Experimental

1. Apparatus and HPLC conditions

A Waters (Milford, MA, USA) high-performance liquid chromatograph was used. The HPLC system consisted of two solvent delivery systems (510), an automatic sample injector (WISP 717), and a detector (486), connected to a computer to monitor chromatographic parameters and process data (Millenium, V 2.15). Separations were performed on a reversed-phase Symmetry C-18 column (250 × 4.6 I.D. mm, 5 µm, 100 Å, Waters) and a Symmetry Guard column (20 × 3.9 I.D. mm, 5 µ, 100 Å, Waters). The mobile phase was distilled water acidified with 0.2% of orthophosphoric acid 0.1 M (solvent A) and acetonitrile (Carlo Erba, HPLC-grade) acidified with 0.2% of orthophosphoric acid 0.1 M (solvent B).
The linear gradient was: solvent A/solvent B; v/v [5 min (95 : 5); 7 min (76 : 24); 27 min (76 : 24); 29 min (95 : 5)]. The flow rate was 1 ml · min^{-1} and the injection volume was 20 µl. Detection was assessed at 280 nm. The run time was 30 min.

2. Samples

Globularia alypum L. leaves were collected in Marseille in October 1997. Half of the leaves were dried at room temperature, the others were dried by micro-waves (4 × 1 min; Panasonic 1330; 50 Hz–2680 watts),
All samples are prepared in a solution of 76% of solvent A and 24% of solvent B. 4-Hydroxybenzoic acid methyl ester (Fluka, 8 mg) was dissolved in 20 ml of solvent (I.S. solution).
Pure globularin was isolated from *Globularia alypum* L. according to a previously reported procedure [5]. A stock solution of globularin with a concentration of 300 µg · ml^{-1} was prepared by dissolving 15 mg of globularin in 50 ml of solvent.
Five standard solutions (10–300 µg · ml^{-1}) were made by further dilution of the stock solution; 1 ml of every standard solution was added to 1 ml of I.S. solution.
Extract solutions were prepared from dried and powdered leaves. Powdered leaves (0.1 g, 0.4 g, 0.6 g, 1.0 g and 2.0 g) were macerated and extracted with 200 ml methanol-water (60 : 40). The extract solutions were diluted to 1/5 with the solvent; 1 ml of every diluted solution was added to 1 ml of I.S. solution.

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Dr. R. Elias, Ph.D.
Laboratory of Pharmacognosy
Faculty of Pharmacy
27 Bd Jean Moulin
13385 Marseille cedex 5
France
Riad.Elias@pharmacie.univ-mrs.fr

Pharmaceutics Laboratory, Department of Pharmaceutical Sciences, Dr. Harisingh Gour Vishwavidyalaya, Sagar, India

Adsorption studies of sugars on calcium hydroxy apatite ceramics and haemoglobin on sugar coated ceramics

S. Patil, A. J. Khopade, S. Nagaich, G. P. Agrawal and N. K. Jain

Whole blood serves a number of important functions but the most critical function is oxygen transfer. In emergency situations like accidents, major surgery, treatment of chronic anaemias, where the supply of whole blood is low, transfusion of blood or oxygen carrying solutions may provide temporary life support. The various potential applications of the oxygen carrying solutions are reviewed by Minato et al. [1]. A number of approaches have been developed during last 5–6 decades, to transport and deliver oxygen effectively and safely using haemoglobin (Hb), crosslinked Hb, perfluorocarbons etc. but all are associated with several limitations. Various carrier systems were developed in order to overcome problems associated with Hb delivery e.g. microcapsules, liposomes, niosomes, multiple emulsions etc. [2]. Aquasomes have also been developed for the same purpose [3].
Aquasomes are colloidal ceramic-carbohydrate composites on which a variety of therapeutic proteins are attached. Basically, they consist of relatively few atoms clustered in solid crystals onto which glassy carbohydrates are adsorbed. This carbohydrate coated core serves as a non-denaturating solid surface for the subsequent attachment of a wide variety of biochemically active molecules like vaccines, therapeutic proteins etc. [4–5]. Carbohydrates (sugars) protect bioactives from degradation and dehydration effects [6]. Aquasomes are prepared by a three step method which involves: production of ceramic cores, adsorption of sugars, and immobilization/adsorption of bioactive protein.
Adsorption is a surface phenomenon which involves weak Van-der-Waal forces. Drug adsorbates for drug delivery and activated powders for detoxification have long been used in pharmaceutical field [7–8].
In the present study, Langmuir and Freundlich adsorption isotherms of various sugars like cellobiose, maltose, sucrose and trehalose were studied on calcium hydroxy apatite (HA) powder prepared by the precipitation-sintering method. The isotherms for the adsorption of haemoglobin on sugar coated ceramics were also studied. The adsorption parameters were calculated from the graphical data and explained on the basis of molecular arrangement.
Adsorption data of sugars on HA powder were found to fit satrisfactorily to both Freundlich and Langmuir isotherms. The regression coefficients of Freundlich isotherm i.e. the logarithm of the equilibrium concentration against the amount adsorbed per gram of adsorbent (Log C vs. Log x/m) and Langmuir isotherm (C vs. C/{x/m}) were in the range of 0.932 to 0.998. The Freundlich adsorption parameters (Log K, n) and the Langmuir adsorption parameters i.e. binding constant (b), and sugar adsorbed per g of HA(Ym) as determined from the graphical data are shown in the Table. The binding constants were found in the order of cellobiose > maltose > sucrose > trehalose while the amount of sugar adsorbed per g of HA followed the reverse profile. This can be explained on the basis of an epitaxial arrangement of sugars adsorbed or the packing of adjacent molecules of sugars adsorbed on HA pow-

