughout this specification the word 'comprise' or variations such as 'comprises' or 'comprising' will be understood to imply the inclusion of a stated element, integer or step or group of elements, integers or steps, but not to the exclusion of any other element, integer or step, or group of elements, integers or steps.

The features and advantages of the present invention will become further apparent from the following description of preferred embodiments.

Description of the Accompanying Drawings

Figure 1: Graphs showing total protein in lipoprotein preparations prepared by a method of the invention as a percentage of the total protein in the starting bovine serum against CaCl_2 concentration;

Figure 2: Graphs showing the ratio of total protein to lipoprotein associated cholesterol by weight in lipoprotein preparations against CaCl_2 concentration; and

Figure 3: Graphs showing the percentage lipoprotein associated cholesterol recovered from bovine serum against CaCl_2 concentration.

Detailed Description of Preferred Embodiments

Generally, bovine serum will be utilised in methods of the invention and may be readily obtained by separating the serum from clotted blood collected freshly at abattoirs.

However, human serum and serum from other mammalian species including those of the porcine, ovine, equine families may be utilised. As will be understood, plasma from any such species may also be readily obtained by collecting the blood into an anti-coagulant such as heparin, and centrifuging the blood to remove blood cells and other cellular material. In particular, separation of the plasma may be achieved by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) or other suitable density gradient.

Particularly preferred fractions of plasma or serum containing lipoprotein that may be used as the starting material in methods of the invention include fibrinogen-poor fractions, and the like. The plasma, serum or fraction thereof may be dialysed against a suitable buffer or diluted by a suitable buffer prior to precipitation of the lipoprotein.

The sulfated polymer for precipitating the lipoprotein in combination with the divalent metal cation may be selected from the group consisting of sulfated polysaccharides, dextran sulfate, sulfated polygalacturonic acid methyl ester, polyanetholsulfonate, sulfated amylopectin, polyvinyl sulfate, and mixtures thereof. Preferably, the polyanion precipitating agent will be dextran sulfate having a molecular weight in a range of from about 5kDa to about 1000 kDa. Most preferably, the dextran sulfate will have a molecular weight in a range of from 250 kDa to about 750 kDa. Typically, the dextran sulfate will be added to the blood component in an amount of 2 g/l or greater and preferably, in an amount in a range of from 2.6 g/l to 3.0 g/l of the blood component. However, the invention is not limited to dextran sulfate and other commercially available physiologically acceptable sulfated polysaccharide polyanion precipitating agents such as heparin may be utilised.

The amount of polyanion precipitating agent required for precipitating the lipoprotein can be determined by adding an amount of the selected precipitating agent to the blood component in the presence of an amount of the selected divalent metal cation, and determining the degree of precipitation of the lipoprotein. This can be repeated a number of times varying the amount of polyanion precipitating agent each time while maintaining the concentration of the divalent metal cation constant, until the desired level of precipitation of the lipoprotein is achieved. This process is then again repeated keeping the determined amount of precipitating agent constant but varying the amount of the divalent metal cation relative to the precipitating agent added, until the amount of albumin

or contaminating protein precipitating with the lipoprotein has been minimised or otherwise reduced to the required level.

The divalent metal cation will usually be selected from Ca^{2+} and Mg^{2+} . However, other divalent metal cations may also be utilised including Be^{2+} , Ba^{2+} , and Mn^{2+} . Preferably, the divalent metal cation will be a Group IIA divalent metal cation.

The selected divalent metal cation will generally be utilised in the form of a salt. The salt will generally comprise halogen, sulfate or phosphate anion(s). Preferably, the salt will be a chloride or sulfate salt of the divalent metal cation and most preferably, CaCl_2 . Increasing the concentration of CaCl_2 above 27.75 g/l when used in combination with dextran sulfate with a molecular weight of 500 kDa at a concentration of 2.85 g/l to precipitate lipoprotein from bovine serum for instance, can result in a significant decrease in the level of precipitation of albumin and other contaminating proteins with the lipoprotein. Preferably, the CaCl_2 will be added to the blood component in an amount greater than about 30 g/l, more preferably, in a range of from about 30 g/l to about 55 g/l or greater and preferably, in a range of from about 35 g/l to about 50 g/l. A concentration of 30g/l corresponds to a concentration of 0.3M. Accordingly, the use of a concentration of 0.3M CaCl_2 or greater and 2.85g/l of dextran sulfate equates to a ratio of moles of Ca^{2+} to dextran sulfate in grams per litre of the blood component of 1:10.55 or greater.

The polyanion precipitating agent and the salt may be dissolved in a minimum amount of a suitable solvent such as distilled or deionised water for being added to the serum. Desirably, the salt will be added directly to the serum undissolved and preferably, before the polyanion precipitating agent is added. Typically, the temperature of the serum or plasma during the precipitation of the lipoprotein will be maintained at a temperature of less than 10°C and typically at 4 – 5° C, but the precipitation step may also be carried out at higher temperatures including room temperature.

The pH of the blood component used will generally not be altered from its natural pH level for the precipitation of the lipoprotein. However, altering the pH of the blood component is not excluded. Typically, the pH of the blood component for precipitation of the lipoprotein will be in range of from about 6.0 – 8.0 and usually, in a range of from about 7.2 – 7.6.

Once the precipitate has formed, it is collected and preferably washed a number of times to remove residual contaminants from the precipitate. The precipitate is then typically dissolved in a suitable dissolving solution such as a solution of potassium chloride and potassium oxalate, and filtered through an appropriate size filter prior to the
5 filtrate containing the dissolved lipoprotein being concentrated by ultrafiltration.

Alternatively, the filtrate may be concentrated by any other conventional method known in the art such as by precipitation using ammonium sulfate or other salts and/or precipitating agent, and adsorption or desorption methods, and subsequently dialysed against a suitable buffer. The filtrate will typically be concentrated to obtain a cholesterol
10 concentration in a range of from about 50 to 3000 mg/dl and more preferably, in a range of from about 1000 to 1500 mg/dl.

In order to enhance shelf life of the concentrated cholesterol product and denature deleterious enzymes and other contaminating proteins present, the concentrate may be subjected to heat treatment. This also eliminates the risk of any contaminating bacteria or
15 other micro-organisms that may be present at this stage. The clarity of the concentrate remains substantially unaffected by the heat treatment. Turbidity of the heat treated concentrate can be determined by measuring percentage transmission of the heat treated concentrate at a wavelength of 650 nm. Typically, the transmission of the heat treated concentrate will be greater than 70% and usually, will be in a range of from 80 – 86%.

20 If desired, the cholesterol may be separated from the protein in the isolated lipoprotein fraction using any method known in the art. Suitable methods include affinity chromatography.

The degree of precipitation of albumin and lipoprotein in the precipitate can be readily evaluated by subtracting the level of the albumin and the lipoprotein in the plasma
25 or serum remaining after precipitation from the original levels prior to precipitation. Similarly, the level of the albumin or lipoprotein can be readily determined using conventional techniques known in the art such as for example, electrophoresis by SDS PAGE or immunodiffusion methods involving comparison with standard samples having a known albumin or lipoprotein content. Similarly, the amount of total protein in the
30 precipitate can be determined by measuring total protein in the plasma or serum before and after precipitation of the lipoprotein. Methods for measuring total protein include

colormetric methods (see for example: Ohnishi, S.T., Barr, J.K., *Anal. Biochem.* 86(193), 321-328 (1978); Lowry, O.H., et al., *J.Biol. Chem.* 193, 265-275 (1951); and Bradford, M., *Anal. Biochem.* 72, 248-254 (1976)).

5 Preferably, the precipitate will contain greater than about 30% or 40% of the lipoprotein associated cholesterol present in the serum or plasma. Most preferably, the precipitate will contain a majority of the cholesterol and most preferably, 60 or 70% of the cholesterol or more. The lipoprotein in the precipitate will generally be primarily high density lipoprotein (HDL) with the remainder of the lipoprotein being low density lipoprotein (LDL). Typically, the precipitated lipoprotein will comprise greater than 70%
10 HDL and more usually, about 76% to about 86% of the lipoprotein. Suitable assays for determining the level of HDL in a sample include enzymatic colorimetric tests (eg. Sugiuchi, H. et al. Direct measurement of high density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated α -cyclodextran. *Clin. Chem.* 41:717-724 (1995)).

15 Preferably, the divalent metal cation will be added in an amount to maintain precipitation of albumin relative to the amount of total protein precipitated below a ratio of albumin to total protein of 1:4 or 1:5 by weight or more preferably, below a ratio of 1:9, 1:19 or 1:49. Most preferably, the ratio will be below 1:99, 1:199, or 1:499 by weight or even lower. Preferably, the albumin in the precipitate will be less than about 5% of the total
20 albumin in the serum or plasma, and more preferably, less than about 2% of the albumin. Most preferably, the precipitate will be substantially free of albumin.

