BINDING OF BILE ACIDS BY CELLULOSE-BASED CATIONIC ADSORBENTS

JURGITA KAZLAUSKE, KRISTINA RAMANAUSKIENE* and JOLANTA LIESIENE

Department of Organic Technology, Kaunas University of Technology, 19, Radvilenu pl., LT-50254 Kaunas, Lithuania
**Department of Pharmacy, Lithuanian University of Health Sciences, 9, A. Mickeviciaus g., LT-44307 Kaunas, Lithuania*

Received April 10, 2013

One of the ways to regulate the cholesterol level in the blood is by adsorption of overproduced bile acids from the gastrointestinal tract. For this purpose, synthetic anion exchangers have been in clinical use for many years. The efficiency of these sorbents is rather low, because of their low binding capacity. In this work, cationic cellulose derivatives of different structure were evaluated as sorbents for binding of bile acids. It was found out that adsorption of bile acids is highly affected by pH. This was explained taking into account dissociation constants of the bile acids, as well as of the functional groups of the adsorbents. It has been showed that the cellulose-based adsorbents are more effective bile acid binders than the commercial ones, due to their more accessible structure. Comparing cellulosic adsorbents, the macroporous adsorbent DEAE-Granocel in all cases showed the best sorption capabilities.

***Keywords**:* cellulose derivatives, cationic adsorbents, sorption of bile acids, cholesterol

INTRODUCTION

Nowadays, one of the most topical health problems associated with coronary heart disease is an increased level of low-density cholesterol in the blood. Although cholesterol is very important for cell membrane formation and serves as a precursor for the biosynthesis of bile acids, steroid hormones and vitamin D, however too high levels of choresterol in the blood cause atherosclerosis. It has been even estimated that, in 2020, artherosclerosis will be the main reason of illnesses and deaths in the world.[1]

Bile acids take part in the cholesterol formation, therefore, one of the ways to regulate the cholesterol level in the blood is by adsorption of overproduced bile acids from the gastrointestinal tract. According to the so-called entherohepatic circulation process, a part of bile acids released into the small intestine works in fat digestion processes, the other and the biggest one is resorbed through the portal vein back to the liver and finally is kept in the gall bladder. With the increase of the bile acid concentration, cholesterol conversion to bile acids is stopped and keeps being high in the body.[2] If the overproduced bile acids are bound, the liver starts to convert more cholesterol into bile acids resulting in a lowered cholesterol level in the blood.

For binding of bile acids, the anion exchangers, such as cholestyramine, colestipol, colesevelam hydrochloride, have been in clinical use for many years. As a food supplement, chitosan is usually recommended to patients. However, the efficiency of these adsorbents is rather low, because of their low binding capacity of bile acids. Consequently, large doses (up to 24 g a day of cholestyramine, 30 g a day of colestipol and 4 g a day of colesevelam) are required for a satisfactory therapeutic response.[3-6] Moreover, the mentioned adsorbents are based on synthetic polymers. For example, cholestyramine is a quaternized styrene-divinilbenzene copolymer; colestipol is synthesised from tetraethylen-pentamine and epichlorohydrin; colesevelam is quaternized poly(allylamine) crosslinked with epichlorohydrin; colestemide is a polymer synthesized from 2-methylimidazol and epichlorohydrin. As known from clinical practice, these adsorbents cause a lot of side effects, such

as metheorism, nausea, ongoing constipation, severe stomach pain, and others.[7,8] It is also well-known that quaternary ammonium compounds are very irritative to the mucuous layer of our guts.

In order to avoid these disadvantages, new more effective and biocompatible adsorbents are searched for. One of the investigated substances is methylan. It is a polysaccharide that is extracted from *Methylobacterium organophilum* bacteria. Methylan was dialkylaminoalkylated and adsorption of bile acids was studied.[9] The results were compared with chorestyramine binding capacity. It was found that DEAE-methylan is 50% more effective than cholestyramine and its daily dose was up to 12 g.

In the attempt to develop bile acid adsorbents with higher binding capacity, we focused our research on a cationic cellulose derivative with amino groups. Cellulose has been chosen due to its good adsorption properties, biocompatibility, stability in the digestive tract and noncitotoxity.[10,11] Previously, Parkinson *et al*.[12] studied the hypolipidemic effects of orally administered dextran and cellulose anion exchangers in cockerels and dogs. The bile acid adsorption capacity *in vitro* and *in vivo* by cellulose derivatives, such as aminoethyl, diethylaminoethyl (DEAE), triethylaminoethyl, guanidoethyl, paraaminobenzyl, ECTEOLA, as well as modified dextran (DEAE-Sephadex), was compared and the DEAE-Sephadex showed the best results. However, the author did not investigate the effect of the adsorbent structure on the binding capacity.

