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Semi-synthetic bile acids as novel drug candidate in liver diseases: physico-chemical characterization and HPLC-ES-MS/MS methods for their quali-quantitative analysis in different experimental animal models

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ABSTRACT

The physico-chemical characterization, structure-pharmacokinetic and metabolism studies of new semi synthetic analogues of natural bile acids (BAs) drug candidates have been performed.

Recent studies discovered a role of BAs as agonists of FXR and TGR5 receptor, thus opening new therapeutic target for the treatment of liver diseases or metabolic disorders. Up to twenty new semisynthetic analogues have been synthesized and studied in order to find promising novel drugs candidates.

In order to define the BAs structure-activity relationship, their main physico-chemical properties (solubility, detergency, lipophilicity and affinity with serum albumin) have been measured with validated analytical methodologies. Their metabolism and biodistribution has been studied in “bile fistula rat”, model where each BA is acutely administered through duodenal and femoral infusion and bile collected at different time interval allowing to define the relationship between structure and intestinal absorption and hepatic uptake ,metabolism and systemic spill-over**.**

One of the studied analogues, 6α-ethyl-3α7α-dihydroxy-5β-cholanic acid, analogue of CDCA (INT 747, Obeticholic Acid (OCA)), recently under approval for the treatment of cholestatic liver diseases, requires additional studies to ensure its safety and lack of toxicity when administered to patients with a strong liver impairment.

For this purpose, CCl_4 inhalation to rat causing hepatic decompensation (cirrhosis) animal model has been developed and used to define the difference of OCA biodistribution in respect to control animals trying to define whether peripheral tissues might be also exposed as a result of toxic plasma levels of OCA, evaluating also the endogenous BAs biodistribution.

An accurate and sensitive HPLC-ES-MS/MS method is developed to identify and quantify all BAs in biological matrices (bile, plasma, urine, liver, kidney, intestinal content and tissue) for which a sample pretreatment have been optimized.

1. Introduction

1.1 Natural Bile Acids

BAs are acidic steroids, end products of cholesterol metabolism playing an important role in the homeostatic control of this molecule, representing the main pathway for its elimination from the body.

Other BAs physiological functions are:

- transport of lipids by forming mixed micelles which promotes intestinal absorption of the products of digestion;
- stimulation of bile flow by osmotic mechanism;
- complexing cations, such as Fe^{2+} and Ca^{2+}, facilitating the intestinal absorption.

Primary BAs are synthesized directly from cholesterol by the liver where they are conjugated with glycine and/or taurine and to a much less extent sulphated or glucuronide. The primary BAs are metabolized to secondary BAs by 7-dehydroxylation by intestinal bacteria.

In humans, the main BAs in bile are glyco and tauro conjugated of the two primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA). A relatively high percentage up to 30-40% are secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), deriving respectively from CA and CDCA (Figure 1) by 7α-dehydroxylation.

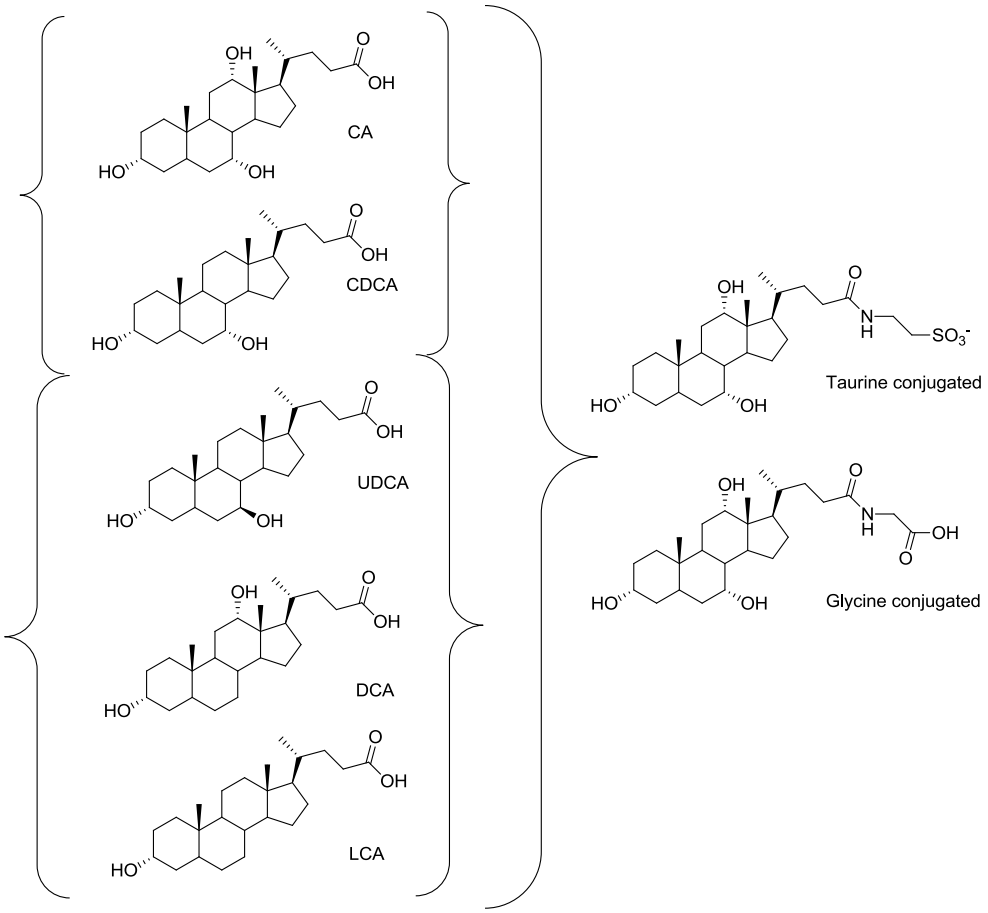


Figure 1: principal human bile acid

1.1.1 Chemical structure

The BAs chemical structure (Figure 2) consists of two parts: the steroidal ring and the side chain.

- Steroid ring: the typical chair conformation is due to the cis junction of the first two steroid rings (A and B), thus the hydrogen in C5 is in β position (β-BAs).

  In lower vertebrates, some BAs can have this junction trans [1a, 1b], so they are called 5α-BAs or Allo-BAs.

  As far as the hydroxyl positions, for natural BAs the most important are in 3α, 6α and 7α as deriving from cholesterol.

In trihydroxylated human BAs, the third hydroxyl group is in 12α position, but in other vertebrates there are other possible positions (C16 and C1).

Regarding murine species, the third hydroxyl group can be also in 6β: these BAs are called muricholic acids [2].

BAs where one hydroxyl group is in configuration β rather than in α (or α instead of β) are defined as epimers.

According the position the hydroxyl group could be differently oriented: axial or equatorial

- Side chain: determines the main BAs classes according to the length, the terminal functional group (alcohols, sulphates or carboxyl) and substituents (usually hydroxyl groups).

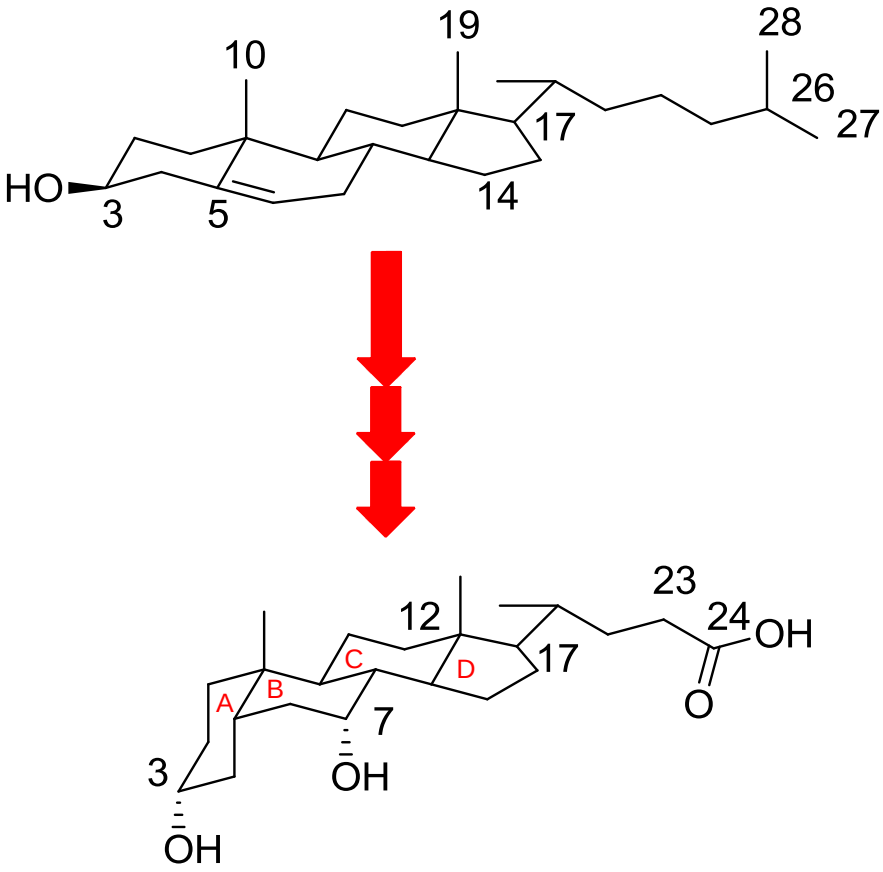


Figure 2: chemical structure of bile acids

1.1.2 Biosynthesis and metabolism

The BAs biosynthesis from cholesterol is divided into two different pathways (Figure 3): the first classic (neutral), occurs in the hepatocyte and the second alternative (acidic), initiates in the liver and macrophages.
In the classic pathway CYP7A1, cholic acid and chenodeoxycholic acid, the two primaries BA in humans, are formed.
In the alternative pathway CYP27A1, mainly chenodeoxycholic acid is formed.

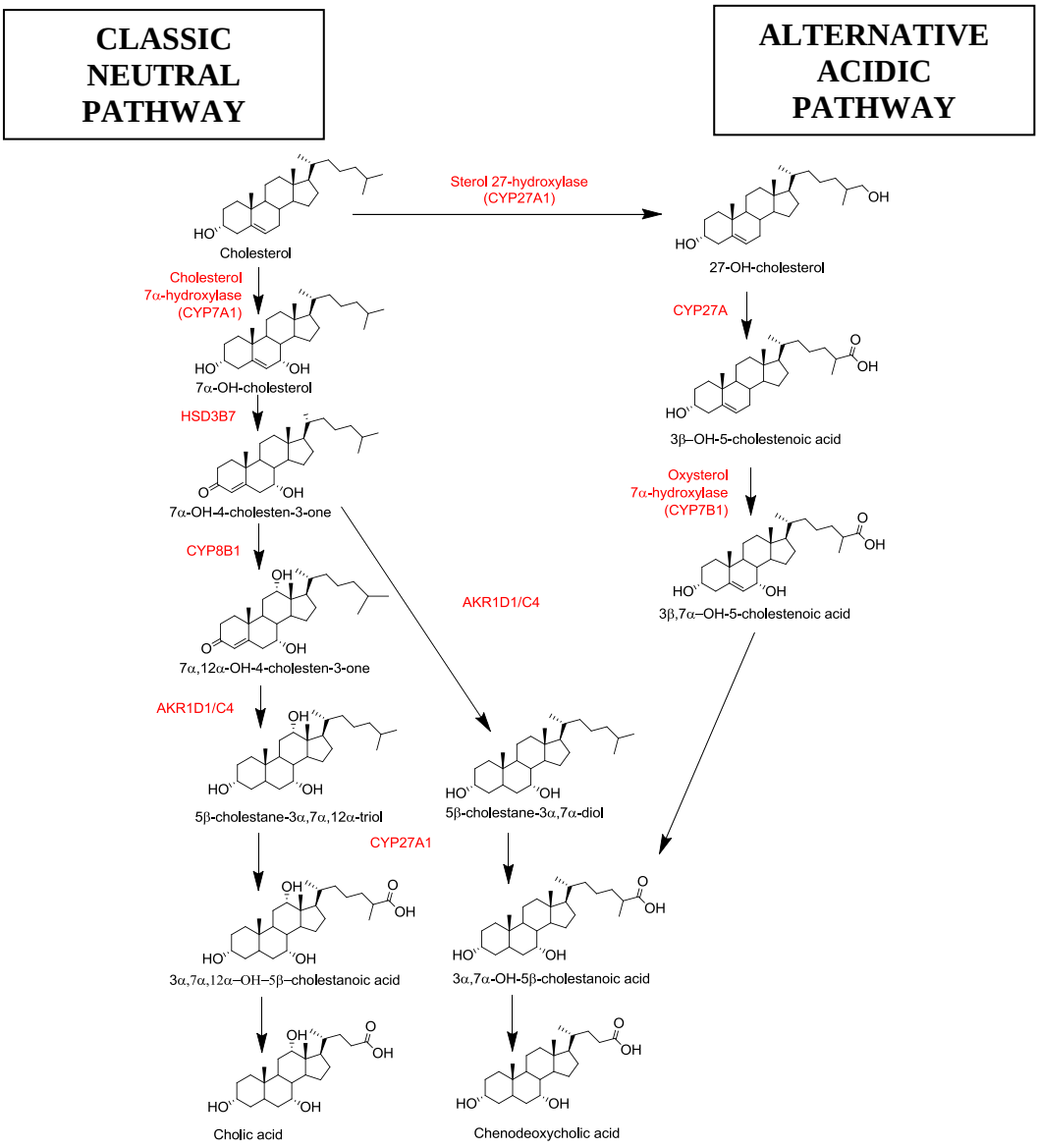


Figure 3: bile acids biosynthesis

In the liver BAs are conjugated with glycine and taurine by BACs and BAT enzymes.

