EXTRACTION OF LIPIDS FROM HUMAN BLOOD PLASMA USING COMPRESSED GASES

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ABSTRACT

Lipids were extracted from human blood plasma using sub- and supercritical fluids. An extraction plant was specifically designed to allow the treatment of plasma with compressed propane, dimethyl ether (DME) and nitrous oxide (N_2O) as solvents. Experiments were performed using sub- and supercritical gases as mild solvents to remove the lipid fraction without denaturing valuable proteins or affecting their activity. Liquid propane, liquid dimethyl ether, and sub- and supercritical nitrous oxide were investigated with regard to their impact on the lipids and proteins in blood plasma at 30 MPa and at temperatures of 25°C to 40°C. Besides the extraction with pure gases the effect of modifiers and liquid solvents were studied. The treatment of plasma with small amounts of tributyl phosphate and Triton X-100 and subsequent extraction with supercritical nitrous oxide showed promising results with respect to a complete removal of plasma lipids. Cholesterol and triglycerides could be removed to an extent of 96 percent whereas the valuable proteins were retained unaffected. Since the removed lipids were shown to cumulate in a foamy layer that is formed during the extraction with supercritical nitrous oxide it is very likely that a selective precipitation of lipoproteins takes place caused by a deformation of their hydrophilic shell. The influence on the activities of the valuable proteins is still under investigation.

INTRODUCTION

Proteins from human blood plasma are widely used in the treatment of life-threatening diseases. Since the supply of specific plasma proteins is not possible by means of plasma transfusion, the fractionation of human blood plasma plays an important role in therapy of hereditary or infectious diseases. Certain steps in the fractionation of blood plasma are affected by the lipids occurring in plasma in the shape of micelle-like complexes called lipoproteins. The rapid inactivation of adsorbents used in fractionation processes is attributed to the lipoproteins in blood plasma. The removal of the lipids prior to the fractionation is assumed to overcome the problem of rapid adsorbent inactivation and hence to be favourable for fractionation processes. Lipids also cause problems in storage of plasma and lyophilisation of plasma products. Hence a removal will generally lead to a decrease in perishability of plasma and less denaturing of proteins during freeze drying.

It is obvious that the transport of the per definition hydrophobic lipids in the aqueous milieu of blood plasma cannot be established in their original free form and is enabled by the formation of lipoproteins. The general assembly is the same for different classes of lipoproteins. The core of the globular lipoprotein complex consists of the more hydrophobic lipids, the triglycerides and the esterified cholesterol, and is encircled by proteins, amphiphilic phospholipids and free cholesterol pointing their charged groups out towards the aqueous environment. Due to this specific structure it is evident that lipoproteins are affected by solvent-detergent treatments which are used to inactivate lipid-enveloped viruses by

disrupting their lipoprotein shell whereas the biologically active plasma proteins remain unaffected. The solvent-detergent method involving the incubation of plasma with 1% tri-n-butyl phosphate (TBP) and 1% Triton X-100 at 30°C for several hours was described by Horowitz et al. [1, 2] who also remarked affections to lipoproteins. Bouzidi et al. [3] used supercritical carbon dioxide and supercritical nitrous oxide to inactivate lipid-enveloped viruses in human blood plasma and also determined denaturation of lipidic components.

In this study quarantine plasma without stabilizing agents was subjected to sub- and supercritical nitrous oxide, to the solvent-detergent treatment described by Horowitz et al. and to combinations of both treatments, in order to determine the effect on the concentration of lipids in the treated blood plasma.

MATERIALS AND METHODS

Pretreatment of plasma: Thawed quarantine plasma was portioned, refrozen in acetone + dry ice and stored at –25°C. Prior to each experiment the plasma portions were thawed in a water bath at 36°C. To study the effect of modifiers 1% detergent (Triton X-100 or Tween 20) and in some experiments additionally 1% tributyl phosphate (TBP) was added to the thawed plasma according to the solvent detergent method for virus inactivation reported by Horowitz et al. [1, 2].