Typically, the precipitate will contain a total protein to total cholesterol ratio of 2.9 by weight or less, preferably a ratio of 2.8 or 2.6 or less, more preferably a ratio of 2.5 or 2.4 or less or most preferably, a ratio of 2.0 by weight or less.

25 The present invention will now be further described by way of a number of examples.

Example 1: Purification and concentration of lipoprotein from bovine serum

1.0 Precipitation of lipoprotein

30 A volume of 1000 litres of pooled bovine serum was transferred to a stainless steel tank and mixed to ensure homogeneity of the serum. An amount of 44 kg of CaCl_2 was then added undissolved to the serum to obtain a serum concentration of the CaCl_2 of

44 g/l. For efficient dissolution and mixing, the CaCl_2 was added in small amounts over the entire surface of the serum and the serum stirred for a minimum of 90 minutes.

An amount of 2.85 kg of dextran sulfate (MW 500,000, Dextran Products Limited, Canada) was then weighed and slowly added to 14 litres of water purified by reverse osmosis (RO water) while mixing. Mixing continued until all the dextran sulfate was dissolved. The dissolved precipitating agent was then added to the serum at a rate of 500 ml/min using a peristaltic pump. To ensure thorough mixing of the dextran sulfate and the serum, the serum was stirred for another 90 min period. The temperature of the serum during the precipitation of the lipoprotein was maintained at 4°C - 5°C .

1.1 Harvesting and washing of lipoprotein precipitate

To assist harvesting of the resulting precipitate, a filter aid solution was prepared by adding 30 kg of diatomaceous earth (Celite 503, Celite Corporation, USA) in 1000 litres of RO water. The pH of the filter aid solution was checked to ensure it was not above 7.5. If necessary, the pH was adjusted to pH 7.5 or just below this value prior to the filter aid being added to the precipitated serum. The precipitated serum was then passed through a filter press (Edwards & Jones 630 mm x 630 mm single screw electromechanical semi-mechanised recessed chamber filter press). The filtrate was checked to ensure it was clear, and was recirculated as necessary through the filter press until clear prior to the filtrate being discarded. To ensure all precipitate was collected, 200 litres of CaCl_2 solution (0.734 kg CaCl_2 /100 litres RO water) was subsequently passed through the filter press.

The precipitate was then collected and washed by adding the precipitate to 500 litres of CaCl_2 solution (0.734 kg of CaCl_2 /100 litres of RO water), and the resulting solution was stirred for a minimum of 60 minutes. Following stirring, the solution was passed through the filter press as described above and the precipitate recollected. A further 200 litres of CaCl_2 solution (0.734 kg CaCl_2 /100 litres RO water) was pumped through the filter press to ensure all precipitate was collected. A second CaCl_2 wash was then performed as described above.

The washed precipitate was then added to 500 litres of RO water in a stainless steel tank and stirred well for a further minimum period of 60 minutes prior to the washed precipitate being collected using the filter press.

1.2 Dissolving of lipoprotein precipitate and filtration of dissolved lipoprotein

A volume of 300 litres of dissolving solution was prepared by dissolving potassium chloride (7.455 kg/100 litres) and potassium oxalate (1.842 kg/100 litres) in RO water. The pH of the dissolving solution was checked and adjusted to 6.5 if necessary. Potassium oxalate removes calcium as an insoluble calcium oxalate and dissociates the dextran sulfate – lipoprotein complex. The ionic strength of the potassium chloride and the pH of the solution allow the lipoprotein to dissolve while maintaining the dextran sulfate insoluble. The washed lipoprotein precipitate was added to the dissolving solution and stirred for a minimum period of 90 minutes to fully dissolve the lipoprotein in the precipitate. The dissolved lipoprotein was then filtered to remove the filter aid and precipitated dextran sulfate, by passing the solution through the filter press and then through a 0.2 micron membrane filter (Cat No. AB2NAZ7PH4, Pall Biopharmaceuticals). The filtrate was retained for further processing.

1.3 Concentration and diafiltration of dissolved lipoprotein

The filtrate retained from Example 1.2 was recirculated through an ultrafiltration unit (Pall Filtron Centrasette 10 fitted with Alpha Open Channel 30K polyethersulfone (PES) membrane cassettes) under the following conditions: a retentate flow rate of 6 litres/min/0.5 m₂ (cassette area); an inlet pressure of 1.7 bar; a retentate pressure of 1.2 bar; and a filtrate pressure of 0.

Once these operating conditions were obtained, the filtrate hose was placed to drain to concentrate the solution to obtain the required volume for a cholesterol concentration of 12 g/litre. The required concentrate volume can be readily determined by measuring the volume of the filtrate retained following filtration of the dissolved lipoprotein and its cholesterol concentration. Any conventional method suitable for measuring cholesterol may be utilised such as by colorimetric methods utilising commercially available kits (eg. Sigma-Aldrich, Cat. No. 401-25P). Once the required concentrated volume was obtained, the concentrate was subjected to diafiltration following the addition of an equal volume of RO water to the concentrate. Diafiltration was continued until the volume of the concentrate was returned to that prior to the addition of RO water. Addition of further RO water and diafiltration was repeated as necessary until the osmolality of the lipoprotein concentrate was less than or equal to 5.

1.4 Heat treatment of lipoprotein concentrate

The pH of the lipoprotein solution resulting from Example 1.3 above was measured and adjusted to 8.0 (± 0.1) if necessary. The lipoprotein solution was subsequently transferred to a heating tank and heated to a temperature in a range of from 80°C to 82°C, and maintained within that temperature range for three hours. At the end of this time period, the temperature of the lipoprotein solution was decreased to a temperature in a range of from 60°C to 62°C. Overall, the lipoprotein solution was at or above that temperature for a total period of 10 hours including the 3 hour period at 80°C to 82°C.

Following the heat inactivation treatment, the cholesterol level of the lipoprotein solution was adjusted to 10.7 g/litre with injection grade water prior to adjusting the pH of the lipoprotein solution to 8.0 (± 0.1) if required. The resulting concentrate was clear.

Example 2: Analysis of lipoprotein concentrate

Further large scale samples of bovine serum were obtained and lipoprotein concentrates prepared by the method described in Example 1. The concentrates were analysed for protein and cholesterol content and the results are shown in Table 1.

As shown in the table, the prepared lipoprotein concentrate was found to contain a ratio of total protein to lipoprotein associated cholesterol of between 2.0 to 3.0 by weight and more typically, a ratio of from 2.39 to 2.86. The total protein in the concentrates ranged from 21.7 g/litre to 34.30 g/litre. As a percentage, the concentrates contained from 2.43% to 3.11% of the total protein in the bovine serum. The amount of lipoprotein associated cholesterol in the concentrates ranged from 9.3 g/litre to 11.1 g/litre with the percentage of the cholesterol recovered from the bovine serum ranging from 52.2% to 61.4%.

The table also shows that the lipoprotein concentrates contained substantially reduced levels of α -globulin and β -globulin proteins compared to levels in the starting bovine serum. In particular, these proteins were reduced to levels ranging from 5% to 8.2% and 11.8% to 13% of that in the bovine serum, respectively.

Albumin could not be detected in any of the lipoprotein concentrates tested for the protein.

Table 1: Protein and lipoprotein associated cholesterol levels in precipitates prepared from bovine serum

SAMPLE	Serum Starting Volume (Litres)	Serum Cholesterol (g/L)	Available Cholesterol (Grams)	Cholesterol Recovered (Grams)	% Cholesterol Recovery	Serum Protein (g/l)	Available Protein (Grams)	Protein Carried Over (Grams)	% Protein Carried Over	Volume Produced (Litres)	Cholesterol (g/L)	Protein (g/l)	Protein: Cholesterol
JT-ABL-0311	920.0	1.85	1702	946	55.6%	81.00	74520	2321	3.1%	91	10.40	25.50	2.45
KT-ABL-0301	1000.0	2.00	2000	1045	52.3%	81.40	81400	2062	2.5%	95	11.00	21.70	1.97
KA-ABL-0302	960.0	1.36	1306	744	57.0%	72.60	69696	2231	3.2%	67	11.10	33.30	3.00
KA-ABL-0304	930.0	1.52	1414	795	56.2%	77.60	72168	1995	2.8%	75	10.60	26.60	2.51
KA-ABL-0305	990.0	1.38	1366	749	54.8%	78.10	77319	1876	2.4%	70	10.70	26.80	2.50
KNZ-ABL-0306	1020.0	1.57	1601	836	52.2%	74.00	75480	1998	2.6%	88	9.50	22.70	2.39
KNZ-ABL-0307	970.0	1.50	1455	791	54.3%	74.00	71780	2066	2.9%	85	9.30	24.30	2.61
KNZ-ABL-0308	1025.0	1.41	1445	843	58.3%	77.00	78925	2414	3.1%	85	9.92	28.40	2.86
KA-ABL-0309	1070.0	1.34	1434	881	61.4%	78.00	83460	2460	2.9%	86	10.24	28.60	2.79
KA-ABL-0310	1050.0	1.41	1481	864	58.4%	79.00	82950	2376	2.9%	90	9.60	26.40	2.75
KA-ABL-0311	980.0	1.44	1411	827	58.6%	78.00	76440	2382	3.1%	83	9.96	28.70	2.88
KA-ABL-0312	2330.0	1.20	2796	1488	53.2%	77.70	181041	5145	2.8%	150	9.92	34.30	3.46
KA-ABL-0313	2610.0	1.20	3132	1800	57.5%	77.20	201492	5382	2.7%	180	10.00	29.90	2.99
KA-ABL-0314	3175.5	1.38	4382	2493	56.9%	76.80	243878	7308	3.0%	242	10.30	30.20	2.93
KA-ABL-0315	3000.0	1.30	3900	2252	57.7%	71.90	215700	6352	2.9%	237	9.50	26.80	2.82

