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Acta Medica Marisiensis 2015;61(4):287-290 DOI: 10.1515/amma-2015-0096

RESEARCH ARTICLE

Determination of Secondary Bile Acids in the Mice Feces. Controversies on their Involvment in the Pathogenesis of Colorectal Cancer

Saracut Claudiu[1,2], Molnar Calin[1,2*], Farczádi L[3], Vlase L[4,5], Tero-Vescan Amelia[6,1],Todoran Nicoleta[7,1], Copotoiu Constantin[1,2]

[1] University of Medicine and Pharmacy Tirgu Mures, Romania
[2] Surgical Clinic 1, Tirgu Mures Emergency County Hospital, Romania
[3] Vim Spectrum SRL, Romania,
[4] Department of Pharmaceutical Technology and Biopharmaceutics Faculty of Pharmacy, Cluj-Napoca, Romania
[5] University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania
[6] Department of Pharmaceutical Biochemistry and the chemistry of environmental factors, Tirgu Mures, Romania
[7] Department of Pharmaceutical Technology, Tirgu Mures, Romania

Objectives: The aim of the study was to determine the level of secondary bile acids (SBA) in the diets and feces of mice and the variation of amount ingested/excreted if these SBA are administered as monotherapy or in 1:1 dose. **Methods**: The mice were divided into 4 groups and fed for 140 days with different diets. The control lot received a normal diet and the others received diets supplemented with 0.25% deoxycholic acid (DCA), 0.25% lithocholic acid (LCA) and 0.125% DCA+0.125% LCA. After 140 days, the mice feces were collected and homogenized to obtain a mixture for each lot from which the determinations of the studied SBA were performed. For the mice food evaluation, portions of 10 g from each of the 4 diets were subjected to the SBA determination. **Results**: The daily ingestion over more than 4 months of DCA or LCA added to the diet and administered as monotherapy determine a significantly increase of the SBA eliminated into the feces (the DCA level was 11x higher, and of the LCA 233x higher). If half of the LCA dose is replaced with DCA, the level of LCA in the feces gets comparable with that of the DCA (their combined amounts represents only 13x higher increase of these two bile acids in feces). **Conclusions**: The simultaneous ingestion and excretion of DCA and LCA can be considered as a particular situation ruled by endogenous mechanisms. This behavior represents an important observation, knowing that the bile acids effects in the colorectal cancer are dose dependent.

Keywords: deoxycholic acid, lithocholic acid, mouse diet, LC-MS/MS

Received: 15 August 2015 / Accepted: 03 September 2015

Introduction

Bile acids (BA) synthesized from cholesterol, into the liver (via two pathways - the classical and the alternative one) are called as primary (cholic acid (CA) and chenodeoxycholic acid (CDCA)). The rate-limiting stepis catalyzed by Cyp7a1. In mice, apart from the two major BA, alpha-and beta-muricholic acids are also synthesized [1]. Before being excreted in the bile, the primary BA are conjugated with glycine or taurine in humans and in mice only with taurine, and then secreted from hepatocytes through the bile canaliculi into the gallbladder, from where they are released within the bile fluid into the duodenum.

Bile contains the *bile acids* in the form of salts. In intestine, these biliary compounds participate in lipids emulsification and absorption, activate the *pancreatic lipase*, prevent precipitation of cholesteroland fatty acids, stimulate the bile secretion and promote absorption of the fat soluble vitamins and of some ions (Ca^{2+}, Fe^{2+}, Cu^{2+}) [2]. Under the action of enzymes secreted by the intestinal microflora, the primary BA are deconjugated by a hydroxylase, 7alpha-dehydroxylated and transformed in secondary BA (lithocholic acid (LCA) and deoxycholic acid (DCA)). Bacteriaof intestinal microflora are capable toepimerizealpha-OH BA to beta-OH BAin order to decrease the toxicity of the compounds. DCA and LCA are predominantly BA in the feces - 7alpha/beta-dehydroxylations require oxidation-reduction reactions which generate energy for microflora bacteria, however the resulting compounds (DCA and LCA) have increased toxicity compared to the parent compounds [3].

Their reabsorption and returning through the portal vein into the liver process is called the enterohepatic circulation of BA. Once in the liver, the secondary BA are hydroxylated and reconjugated to the primary BA and then re-secreted.

The role of dietary factors such as the high in fats and carbohydrates and low in fibers and vegetables nutrition is well-known in the pathogenesis of colorectal cance r(CRC), but the BA involvement and the use of their rate of excretion into feces as biomarker in CRC is still a subject of controversy [4]. Thus, if it is well known the fact that the secondary BA in excess at the intestinal level increase the risk of CRC, the ursodeoxycholicacid (UDCA), asynthetic secondary BA, reduces the risk of dysplasia and CRC by decreasing the concentration of BA in the colon, reducing the oxidative stress and the *cyclooxygenase*-2 gene expression [5].