Nichifor *et al*. systematically studied the application of aminated polysaccharides as bile acid adsorbents.[13-15] They prepared cationic adsorbents based on cross-linked dextran, pullulan and microcrystalline cellulose, containing tertiary amino and/or quaternary ammonium groups. The sorption capacity and the affinity of these adsorbents for cholic acid have been investigated considering the nature of the polymeric matrix, the swelling porosity of the adsorbents, and the basicity of cationic groups. It was demonstrated that sorption capacity increases with the increase in the content of cationic groups, their basicity, and the length of alkyl substituents in functional groups. The authors showed that all polysaccharide adsorbents have a better affinity for bile acids than cholestyramine, and among polysaccharides, dextran-based adsorbents were the best. The same group of researchers has prepared dextran microspheres containing quaternary ammonium groups with different chemical structure.[16,17] They studied the influence of the chemical structure of functional groups on the sorption capacity and affinity for cholic acid. It was concluded that the ionic linkage is the predominant interaction in the binding of cholic acid, but a hydrophobic interaction between the alkyl chain of the substituent and the steroid skeleton of cholic acid can occur simultaneously. It was also shown that the structure of the adsorbent plays a very important role in the sorption capacity for bile acids.

We have prepared macroporous cellulose, whose pores are accessible to proteins up to 1000 kDa. It was interesting to evaluate it as a matrix for the preparation of the bile adsorbent. Thus, the aim of this work was to compare the binding of bile acids by different cellulose-based adsorbents and to investigate the effect of their structure on the sorption capacity. The binding capacity of anion exchangers based on different kinds of cellulose, namely macroporous (DEAE-Granocel), microgranular (DE-52), fibrous (DEAE-CF), and microcrystalline (DEAE-MKC), was studied and compared with that of commercial adsorbents.

EXPERIMENTAL

Materials

Cholestyramine resin, cholic acid (≥98.0), sodium deoxycholate (≥97.0), sodium glycocholate hydrate (≥97.0), sodium taurocholate hydrate (≥97.0), and 2-diethylaminoethyl chloride hydrochloride (98.0%) were purchased from Sigma. Chitosan was obtained from UAB Aconitum (Lithuania). Cellulose diacetate was received from Roshal plant (Russia), microcrystalline cellulose (MKC) – from Chemapol (Czech Republic), cotton cellulose (CF) – from Polimersintez (Russia) and cellulose-based anion-exchanger DE52 – from CDR (Whatman, Great Britain). All reagents were used as received without further purification.

Preparation of cellulose derivatives

Granocel cellulose-based gel was prepared by the regeneration of cellulose from cellulose diacetate according to patent.[18] 100 g of cellulose diacetate was dissolved in 1100 mL of acetone ammonia mixture and kept until formation of the gel. The obtained gel bulk was washed with water, cut mechanically and fractionated by sieving. The particles of 200-315 μm in size were used for the experiments.

DEAE ligands were attached to the cellulose-based matrices by the reaction of the cellulose with 2-diethylaminoethyl chloride hydrochloride (DEAECl) in the presence of sodium hydroxide. The amounts of

reagents are presented in Table 1. The suspension was stirred at 50 °C for 1 h. The obtained product was washed with water thoroughly.

Table 1
Synthesis of DEAE-Granocel, DEAE-CF and DEAE-MKC

Cellulose	Reaction mixture			
	Cellulose, g	DEAE, g	NaOH, g	H_2O, g
Granocel	10	1	0.35	12
CF	10	8	3.2	37
MKC	10	8	3.2	37

Characterisation of adsorbents

The total nitrogen content *N* was determined according to the Kjeldahl method.

Total ion exchange capacity of the adsorbent was determined by volumetric titration. 0.5 g of adsorbent (in OH^- form) was placed in 20 mL of 0.1 mol L^{-1} HCl solution (40 mL and 60 mL in the case of cholestyramine and chitosan, respectively) and left overnight. Afterwards, the dispersion was filtrated and the adsorbent was washed with 10 mL of water. The collected filtrate was titrated with 0.1 mol L^{-1} NaOH solution, in the presence of the Kjeldahl indicator. Total ion exchange capacity (TIEC) was determined:

$$TIEC = \frac{(V_1 - V_2) \cdot N_{NaOH}}{g}, \text{ mmol/g} \quad (1)$$

where: V_1 – volume of NaOH solution used for titration of the blank sample; V_2 – volume of NaOH solution used for titration of the filtrate, mL; N_{NaOH} – normality of NaOH solution; g – sample weight, g.