Sample	Serum Albumin g/l	Serum α -globulin g/l	Total α -globulin Available g/l	Serum β -globulin g/l	Total β -globulin Available g/l	Lipo α -globulin g/l	α -globulin carried over g/l	Lipo β -globulin g/l	β -globulin Carried Over g/l	Final product α -globulin %	Final product β -globulin %
KA-ABL-0309	38.2	11.1	11877	9.7	10379						
KA-ABL-0310	33.8	15.1	15855	9.9	10395						
KA-ABL-0313	33.8	15.1	39411	9.9	25839	11	1980	17	3060	5.0%	11.8%
KA-ABL-0314	38.2	11.1	35248.05	9.7	30802.35	12	2904	15	3630	8.2%	11.8%
KA-ABL-0315	38.2	11.1	33300	9.7	29100	11	2607	16	3792	7.8%	13.0%

Example 3: Effect of varying the concentration of divalent metal cation

A study was conducted to evaluate the effect of varying the CaCl_2 relative to the concentration of the dextran sulfate on the precipitation of albumin and other protein protein with the lipoprotein. Comparisons between adding the dextran sulfate with the CaCl_2 and delaying the addition of the dextran sulfate for 20 minutes were also made.

Briefly, bovine serum samples from a number of different sources were obtained. Each sample was divided into 0.8 litre aliquots. For the purpose of precipitation of the lipoprotein, the aliquots were added to 1 litre centrifuge buckets and dextran sulfate at a concentration of 2.85g/litre was added to each. The concentrations of the CaCl_2 added ranged from 25g/l to 56.3 g/l. The precipitate was collected by centrifuging the buckets and decanting off the supernatant.

The lipoprotein was precipitated and processed generally following the protocol described in Example 1 except that the dissolved lipoprotein was not concentrated due to the small volumes involved and the lipoprotein was subjected to dialysis to purify the lipoprotein rather than ultrafiltration. Diatomaceous earth was also not used except for one of the samples.

The results are shown in Table 2. As can be seen, increasing the concentration of the CaCl_2 relative to the dextran sulfate resulted in a decrease in the total amount of protein precipitated. While the amount of total protein decreased, the percentage of lipoprotein associated cholesterol recovered from the bovine serum remained substantially unaffected or in some cases was observed to increase with the increase in CaCl_2 concentration, indicating that the amount of contaminating protein precipitated decreased as the concentration of the CaCl_2 increased. This general trend was observed for all the different bovine serum samples tested. However, in some of the samples the trend levelled off at the higher CaCl_2 concentrations although the ratio of total protein to cholesterol observed for these concentrations remained well below the ratio observed for the lower concentrations of CaCl_2 used.

In particular, the percentage of total protein precipitated with a concentration of 30g/l or more ranged from 1.75% of the total protein in the bovine serum to 4.73% and typically, between about 2.09% and 3.89%. The recovery rate of the lipoprotein associated cholesterol from the bovine serum ranged from 54.4% to 77.3% for these CaCl_2

concentrations. The ratio of total protein to lipoprotein associated cholesterol recovered from the bovine serum was dependent on the initial concentration of the cholesterol in the bovine serum samples assayed. That is, higher ratios of protein to cholesterol were found for those samples that had a lower initial concentration of cholesterol.

Table 2: Effect of varying calciumion concentration relative to dextran sulphate concentration on precipitation of other protein with lipoprotein from bovine serum

Trial No.	Sample ID	Salt Precipitation Conc (g/L)	Starting Volume (Litres)	Serum Cholesterol (g/L)	Available Cholesterol (Grams)	Cholesterol Recovered (Grams)	% Cholesterol Recovery	Serum Protein (g/l)	Available Protein (Grams)	Protein Carried Over (Grams)		% Protein Carried Over	Final Product Volume (Litres)	Final Product Cholesterol (g/L)	Final Product Protein (g/l)	Protein: Cholesterol
										Over	Over					
Trial 1	1	25.0	0.80	2.00	1.60	1.14	71.1%	80.00	64.00	3.36	3.36	5.3%	0.30	3.79	11.20	2.96
	2	31.3	0.80	2.00	1.60	1.23	76.7%	80.00	64.00	3.03	3.03	4.7%	0.30	4.09	10.10	2.47
	4a	37.5	0.80	2.00	1.60	1.20	75.0%	80.00	64.00	2.49	2.49	3.9%	0.30	4.00	8.30	2.08
	4b	37.5	0.80	2.00	1.60	1.21	75.4%	80.00	64.00	2.40	2.40	3.8%	0.30	4.02	8.00	1.99
	4c	37.5	0.80	2.00	1.60	1.20	75.0%	80.00	64.00	2.49	2.49	3.9%	0.30	4.00	8.30	2.08
	5	43.8	0.80	2.00	1.60	1.24	77.3%	80.00	64.00	2.10	2.10	3.3%	0.30	4.12	7.00	1.70
Trial 2	20	25.0	0.80	2.00	1.60	0.86	53.8%	80.00	64.00	3.32	3.32	5.2%	0.30	2.87	11.07	3.86
	25	31.3	0.80	2.00	1.60	0.87	54.4%	80.00	64.00	2.84	2.84	4.4%	0.30	2.90	9.46	3.26
	30	37.5	0.80	2.00	1.60	0.94	58.7%	80.00	64.00	2.39	2.39	3.7%	0.30	3.13	7.95	2.54
	35	43.8	0.80	2.00	1.60	0.98	61.5%	80.00	64.00	2.13	2.13	3.3%	0.30	3.28	7.09	2.16
	40	50.0	0.80	2.00	1.60	0.98	61.3%	80.00	64.00	1.97	1.97	3.1%	0.30	3.27	6.58	2.01
	45	56.3	0.80	2.00	1.60	0.98	61.3%	80.00	64.00	2.25	2.25	3.5%	0.30	3.27	7.51	2.30
DE Added	Set 1 (20)	25.0	0.80	2.00	1.60	1.03	64.1%	80.00	64.00	2.31	2.31	3.6%	0.30	3.42	7.71	2.25
	Set 1 (25)	31.3	0.80	2.00	1.60	1.03	64.5%	80.00	64.00	1.84	1.84	2.9%	0.30	3.44	6.14	1.78
	Set 1 (30)	37.5	0.80	2.00	1.60	1.15	72.0%	80.00	64.00	1.89	1.89	3.0%	0.30	3.84	6.31	1.64
	Set 1 (35)	43.8	0.80	2.00	1.60	1.01	63.2%	80.00	64.00	1.34	1.34	2.1%	0.30	3.37	4.45	1.32
	Set 1 (40)	50.0	0.80	2.00	1.60	1.00	62.3%	80.00	64.00	1.22	1.22	1.9%	0.30	3.32	4.06	1.22
	CaCl dissolv															
20 min	Set 1 (45)	56.3	0.80	2.00	1.60	1.03	64.3%	80.00	64.00	1.12	1.12	1.7%	0.30	3.43	3.73	1.09