In the intestine, there are regions full of bacteria (terminal ileum and caecum) where BAs are subjected to enzymatic reactions [3]:

- side-chain deconjugation of glycine and taurine conjugated BAs by choloylglycine hydrolase;
- oxidation of the hydroxyl groups in positions C3, C6, C7 and C12 to form oxo-derivatives by different bacterial dehydrogenases;
- 7α-dehydroxylation of primary BAs, CA and CDCA, to form the two main secondary BAs, in human, deoxycholic acid (DCA) and lithocholic acid (LCA). Usually, the 7α-dehydroxylase is less active for hydroxylated in 7β position [4] and has no effect on BAs conjugated with glycine or taurine [5].

1.1.3 Enterohepatic circulation

The BAs biodistribution in humans is largely confined to the liver, bile, bowel and circulatory system [6] defined as enterohepatic circulation.

In peripheral blood the BAs concentration is very low (μM level), about 1000 times lower than that in the bile. Even in the liver, in physiological conditions, their concentration is very low as a result of a low residence time due to an efficient secretion into the bile.

In blood, BAs are present mainly in the form of monomers partially bound to proteins (albumin).

Enterohepatic circulation (Figure 4) of BAs is a dynamic entity in which the "pool" of BAs (their mass) constantly recirculates, realizing its physiological functions and undergoing synthesis processes as well as transformations at both hepatic and intestinal [7]. BAs have a first-pass hepatic uptake and they are secreted exclusively with bile; then they are absorbed efficiently in the ileum with an active mechanism, if conjugated with glycine and taurine and throughout the intestine by a passive

mechanism if free. Their elimination is mainly by faeces and the bacterial metabolites of some BAs absorbed, are relatively hepatotoxic. Metabolites as glucuronide and sulphates, are not absorbed because the transport is specific for ileal BAs conjugated with glycine or taurine and thank to their high hydrophilicity are not passively absorbed.

These molecules can then perform their physiological function in the entire intestine before being absorbed in the terminal ileum.

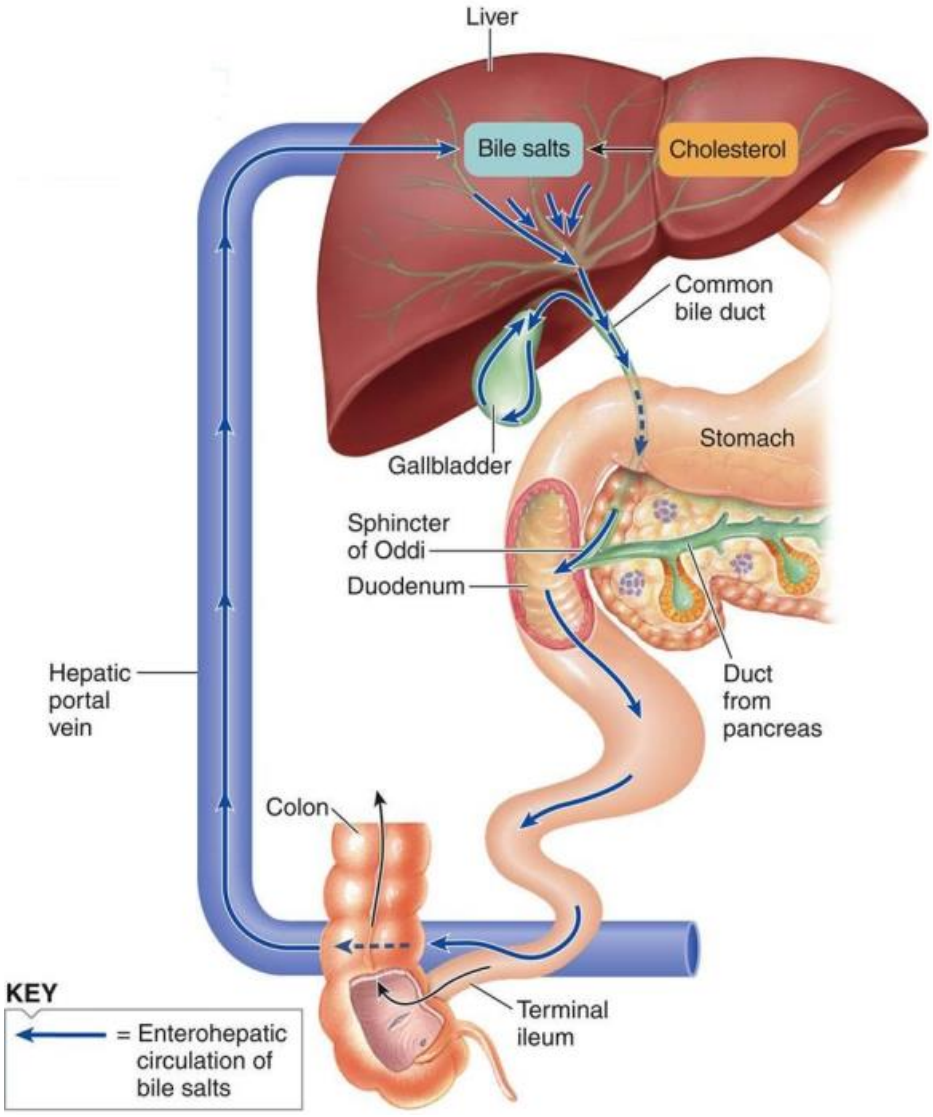


Figure 4: enterohepatic circulation

1.1.4 Receptorial activity (FXR and TGR5)

Farnesoid X receptor (FXR), shown in Figure 5, is a negative feedback nuclear regulator in the BAs hepatic synthesis from cholesterol through the repression of cholesterol 7α-hydroxylase (CYP7A1), limiting step of the entire biosynthesis, conjugation and BAs transport of [6].

The membrane receptor TGR5 coupled to protein G [9] is expressed in gut cells specialized in the production of enteroendocrin hormones, controls secretion of glucagon-like peptide 1 (GLP-1), that controls the pancreas function and the regulation of sugar levels in the blood (Figure 5).

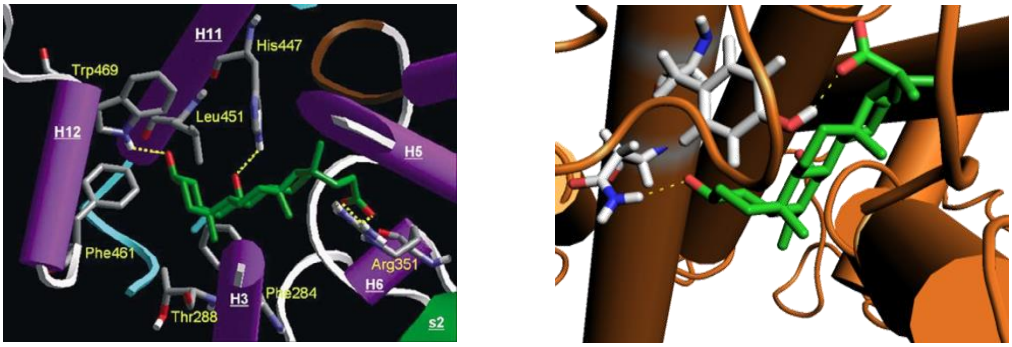


Figure 5: Docking images of receptors FXR (left) and TGR5 (right)

It has been demonstrated [10] that BAs are natural agonist for FXR or TGR5. In particular the primary BA chenodeoxycholic acid (CDCA) is the most important ligand for the first and cholic acid (CA) for the second, as shown in Table 1 [11]. Moreover lithocholic acid (LCA) is the more potent when in vitro data are generated but this secondary BA is present in low concentration representing not more than 5% of total bile acids pool.

Table 1: activity values of natural bile acid for TGR5 and FXR

BA	TGR5** agonist potency	FXR* agonist potency
	EC_{50} (μM)	
CDCA	30	13
CA	40	NA
LCA	0.58	20

**AlphaScreen FXR **FRET (cAMP)NCL-H717 cells TGR5*

1.2 Semi-synthetic bile acids

1.2.1 Receptorial activity (FXR and TGR5) and therapeutic perspective

In recent few years [12,13], a series of CDCA and CA semi synthetic analogues have been synthesized, in order to produce tools for the discovery new lead candidate more potent and specific agonists for FXR or TGR5.

New therapeutic targets for the treatment of cholestatic liver diseases like primary biliary cirrhosis (PBC), by acting on FXR, or diabetes, atherosclerosis and metabolic syndrome, by acting on TGR5 are highly demanded [14].

The two receptors differ in some amino residues, therefore it is possible to develop different BAs semi-synthetic derivatives with different selectivity for FXR or TGR5.

In addition recent studies have shown that some of these new synthetic BAs are specific ligands for both receptors, opening new therapeutic opportunities for the contemporary treatment of cholestatic liver diseases and metabolic disorders as obesity, diabetes, dyslipidemia and metabolic syndrome [15].

1.2.2 Structure-activity relationship

It has been demonstrated [16, 17, 18], that small modifications in BAs structure modify their biological activity. For this reason it is necessary to accurately evaluate the relationship between structure and activity in particular the interaction with these receptors.

About FXR, it has been showed [12] the existence of a small hydrophobic pocket, consisting on Phe284, Thr288, Leu451 and Phe461 residues (above in Figure 5), in which bile acids with alkyl group in 6α position could be hosted more easily.

Moreover, glyco and tauro conjugates of this analogue could activate FXR too because of the largest pocket near the carboxylic group that could easily guest the glycine and taurine [19, 20].

About TGR5, the analysis of structure–activity relationship indicates some positions that are important for potency and selectivity for this receptor [21]:

- Side chain length (if it is shorter reduces their potency on TGR);
- substituent in C23-position of the side chain (i.e. (S)-methyl group increases potency and provides higher selectivity for TGR5 against FXR);
- Bioisosters, substituents or groups with physical-chemical and biological properties similar to carboxyl group, approach used in drug design to reduce toxicity or alter the metabolism of the reference compounds;
- Number of hydroxyl groups (TGR5 shows a binding pocket with three hydrogen bond acceptors to anchor three hydroxyl groups of BA).

Considering these informations, some examples of new BAs analogues with small structural modifications are reported in Table 2 and their respective receptorial activities are reported in Table 3.