Potentiometric titration of cationic celluloses was carried out with 0.1 mol L^{-1} HCl in the presence of 0.1 mol L^{-1} NaCl at 25 °C in nitrogen atmosphere, using a microprocessor pH-meter and a 744 pH-meter (Methrom, Switzerland). The capacity of small ions was calculated from equivalence points. Equivalence points for each type of group were determined graphically from tangential lines drawn through inflection points. The pK_a value of amino groups was calculated by the equation of Hendelsson-Haselbach at a dissociation degree $\alpha = 0.5$. All solutions were prepared in deionised and degassed water, which was obtained through boiling and subsequent cooling under nitrogen atmosphere.

The pore size of DEAE-Granocel was determined using the inverse size exclusion chromatography.

Table 2
Characteristics of adsorbents

Adsorbent	Functional groups	Nitrogen content, %	Ion-exchange capacity calculated from nitrogen content, meq/g	Experimentally determined ion-exchange capacity, meq/g	Water retention, g/g
DEAE-Granocel	Diethylaminoethyl	1.2	0.86	0.8	5.9
DEAE-MKC	Diethylaminoethyl	0.8	0.57	0.5	4.4
DEAE-CF	Diethylaminoethyl	0.9	0.64	0.6	2.9
DE52	Diethylaminoethyl	1.3	0.93	0.7	4.2
Cholestyramine	Benzyltrimethylammonium	5.2	3.71	1.2	2.5
Chitosan	Primary amino	8.1	5.79	3.3	2.0

Water retention

The adsorbents were kept in water at room temperature overnight. Later, the samples were centrifuged at 2000 rmp for 15 min, weighed and heated for 3 h at 105 °C. After heating, they were weighed again. Water retention *Q* was calculated:

$$Q = \frac{m_w}{m_d}, \text{ g/g} \quad (2)$$

where: m_w– mass of wet adsorbent, g; m_d – mass of dry adsorbent, g.

Adsorption and desorption of bile acids

The experiments of bile acid adsorption *in vitro* were performed in the 0.025 mol L^{-1} phosphate buffer solution at different pH ranging between 4 and 6. Each bile acid was dissolved in 220 mL of the phosphate buffer and pH was adjusted to the required one by adding solutions of phosphoric acid or sodium

hydroxide. The concentration of the cholic acid solution used for the experiments was 0.1 mmol L^{-1}, while the concentration of sodium deoxycholate, sodium glycocholate and sodium taurocholate was 0.2 mmol L^{-1}. 0.1 g of the dry adsorbent was added to the solution of each bile acid and the dispersion was constantly mixed with the magnetic stirrer. The experiments were performed at room temperature. Periodically, 1 mL aliquots of the mixture were taken and the concentration of bile acids was determined quantitatively by the spectrophotometric method.[19] Absorption was measured at 389 nm using Cary 50 Scan spectrophotometer (USA), after the reaction of bile acids with sulphuric acid. Kinetic experiments were done until the equilibrium was reached. Desorption was studied in the same manner at pH 7.

RESULTS AND DISCUSSION

Characterization of adsorbents

Cationized cellulose derivatives containing diethylaminoethyl groups were evaluated as adsorbents. Their sorption capacity was compared with that of commercial adsorbents cholestyramine and chitosan. For the preparation of the adsorbents, three types of cellulosic materials of different morphology were used, namely, microcrystalline cellulose (MKC), cotton fibers (CF), and macroporous regenerated cellulose Granocel. A commercial microgranular cellulosic anion-exchanger DE52 was also studied. The porosity of Granocel is approx. 95%. The pores determined by means of the inverse gel-chromatography are accessible to proteins up to 1000 kDa, which means that the pores could be assigned to macropores, whereas MKC and CF are of microporous structure. CF is polymorphous, while MKC is of crystalline structure. The cellulose-based matrices were aminated with 2-diethylaminoethyl chloride hydrochloride (DEAECl). By means of a potentiometric titration (data not given), it was determined that aminated celluloses contained a mixture of amino groups, namely tertiary amino groups with the dissociation constant (pK_a) of about 8.5 and groups of weak basicity (pK_a approx. 6).[10,20] Some characteristics of the adsorbents are given in Table 2.

As it appears from the data presented in Table 2, the experimentally determined ion-exchange capacity of the cellulose-based adsorbents approximately corresponds to that calculated from the nitrogen content. This means that almost all amino groups are accessible to small Cl^- ions. Contrarily, just a part of amino groups of cholestyramine and chitosan participates in the ion exchange process. Supposedly, the main reason is the restricted accessibility of ionogenic groups, due to a dense and porousless structure of those adsorbents. This presumption is supported by the low water retention of those adsorbents (Table 2). The retention of water by cholestyramine and chitosan is very low.