Analytical methods: Due to severe scattering of the analytical results with test kits for triglycerides and cholesterol, it was decided to charge a medical laboratory with the analyses. Besides the analyses for triglycerides and cholesterol in later experiments also the content of total protein (electrophoresis) and IgG (nephelometry) was analyzed. Triton X-100 was determined in the extracts by HPLC qualitatively. TBP could be determined in raffinates and in extracts with a gas chromatographic method quantitatively.

Extraction with compressed gases: For the extraction with liquid and supercritical gases (below termed as solvent) two different high pressure liquid gas contactors were used. Cell 1 is a horizontal stainless steel high pressure liner of 25mm ID with sapphire windows at both ends. Plasma was pumped into the pressurized cell until half of the volume of the cell was filled with plasma. Using a high pressure gear pump the solvent was conveyed across the phase boundary surface from one end of the liner to the other while the plasma was stirred by means of a magnetic stirrer.

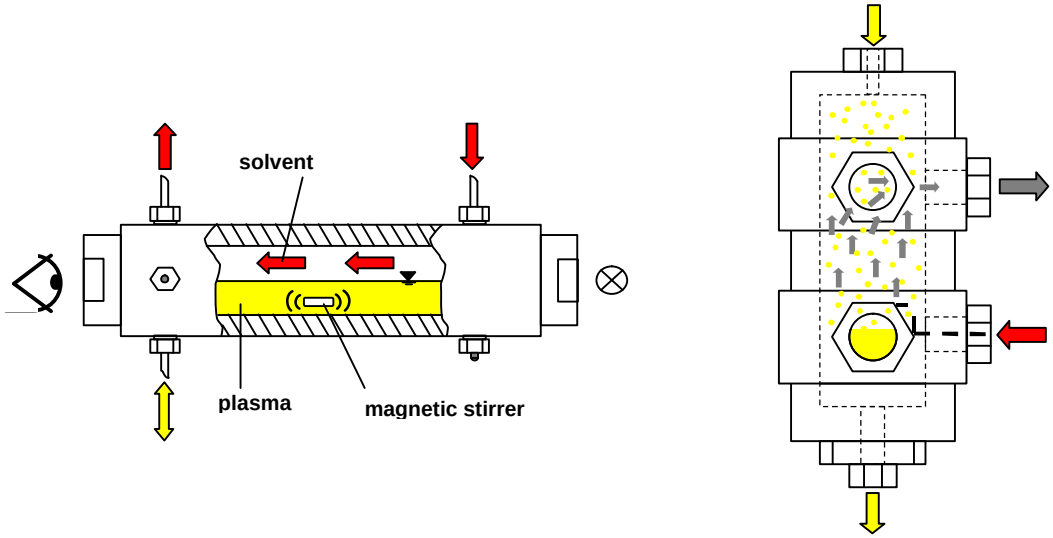

Figure 1: High pressure extraction cells for the extraction of blood plasma with compressed gases. Cell 1 (left) and cell 2 (right).

Cell 2 consists of a vertically placed stainless steel liner of 25mm ID with two pairs of sapphire windows. Between the pairs of sapphire windows a stainless steel gauze packing (Sulzer CY) was placed to increase the mass transfer surface. Plasma was pumped into the pressurized cell until the phase boundary line was in the middle of the lower pair of sapphire windows. Plasma was withdrawn at the bottom and fed to the top of the cell with an HPLC pump and hence circulated through the gauze packing. The solvent enters the cell directly above the lower pair of sapphire windows and is withdrawn at the level of the upper pair of windows. By this technique a countercurrent flow of solvent and plasma was established. In Figure 1 a sketch of the cells is shown. Both cells were incorporated into a closed high pressure system consisting of a 300 ml buffer autoclave, a high pressure syringe pump, and a compressor generator. The cell, the buffer autoclave and the gear pump were immersed in a water bath that was temperature controlled by means of a thermostat. The high pressure syringe pump (Isco 260 D) was connected to a pressure transducer and programmed to maintain constant pressure during sampling. The controller of the syringe pump was simultaneously used to indicate the pressure of the system.