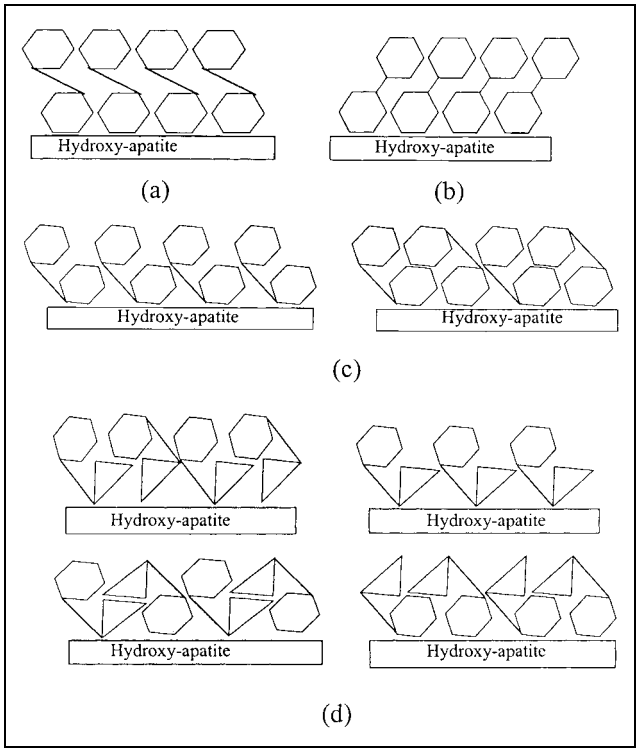

Fig.: Possible packing of sugars on hydroxy-apatite powder
a) cellobiose, b) maltose, c) trehalose, d) sucrose

der. The possible molecular arrangement of adsorption is shown in the Fig.

It is revealed from the Fig. that the packing of cellobiose and maltose is comparatively greater than that of sucrose and trehalose. There are multiple ways in which sucrose and trehalose can be adsorbed. They try to arrange themselves in a way that the lowest energy of adsorption is achieved. Their packing involves adsorbed as well as intercalated molecules. The intercalated molecules may be removed easily in stressful conditions which may account for their low binding constants. The Freundlich adsorption parameters were almost the same for all sugars studied probably because of their similar structure.

The Langmuir and Freundlich adsorption parameters of Hb on sugar adsorbed HA powder were also studied. The adsorption isotherms of Hb on sugar coated ceramics showed regression coefficients in the range of 0.951 to 0.989 for both the isotherms. The binding constants were in the trehalose > sucrose > maltose > cellobiose. This can again be explained by the interaction between the sugar and Hb. The Fig. shows cavity portions on the sugar adsorbed layer. Hb may closely fit into those cavities thereby achieving a better adsorption. Cellobiose which has the closest packing during adsorption has the lowest binding constant because of the less number of functional groups available for binding. The amount of Hb adsorbed was, however, higher than with trehalose. The values for sucrose and maltose varied between the values of cellobiose and trehalose. The Freundlich adsorption parameters were also the highest for trehalose and the lowest for cellobiose.

The study provides a clue about the stability and loading of sugars and Hb on HA ceramics. The Hb loading seems to be low with the present method to prepare aquasome formulations for its use as oxygen carrier. Studied are underway to improve the loading of Hb as well as the sugars.

Table: Langmuir and Freundlich adsorption parameters of sugars on hydroxy-apatite powder and haemoglobin on sugar coated hydroxy-apatite powder

Sugar	Sugar on HA				Hb on sugar-coated HA			
	b	Ym	Log K	n	b	Ym	Log K	n
Cellobiose	6.89	3.38	2.44	1.7	2.84	134.8	0.97	2.00
Maltose	6.54	3.77	2.44	2.8	5.03	121.3	0.96	2.17
Sucrose	2.91	4.90	2.41	1.8	4.22	111.9	1.00	2.37
Trehalose	2.13	4.91	2.44	1.7	9.53	99.3	1.02	3.60

HA: Hydroxy apatite; Hb: Haemoglobin

Experimental

1. Materials

Anthrone, haemoglobin (Loba Chemie), benzidine (Thomas Baker), maltose, sucrose, cellobiose, trehalose (Hi-Media). All other reagents and chemicals were of A. R. grade and distilled deionized water was used in all the experiments.