Characterization of bile acids

The bile acids (Fig. 1) possess a hydrophobic steroid skeleton, to which various hydrophilic groups are attached, in particular, hydroxyl groups and side chain carboxylic acid or sulfonic acid groups. Cholic acid is the main component of human bile acids.[21] A part of it makes conjugates with glycine and taurine and exists in the form of so-called glycocholic and taurocholic acids. In this work, four most common bile acids or their salts, such as cholic acid, sodium deoxycholate (NaDCA), sodium glycocholate (NaGCA), and sodium taurocholate (NaTCA), were used for adsorption experiments. A chemical structure and the pK_a of the studied bile acids are given in Table 3.

Adsorption of bile acids

Adsorption of bile acids *in vitro* was performed at different pH ranging between 4 and 6, because bile acids are found in the small intestine, where acidic pH changes to neutral. In all cases, the sorption equilibrium on cellulose-based adsorbents was reached in about 30 min (Figs. 2-4), whereas on cholestyramine and chitosan, the sorption maximum was achieved only after approx. 1 hour (Figs. 5, 6).

Table 3
Chemical structure and pK_a of bile acids[22-24]

Bile acid	R^1	R^2	pK_a
Cholic	-OH	-OH	4.98
Deoxycholic (DCA)	-H	-OH	6.58
Glycocholic (GCA)	-OH	$-NHCH_2COOH$	2.35
Taurocholic (TCA)	-OH	$-NHCH_2CH_2SO_3H$	1.40

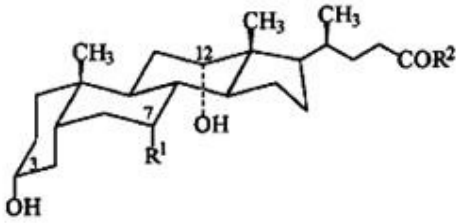

Figure 1: Chemical structure of the bile acids (R^1 and R^2 are indicated in Table 3)