Trial No.	Sample ID	Salt Precipitation Conc (g/L)	Starting Volume (Litres)	Serum Cholesterol (g/L)	Available Cholesterol (Grams)	Cholesterol Recovered (Grams)	% Recovery	Serum Protein (g/l)	Available Protein (Grams)	Protein Carried Over (Grams)	% Protein Carried Over	Final Product Volume (Litres)	Final Product Cholesterol (g/L)	Final Product Protein (g/l)	Protein: Cholesterol
delay	Set 2 (25)	31.3	0.80	2.00	1.60	1.15	72.0%	80.00	64.00	1.81	2.8%	0.30	3.84	6.03	1.57
	Set 2 (30)	37.5	0.80	2.00	1.60	1.14	71.1%	80.00	64.00	1.76	2.8%	0.30	3.79	5.88	1.55
	Set 2 (35)	43.8	0.80	2.00	1.60	1.16	72.4%	80.00	64.00	1.54	2.4%	0.30	3.86	5.13	1.33
	CaCl dissolv														
	Set 2 (40)	50.0	0.80	2.00	1.60	1.12	69.9%	80.00	64.00	1.74	2.7%	0.30	3.73	5.80	1.55
	Set 2 (45)	56.3	0.80	2.00	1.60	1.10	68.6%	80.00	64.00	1.49	2.3%	0.30	3.66	4.96	1.36
Trial 4	25	25.0	0.80	1.21	0.97	0.64	65.7%	75.00	60.00	2.44	4.1%	0.24	2.65	10.16	3.83
	30	30.0	0.80	1.21	0.97	0.63	65.2%	75.00	60.00	2.20	3.7%	0.24	2.63	9.17	3.49
	35	35.0	0.80	1.21	0.97	0.66	67.7%	75.00	60.00	2.21	3.7%	0.24	2.73	9.19	3.37
	40	40.0	0.80	1.21	0.97	0.65	66.9%	75.00	60.00	1.87	3.1%	0.24	2.70	7.79	2.89
	45	45.0	0.80	1.21	0.97	0.63	65.5%	75.00	60.00	1.72	2.9%	0.24	2.64	7.15	2.71
	50	50.0	0.80	1.21	0.97	0.63	65.2%	75.00	60.00	1.57	2.6%	0.24	2.63	6.55	2.49
Trial 5	TYB (1)	44.0	0.80	1.46	1.17	0.79	67.4%	75.60	60.48	1.95	3.2%	0.24	3.28	8.13	2.48
	TYB (2)	44.0	0.80	1.46	1.17	0.82	69.9%	75.60	60.48	2.27	3.8%	0.24	3.40	9.47	2.79
	TYB O/N (3)	44.0	0.80	1.46	1.17	0.80	68.8%	75.60	60.48	2.40	4.0%	0.24	3.35	9.98	2.98
	TYB O/N (4)	44.0	0.80	1.46	1.17	0.80	68.6%	75.60	60.48	2.07	3.4%	0.24	3.34	8.63	2.58
	TYB (5)	48.0	0.80	1.46	1.17	0.81	69.2%	75.60	60.48	1.95	3.2%	0.24	3.37	8.13	2.41
	TYB (6)	48.0	0.80	1.46	1.17	0.76	65.1%	75.60	60.48	1.90	3.1%	0.24	3.17	7.91	2.50
	TYB (7)	52.0	0.80	1.46	1.17	0.75	64.1%	75.60	60.48	1.64	2.7%	0.24	3.12	6.83	2.19
	TYB (8)	52.0	0.80	1.46	1.17	0.75	64.1%	75.60	60.48	1.69	2.8%	0.24	3.12	7.03	2.25

Trial No.	Sample ID	Salt Precipitation Conc (g/L)	Starting Volume (Litres)	Serum Cholesterol (g/L)	Available Cholesterol (Grams)	Cholesterol Recovered (Grams)	% Recovery	Serum Protein (g/l)	Available Protein (Grams)	Protein Carried Over		Final Product Volume (Litres)	Final Product Cholesterol (g/L)	Final Product Protein (g/l)	Protein: Cholesterol
										Protein Carried Over (Grams)	% Over				
Trial 6	GL (1)	44.0	0.80	2.28	1.82	1.09	59.9%	79.70	63.76	2.17	3.4%	0.24	4.55	9.04	1.99
	GL (2)	44.0	0.80	2.28	1.82	1.18	64.9%	79.70	63.76	2.40	3.8%	0.24	4.93	10.02	2.03
	GL (3)	48.0	0.80	2.28	1.82	1.15	63.3%	79.70	63.76	2.21	3.5%	0.24	4.81	9.21	1.91
	GL (4)	48.0	0.80	2.28	1.82	1.18	64.6%	79.70	63.76	2.25	3.5%	0.24	4.91	9.38	1.91
	GL (5)	52.0	0.80	2.28	1.82	1.27	69.5%	79.70	63.76	2.26	3.5%	0.24	5.28	9.42	1.78
	GL (6)	52.0	0.80	2.28	1.82	1.16	63.7%	79.70	63.76	2.15	3.4%	0.24	4.84	8.95	1.85
Trial 6	CaCl ₂ (1)	44.0	0.80	1.31	1.05	0.62	59.3%	76.40	61.12	2.26	3.7%	0.30	2.07	7.54	3.64
	CaCl ₂ (2)	48.0	0.80	1.31	1.05	0.61	58.4%	76.40	61.12	1.92	3.1%	0.30	2.04	6.40	3.14
	CaCl ₂ (3)	52.0	0.80	1.31	1.05	0.52	49.5%	76.40	61.12	1.67	2.7%	0.30	1.73	5.57	3.22
	CaCl ₂ (4)	56.0	0.80	1.31	1.05	0.52	49.8%	76.40	61.12	1.73	2.8%	0.30	1.74	5.77	3.32
	MnCl ₂ (1)	44.0	0.80	1.31	1.05	0.61	58.4%	76.40	61.12	5.13	8.4%	0.30	2.04	17.11	8.39
	MnCl ₂ (2)	37.7	0.80	1.31	1.05	0.61	58.1%	76.40	61.12	5.56	9.1%	0.30	2.03	18.52	9.12

The results are best visualised in Fig.1 and Fig. 2 which show the effect of increasing CaCl_2 concentration on the total protein precipitated and the ratio of total protein to lipoprotein associated cholesterol, respectively. Graphs illustrating the effect of increasing the CaCl_2 concentration relative to dextran sulfate concentration on the percentage of lipoprotein associated cholesterol recovered from the starting bovine serum are shown in Fig. 3.

Delaying the addition of the dextran sulfate for 20 minutes after the CaCl_2 was added did not have any noticeable overall impact on the results obtained.

10 **Example 4: Effect of different divalent metal cations on precipitation of lipoprotein from bovine serum**

A comparison was made between the capacity of different divalent metal cations to precipitate lipoprotein from bovine serum as well as the impact of the anion in the salt used. The lipoprotein was precipitated following the same protocol as that used in Example 3. As in Example 3, dextran sulfate was added to the serum at a concentration of 2.85 g/l. The results are shown in Table 3.

Similar results to those observed in Example 3 were obtained using CaCl_2 , MgCl_2 and MgSO_4 for all parameters measured. Moreover, a decreasing ratio of total protein to lipoprotein associated cholesterol in the precipitates were also obtained with increasing concentrations of the salts. In contrast, substantially all the protein was precipitated from the bovine serum using CaSO_4 , CaPO_4 and CaCO_3 and only low levels of lipoprotein were recovered from the precipitate.

Table 3: Use of different salts of divalent metalions for lipoprotein precipitation from bovine serum

Trial No.	Sample ID	Salt Precipitation Conc (g/L)	Starting Volume (Litres)	Serum Cholesterol (g/L)	Available Cholesterol (Grams)	Cholesterol Recovered (Grams)	% Recovery	Serum Protein (g/l)	Available Protein (Grams)	Protein		Final Product Volume (Litres)	Final Product Cholesterol (g/L)	Final Product Protein (g/l)	Protein: Cholesterol
										Carried Over (Grams)	% Over				
Trial 7	CaCl ₂ (1)	44.0	0.80	1.54	1.23	0.74	59.9%	72.50	58.00	2.09	3.6%	0.30	2.46	6.95	2.83
	CaCl ₂ (2)	32.0	0.80	1.54	1.23	0.76	61.6%	72.50	58.00	2.79	4.8%	0.30	2.53	9.30	3.68
	MgCl ₂ (1)	44.0	0.80	1.54	1.23	0.75	61.1%	72.50	58.00	1.29	2.2%	0.30	2.51	4.30	1.71
	MgCl ₂ (2)	61.0	0.80	1.54	1.23	0.76	61.4%	72.50	58.00	1.25	2.2%	0.30	2.52	4.17	1.65
	MgSO ₄ (1)	44.0	0.80	1.54	1.23	0.60	48.9%	72.50	58.00	2.02	3.5%	0.30	2.01	6.73	3.35
	MgSO ₄ (2)	74.0	0.80	1.54	1.23	0.75	60.9%	72.50	58.00	1.62	2.8%	0.30	2.50	5.40	2.16
	CaSO ₄ (1)	44.0	0.80	1.54	1.23	0.06	5.0%	72.50	58.00	0.99	1.7%	0.30	0.21	3.31	16.15
	CaSO ₄ (2)	51.7	0.80	1.54	1.23	0.07	5.7%	72.50	58.00	0.97	1.7%	0.30	0.24	3.24	13.79
	CaPO ₄ (1)	44.0	0.80	1.54	1.23	0.01	0.5%	72.50	58.00	0.28	0.5%	0.30	0.02	0.94	47.00
	CaPO ₄ (2)	40.8	0.80	1.54	1.23	0.01	0.5%	72.50	58.00	0.24	0.4%	0.30	0.02	0.81	40.50
	CaCO ₃ (1)	44.0	0.80	1.54	1.23	0.01	0.5%	72.50	58.00	0.00	0.0%	0.30	0.02	0.00	0.00
	CaCO ₃ (2)	30.0	0.80	1.54	1.23	0.01	0.5%	72.50	58.00	0.08	0.1%	0.30	0.02	0.26	13.00

Although the present invention has been described hereinbefore with reference to a number of preferred embodiments, the skilled addressee will appreciate that numerous changes and modifications are possible without departing from the spirit or scope of the invention. The present embodiments described are, therefore, to be considered in all
5 respects as illustrative and not restrictive.