* Correspondence to: Călin Molnar
E-mail: molnar.calin@yahoo.com

The aim of our study was to determine the basal level of secondary BA (LCA and DCA) in the feces of mice and the changes occurring in their elimination by the increased food intake (hard pellets with addition of LCA or DCA), as well as the variation of amount ingested/amount excreted in the feces ratio if these BA are administered as monotherapy or in 1:1 LCA:DCA as compared to a control group.

Material and methods

Experimental procedures on animals

The experiments conducted on lab animals were approved by the Research Ethics Committee of the University (no. 43/ 2.07.2014).

Experimental animals were divided into 4 groups treated as follows:

– lot 1 (5 mice) - animals were fed 140 days with the basic diet consisting in the normal caloric balanced diet for mice, in form of hard pellets (Cantacuzino Institute, Bucharest, Romania);
– lot 2 (5 mice) - animals were fed 140 days with the basic diet to which it was added: 0.25% deoxicholic acid (r.s.);
– lot 3 (5 mice) - animals were fed 140 days with the basic diet to which it was added: 0.25% lithocholic acid (r.s.);
– lot 4 (5 mice) - animals were fed 140 days with the basic diet to which it was added: 0.25% deoxycholic acid/ lithocholic acid (1:1) (r.s.).

After 140 days, *the mice feces* were collected three consecutively days, dried to constant mass and homogenized to obtain a mixture for each lot from which the quantitative determinations of the studied BA were performed. For *the mice food* evaluation, portions of 10 g from each of the 4 administered diets were subjected to the BA quantitative determination.

Quantitative determination of BA

HPLC LC-MS/ MS method - it was developed a method by high performance liquid chromatography with MS detection in order to evaluate the amount of BA administered/lot/day from forage and of BA excreted/day/lot from feces.

Bile acids extraction from the studied samples:

– *From the dried mice food:* 50 ml water was added to the entire quantity of sample (the daily food/lot of mice) and the mixture was stirred until hydration and total disintegration of food pellets. Then, 80 ml absolute methanol was added and the obtained mixture was kept 30 minutes in ultrasonic bath and then filtered and brought to a volume of 150 ml with methanol, by washing the filter. A sample of 0.1 ml of the mixture was diluted with 0.9 ml methanol, centrifuged six minutes at 10,000 rpm and a volume of 0.15 ml of the supernatant was inserted into an autosampler vial for analysis and 1 μL was injected into the chromatographic system.
– *From the dried mouse feces:* 0.5g of mouse feces are weighed and transferred to a mortar. After 3 minutes of trituration, 5 mL of methanol were added and trituration was continued for 3 more minutes. Solution was filtered into a 25 mL volumetric flask and volumetric diluted with methanol. 1 mL of solution was centrifuged for 6 minutes (10000 rpm). 0.15 mL of supernatant was transferred to an autosampler vial and 1 μL was injected into the chromatographic system.

Results

Calibration of the liquid chromatography instrument

The analytical quantifications were performed on the food samples (the total daily intake/lot) and on the feces samples (the excreted bile acids amounts/g of dried mice feces). Due to the endogenous nature of the excreted bile acids in feces, a biological blank matrix was not possible to be used. For this reason, all concentrations of the samples (food supplemented with bile acids r.s. or feces containing endogenous excreted bile acids) were being reported to the two standard solutions of the analyzed compounds (deoxycholic acid r.s. and lithocholicacid r.s.) prepared in absolute methanol.

The typical calibration curves of the two BA prepared in methanol (n=5) are shown in figure 1 and figure 2, in both cases the coefficient of determination is higher than 0.99.