Table 2: different structural modification of bile acid analogues

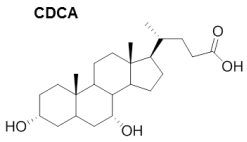
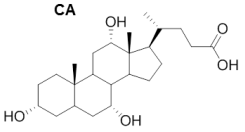
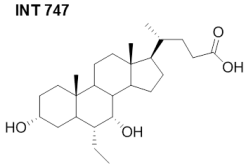
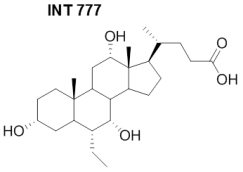
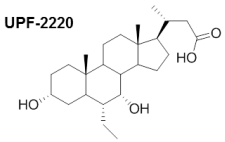
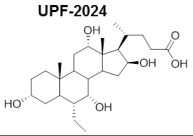
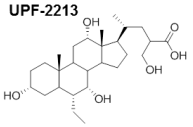
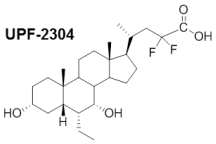
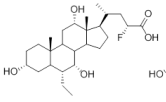
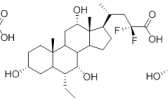
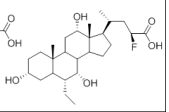
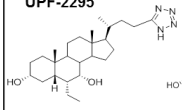
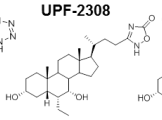
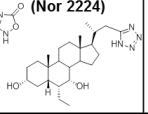
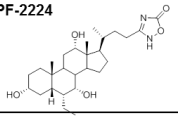
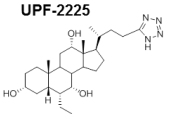
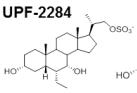
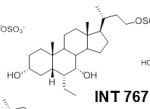
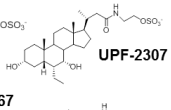
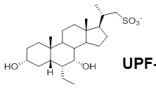
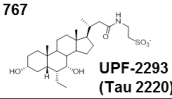
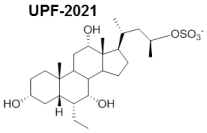
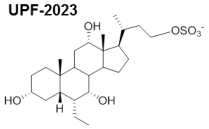
ENDOGENOUS	CDCA 	CA 
6α-ETHYL	INT 747 	INT 777 
CARBOXYLIC	UPF-2220 	UPF-2024  UPF-2213 
FLUORIDE	UPF-2304 	UPF-2227  UPF-2207  UPF-2226 
BIOISOSTERS	UPF-2295  UPF-2308  UPF-2289 (Nor 2224) 	UPF-2224  UPF-2225 
SULFATE-SULPHONATE	UPF-2284   UPF-2307  INT 767  UPF-2283  UPF-2293 (Tau 2220)	UPF-2021  UPF-2023 

Table 3: activity values of synthetic bile acid for TGR5 and FXR

BA	TGR5**agonist potency	FXR* agonist potency
	EC_{50} (μM)	
6α-ETHYL		
INT 747	15	0.15
INT 777	0.9	175
CARBOXYLIC		
UPF 2024	0.68	68
UPF 2220	0.8	0.5
UPF 2213	7.5	80
FLUORIDE		
UPF 2304	0.34	0.2
UPF 2227	22	2.9
UPF 2207	1.5	4.3
UPF 2226	7	5.6
BIOISOSTERES		
UPF 2295	0.72	0.15
UPF 2308	0.1	0.45
UPF 2289	0.48	0.075
UPF 2224	2	1
UPF 2225	4	1.7
SULPHATE-SULPHONATE		
INT 767	0.4	0.033
UPF 2284	0.76	0.24
UPF 2307	0.016	1.5
UPF 2293	0.2	1.3
UPF 2283	0.93	0.49
UPF 2021	1.1	3
UPF 2023	1.6	0.65

**AlphaScreen FXR **FRET (cAMP)NCL-H717 cells TGR5*

1.2.3 Obeticholic acid as potential novel drug

The 6α-ethyl chenodeoxycholic acid (Figure 6), later became Obeticholic acid (OCA), is the most potent FXR agonist [12].

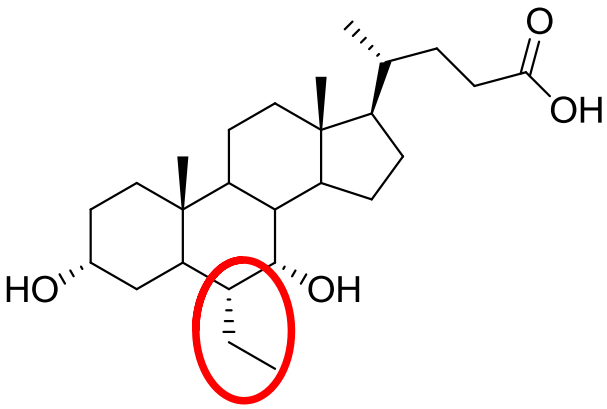


Figure 6: chemical structure of Obeticholic acid (OCA)

As previously reported, the presence of ethyl in 6α position allows to increase the activity in FXR dependent processes as therapeutic targets for human disease.

It is the first semi-synthetic BA to be undergoing in human studies for specific liver and gastrointestinal disorders [22].

Moreover, it has been demonstrated [23, 24] its efficacy in primary biliary cirrhosis (PBC) treatment (ClinicalTrial.gov: http://www.clinicaltrials.gov NCT01473524), type 2 diabetes with non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH) (ClinicalTrial.gov: http://www.clinicaltrials.gov NCT01265498).

1.3 Physico-chemical properties of natural and semi-synthetic BAs

It is well know that most of the BAs biological properties can be explained and also predicted knowing their physico-chemical properties [25].
BAs are present in bile and intestinal content at mM level, so they can self-aggregate to form micelles depending on their physical-chemical properties, as for common anionic detergents. Their structure and the number and position of hydroxyl groups, the length and substituent in the side chain play a major role in their amphipaticity and in the tendency to form aggregates at a given critical micellar concentration.
The main physico-chemical properties in aqueous solution, including solubility, detergency, lipophilicity, and binding affinity with albumin) have been evaluated [25] on BAs as a protonated non-ionized species (bile acids) and ionized species (bile salts).

1.3.1 pK_a

The BAs pK_a plays the major role in determining the presence of protonated form in respect to the ionized one at a given pH (*in vivo* changes within the gastrointestinal tract)
It has been demonstrated [26] that the pK_a values for all common carboxylate bile acids is 5.00, as isopentanoic acid and this means that steroid ring substituents do not influence ionization.
Therefore, at physiological pH > 5.00 (in hepatic bile pH is about 8.00), they are present in ionized form, i.e. as bile salts.
The glycine and taurine conjugations lead to a lower of pK_a of about 2.4 and 5 units respectively, due to inductive effect of the amide bond on the carbonyl group.
Taurine conjugated BAs have a pK_a value about 0, because of the extremely acidic nature of the sulfonic acid group, and for this reason taurine conjugated BAs are fully ionized at all physiological pH. The

glycine conjugated with a pK_a=3.6 are fully ionized only if the pH in 2 units higher than their pK_a and vice versa at lower pH.

1.3.2 Lipophilicity

The lipophilicity is the BAs ability to distribute in a lipid domain (as lipidic cell membranes) and it is experimentally measured with the 1-octanol/water partition coefficient, $LogP_{o/w}$, that strongly depends on solution pH and molecule pK_a.

$LogP_{o/w} = LogD_{o/w} + Log\ (1+10^{pH-pKa})$,

where distribution coefficient D is ratio between the total concentrations (protonated and ionized form) in the two phases and partition coefficient P is referred to a single chemical species.

For in vitro experiments, 1-octanol is officially used as non polar solvent because of its similarity to cell membrane lipid layer and aqueous solution at physiological pH (about 7.20) is used to consider all BAs fully ionized.

BAs that display some preference for the nonpolar phase (LogP > 1, as unconjugated BAs), are defined lipophilic or hydrophobic, while BAs are hydrophilic or lipophobic when LogP < 0 (as taurine conjugated).

1.3.2 Water solubility

BAs aqueous solubility is measured for the protonated non-ionized species (almost insoluble). When fully ionized BAs are usually much more soluble, the pK_a and the solution pH play a major role in the monomer water solubility.

BAs solubility is influenced by [26]:

- steroidal ring substituents (number and orientation): high number or β-orientation of hydroxyl group increase solubility;
- side chain structure (length and substituent): shorter side chain or presence of sulphated or hydroxyl groups increase solubility.

In vitro experiments are performing by solubility measurements at pH=1.00 four units below the pK_a of the common carboxylate BA (about 5.00) and

even when amidated with glycine (pK_a= 3.9) order to consider only protonated species. For the taurine conjugate the protonated form cannot be produced having a very low pKa and therefore their solubility is that of the ionized form which on turn is not affected by the pK_a.
The BAs solubility and its variation with the pH are very important to define if the drug is present in solution in a given biological fluid and therefore suitable to be absorbed. In addition it is very important to evaluate the solubility at stomach pH (acid) when orally administration.

1.3.3 Detergency

BAs are amphipathic molecule with the steroid ring divided in two regions: the hydrophilic α face containing the 3α, 7α, 12α oriented hydroxyl groups and the hydrophobic β face (i.e. Cholic acid, Figure 6a) that lead to self-association to form micelles via back to back interaction leaving the hydroxyl groups oriented toward the solution. Additional interactions with other dimers via hydrogen bound of the hydroxyl allow to increase the aggregation number step-wise.
The hydrophobic/hydrophilic ratio determines the CMC and the micelles dimensions. The side chain play a minor role in the aggregates but usually the increased length reduces the CMC value.
In respect to typical anionic detergents, BAs self-association occurs in a larger concentration range and it is more stepwise passing through dimers (i.e. Cholic acid, Figure 6b), tetramers and so on [27, 28].
The concentration at which BAs begin to self-associate is called Critical Micellar Concentration (CMC).
The methods used to calculate this parameter [29] consist of:
-dynamic measurements of surface tension using maximum bubble pressure principle;
-dye solubilization in BA solution and absorbance measures at the dye absorption wavelength.

The data previously obtained shows that the key factor influencing self-association is the contiguous hydrophobic area (β face): any decrease in lipophilicity of this area increase the CMC values.

As far as the side chain, a reduction in length decreases the CMC. Regarding the conjugation with glycine or taurine, this lead to small reduction of CMC values in respect to free BA: the polarity of the amide bond is compensated by the increased length of the side chain.

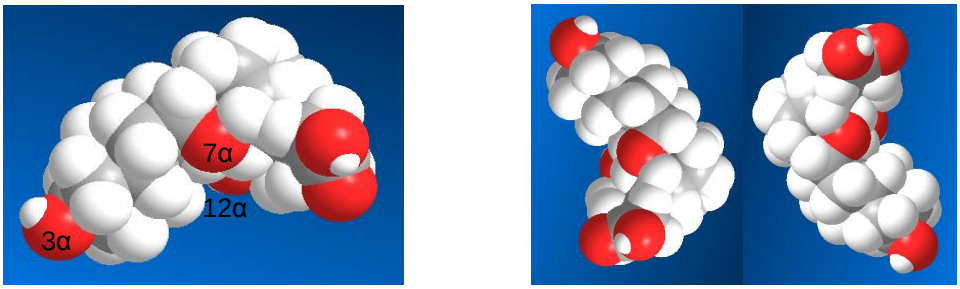
Natural BAs possess relatively low CMC values (< 10 mM) because of the hydroxyl groups in the same surface (α orientation) and their negative charged side chain that facilitate the back-to-back micelles formation (i.e. Cholic acid, Figure 6b).

For this reason, anionic form solubility (pH>7.00) increases because of the micelles formation and the consequent greater solubilization of the anionic forms inside the micelles.

Indeed, another parameter also important is critical micellar pH (CMpH), that is the pH above which a solid BA dissolves in a micellar phase and depends directly on the pKa, the CMC and the solubility of the protonated form (pH=1.00).

The CMpH value has a direct impact with intestinal and stomach processes: when a BA orally administrated passes from stomach (pH<3) to intestine, the absorption is promoted by a pH ≥ CMpH.

For BAs administered in concentration higher than their CMC, this can be more easily verified after meal, when intestine pH increases.



(a) **(b)**

Figure 6: Cholic acid monomer (a) and dimer with back to back self-association (b)

1.3.4 Affinity binding to serum albumin

The affinity binding to serum albumin is an important parameter that plays a role in the systemic transport and also the bioavailability of BAs as drugs because it determines the percentage of BAs present in blood in a free unbound form, potentially more pharmacologically active.

On the other hand, it has been previously demonstrated [30, 31, 32] that there is a receptor for albumin on the hepatocytes that could be responsible for the more efficient hepatic extraction of BAs when more bound to the albumin.

The interaction of human serum albumin with BAs has been measured by equilibrium dialysis technique [31] and the affinity constant depends on: number of hydroxyl groups (decrease if they are less) and substitution of 7α-hydroxyl group with 7-keto group.

Furthermore, the unconjugated bile acids have binding constants higher than the conjugated forms [33, 34].

1.4 Bile fistula rat model

The BAs structure-activity relationship as drugs need to be verified using suitable experimental animal models able to determine their pharmacokinetic and metabolism and predictive to human situation.

For this reason, hepatic metabolism and biliary secretion have been evaluated using the animal model "*bile fistula rat*", where the bile duct is cannulated in order to collect bile at different time interval during the acute administration by one hour infusion either intravenously and orally (id by gavage).

During this study, plasma is also collected in order to evaluate the hepatic uptake and the systemic spillover. When possible at the end of the experiment the main target organs (liver, kidney, intestine tissue and content and stools) will be collected.

This model consist on BA administration with intravenous and intraduodenal (by gavage) infusion (Figure 7), preferred over the single bolus because in the anesthetized animal, the intestinal motility is hampered so the infusion allows the progression of the substance.