Claims

1. A method for preparing lipoprotein from a liquid blood component containing albumin, the method comprising:
5 adding a divalent metal cation and a polyanion precipitating agent to the blood component;
mixing the divalent metal cation and the precipitating agent with the blood component to obtain a precipitate containing lipoprotein; and
collecting the precipitate from the blood component for further purification of the lipoprotein;
10 wherein the divalent metal cation is added to the blood component in an amount relative to the precipitating agent such that less than 10% of the albumin in the blood component is precipitated with the lipoprotein, and the precipitate obtained has a ratio of albumin to total protein below 1:3 by weight and greater contains than 20% of the lipoprotein associated cholesterol present in the blood
15 component.
2. A method according to claim 1 wherein the lipoprotein is precipitated from the blood component in a single stage precipitation process.
3. A method according to claim 1 or 2 wherein the lipoprotein in the precipitate consists primarily of high density lipoprotein (HDL).
- 20 4. A method according to claim 3 wherein greater than 70% of the lipoprotein in the precipitate is high density lipoprotein.
5. A method according to claim 3 or 4 wherein greater than 80% of the lipoprotein in the precipitate is high density lipoprotein.
6. A method according to any one of claims 1 to 5 further comprising maintaining the
25 temperature of the blood component below about 10°C while the precipitate is precipitated from the blood component.
7. A method according to any one of claims 1 to 6 wherein the collecting of the precipitate comprises:

adding a filter aid to the blood component following the precipitation of the precipitate to provide a solution of the filter aid and the precipitate; and recovering the filter aid and precipitate from the solution.

8. A method according to claim 7 wherein the filter aid comprises diatomaceous earth.
- 5 9. A method according to any one of claims 1 to 8 further comprising purifying the lipoprotein collected from the blood component, wherein the purification of the lipoprotein comprises:
 - preparing a dissolving solution;
 - dissolving the lipoprotein in the dissolving solution from the precipitate;
 - 10 and
 - separating the dissolved lipoprotein from undissolved matter.
- 10 10. A method according the claim 9 wherein the separation of the dissolved lipoprotein from the undissolved matter comprises passing the dissolved lipoprotein through a filter to trap the undissolved matter and provide a filtrate containing the dissolved lipoprotein.
- 15 11. A method according to claim 9 or 10 wherein the dissolving solution comprises an agent for inhibiting the undissolved matter dissolving with the lipoprotein.
12. A method according to any one of claims 1 to 11 further comprising concentrating the lipoprotein to a desired concentration level in a range of from about 50mg/dl to
20 about 3000mg/dl.
13. A method according to any one of claims 1 to 12 further comprising heat heating the lipoprotein to provide a heat treated product with a transmission at a wavelength of 650 nm of 70% or greater.
14. A method according to any one of claims 1 to 13 wherein the divalent metal cation
25 is a Group IIA divalent metal cation.
15. A method according to claim 14 wherein the divalent metal cation is selected from the group consisting of Ca^{2+} , Mg^{2+} , and mixtures thereof.

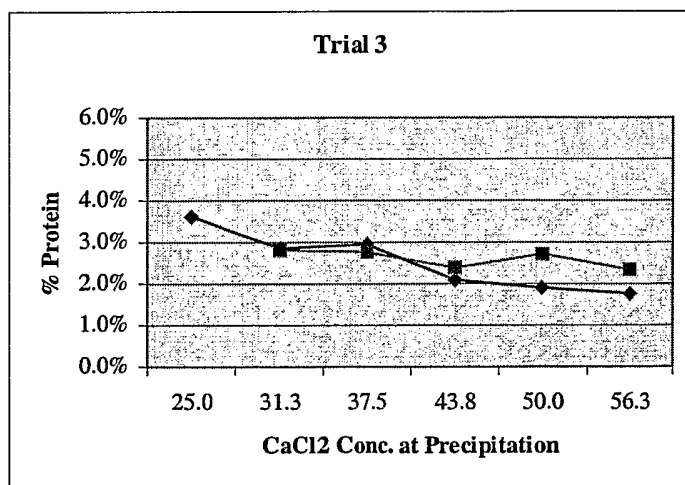
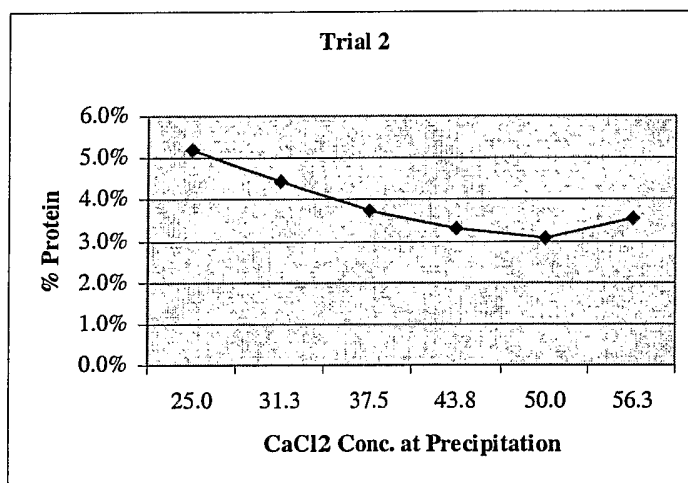
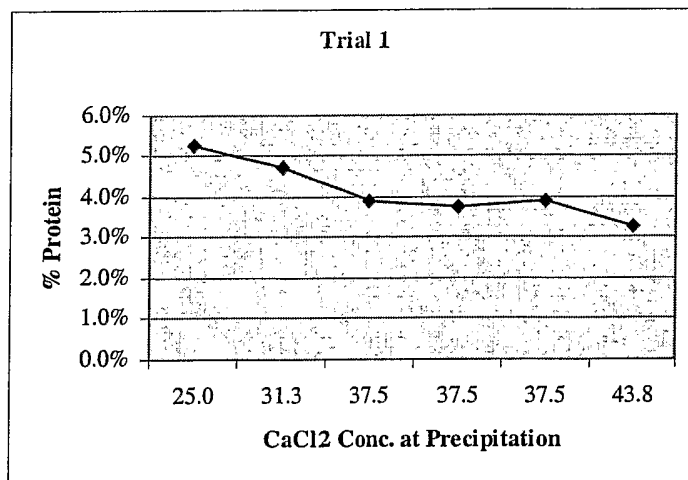
16. A method according to any one of claims 1 to 15 wherein the divalent metal cation is added to raise a level of the divalent metal cation in the blood component by a concentration of 0.3 M or greater.
17. A method according to claim 20 wherein the divalent metal cation is added to raise the level of the divalent metal cation by a concentration in a range of from about 0.3 M to about 0.5 M.
18. A method according to claim 17 wherein the divalent metal cation is added is added to raise the level of the divalent metal cation by a concentration in a range of from about in a range of from 0.35 M to about 0.50 M.
19. A method according to any one of claims 1 to 18 wherein the polyanion precipitating agent is selected from the group consisting of sulfated polymers, sulfated polysaccharides, dextran sulfate, heparin, sulfated polygalacturonic acid methyl ester, polyanetholsulfonate, sulfated amylopectin, polyvinyl sulfate, and mixtures thereof.
20. A method according to claim 19 wherein the polyanion precipitating agent is a sulfated polysaccharide.
21. A method according to claims 20 wherein the sulfated polymer is dextran sulfate.
22. A method according to claim 21 wherein the dextran sulfate has a molecular weight in a range of from of about 5kDa to about 1000 kDa.
23. A method according to any one of claims 1 to 22 wherein less than 5% of the albumin in the blood component is precipitated from the blood component with the lipoprotein.
24. A method according to claim 23 wherein less than 2% of the albumin is precipitated with the lipoprotein.
25. A method according to any one of claims 1 to 24 wherein the ratio of albumin to total protein in the precipitate is below 1:49 by weight.
26. A method according to claim 25 wherein the ratio of albumin to total protein in the precipitate is below 1:99.