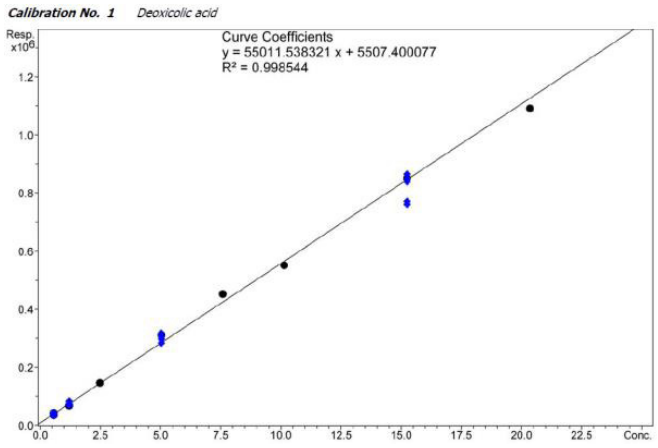

Fig. 1. A typical calibration curve of deoxycholic acid

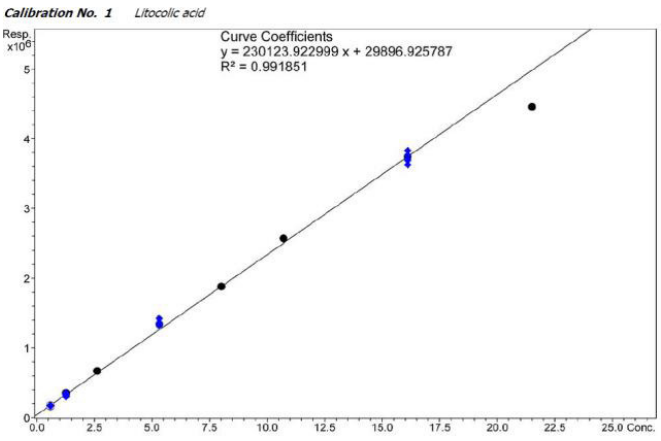

Fig. 2. A typical calibration curve of lithocholic acid

Analytical levels quantified in the studied samples

The determined levels of deoxycholic acid and lithocholic were obtained by analyzing all samples in triplicate. In figure 3 is shown an example of a food sample chromatogram and in figure 4 an example of a feces sample chromatogram.

LCA and DCA quantities calculated based on the analysisof the determined chromatogramsare presented in table I.

Discussions

The experimental determined data show the following: Compared to the control lot the elimination of DCA and LCA in feces is significantly increased if these two bile acids are additionally intake as monotherapy ($p<0.005$). In case of the lot 2 (additional intake in food of DCA) the quantity of LCA in feces (basal level) decreases below the detection limit of the method that can suggest an interference regarding LCA elimination along with large amounts of DCA, the difference being significant compared to the control group ($p <0.001$). In case of the lot 3 (additional supply of LCA) the amount of DCA excreted in feces is similar to the control group, the difference being not statistically significant.

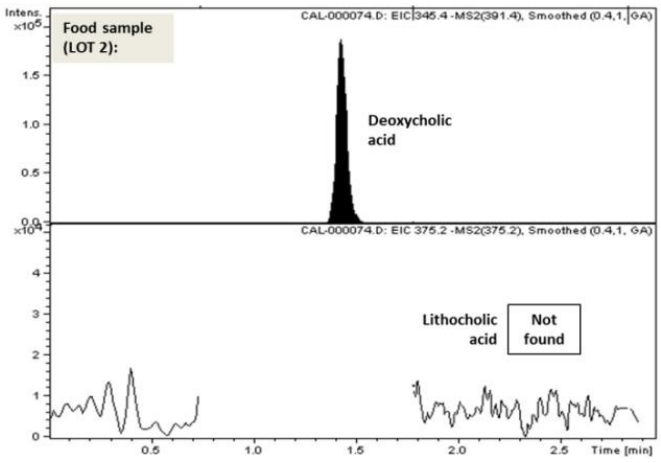

Fig. 3. Example of chromatogram quantifying the bile acids extracted from a mice food sample

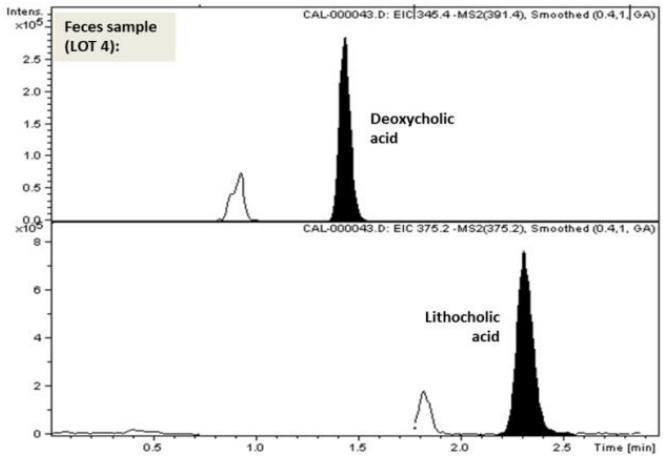

Fig. 4. Example of chromatogram quantifying the bile acids extracted from a mouse feces sample

Taking into account the bile acids biochemistry and the cellular changes that occur in CRC, enterohepatic circulation of BA is strongly changed. Physiologically, the removal of the -OH group in position 7 and the amide bond hydrolysis occur under the action of some enzymes coming from the intestinal microbial flora. The secondary bile acids are generated, from cholicacid → deoxycholicacid, and from chenodeoxycholicacid→lithocholicacid. In CRC, the conjugation capacity of BA is affected and this leads to the increase in serum levels and then to an increased elimination into feces. The study of *Dongfeng D.et al* concluded that the conjugated BA serum levels decrease and those of the free BA increase in patients with CRC compared to healthy volunteers [6].