After flushing the system two times with the solvent the system was pressurized up to 5 MPa using the compressor generator. Prior to the adjustment of the final extraction pressure the plasma was pumped into the cell by a HPLC pump. When the desired extraction pressure was reached the gear pump was started to circulate the solvent through the cell. In case of using cell 1 the magnetic stirrer was started at the same time. For experiments with cell 2 the HPLC pump was started to circulate the plasma through the gauze packing. Each experiment was prepared the preceding afternoon and the solvent was circulated overnight. 30 minutes before sampling the gear pump and the magnetic stirrer were switched of and the valve for the gas phase sampling was heated to 50°C to prevent freezing due to the Joule-Thomson effect.

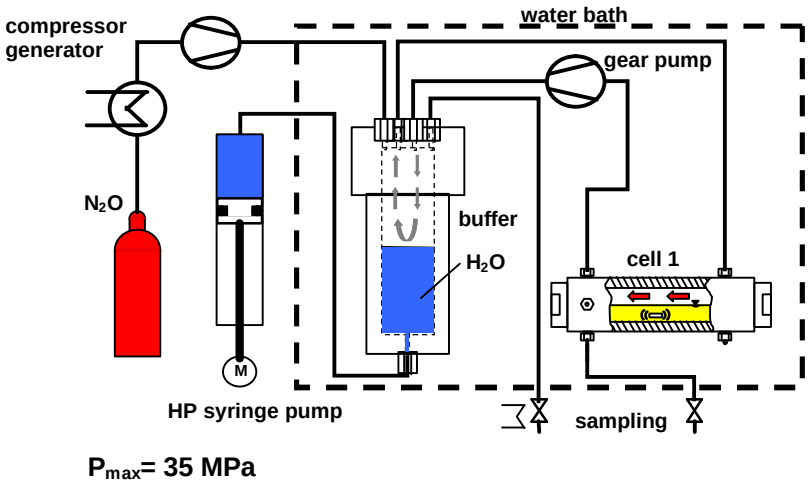

Figure 2: System for batch extraction of human blood plasma

The plasma sample was directly expanded to plastic test tubes as delivered from the laboratory performing the analyses of triglycerides and cholesterol. In some experiments the amount of gas dissolved in the plasma was evaluated by passing the expanded gas to a burette system.

The sampling unit for the gas phase consisted of a trap-like sample vial which was connected to a gas meter by a hose. The solvent phase was expanded to ambient pressure into the sample vial and the gas was passed through the subsequent gas-meter to evaluate the amount of expanded gas. In some experiments an additional cooling trap immersed in an aceton+dry ice bath was used to retain residual water from the expanding gas.

Experiments with Triton, Tween and TBP exhibited formation of a foam layer at the phase boundary during extraction. In order to analyze the foam, Ringer's solution was pumped into

the cell after removal of the liquid plasma and stirred for two hours to dissolve the foam and withdraw it together with the Ringer's solution.
For all high pressure batch extractions the solvent to feed ratio was approximately 10 (vol/vol).

Liquid extractions: Liquid extractions according to the solvent/detergent-method (SD) were performed in 25 ml flat-bottomed flasks at 30°C in a water-heated shaker. After shaking the spiked plasma samples (TNBP, Triton X-100, Tween 20) for 30 minutes germ oil or hexane was added and the mixture was again shaken for 30 minutes. Subsequently the plasma was filled in 25 ml centrifugal flasks and centrifuged for 10 minutes at 4000 g. The solvent layer and denatured protein was withdrawn and the plasma was sent to the analytical laboratory.

RESULTS

Experiments with N_2O and liquid extractions : Experiments with untreated plasma and N_2O were only successful using the vertically placed cell 2 with countercurrent flow of plasma and solvent. By this technique the content of cholesterol and TG could be reduced to 50 % of the original concentration in one case. Since cholesterol and TG could not be detected in the extract and the cell and the buffer were filled with foam the mode of action is not clear. Also the raffinates from the experiments looked turbid and grayish and the results were not reproducible. Using cell 1 the reduction of cholesterol and TG was not possible without pretreatment of plasma with detergent (Triton-X 100, Tween 20).
Figure 3 shows some results from experiments with pure N_2O and with untreated and detergent-spiked plasma. In Figure 4 the results of the SD methods with the different extracting agents are depicted.