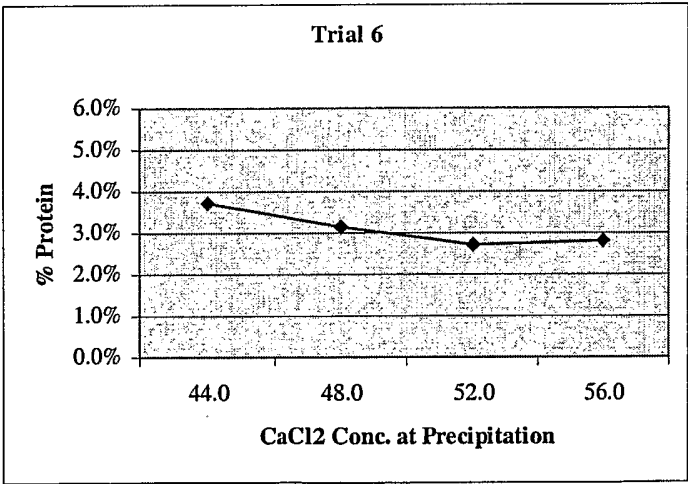
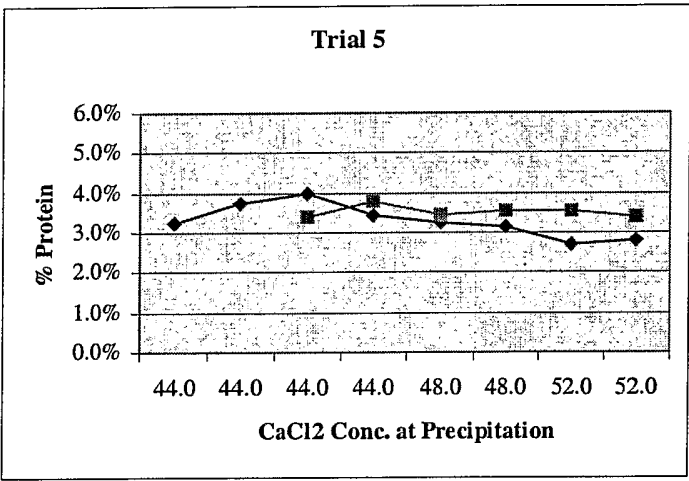
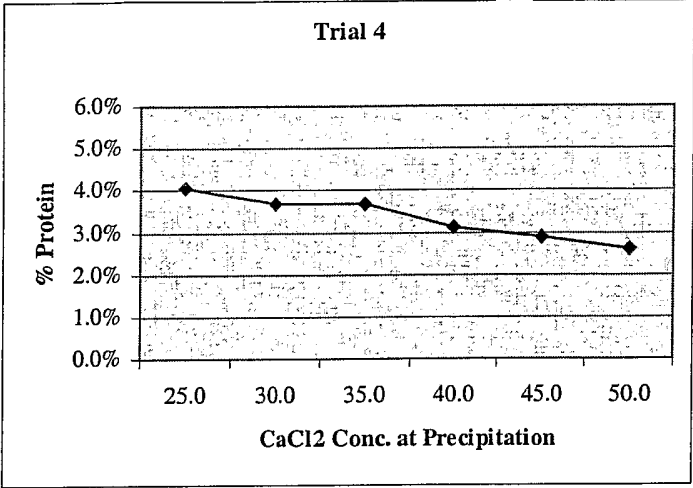
27. A method according to any one of claims 1 to 25 wherein the precipitate is substantially free of albumin.
28. A method according to any one of claims 1 to 27 wherein the precipitate has a ratio of total protein to lipoprotein associated cholesterol of 3:1 by weight or less.
- 5 29. A method according to claim 28 wherein the ratio of total protein to lipoprotein associated cholesterol is 2.5:1 by weight or less.
30. A method according to claim 29 wherein the ratio of total protein to lipoprotein associated cholesterol is 2:1 by weight or less.
- 10 31. A method according to any one of claims 1 to 30 wherein the divalent metal cation is a divalent metal cation of a salt and the method comprises selecting the salt and adding the salt to the blood component.
32. A method according to claim 31 wherein the salt is a Group IIA metal salt.
33. A method according to claim 32 wherein the salt is selected from a group consisting of CaCl_2 , MgCl_2 and mixtures thereof.
- 15 34. A method according to any one of claims 1 to 33 wherein the blood component contains albumin at a concentration greater than 24 g/litre.
35. A method according to claim 39 wherein the concentration of the albumin is 30g/litre or greater.
- 20 36. A method according to any one of claims 1 to 35 wherein the blood component is serum or plasma.
37. Lipoprotein prepared by a method as defined in any one of claims 1 to 35.
38. Cholesterol purified from lipoprotein prepared by a method as defined in any one of claims 1 to 35.
- 25 39. A method for preparing lipoprotein from a liquid blood component, the method comprising:
(a) adding a divalent metal cation and dextran sulfate to the blood component;

- (b) mixing the divalent metal cation and the dextran sulfate with the blood component to obtain a precipitate containing lipoprotein; and
- (c) collecting the precipitate from the blood component for further purification of the lipoprotein;
- 5 wherein the divalent metal cation and the dextran sulfate are added to the blood component in a ratio of moles of the divalent metal cation to dextran sulfate in grams per litre of the blood component of 1:9.5 or greater, and the precipitate contains less than 10% of the albumin in the blood component and greater than 20% of the lipoprotein associated cholesterol present in the blood component.
- 10 40. A method according to claim 39 the divalent metal cation is added to the blood component in an amount of 0.3g per litre of the blood component or greater.
41. A method according to claim 40 wherein the divalent metal cation is added to the blood component in an amount in a range of from 0.3 moles to 0.5 moles per litre of the blood component.
- 15 42. A method according to claim 41 wherein the divalent metal cation is added to the blood component in an amount in a range of from 0.35 to 0.5 moles per litre.
43. A method according to any one of claims 39 to 42 wherein the divalent metal cation is a Group IIA divalent metal cation.
44. A method according to claim 43 wherein the divalent metal cation is a divalent
20 metal cation of salt and the method comprises selecting the salt and adding the salt to the blood component.
45. A method according to claim 44 wherein the salt is selected from the group consisting of CaCl_2 , MgCl_2 , and mixtures thereof.
46. Lipoprotein prepared by a method as defined in any one of claims 40 to 45.
- 25 47. Cholesterol purified from a lipoprotein prepared by a method as defined in any one of claims 40 to 45.

48. A method for determining an amount of a divalent metal cation for preparing lipoprotein from a liquid blood component containing albumin in combination with a polyanion precipitating agent, the method comprising:
- 5 (a) adding an initial amount of the divalent metal cation and an amount of the polyanion precipitating agent to a sample of the blood component;
- (b) mixing the divalent metal cation and the polyanion precipitating agent with the blood component to obtain a precipitate from the liquid blood component containing lipoprotein comprising greater than 20% of the lipoprotein associated cholesterol present in the blood component;
- 10 (c) repeating steps (a) and (b) one or more times using a further sample of the liquid blood component and varying the amount of the divalent metal cation relative to the amount to the polyanion precipitating agent in the further blood component each time, respectively; and
- (d) determining the amount of the divalent metal cation relative to the
- 15 polyanion precipitating agent required to obtain a precipitate containing less than 10% of the albumin in the blood component and having a ratio of albumin to total protein in the precipitate below 1:3 by weight.
49. A method according to claim 48 wherein the amount of the polyanion precipitating agent is maintained substantially constant in step (c) while the amount of the
- 20 divalent metal cation added to the blood component is varied.
50. A method according to claim 48 or 49 wherein the polyanion precipitating agent is selected from a group consisting of sulfated polymers, sulfated polysaccharides, dextran sulfate, heparin, sulfated polygalacturonic acid methyl ester, polyanetholsulfonate, sulfated amylopectin, polyvinyl sulfate, and mixtures thereof.
- 25 51. A method according to claim 50 where the polyanion precipitating agent is dextran sulfate.
52. A method according to claim 51 wherein the dextran sulfate has a molecular weight of from about 5 kDa to about 1000 kDa.
53. A method according to claim 52 wherein the dextran sulfate has a molecular weight
- 30 of about 500 kDa.