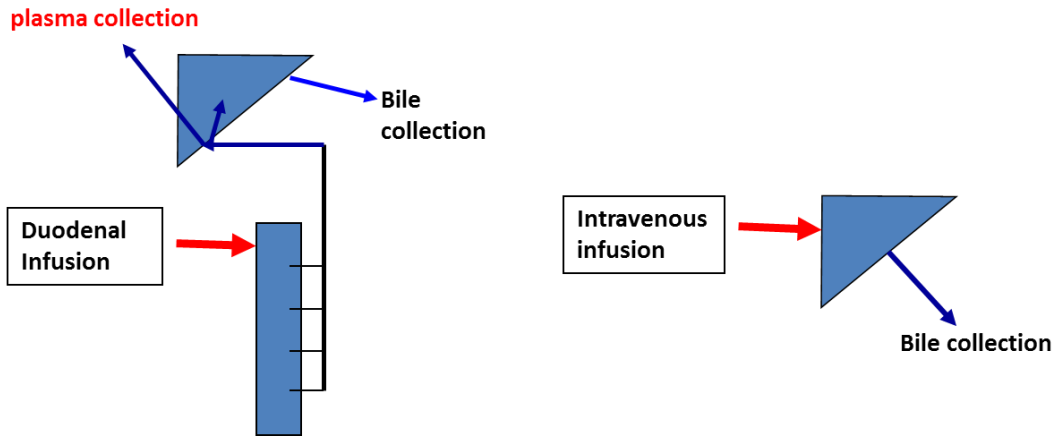


Figure 7: intraduodenal and intravenous infusion in bile fistula rat model

1.5 OCA in cirrhosis treatment: biodistribution and metabolism *in vivo*

In previous studies carried out in bile fistula rat model [35] it has been demonstrated that the pharmacokinetic and metabolism of OCA is quite similar to CDCA with the only exception that OCA is not 7-dehydroxylated by intestinal bacteria, but it is still conjugated with glycine and taurine in the liver.

The overall physico-chemical and biological properties of OCA are comparable to the natural analogues in rat and therefore it is expected the same for humans.

Recent clinical studies demonstrated the efficacy of OCA in the treatment of primary biliary cirrhosis (PBC), and high plasma levels of OCA and its glycine and taurine conjugate forms have been found in these patients. During cholestasis, OCA will increase in blood exactly like endogenous BAs as a result of the different compartmentalization in the enterohepatic circulation, caused by an impaired hepatic uptake or biliary secretion (cholestasis).

OCA is not subjected to 7-dehydroxylation by enzyme 7α-dehydroxylase in intestinal bacteria because of the steric hindrance of 6 ethyl group. For this reason, OCA do not produce mono-hydroxylated BAs (such as lithocholic acid from CDCA) that are potentially toxic at high concentration; on the other hand the formation of mono-hydroxyl BAs (secondary BAs) is an excretory mechanism since they are the main constituents of BA stools.

An appropriate balance between BA excretion and the feedback mechanism of their synthesis from cholesterol keeps the equilibrium in the overall enterohepatic circulation which drives the biodistribution of the endogenous BA and OCA, not only in bile but in almost all target organs including liver, the overall intestinal tract, blood, urine and stools.

Any alteration in the physiology of the involved organs (due to a pathology, like cirrhosis) determines a different biodistribution among them, resulting in an increased concentration or accumulation in a given fluid, like blood in the specific case of liver impairment, because of a poor liver uptake.

For this reason it is necessary to achieve more accurate informations, predictive for human, regarding OCA biodistribution and metabolism in all the involved organs particularly in liver disease.

2. Aim and rationale

Despite numerous studies on physico-chemical properties, metabolism, physiology and structure-activity relationship of endogenous BAs, many aspects have not yet been fully elucidated.

This limitation is probably due to the complexity of the BA metabolism which is different in human and animal species.

Indeed the high variability of BAs composition in different organs and the several metabolites produced in low concentrations could lead to analysis procedures very difficult and time consuming an often poor accurate and precise.

Moreover, the recent discovery of BAs role as agonists of receptors FXR and TGR5 opens new therapeutic target for the treatment of cholestatic liver diseases, as primary biliary cirrhosis (PBC) and Nonalcoholic steatohepatitis (NASH), or metabolic disorders, as obesity, diabetes, dyslipidemia and metabolic syndrome.

In this context, the collaboration with Prof. Roberto Pellicciari's laboratory, (University of Perugia), Intercept Pharmaceuticals and Servier, has been set-up properly design new candidate BAs analogues for the discover of promising novel drugs.

This research activity joined with this project by focusing on physico-chemical characterization and metabolic studies of new synthetic analogues of BAs with structural changes from cholic and chenodeoxycholic acid natural analogues.

The rationale of this project is to design compounds that are active on the target receptors and in part more metabolically stable, such as resistant to intestinal bacterial 7-dehydroxylation responsible for the formation of potentially toxic metabolites. Moreover it is important to maintain an efficient hepatic uptake and intestinal absorption.

In the last few years, up to twenty new synthetic analogues have therefore been synthesized and studied.

They can be divided into two main groups: unconjugated and steroid modified BAs and BAs conjugated with taurine or glycine or pseudo conjugates (sulphonate and sulphate analogue).

The first group includes derivatives with modifies from CDCA or CA by introducing an ethyl group in 6α position and a substituent in 23 position (methyl, methoxyl, fluorine).

These derivatives are selected according with their potency toward on FXR rather than TGR5 and on the mean time the increase of metabolic stability toward 7α-dehydroxylation and side chain conjugations.

The second group consists on modifies in the terminal functional group of synthetic analogues side chain (sulphonate, sulphate or taurine conjugated), in order to have an efficient secretion of bile and high metabolic stability.

The new BA analogues studied are: UPF 2024, UPF 2220, UPF 2213, UPF 2207, UPF 2226, UPF 2227, UPF 2304, UPF 2224, UPF 2225, UPF 2295, UPF 2289, UPF 2308, UPF 2021, UPF 2023, UPF 2284, UPF 2283, UPF 2293, UPF2307.

The results were compared with those obtained in previous years using the same experimental protocol in the laboratory of Professor Roda for endogenous BAs (CDCA, CA, TCDCA) and analogues INT-747, INT-767, INT-777.

In order to achieve more accurate informations, predictive for human, regarding its biodistribution and metabolism, it would be necessary to develop an animal model causing cirrhosis with hepatic decompensation.

Indeed, it should be evaluated the biodistribution and mass balance of OCA and its metabolites (taurine and glycine conjugates) in CCl_4-induced decompensated cirrhotic rats after a single oral dose of 30 mg/kg, quantifying them in the biological fluids and organs.

Aim of the study is to evaluate whether in decompensated liver disease, peripheral tissues (liver, kidney and intestine) might be also exposed as a result of toxic plasma levels of OCA due to porto-systemic shunting, liver metabolism impaired and cholestasis.

In addition, endogenous BA quali-quantitative composition in the studied biological fluids and organs has been quantified in order to ascertain the extent of biodistribution of these endogenous BA as a consequence of liver failure and to compare data with OCA and its metabolites in cirrhotic and control animals.

3. Materials and methods

3.1 Materials and reagents

All solvents are purchased from LiChrosolv® for HPLC, Merck (Darmstadt, Germania) with high purity analytical grade. Silica C18 cartridge for SPE (Isolute ®, 53 Å, 6mL) are purchased from StepBio S.r.l. (Bologna, Italy).
Each semi-synthetic BA powder is synthesized and purified (purity>99 %) from group of Prof. R. Pellicciari (Institute of Pharmaceutical Chemistry, University of Perugia, Italy).

3.2 HPLC-ESI-MS/MS method

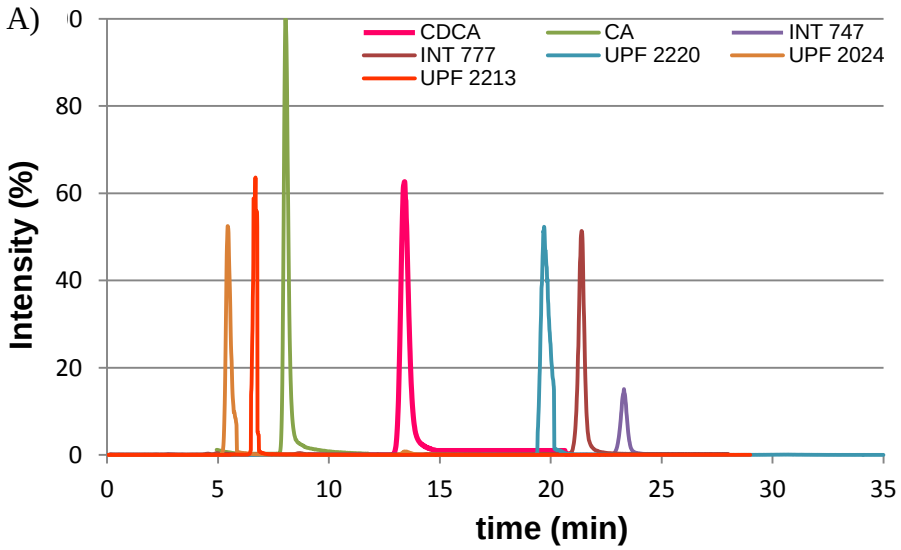
3.2.1 HPLC optimization parameters

All samples are analyzed by liquid chromatography using Waters Alliance 2695 system. The column used for BAs quantitative analysis is XSelect Phenyl-Hexyl CSH Column 5µm, 150x2.1 mm (protected with SecurityGuard Phenyl-Hexyl 2.1x10mm i.d. guard column) from Waters S.p.a. (Milano, Italy).
Elution is performed at flow rate of 0.15 mL/min, sample injection volume is 5 µL and the column is maintained at 45°C.
The elution gradient is optimized (see Table 4) with mobile phase using 15mM ammonium acetate buffer (pH=8.00) as aqueous phase and acetonitrile:methanol, 75:25, v/v as organic phase.

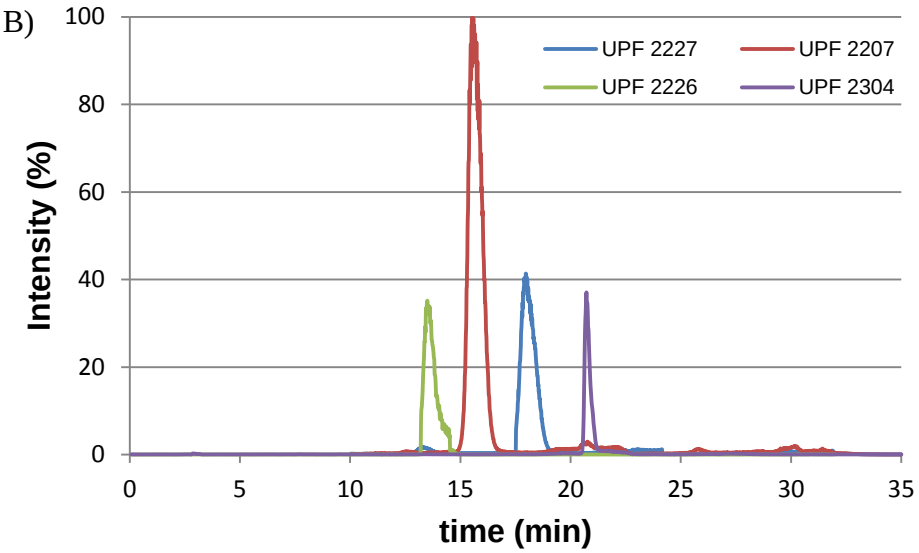
Table 4: Elution gradient

Time (min)	A%	B%	Flow (mL/min)	Curve
0.00	65	35	0.150	1
10.00	65	35	0.150	1
10.30	55	45	0.150	6
21.00	55	45	0.150	1
21.30	0	100	0.150	6
23.30	0	100	0.150	1
24.00	65	35	0.150	6
35.00	65	35	0.150	1

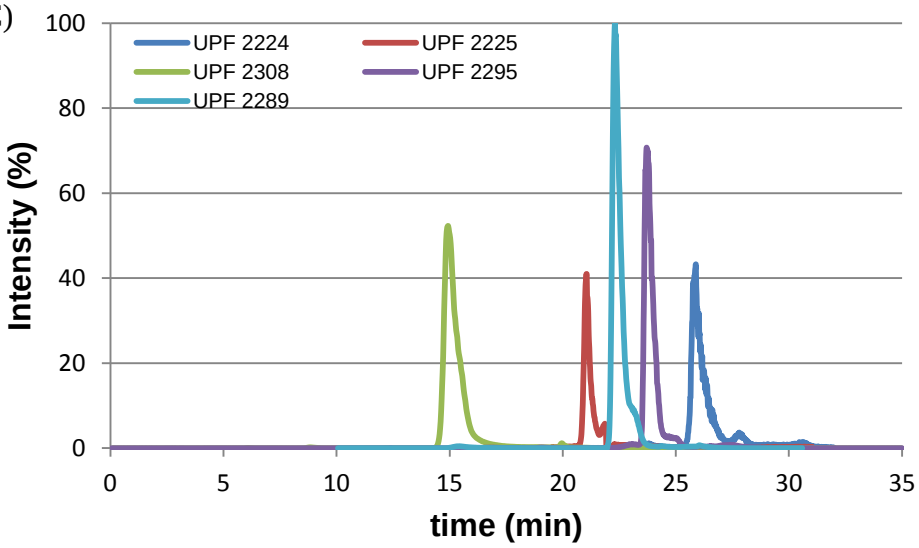
This gradient allows to separate all the BAs studied as shown in Figure 8.