2. Preparation of hydroxy-apatite ceramic cores

Diammonium hydrogen phosphate solution (0.19 mol/l) was added to continuously stirred calcium nitrate solution (0.32 mol/l) in a flask with one charge funnel, a thermometer and a reflux condenser fitted with a CO_2 trap. The temperature was kept constant at 75 °C using a glycerol thermostat bath. During the addition the pH was kept constant at 8–10 using concentrated ammonia solution. The mixture was stirred for 4–6 h at same temperature and pH. The precipitate was filtered, washed throughly with distilled deionized water and dried at 100 °C overnight. The powder was sintered at 900 °C [9].

3. Adsorption of sugars on hydroxy-apatite ceramics

The solutions of different sugars e.g. trehalose, cellobiose, maltose, sucrose were prepared in the concentration of 5 mg/ml in distilled deionised water. About 1 g of HA powder was taken in series of iodine flasks and sugar solution was added to each of them in increasing amounts and volume was made up with water to 50 ml. The flasks were stoppered and vigourously shaken for 60 min. The suspensions were taken and centrifuged at 2000 rpm for 5 min and the aliquots from the supernatent were estimated for sugar content using anthrone reagent [10] on a UV spectrophotometer at 624 nm (Shimadzu UV 1601). Langmuir and Freundlich adsorption isotherms were plotted and the respective parameters were calculated from the data.

4. Adsorption of haemoglobin on sugar adsorbed hydroxy-apatite cores

Adsorption of haemoglobin was studied similarly on the selected sugar coated ceramic. Haemoglobin solution (1%) was used for the study and the benzidine method [11] was used for hemoglobin analysis.

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Prof. N. K. Jain
Pharmaceutics Laboratory
Department of Pharmaceutical Sciences
Dr. Harisingh Gour Vishwavidyalaya
Sagar-470 003 (M.P)
India

Institute of Chemistry and Clinical Biochemistry[1], Medical Faculty, Department of Pharmacology and Toxicology[2], Faculty of Pharmacy, Commenius University, Bratislava, Slovak Republic

Effect of a phytosterol mixture diet on the plasma level of fatty acids in hypercholesterolaemic rats (PHHC)

Ž. AMBROŠOVÁ[2], I. VOZÁR[1], M. ČIERNIK[2], P. ŠVEC[2] and J. KYSELOVIC[2]

The process of atherogenesis can result in severe atherosclerotic lesions at predisposed sites of the artheries [1]. Such lesions are typically characterized by the presence of inflammatory and fibroproliferatory mechanisms of varying levels [2]. Chemical, mechanical (shear stress) and biological (virus, endotoxin) stimuli as well as contineous abundant plasma levels of cholesterol can develop atherogenetic processes [3, 4].

At present, one of the possible approaches of the therapy is a phytosterol diet. The primary aim of clinical and experimental studies of phytosterol supplementation is the reduction of pathologically increased cholesterolaemia. This beneficial effect in hypercholesterolamic subjects is usually accompanied by the restoration of abnormal lipoprotein metabolism and regression of atherogenesis. The generally accepted mechanism of the phytosterols is the inhibition of cholesterol absorption from the intestine [5].

The changes in plasma levels of free and bound fatty acids associate with hypercholesterolaemia and progresion of atherogenesis [1, 3]. Increased concentrations of free oleic and linolic acids were reported during stage IV of atherogenesis [3]. The saturated fatty acids increase LDL cholesterol and decrease HDL cholesterol and may therefore increase coronary risk [6]. Long-chain fatty acids may influence human susceptibility to pathological processes which involve the interaction of leukocytes with endothelial cells, such as in atherogenesis and in inflammation [7]. In cholesterol-lowering diets, saturated fatty acids can be replaced be different classes of unsaturated fatty acids [6].

It is worth to know how these metabolic effects are modified by chronic supplementation with photysterols. Therefore we decided to evaluate the effects of phytosterol supplementation on changes in plasma concentrations of free and bound oleic, linolic, linoleic and arachidonic acid. To confirm potential effects of phytosterol on this physiological paramethers, we carried out an experimental study of a 60-days lasting diet supplementation with a phytosterol mixture (β-sitosterol 65%, stigmastanol 18%, campesterol 14% and campestanol 3%) in hypercholesterolaemic (PHHC) rats ($n = 12$) and normocholesterolaemic Wistar rats ($n = 12$). The biochemical profile of the PHHC rat, an experimental model of hypercholesterolaemia, is very similar to the human form of the disease [8].

Wistar and PHHC rats were divided into two experimental groups. Each group was maintained on a normal diet and on a diet containing 21 mg/kg of the phytosterol mixture. After 60 days, plasma samples were collected and the profiles of plasma fatty acids were analysed using GC. We focused on the ratio of saturated/unsaturated fatty acids and on changes of the profile of free and bound fatty acids typical for each stage of atherogenesis.

Supplementation of phytosterols to hypercholesterolaemic rats significantly changes plasma levels of fatty acids. The plasma levels of saturated acids (palmitic and stearic) were partially decreased in both rat strains. While in