Figure 1





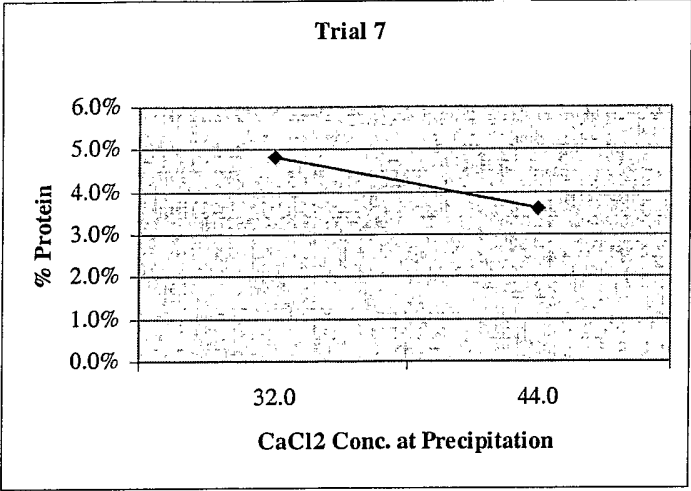
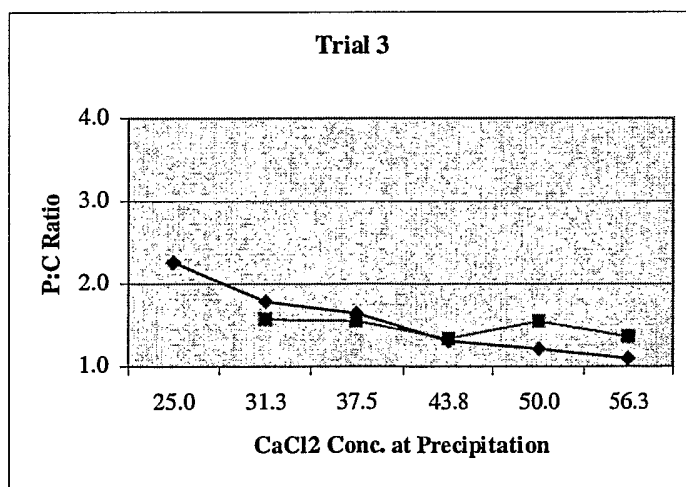
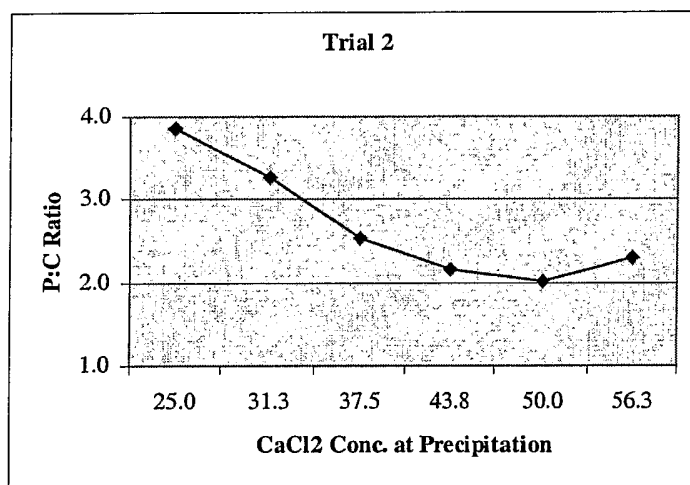
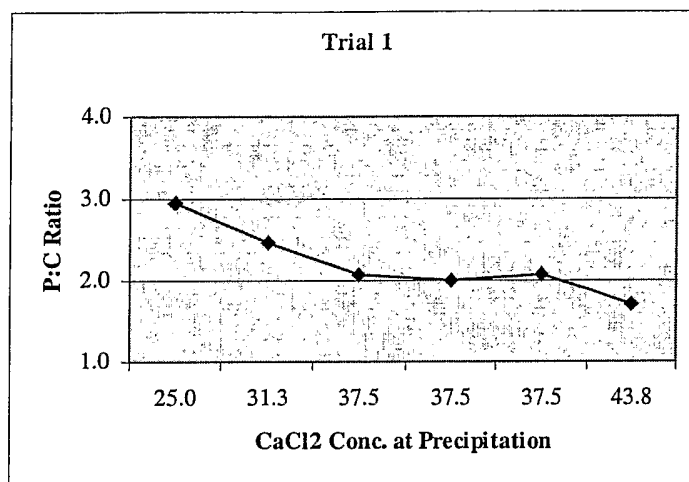
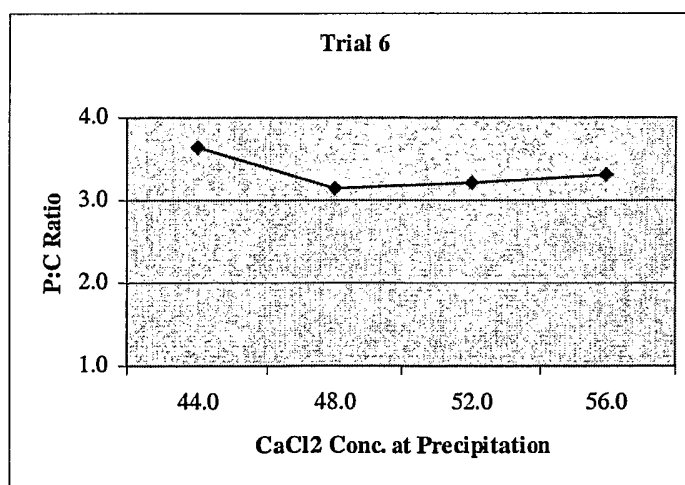
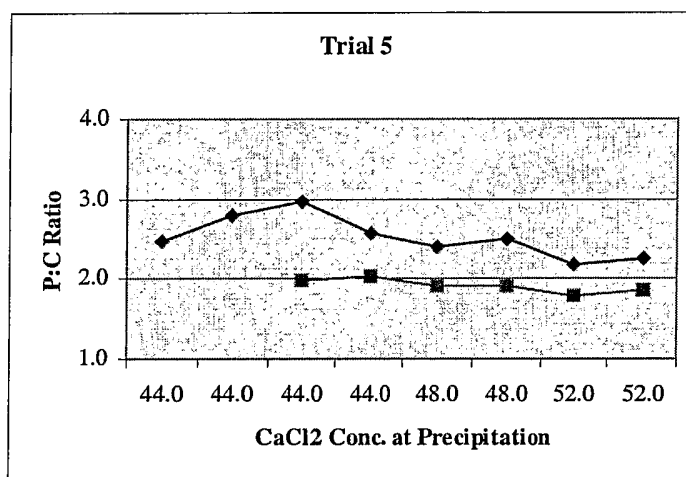
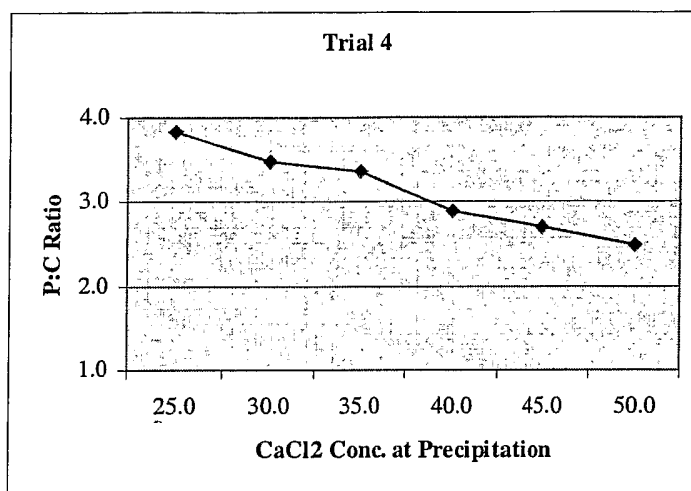