Reports in the literature on the inducing of carcinogenesis through repeated exposure to the BA of the gastrointestinal tract following a high-fat diet are controversial by studies showing the protective role of the BA additional intake in the CRC chemoprevention. In contrast to hepatocytes, colonocytes apoptosis through the extrinsic mechanism is only a secondary pathway; BA apparently induce"cell death" by altering the oxidative function of mitochondria, by inducing the oxidative stressespecially on the cellular and the mitochondrial DNA [7]. On the other hand the studies show that the "pro-death" or "pro-survival" role of BA in CRC is dose dependent- if their massive release from bile produces an increase of CRC risk, in small doses they present a colon-protective effect [8].

Besides of the amount of lipids ingested, it appears that the type of fat ingested is also very important– the fats rich in polyunsaturated fatty acids type of omega3 (eicosapentaenoicacidanddocosahexaenoic) commonly found int he Mediterranean diet have practically no protumoral effect [9], while the percentage of saturated fats which need

Table I. Amounts of bile acids quantified as consumed and excreted

Mice lot	Diet intake		Gastrointestinal excretion	
	mg/g food ± DS (CV %)		µg/g feces ± DS (CV%)/ statistical significance	
	DCA	LCA	DCA	LCA
Lot 1 (control)	0.000..	0.000..	160.60 ± 69.61 (43.35)	17.10 ±2.94 (17.16)
			-	-
Lot 2	2.168 ± 0.186 (8.61)	0.000..	1854.20 ± 314.57 (16.96)	0.00
			*p<0.005	*p<0.001
Lot 3	0.000..	1.762 ± 0.079 (4.39)	126.20±22.31 (17.69)	4664.20 ±852.87 (18.29)
			**p>0.05	*p<0.005
Lot 4	1.158 ± 0.143 (12.36)	0.998 ± 0.072 (7.31)	1167.80±153.24 (13.12)	1250.70±335.58 (26.83)
			*p<0.005	*p<0.01

DCA= deoxicholic acid; LCA= lithocolic acid
SD= standard deviation; CV= coefficient of variation
Anova test:*statistically significant ($p<0.05$); **statistically insignificant ($p>0.05$)

increased amounts of BA for digestion and emulsification (BA increase the activity of *pancreatic lipase* and promote the absorption of triglycerides as free fatty acids and 2-monoglycerides) and vegetable oils rich in omega 6 fatty acids-linoleic acid demonstrated inductor effect in CRC [10].

A recent study shows that UDCA manifests a tumorosupressor effect in the gastric cancer cisplatin-resistant by inducing the apoptosis of the autophagic cells [11].

The meta-analysis published in 2012 by *SerfatyL* shows that the UDCA has chemopreventive effect in CRC by inhibiting the development of azoxymethaneordextran-induced tumors in experimental animals, that UDCA has the opposite effects (DCA-tumor promotion and UDCA-tumor inhibition) of deoxycholic acid on the receptors of the epidermal growth factor and the gene expression of *cyclooxygenase-2* and that 5 out of 10 retrospective studies in human patients with operated colorectal adenoma prove the beneficial effects of UDCA [12].

In the same year (2012) *Carey EJ* and *LindorKD* deny the use of UDCA in the CRC chemoprevention, considering that the studies are retrospective, carried out on a small number of participants and lack of scientific evidence [13].

Conclusions

Daily ingestion of deoxicholic or lithocholic acids added to a normal diet and administered as monotherapy in doses corresponding to at about 3 μg/kg of mouse body weight, daily ingested over a period longer than three months determine a significantly increase of the similar but endogenous secondary bile acids eliminated into the gastrointestinal tract and which are excreted into the feces (the DCA level becomes approximately 11 times higher, and of the LCA 233 times higher). If half of the LCA dose is replaced with DCA, the level of LCA in the feces gets comparable with that of the DCA (as their combined amounts represents only 13 times higher increase of these two bile acids in the feces). Therefore, the simultaneous ingestion and excretion of DCA and LCA can be considered as a particular situation ruled by endogenous mechanisms. This behavior represents an important observation, knowing (from published data) that the bile acids effects in the colorectal cancer (risk factor or protective) are dose dependent.

Acknowledgements

This paper was published under the frame of European Social Found, Human Resources Development Operational Program 2007-2013, project no. POSDRU/159/1.5/S/136893.

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