B)


100
80
60
40
20
0
Intensity (%)
0
5
10
15
20
25
30
35
time (min)
UPF 2227
UPF 2207
UPF 2226
UPF 2304

C)


100
80
60
40
20
0
Intensity (%)
0
5
10
15
20
25
30
35
time (min)
UPF 2224
UPF 2225
UPF 2308
UPF 2295
UPF 2289

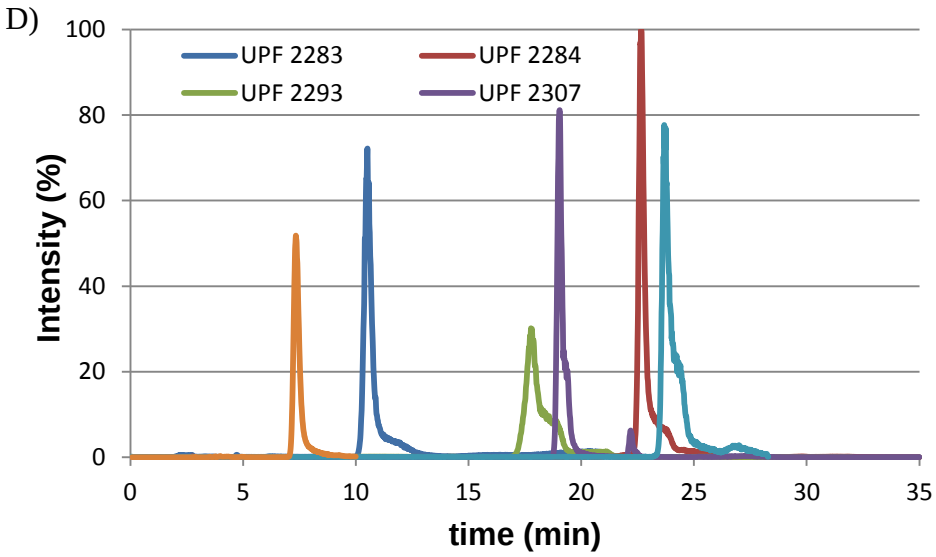


Figure 8: chromatograms of the BAs analyzed divided into carboxylic (A), fluorine substituted (B), bioisosters (C) and sulphates/sulphonates (D)

The HPLC is coupled with electrospray source (ES) – triple quadrupole Mass Spectrometer with the following parameters.

3.2.2 ES- Mass spectrometry optimization parameters

The HPLC system was connected with Quattro-LC 9007 (MicroMass): triple quadrupole analyzer operating with an electrospray (ES) ionization source in negative mode. The following parameters (Table 5) are performed and optimized by direct infusion of each BA, using both single MS and tandem MS/MS configuration.

Table 5: Tuning parameters

Instrument parameters	Value
Capillary	3. 0 KV
Cone	60 V
Extractor	1 V
RF lens	0.50 V
Source block temp.	120°C
Desolvation temp.	180°C
MS	
Entrance	1.0 V
Exit	10 V
Ion energy	1.5 V
LM resolution	12.0
HM resolution	12.0
MS2	
Ion energy	3.0 V
LM resolution	12.0
HM resolution	12.0
Pressure	
Analyzer vacuum	3.4e-5 mBar
Gas cell	2.6e-3 mBar
Flows	
Nebulizer (Nitrogen)	87 l/h
Desolvation gas (Nitrogen)	800 l/h
Multiplier	650 V

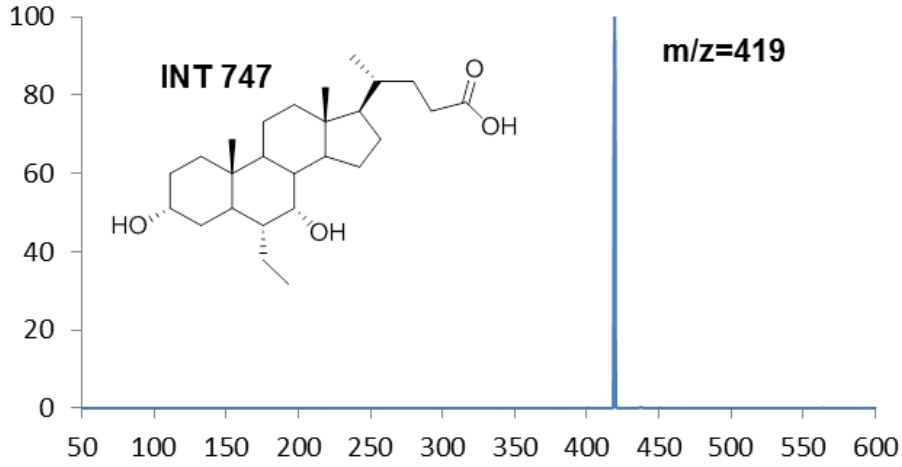
The acquisition mode used in this method is multiple reaction monitoring (MRM) mode. It allow to achieve more sensitivity and selectivity, in particular with isomers (BAs that have the same molecular weight but different specific fragmentation pattern)

For the qualitative analysis (identification of new entities) the acquisition mode single ion monitoring (SIM) is also used.

MassLynx software version 4.0 is used for data acquisition and processing.

Mass spectra of several semi-synthetic BAs standards (one of each principal group), obtained with tandem MS acquisition, are reported below (Figure 9).

A)



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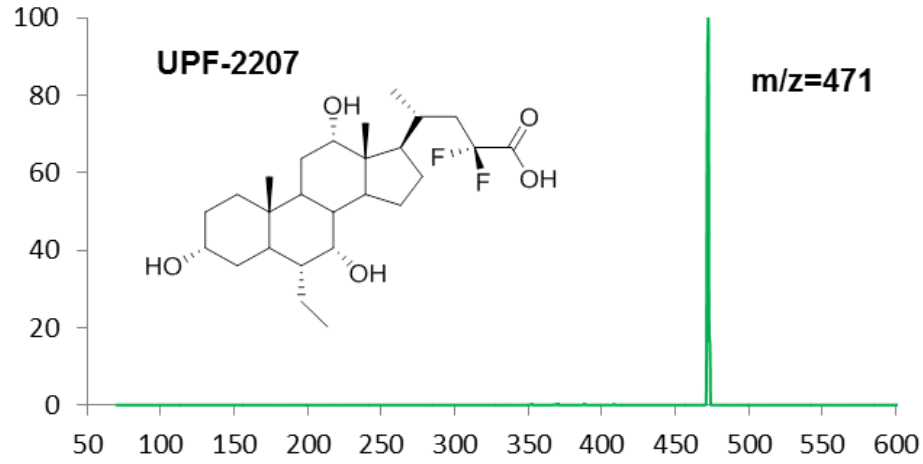
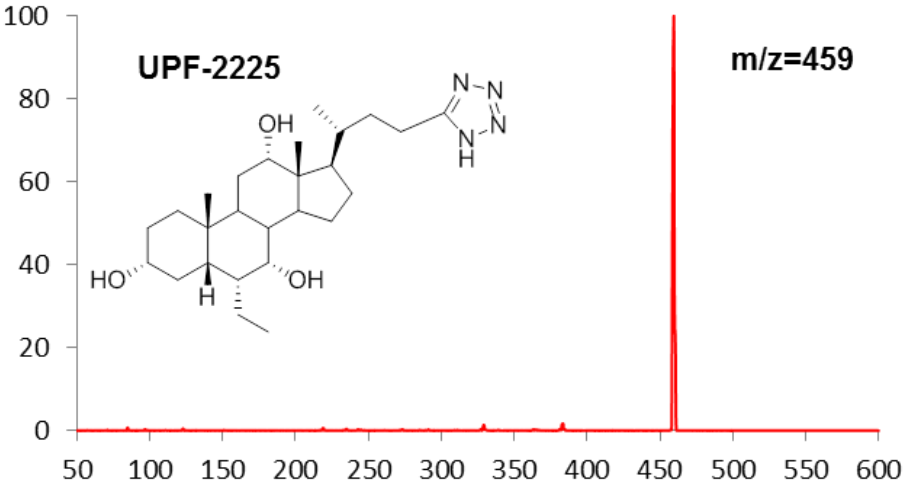
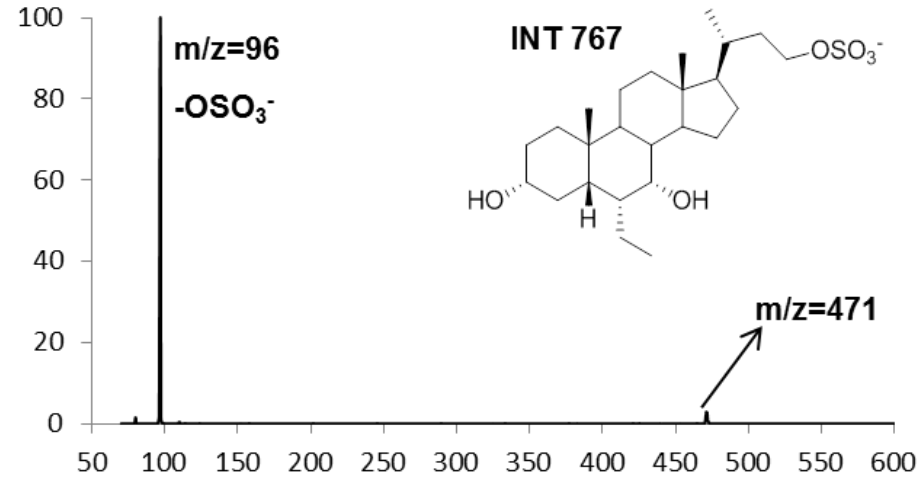


C)


UPF-2225
m/z=459
OH
HO
OH
H
N
N
N
N
H
100
80
60
40
20
0
50
100
150
200
250
300
350
400
450
500
550
600

D)


m/z=96
-OSO3-
INT 767
OSO3-
HO
OH
H
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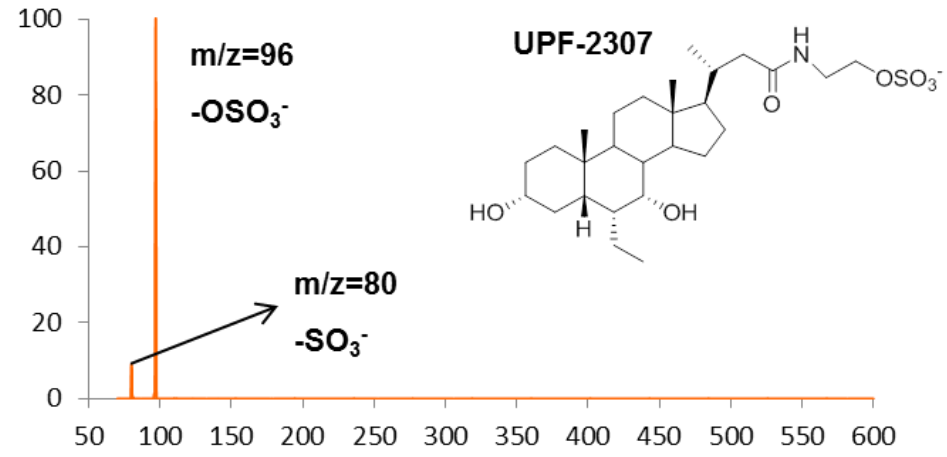
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UPF-2307
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N
O
OSO3-
HO
OH
H
m/z=80
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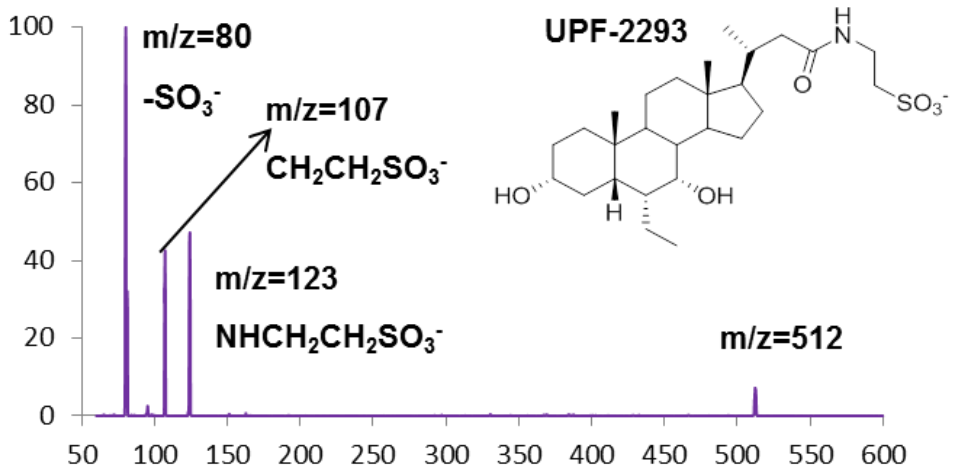


Figure 9: mass spectra of the BAs analyzed divided into carboxylic (A), fluorine substituted (B), bioisosters (C) and sulphates/sulphonates (D)

3.2.3 Method validation

The limit of detection (LOD) of the different BAs (for each biological matrix) is calculated as three times signal-to-noise ratio and the limit of quantification (LOQ) as three times LOD.