Figure 2





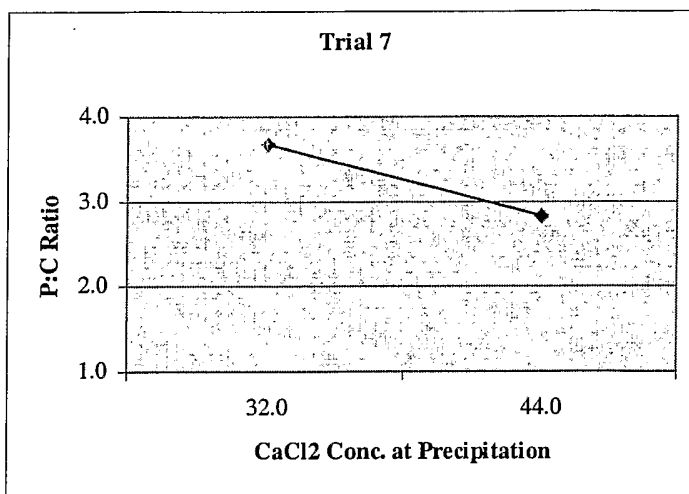
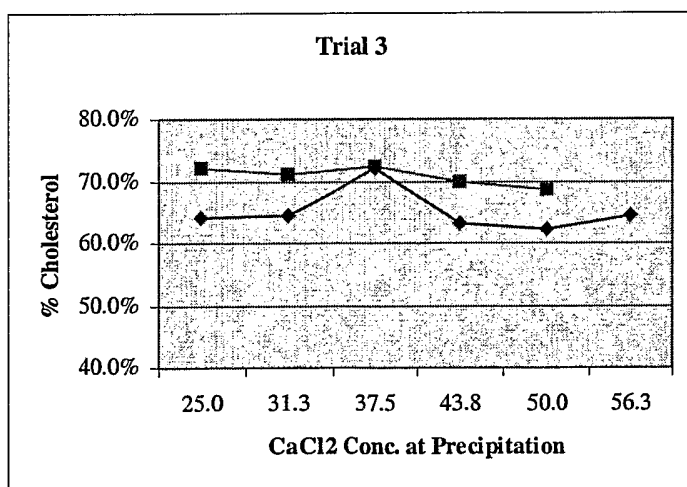
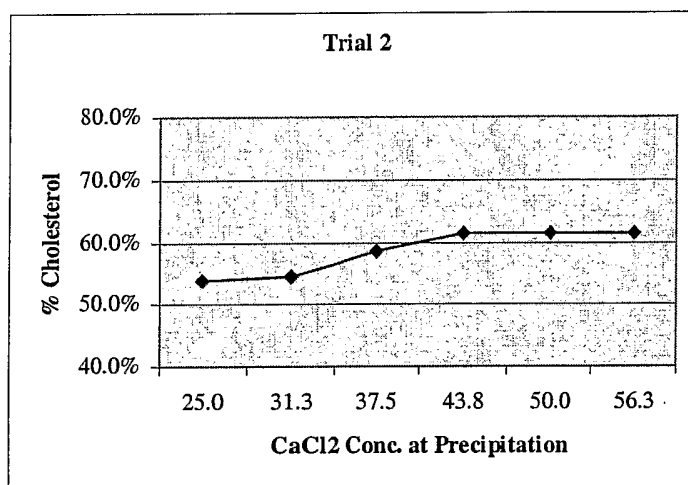
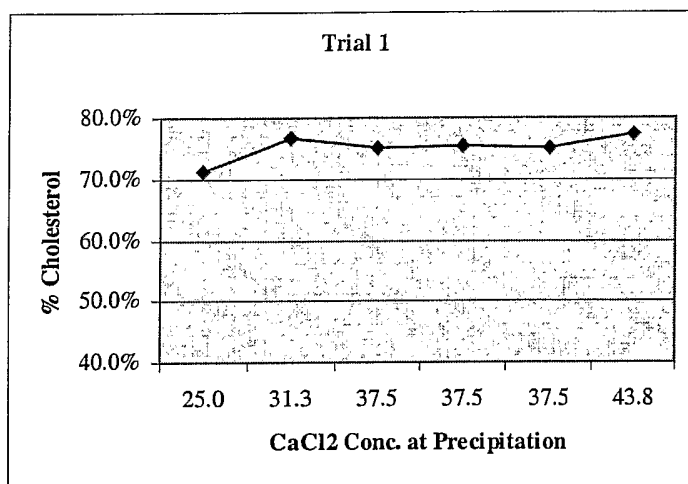
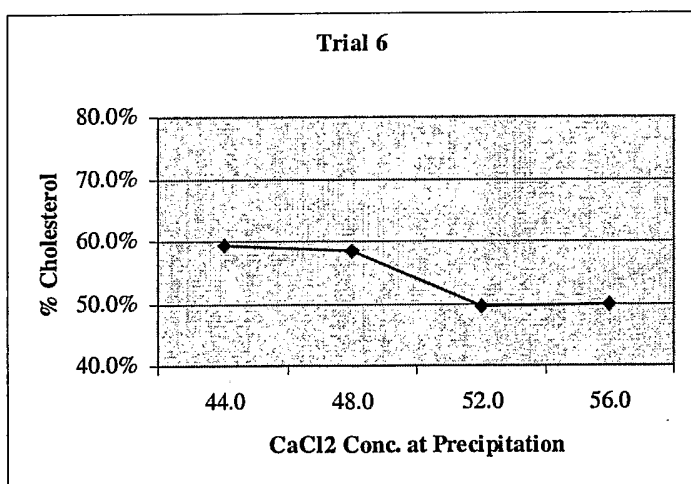
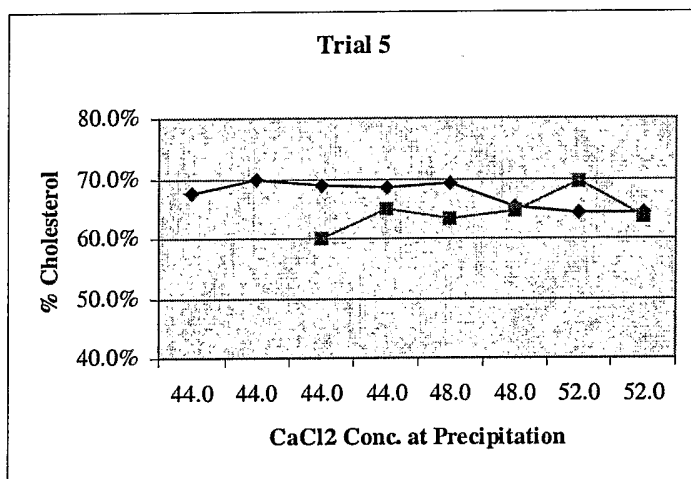
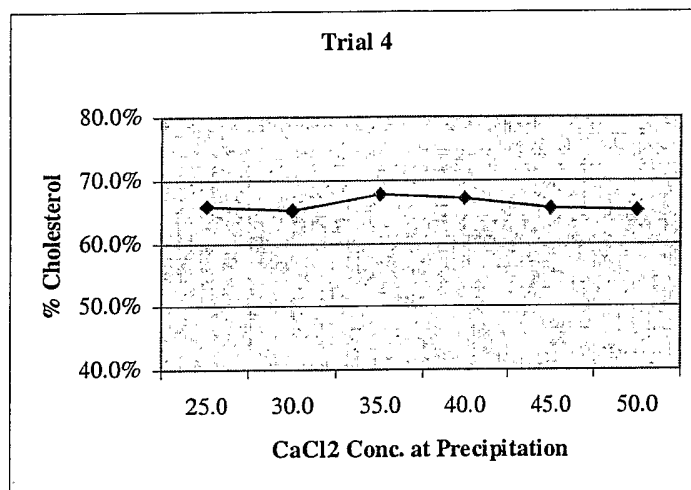
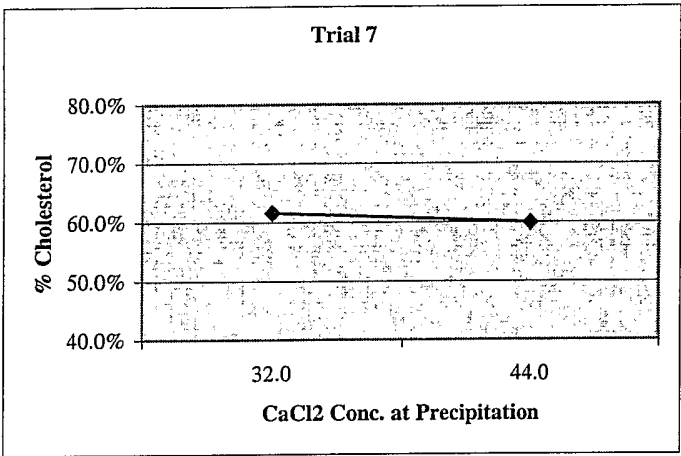


Figure 3







INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/01013

A. CLASSIFICATION OF SUBJECT MATTER												
Int. Cl. ⁷ : C07K 1/14, B01D 11/04												
According to International Patent Classification (IPC) or to both national classification and IPC												
B. FIELDS SEARCHED												
Minimum documentation searched (classification system followed by classification symbols)												
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched												
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN: File Medline, CA, WPIDS, Biosis. Keywords: lipoprotein, divalent, magnesium, calcium, polyanion?, dextran(w)sulfate, heparin, purif?, isolat?, precipit?, extract?, blood, serum, and similar terms.												
C. DOCUMENTS CONSIDERED TO BE RELEVANT												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X	US 4045176 A1 (Proksch et al) 30 August 1977 See whole document, in particular Example 1	1-53										
X	JP 07-294532 A (EIKEN KAGAKU KK), 10 November 1995 Abstract	1-53										
X	JP 55-093065 A (WAKO PURE CHEM IND LTD), 15 July 1980 Abstract	1-53										
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex												
* Special categories of cited documents: <table border="0"> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention											
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone											
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art											
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family											
"P" document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search 16 September 2003		Date of mailing of the international search report - 5 DEC 2003										
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer IAN DOWD Telephone No : (02) 6283 2273										

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/01013

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Lamplugh SM et al "Selective Precipitation of Very Low Density Lipoproteins from Fasting Sera by Heparin and Manganese Ions" Clinica Chimica Acta, 86(1978) 31-36	1-53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU03/01013

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
US	4045176	US	3955925
JP	7294532	NONE	
JP	55093065	NONE	
END OF ANNEX			

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/01013

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos : 1-53 (all in part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Due to economic reasons, the search was limited to incorporate the specific embodiments only.
3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.