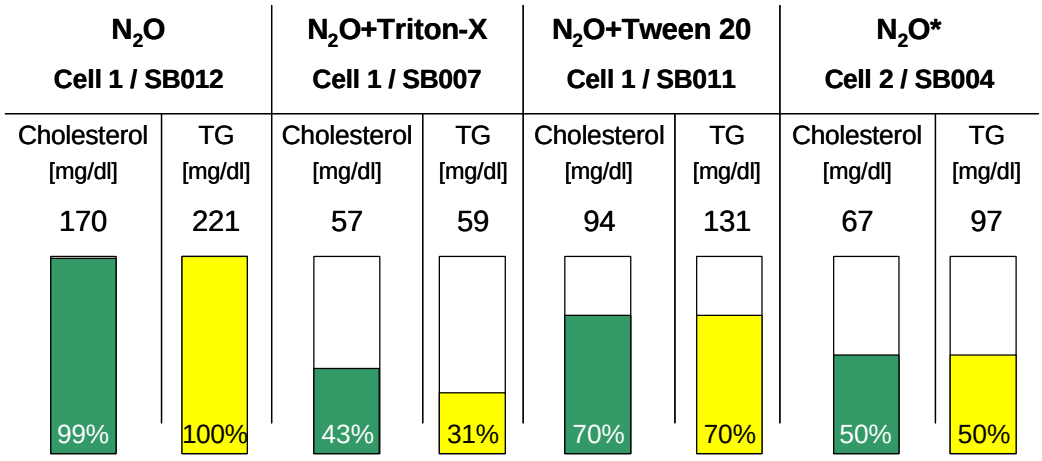

Figure 3: *Cholesterol and triglyceride concentrations of raffinates after contact with N_2O at 30 MPa and 38°C (* 25 MPa, 40°C). Feed concentrations: Cholesterol 134-170 mg/dl, triglycerides 180-220 mg/dl. SBxxx indicates the number of experiment.*

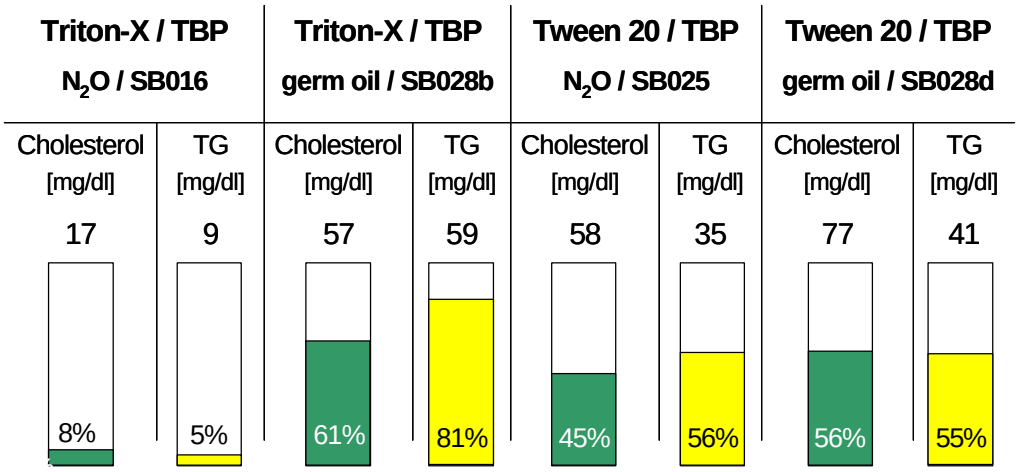

Figure 4: *Lipid concentrations of plasma after extraction with N_2O (30 MPa, 30°C) or centrifugation (ambient pressure, 30°C). Feed concentrations: Cholesterol 128-221 mg/dl, triglycerides 63-177 mg/dl. TBP and detergents 1 % (vol) respectively.*

Experiments with detergent-spiked plasma exhibited the formation of a foam layer during the extraction which was dissolved in Ringer's solution and analyzed. The diluted foam showed remarkable Cholesterol and TG concentrations and also noticeable protein concentrations. Since no lipids were found in the extracts this led to the assumption that the lipoproteins were selectively precipitated during the extraction process at the phase boundary originating the foamy layer.
Considerable amounts of extracts were detected in experiments with Triton and/or TBP spiked plasma only. The existence of Triton and/or TBP was determined qualitatively by HPLC- and GC-Analysis. Triton and TBP seem to be the principal constituents of these extracts.