The LOQ values obtained for each BA for each matrix are between 0.005 and 0.01µM and the LOD values were between 0.001 and 0.02 µM.

In addition, intra-day and inter-day precision and accuracy are calculated for each BA by analyzing them in three different quality controls (QCs) corresponding low (0.3 µmol/l), medium (8.0 µmol/l) and high (17.5 µmol/l) representative concentrations.

The internal standard concentration (1 µmol/l for OCA, G-OCA, T-OCA and 1µmol/l for INT-777, T-INT-777 and G-INT-777 when analyte is OCA) is constant in all the QCs.

Intra-day and inter-day accuracy, measured as RME, ranged from 7.7% to 8.0%. Precision, measured as CV (%), is below 10.0% and 9.4% for intra- and inter-day, respectively.

The total recovery is determined as the ratio of the analyte in mobile phase directly injected with respect to the analyte in mobile phase injected after the clean-up procedure (previously described in 3.3)

The total recovery after pretreatment was about 95% for each analyte.

The matrix effect of each biological matrix was calculated as the ratio of the analyte in mobile phase with respect to the analyte in the real sample (collected at time zero and diluted 1:10, v/v with mobile phase) fortified at the end of the clean-up.

The average recovery due to the effect matrix for all analytes is 97% while for the internal standards are 100% (CV% < 2), thus confirming the absence of the matrix effect in optimized conditions.

The calibration curves show good linearity in the range of concentration between 0.1and 20 μM, suitable to analyze all BAs in all biological matrices. In terms of linearity, the regression coefficients for all the calibration curves of the BA are higher than 0.992.

Table 6 shows the parameters for the calibration curve derived from the statistical analysis of six calibration curves (in duplicate) obtained in mobile phase.

Table 6: Calibration curve for some BAs representing the principal groups

Bile Acid	**Intercept**	**Slope**	**R^2**
OCA	2.74 e-3±0.07e-3	4.2e-1±0.1e-1	0.992±0.006
UPF 2304 (23-fluorine substituted)	5.56e-4±0.09e-4	2.3e-4±0.05e-4	0.984±0.004
UPF 2295 (bioisoster)	3.67e-3±0.07e-3	4.04e-2±0.08e-2	0.997±0.008
UPF 2293 (sulphonates)	2.20e-3±0.06e-3	5.04e-2±0.08e-2	0.986±0.005
UPF 2284 (sulphates)	2.40e-3±0.06e-3	4.03e-2±0.07e-2	0.997±0.008
UPF 2220 (shorter side chain)	6.0e-4±0.1e-4	2.11e-1±0.05e-1	0.996±0.007

3.3 Physico-chemical properties

3.3.1 pK_a and lipophilicity

The pK_a values are calculated *in silico* for each semi-synthetic BA with Epik module version 2.2 from Schrödinger Suite 2010, with water as the solvent.

Standard solution of each semi-synthetic BA is prepared dissolving about 11 mg (MW =400-500 u) in 500µL NaCl 0.15 M to obtain a 50mM solution. For semi-synthetic bile acid with carboxylic group in the side chain, salification is done with an excess of $NaHCO_3$ (1:1, mol : mol).

The procedure [29] to measure the partition coefficient 1-octanol/water for ($LogP_A^-$) is carried out for each BA using a 100µM solution with phosphate buffer 0.1 M pH= 8.00 (all BAs were full ionized at this pH value), previously saturated with 1-octanol.

Then 2 mL of this solution is added to 2 mL of 1-octanol previously saturated with phosphate buffer 0.1 M pH= 8.00 and incubated at room temperature.

After about one week, the mixture is centrifuged to have a clean separation of two immiscible phases.

The buffer solution thus obtained is diluted in mobile phase and injected in HPLC-MS/MS system for bile acids quantification, as written below (in 3.4). 1-octanol/water partition coefficient is expressed as logarithmic ratio of the concentrations:

$$LogP = Log\frac{[BA]initial - [BA]final}{[BA]final}$$

3.3.2 Water solubility

The water solubility is determined only for semisynthetic BA having a carboxylic group in the side chain because their protonated forms (at pH<pK_a-2) could be poor soluble.

The sulphate and sulphonate analogues are highly water soluble being fully ionized at a pH range from 0 to 12.

A small amount of each bile acid was suspended, without preliminary salification, in 5 mL of 0.1M HCl (pH about 1). After one week of incubation at 25 °C, the saturated solutions were filtered on a Millipore filter (0.22 µm) and then injected in HPLC-ES-MS/MS system for BA quantification, as reported below (in 3.4). Water solubility was expressed as concentration (µmol/l).

3.3.3 Detergency

The detergency was evaluated by surface tension (ST) measurements and dye solubilization technique [36] and the critical micellar concentration (CMC) was calculated.

Standard solution of each semi-synthetic BA is prepared dissolving about 11 mg (MW= 400-500 a.m.u.) in 500µL NaCl 0.15 M to obtain a 50mM solution. For semi-synthetic BA with carboxylic group in the side chain, salification is done with an excess of $NaHCO_3$ (1:1, mol : mol).

In the first method, the relationship between surface tension and BAs CMC is measured by an improved maximum bubble pressure method with a tensiometer (Sensadyne 6000, Chem-Dyne Research Corp., Milwaukee, WI) consisting of two glass probes connected to a source of compressed air. The bubble frequency was 1 bubble/sec in distilled water at room temperature (P=2.7 atm) and the calibration was made with water and methanol.

The ST of each BA solution, as sodium salt, is measured at various concentrations between 50 mM and 0.1 mM with appropriate dilutions.

Then CMC values are calculated from the plot ST values versus logarithm of the BA concentration: the abscissa value corresponding to the point where the two regression lines (corresponding respectively to monomeric and micellar phase) intersect.

The second method is based on the fact that some dyes, specifically Orange OT (purchased from Intercept Pharmaceuticals S.p.a., San Diego, CA), are almost insoluble in water but dissolve in solutions with micellar aggregates that incorporate them; thus, the absorbance of the solution due to the amount of the dye dissolved increases with BA concentration (after CMC achievement). The amount of dye solubilized in relation to BA concentration is determined with absorption spectroscopy.

For each BA, various solutions at different concentrations, between 50 mM and 0.1 mM with appropriate dilutions, are incubated under stirring at room temperature for 3 days with an excess of Orange OT. Then all the solutions are centrifuged and filtered through a 0.22 µm Millipore filter (Millipore Corp., Bedford, MA).

Preliminary experiments show that 2 days are sufficient for saturation of the micellar solutions to occur, and that the solubilized dyes do not adsorb to the membrane filters.

Absorbance of each solution is measured at 483 nm (typical wavelength of Orange OT absorption) with Spectrophotometer (Wellwarm, Labsystems, Cambridge, UK).

The CMC value is the intersection point between the line parallel to the abscissa through the first several points (reflecting the solubility of the dye at lower concentration of bile acid) and the line through the other points (when the dye solubilization increase).

3.3.4 Affinity binding to serum albumin

Standard solution of each semi-synthetic bile acid is prepared dissolving about 11 mg (MW =400-500 u) in 500 µL of NaCl 0.15M to obtain a 50mM solution. For semi-synthetic BAs with carboxylic group in the side chain, salification is done with an excess of $NaHCO_3$ (1:1, mol : mol).

The percentage of affinity binding to serum albumin is evaluated by equilibrium dialysis at a fixed BA albumin ratio [31, 34, 36]. Each BA is dissolved at a concentration of 100 µM in a water solution with NaCl 0.15M and 5% bovine serum albumin (BSA) fatty acid free, from Sigma Aldrich (St Louis, MO), and incubates for one day at room temperature.

Two ml of this solution are placed in a cellulose membrane (cut-off=12,000-14,000 Dalton), from Spectra/Por, Spectrum Medical lndustries lnc. (Los Angeles, CA) and then dialyzed against 25 ml of water solution with NaCl 0.15M (pH 7.20).

The system is equilibrated by gently shaking for about three days at room temperature.

The BA concentration of the dialyzed solutions (corresponding to the free unbound fraction) is determined with HPLC-ESI-MS/MS system for bile acids quantification, as reported below (in 3.4).

The percentage of albumin binding is calculated with the ratio between initial BA concentration and the unbound concentration in the dialyzed fraction.

3.4 Bile fistula rat model experiments

The In vivo experiments have been performed by Dr. Rita Aldini, Department of Pharmacy and Biotechnologies, University of Bologna.

The procedure is conducted in Wistar-Han rats anesthetized: the bile duct was cannulated, and the BA was administered with intravenous (IV) or intraduodenal (ID) infusion [35].

Each rat is subjected to one kind of infusion with one BA dose.

For both the infusions, the dose of 1 µmol/min/kg is injected over 1 hour at 2.5 ml/hour. The BA was dissolved in saline solution (NaCl 0.1M) for ID administration and in saline solution with 3% of albumin (BSA fatty acid free, Sigma Aldrich, MI, Italy) for IV administration.

Bile and plasma were collected respectively at 15-minute and 30-minutes time intervals throughout the infusion and for the next two hours.

BAs secretion and the following parameters have been calculated: bile flow (SV_{max}) gravimetrically, maximum secretion rate (SB_{max}) of the biliary secretion rate values over the 3 hours of bile collection, plasma concentration of BA and its metabolites over the three hours.

3.5 CCl_4 induced cirrhosis rat model: Biodistribution experiments

Experimental liver cirrhosis has been inducted in male Wistar-Han rats (Charles River Laboratories, Calco, LC, Italy), weighing 225-250 g, by one week phenobarbital (0.3 g.L−1 in drinking water) administration, Cirrhosis was induced by carbon tetrachloride (CCl_4) inhalation [37].

Phenobarbital was continued throughout the study as an enzyme induced. Animals are housed in a controlled environment (22–24°C), maintained on a standard 12-h light/dark cycle (lights on at 07.00 h) and had free access to food and water throughout the study.

After one week exposure to phenobarbital, inhalation of CCl_4 was started. The rats were placed in a gas chamber (70 × 25 × 30 cm) and compressed air, bubbling through a flask containing CCl_4, was passed into the gas chamber via flow meter (1 L•min−1). Animals were exposed to the gas atmosphere twice a week (on Mondays and Fridays), starting with 0.5 min of bubbling and 0.5 min in the gas atmosphere. Afterwards, the time was increased to 1 min and then by 1 min until 5 min of air flow and 5 min in gas atmosphere were reached. The mortality rate associated with this induction method was approximately 13%. The animals were fasted for 8 hours before dosing.

Then the rats were treated by oral administration (gavage, in conscious animals) of OCA at a dose of 30 mg/kg dissolved in 300 µL saline and immediately randomized. (Because animals were similar in weight, randomization was done after dose administration, to avoid any bias).

Then the main organs (liver, kidneys, small intestine and colon tissue, small intestine and colon content, faeces), and fluids (plasma and urine) were collected after 1, 2, 4,8, 24 and 48 hours after the administration.

OCA and its main metabolites have been quantified by the use of HPLC-ES-MS/MS method previously reported.

The same dose of OCA was administered to healthy rats and the same organs and fluids are collected after 24 and 48 hours in order to compare their biodistribution with that of cirrhotic rats.

3.6 BA and OCA extraction and clean-up from biological matrices

3.6.1 Bile samples

Rat bile samples are brought to room temperature and diluted 1:1000, (v/v) (bile samples from femoral infusion and from duodenal infusion in bile fistula rat model) with mobile phase used for HPLC method.
The final solution is injected in the HPLC-ES-MS/MS system for BAs quantification, as reported below (in 3.4).
When samples are out of the linearity range, they were diluted and reanalyzed [35].

3.6.2 Plasma and urine samples

Plasma and urine samples (400 µl) with 10 µL of internal standard (mix 1µM of OCA, T-OCA; mix 1µM of INT 777 ant T-INT 777 when the analyte is OCA) are diluted with 2mL of NaOH 0.1 N at 64°C for 30 minutes. The cartridge is conditioned before to sample loading.
Plasma samples were loaded in the solid phase (SPE) C18 cartridge, previously conditioned with 5 ml of methanol and 5 ml of water, and then washed with 10 ml water.
After that, the BAs were eluted with 5 ml methanol, the eluate dried under vacuum, reconstituted with 100 µl of the mobile phase and injected into the HPLC-ES-MS/MS system for BAs quantification, as reported below (in 3.4) [36].