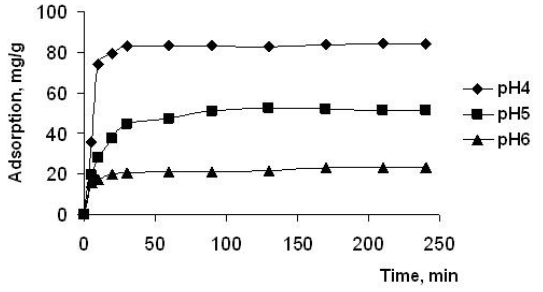

Figure 2: Kinetics of cholic acid adsorption on DEAE-Granocel at different pH

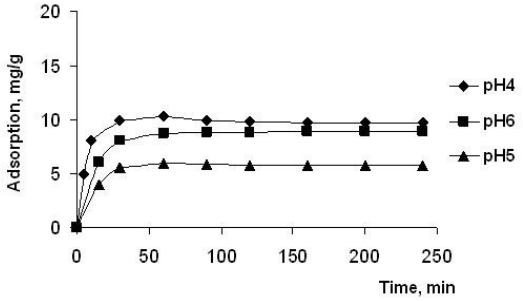

Figure 3: Kinetics of cholic acid adsorption on DE52 at different pH

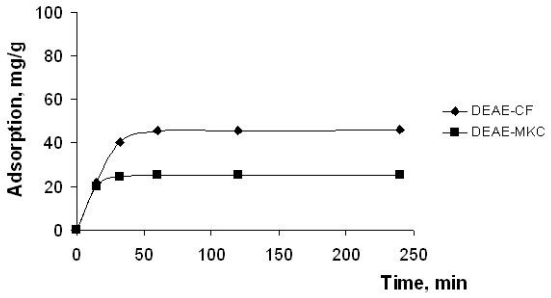

Figure 4: Kinetics of cholic acid adsorption on DEAE-CF and DEAE-MKC at pH 4

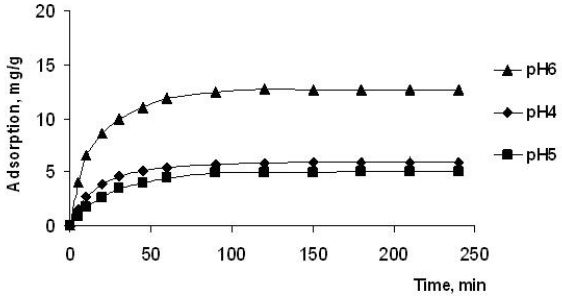

Figure 5: Kinetics of cholic acid adsorption on cholestyramine at different pH

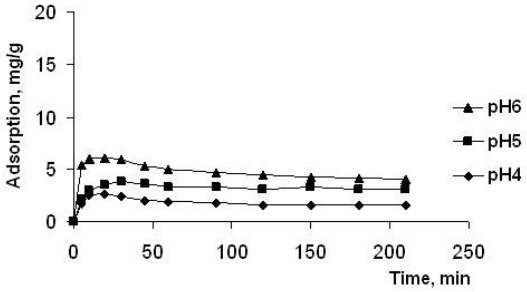

Figure 6: Kinetics of cholic acid adsorption on chitosan at different pH

Table 4
Adsorption capacity for different bile acids

Bile acid	Adsorption capacity, mg/g								
	DEAE-Granocel			Cholestyramine			Chitosan		
	pH 4	pH 5	pH 6	pH 4	pH 5	pH 6	pH 4	pH 5	pH 6
Cholic	84	55	20	5	4.5	12.5	1.8	3.5	6
NaDCA	152	65	30	10	25	36	10	18	26
NaGCA	20	40	70	6	39	11	60	42	30
NaTCA	116	70	50	5	10	23	82	41	22

It was found that the adsorption of bile acids was affected by pH. Since the alkyl chains in the amino functional group are rather short, it may be supposed that the adsorption predominantly occurs due to an electrostatic interaction between the dissociated bile acids and amino groups of the adsorbent. Thus, the differences in the adsorption results could be explained considering the dissociation constants of the bile acids, as well as of the amino groups of adsorbents. According to the pK_a of bile acids, taurocholic, as well as glycocholic acids, are dissociated in all ranges of the studied pH, whereas the acidic groups of cholic acid are fully protonized only at a pH above 5.

DEAE-celluloses contain a mixture of amino groups. Tertiary amino groups are dissociated below pH 8.5, while amino groups of weak basicity – below pH 6. Quaternized cholestyramine should be able to participate in the ion-exchange in a wide range of pH, but primary amino groups of chitosan are dissociated only below pH 6. On the other hand, it should be taken into account that the local pH in the pores of the adsorbent is higher than in the surrounding medium. This could explain a quite high sorption of cholic acid at pH 4, when its carboxylic groups are not dissociated. The accessibility of the binding sites of the adsorbents at the different pH to large ionic species may also be important in determining their affinity for bile acids. Due to all these factors, different sorption dependence of different bile acids on pH was achieved (see Table 4).

In all cases, the binding capacity of cellulose-based adsorbents was higher than that of cholestyramine or chitosan. Comparing cellulosic adsorbents, the impact of the porosity on the sorption capacity was evident. The macroporous adsorbent DEAE-Granocel showed the best sorption capabilities (Fig. 2; Table 5).

Table 5
Maximum cholic acid sorption capacity in pH range of 4-6

Adsorbent	Adsorption capacity	
	mg/g	mmol/g
DEAE-Granocel	84.0	0.206
DEAE-MKC	25.3	0.062
DEAE-CF	46.0	0.113
DE52	11.0	0.027
Cholestyramine	12.5	0.031
Chitosan	6.0	0.015

In order to evaluate the impact of electrostatic interaction on the sorption of bile acids, the experiments were done at pH 2 and 3. The sorption of cholic acid at pH 2 and 3 was much lower on all studied adsorbents except cholestyramine, whereas NaDCA was not adsorbed at all. NaGCA and NaTCA at pH 2 and 3 were adsorbed at approximately the same level as at pH 4 (data not presented).

To evaluate how strong was the interaction between bile acids and adsorbents, the desorption of bile acids in a model bowel fluid was studied. No desorption during 2 hours of keeping in the phosphate buffer, pH 7, was found. The results reveal that bile acids are adsorbed by means of electrostatic interaction.

CONCLUSION

It has been found that the cellulose-based adsorbents are more effective bile acid binders than cholestyramine or chitosan. Thus, using the cationized cellulose as an adsorbent in the remedies against cholesterol, the daily doses for patients could be sharply reduced. Comparing the efficiency of the adsorbents, the impact of the porosity on the sorption capacity was evident. The macroporous adsorbent DEAE-Granocel in all cases showed the best sorption capabilities. The electrostatic interaction was proven to be the main driving force for the bile acid binding by the cationized cellulose adsorbents with diethylaminoethyl groups.

ACKNOWLEDGEMENT: The work was supported by grant MIP117/2011 from the Research Council of Lithuania.

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