It is obvious that the reduction of cholesterol and triglycerides is best using Triton and TBP with subsequent extraction with N_2O. A time observation of the extraction of Triton/TBP-spiked plasma is shown in Table 1. The concentration of Triton-X could not be determined quantitatively. Analysis of the concentration of immunoglobulins in the lipid reduced plasma fraction showed only a slight decrease in IgG.

Extraction time	**TBP**	**Triton-X 100**	**Cholesterol**	**Triglycerides**	**Protein**	**IgA**	**IgG**	**IgM**
[min]	[mg/ml]	[mg/ml]	[mg/dl]	[mg/dl]	[g/l]	[mg/dl]	[mg/dl]	[mg/dl]
Feed								
0	10,046	10	137	75	54	163	939	42
10	2,66	-	137	78	57	-	-	-
30	2,42	-	136	79	56	-	-	-
90	0,86	-	33	17	52	160	903	40
150	0,125	-	18	11	53	160	919	42

Table 1: Time observation of extraction with N_2O (30 MPa, 30°C) of Triton/TBP-spiked plasma. Solvent to feed ratio is reduced with each sampling of 5 ml.

Experiments with propane, DME, and carbon dioxide: Propane as solvent did not show any reduction of relevant lipids in the plasma fractions. Neither the treatment of plasma in cell 1 nor in cell 2 caused any relevant effect. The addition of 10 % DME (gas phase) showed a higher solubility of DME in the aqueous plasma phase than that of propane (79 % of the gas dissolved in the plasma was shown to be DME) but also caused no effect regarding the reduction of TG and cholesterol. Experiments with pure DME as well as experiments with carbon dioxide resulted in complete denaturing of plasma proteins.

CONCLUSION

Extraction of cholesterol and triglycerides using compressed gases could only be achieved successfully so far with addition of agents known from SD viral inactivation technology. The application of liquid propane did not show any reduction of triglyceride concentration although it exhibits a high dissolving power for triglycerides like triolein and tripalmitin [4, 5]. The main obstacle that hinders the direct extraction of lipids is presumably the micellar structure of the lipoproteins.

Best results were obtained when plasma was incubated with Triton X-100 and TBP (1 % (vol) each) and subsequently extracted with N_2O at 30 MPa and 30°C. With the latter procedure reduction of cholesterol and triglycerides in the plasma down to 8% and 5% of the original amount was accomplished, respectively. The solubility of substances in the compressed N_2O was rather low and the amounts of extract small. Lipids could not be detected in the extracts but in a foamy layer at the phase boundary which was only observed in experiments with successfully reduced lipid content and seems to be constituted of precipitated lipoproteins. The change of temperature between 30°C and 38°C and hence the change of state of the nitrous oxide between sub- and supercritical had no remarkable effect.

Since the plasma proteins remain mainly dissolved in the aqueous plasma phase the precipitation seems to be selective for lipoproteins. The mode of action for this selective precipitation is thought to be an interfacial phenomenon which is connected to the substances active at the interface like Triton X and Tween. These substances probably penetrate the micelle structure and lead to the disruption of the structure during extraction with TBP and N_2O and hence to the precipitation of the lipoproteins at the phase boundary. It has to be noted that the reduction of lipids in blood plasma during subjection to nitrous oxide is strongly dependent on sufficient agitation by means of the magnetic stirrer and the gear pump which leads to the assumption that the precipitation of lipoproteins is partially established during the formation of foam and might be partially reversible.

Further investigations now focus on the influence of the treatment on the biological activity of labile plasma proteins like Factor VIII and fibrinogen.

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