3.6.3 Liver and kidneys samples

Liver and kidneys samples (0.5 g each), previously minutely chopped, were homogenized in 1 ml of phosphate buffer (0.005 M, pH 7.2) using a potter and then washed with methanol (3 × 0.5 mL).
The mixture, was sonicated, vortexed, incubated at 37 °C for 20 minutes and centrifuged at 3000 rpm for 15 minutes.
Four hundred µl of the supernatant with 10 µl of internal standard working was dried under vacuum and then reconstructed 2 ml of NaOH 0.1 N at 64°C for 30 minutes. The SPE was carried out as shown above and the eluate is dried under vacuum, reconstituted with 100 µl of mobile phase and injected into the HPLC-ES-MS/MS system for BAs quantification, as reported below (in 3.4) [35].

3.6.4 Intestinal contents and faeces samples

Small intestine, colon contents and faeces samples was minutely chopped, weighted (0.3 mg) and homogenized with 3mL of isopropyl alcohol using a mixer. The obtained mixture is vortexed and centrifuged at 3000 rpm for 10 minutes.
Ten µl of supernatant were diluted with 180 µl of mobile phase and 10 µl of internal standard (1:10 v/v dilution) with mobile phase and injected into the HPLC-ES-MS system for BAs quantification, as reported below (in 3.4) [35].

4. Results and discussion

4.1 Physico-Chemical properties

The physico-chemical properties results for endogenous BAs and the first semi-synthetic analogues (i.e. INT 747, INT 777 and INT 767) were used as a reference to compare the structure-activity and metabolism with the new more recently synthesized analogues. The more relevant properties in aqueous solution refer to the acidity properties and to lipophilicity.

4.1.1 pK_a

The pK_a values measured in silico and the LogP values of each BA in the ionized form (A^-) for the new semi-synthetic analogues are reported in Table 7. The Log P refers only to ionized species since the protonated one is often almost water insoluble and at the physiological pH the ionized is the predominant with the exception of the gastric acid content referring to the carboxylate BAs.

About pK_a, data shows that the 6α-ethyl substituted BAs maintain the same pK_a of the endogenous analogues (CA and CDCA) and it is also true for the bioisosteres.

However, the 23-fluorine substituted BAs have a pK_a nearer the taurine conjugated analogues because the strong electronegativity of the fluorine atom allows to ionize easily the carboxylic group by electron windrowing effect.

Moreover, as expected, the sulphates and sulphonate analogues have the same pK_a of taurine conjugate endogenous BAs (i.e. TCDCA) being to fully ionized at all pH.

4.1.2 Lipophilicity

The lipophilicity ($LogP_A^-$) decreases by increasing the number of hydroxyl group in the steroid ring and also the nature of the substituents introduced in the side chain plays a major role.

In the 6α-ethyl substituted group, UPF 2024 and UPF 2213 present a negative $LogP_A^-$, probably due to one more hydroxyl group in position 17β for the first and one methoxyl group in 23 position for the second in respect to UPF 2220 (Fig 10)

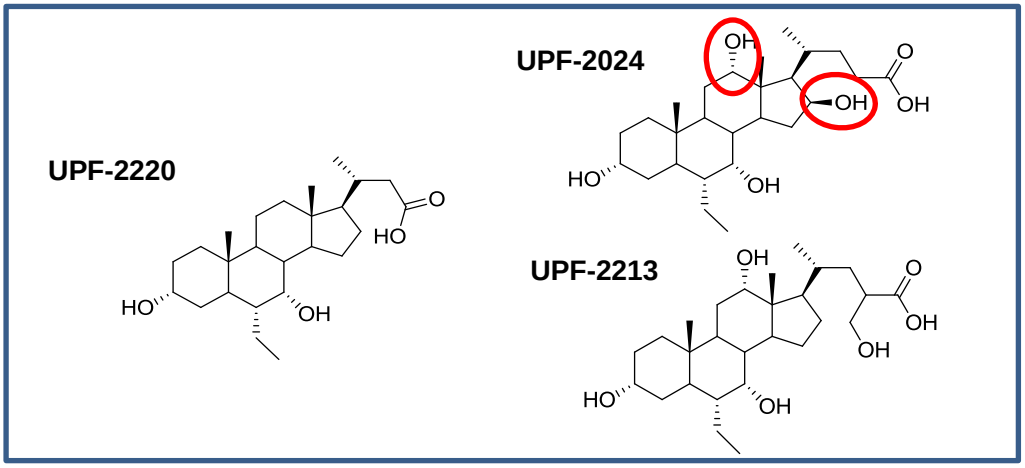


Figure 10: structure differences between UPF 2220 and UPF 2024/UPF 2213

These chemical modifications contribute to make these semi-synthetic BAs more hydrophilic than their analogues (CDCA, CA, INT 747).

The same difference can be observed in the group of bioisosteres analogues between UPF 2225 and UPF 2295 and in the group of fluorine substituted between UPF 2207 and UPF 2304, because the first ones have one more hydroxyl group in 12α position (Fig 11).

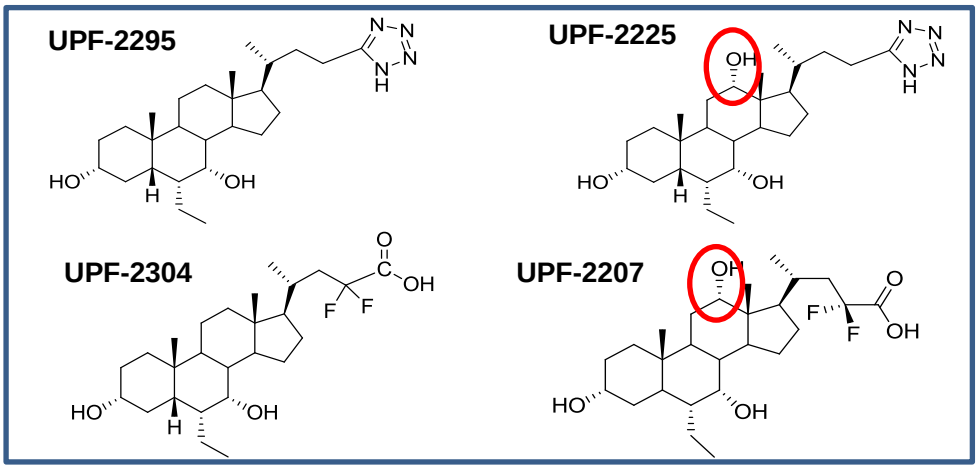


Figure 11: structure differences between UPF 2295/UPF 2304 and UPF 2225/UPF 2207

Moreover UPF 2220 is less lipophilic than its analogue INT 747 due to its shorter side chain (one methyl less). The same difference can be seen between INT 767 and UPF 2283 in the group of sulphates analogues.

Table 7: pKa and $LogP_A^-$ values reported for the new semi-synthetic BAs and their main analogues

BA	pK_a	$LogP_A^-$	BA	pK_a	$LogP_A^-$
CDCA	5	2.2±0.2	**BIOISOSTERES**		
TCDCA	<1	0.9±0.1	**UPF 2295**	5.59	1.2±0.1
CA	5	1.1±0.2	**UPF 2308**	5.71	2.0±0.3
6α-ETHYL			**UPF 2289**	5	1.9±0.2
INT 747	5	2.5±0.4	**UPF 2224**	5.94	1.6±0.3
INT 777	5	1.4±0.2	**UPF 2225**	5.59	1.5±0.3
UPF 2024	5.29	-0.05±0.01	**SULPHATE-SULPHONATE**		
UPF 2220	5.24	1.3±0.3	**INT 767**	<1	2.0±0.2
UPF 2213	4.36	-0.20±0.07	**UPF 2284**	<1	1.4±0.1
FLUORIDE			**UPF 2307**	<1	0.9±0.1
UPF 2304	1.10	1.4±0.3	**UPF 2293**	<1	0.9±0.1
UPF 2227	2.82	0.20±0.05	**UPF 2283**	<1	0.80±0.04
UPF 2207	1.10	1.0±0.2	**UPF 2021**	<1	0.70±0.03
UPF 2226	2.82	0.10±0.04	**UPF 2023**	<1	0.60±0.03

4.1.3 Water solubility

The relationship with BA structure and presence of different substituents is not so directly evident since the solubility of a molecule is mostly related to the stability of the solid state crystalline structure and additional studies should be required starting from the measurement of the melting point. The water solubility data of the new semi-synthetic BAs and their main analogues are reported in Table 8.

Table 8: Ws values, expressed in μM, reported for the new semi-synthetic BAs and their main analogues

BA	Ws (μM)	BA	Ws (μM)
CDCA	32±4	**BIOISOSTERES**	
TCDCA	hs*	**UPF 2295**	135±9
CA	273±10	**UPF 2308**	5±1
6α-ETHYL		**UPF 2289**	hs*
INT 747	9±2	**UPF 2224**	392±11
INT 777	99±3	**UPF 2225**	517±14
UPF 2024	290±11	**SULPHATE-SULPHONATE**	
UPF 2220	75±4	**INT 767**	hs*
UPF 2213	hs*	**UPF 2284**	hs*
FLUORIDE		**UPF 2307**	hs*
UPF 2304	16±2	**UPF 2293**	hs*
UPF 2227	469±15	**UPF 2283**	hs*
UPF 2207	225±10	**UPF 2021**	hs*
UPF 2226	971±18	**UPF 2023**	hs*

*hs=high solubility when value >1000μM

These results shows that all the sulphates and sulphonates BAs have a high solubility (>1000μM), due to the full ionization in all the range of pH

from 0 to 12. About the carboxylic BAs, the water solubility at pH=1 was measured to ensure a complete protonation. As seen, the solubility decrease with the presence of 6α-ethyl in respect to the natural analogues CA and CDCA and on the contrary increase with the presence of more hydroxyl groups (UPF 2207 and UPF 2225 compared with UPF 2304 and UPF 2295) or fluorine atom (UPF 2227 compared with INT 777).

4.1.4 Detergency

The critical micellar concentrations (CMC) of new semi-synthetic BAs and their endogenous analogues are obtained from the plot concentration (mM) VS surface tension (ST) in Figure 12a and concentration (mM) vs absorbance (u.a.) in Figure 12b.

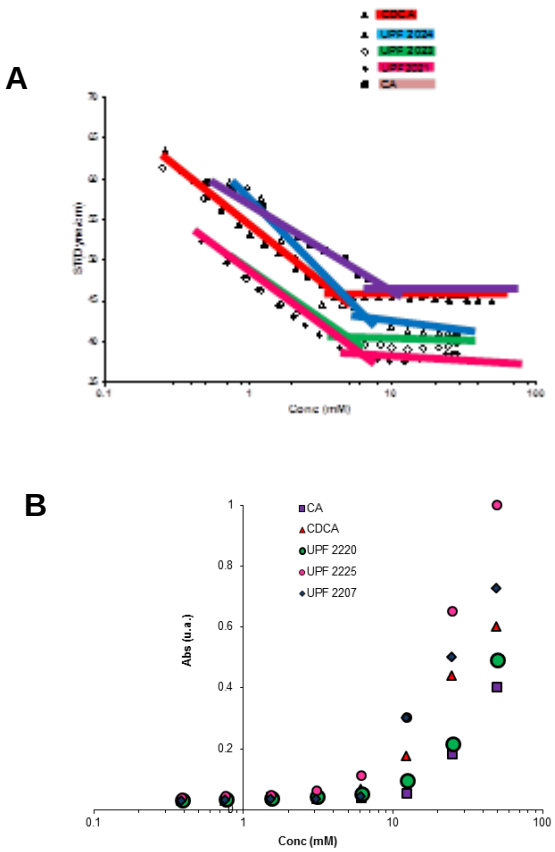


Figure 10: plot with surface tension (A) and absorbance (B) in respect to concentration (mM) of each BA

The CMC values for each BA and their calculated CMpH are reported in Table 9.

Table 9: CMC values, expressed in mM, reported for the new semi-synthetic BAs and their main analogues

BA	CMC (mM)	CMpH	BA	CMC (mM)	CMpH
CDCA	3.2±0.9	7	**BIOISOSTERES**		
TCDCA	3±1	-	**UPF 2295**	nd*	nc**
CA	9±2	6.5	**UPF 2308**	11±3	9.0
6α-ETHYL			**UPF 2289**	10±2	5.0
INT 747	1.3±0.5	7.2	**UPF 2224**	5±1	7.0
INT 777	2.0±0.7	6.1	**UPF 2225**	5±1	6.6
UPF 2024	6.3±0.7	6.5	**SULPHATE-SULPHONATE**		
UPF 2220	10±2	7.1	**INT 767**	1.3±0.4	-
UPF 2213	5±1	4.5	**UPF 2284**	3.0±0.6	-
FLUORIDE			**UPF 2307**	4.0±0.6	-
UPF 2304	8.5±0.5	3.7	**UPF 2293**	5.0±0.7	-
UPF 2227	3.9±0.6	3.9	**UPF 2283**	10±2	-
UPF 2207	3.6±0.6	2.7	**UPF 2021**	6±1	-
UPF 2226	6.0±0.9	3.6	**UPF 2023**	4.1±0.9	-

*not detected **not calculated

As shown, for the carboxylic BAs, the CMC increases with a decrease of side chain length and presence of substituents: when it is shorter (UPF 2220 in respect to INT 747) or when it has fluorine substituent (UPF 2207 and 2304 in respect to INT 777 and INT 747).

This phenomenon is on line with the behavior of the classic anionic detergent in solution where the side chain play a role in the self-aggregation of the lipophilic tail. [29]

Concerning the bioisosteres, the higher CMC of UPF 2289 confirms the importance of the side chain length in BAs detergent properties. Moreover, the CMC for UPF 2295 cannot be measured due to its low solubility.
Concerning the CMpH, i.e. the pH value above which a micellar phase dissolution is observed, it cannot be calculated for sulphates and sulphonates derivatives, as their endogenous analogues taurine conjugated (TCDCA) since their pKa cannot be calculated accurately and is very low.
This is due to the fact that they are fully ionized (1>pH<12) and therefore their solubility is always higher than that of their free analogues and it doesn't depend on micelles formation.

4.1.4 Affinity binding to serum albumin

The binding of the studied BAs has been performed at a fixed and defined initial BA concentration and excess of BSA (Bovine Serum Albumin). Therefore the obtained percentage of BA bound to albumin is not the thermodynamic value and therefore these data are only used for inter-comparative studies.
The measurement of the affinity constant (K_{aff}) requires studies with different initial concentrations and the data should be extrapolated by a Statchard plot [31, 36].
The results obtained for the percentage of albumin bound to each BA are reported in Table 10.
The data show that there are no significant differences in affinity with albumin for the new synthetic BAs in respect to their endogenous analogues.

Table 10: Albumin binding values, expressed in %, reported for the new semi-synthetic BAs and their main analogues

BA	Albumin binding (%)	BA	Albumine binding (%)
CDCA	90±5	**BIOISOSTERES**	
TCDCA	76±2	**UPF 2295**	76±2
CA	88±3	**UPF 2308**	90±5
6α-ETHYL		**UPF 2289**	99±5
INT 747	96±5	**UPF 2224**	84±4
INT 777	62±2	**UPF 2225**	83±4
UPF 2024	47±1	**SULPHATE-SULPHONATE**	
UPF 2220	97±4	**INT 767**	85±4
UPF 2213	55±2	**UPF 2284**	80±4
FLUORIDE		**UPF 2307**	86±5
UPF 2304	66±2	**UPF 2293**	68±3
UPF 2227	89±3	**UPF 2283**	45±1
UPF 2207	99±4	**UPF 2021**	71±2
UPF 2226	84±3	**UPF 2023**	74±2

4.2 Bile fistula rat model

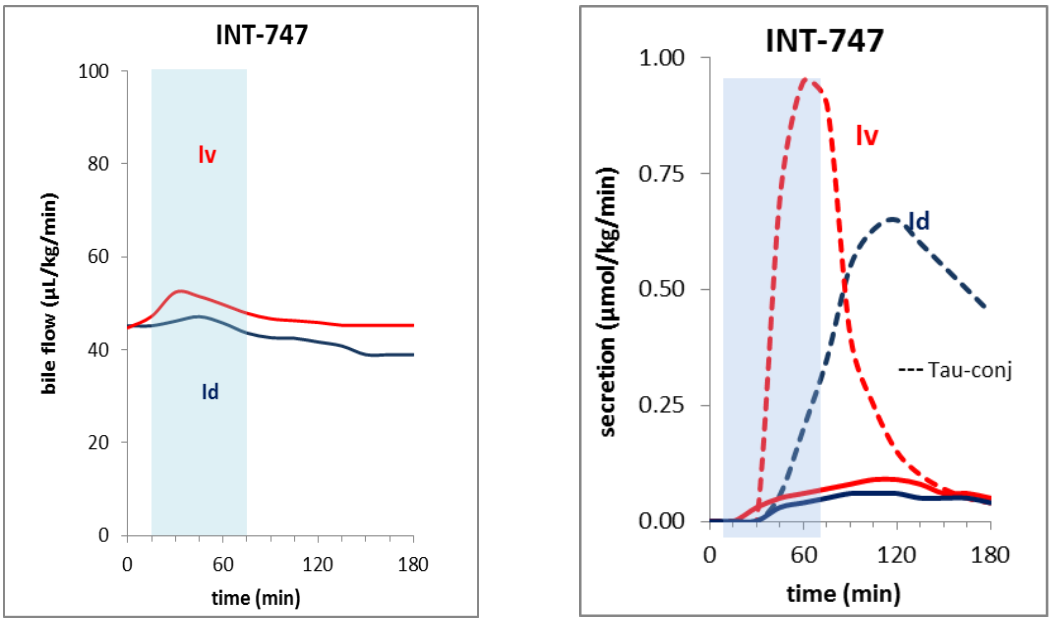
For each semi-synthetic BA, intraduodenal and intravenous acute administration experiments have been performed to obtain informations about bile flow, BA biliary secretion and simplified pharmacokinetic (plasma levels, hepatic and intestinal metabolism).

4.2.1 Iv and id administration: bile flow and BA secretion, plasma levels

Figure 13a shows a typical example of bile flow calculated by bile volume for each collected sample. The mean value of bile flow in control untreated animal in the pre-infusion period is about 40-60 µl/min/Kg: a cholestatic or choleretic effect can be observed depending on whether the maximum values measured is lower or higher than the above range.

The Figure 13b reports the BA biliary secretion calculated from the expression:

$$\frac{BA\ concentration\ (\mu M) \cdot bile\ volume\ (L) \cdot rat\ weight\ (Kg)}{collection\ time\ (\mathrm{min})}$$



(a) **(b)**

Figure 13: example of bile flow (a) and bile secretion (b) trends during each intraduodenal (blue) and intravenous (red) experiment (180 min)

In Table 11 all the maximum secretory (SBA_{max}) and maximum bile flow (SV_{max}) are reported for each IV and ID experiment for each BA.

Table 11: SV_{max} (a) and SBA_{max} (b) in the intraduodenal (ID) and intravenous (IV) experiments

	SV_{max} IV	SV_{max} ID	SBA_{max}IV	SBA_{max}ID
	µl/min/kg		µmol/min/kg	
UPF 2308	34±2	35±2	0.04±0.01	0.05±0.02
UPF 2307	23±2	24±1	0.55±0.05	0.05±0.02
UPF 2306	42±3	48±3	0.07±0.02	0.32±0.03
UPF 2304	31±1	37±3	0.10±0.03	0.002±0.005
UPF 2295	20±1	50±4	0.07±0.02	0.21±0.03
UPF 2289	22±1	14±1	0.07±0.02	0.03±0.01
UPF 2213	30±2	20±1	0.94±0.06	0.82±0.06
UPF 2207	41±3	40±3	0.20±0.04	0.0010±0.0007
UPF 2227	49±3	27±2	0.82±0.06	0.0010±0.0006
UPF 2224	67±4	23±1	0.35±0.03	1.00±0.09
UPF 2225	1.0±0.3	20±1	0.36±0.03	1.00±0.09
UPF 2226	62±4	75±5	0.660.05	0.03±0.01
UPF 2293	18±1	38±2	0.99±0.09	0.30±0.03
UPF 2284	31±2	39±3	0.25±0.03	0.013±0.007
UPF 2283	30±2	30±2	0.35±0.03	0.02±0.01
UPF 2220	30±2	20±1	0.005±0.001	0.0010±0.0005
UPF 2024	52±3	47±3	0.99±0.09	0.08±0.03
UPF 2023	63±3	53±3	0.70±0.07	0.06±0.01
UPF 2021	73±4	40±3	0.79±0.08	0.004±0.001
INT 777	130±7	115±7	0.92±0.09	0.28±0.07
INT 767	65±4	58±3	0.60±0.06	0.30±0.03
INT 747	83±5	70±4	0.96±0.09	0.80±0.08
TCDCA	41±3	44±3	0.63±0.06	0.08±0.01
CDCA	63±4	65±4	0.98±0.08	0.90±0.06
CA	105±6	90±5	1.00±0.08	0.85±0.08

In Table 12 the mean maximum plasma concentration are reported for the ID experiment for each BA.

Table 12: maximum plasma concentration in the intraduodenal (ID) and experiments

BA	Plasma ID (μM)	BA	Plasma ID (μM)
TCDCA	0.07±0.03	**UPF 2308**	1.2±0.1
CDCA	-	**UPF 2307**	31±3
CA	-	**UPF 2306**	6.2±0.6
INT 777	0.20±0.07	**UPF 2304**	15±2
INT 767	0.0010±0.0005	**UPF 2295**	2.9±0.3
INT 747	0.010±0.007	**UPF 2289**	3.9±0.4
UPF 2293	0.05±0.01	**UPF 2213**	59±3
UPF 2284	1.9±0.4	**UPF 2207**	0.10±0.08
UPF 2283	0.5±0.1	**UPF 2227**	9.9±1
UPF 2220	85±4	**UPF 2224**	0.3±0.1
UPF 2024	0.010±0.008	**UPF 2225**	6.3±0.6
UPF 2023	1.6±0.3	**UPF 2226**	4.2±0.4
UPF 2021	0.5±0.1		

BAs with a high SBAmax and a low plasma concentration (i.e. INT 747, UPF 2293) could be subjected to the enterohepatic circulation like natural occurring BA with a preferential distribution in the bile and small intestine.

BAs with low SBAmax and a high plasma concentration (i.e. UPF 2220) could have systemic behavior and their plasma exposure will be responsible for the action in the organs involved.

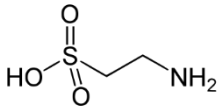
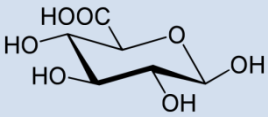
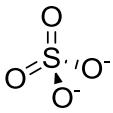
For BAs with low SBAmax and a low plasma concentration it can be hypothesized two different behaviors:

- they could have a high intrahepatic concentration due to a relevant hepatic back diffusion to blood and poor biliary secretion, causing dose-related potential hepatotoxicity/cytotoxicity) for the presence of high hepatic concentration (i.e. UPF 2213 and UPF 2304);
- they could be gastrointestinal (GI) restricted, poorly absorbed either actively or passively by the intestine, excreted with stool unmodified, like sulphate/sulphonate (i.e. UPF 2283 and UPF 2023) and taurine conjugated (i.e. UPF 2295) because of the less active intestinal transport for poor recognition by ileal transporters.

4.2.2 Hepatic and intestinal metabolism in vivo: identification of new metabolites

In rat, semi-synthetic BAs are preferentially conjugated with taurine by the liver, as their natural analogues such as cholic and muricholic acid.
Furthermore, during intraduodenal administration, the poorly absorbed BAs in the small intestine reach the terminal ileum and the colon where they are metabolized by the intestinal bacteria micro flora, and dehydroxylation, oxidation, epimerization process can be observed as indicated by their presence in stools. In the liver and small intestine conjugation with glucuronic acid or formation of sulphates at the hydroxyl groups or side chain carboxyl in order to produce more hydrophilic compounds better excreted with stools or urine
These expected metabolites have been investigated and identified according to the m/z values thanks to HPLC-ES-MS/MS method previously described (see Table 13).
Since the standards necessary for their precise quantification were not available, a calibration curve with more similar natural analogues is used.
The SBA_{max} values and plasma levels previously reported in Table 11 and 12 are expressed including also metabolites concentration for each BA.

Table 13: main metabolites of natural bile acid hypothesized also for new semi-synthetic BAs in bile

METABOLITE	m/z	HPLC RETENTION TIME	MS ACQUISITION MODE
Epimer	**$[M-H]^-_1 = [M-H]^-_0$**	$tr_1 \neq tr_0$	MRM/SIM
Taurine	**$[M-H]^-_0$ +107** 	$tr_1 < tr_0$	MRM
Glucuronide	**$[M-H]^-_0$ +176** 	$tr_1 << tr_0$	MRM/SIM
Sulphate	**$[M-H]^-_0$ +80** 	$tr_1 << tr_0$	SIM
Dehydroxylated	**$[M-H]^-_0$ -16** - OH	$tr_1 > tr_0$	SIM
Oxidized	**$[M-H]^-_0$ -2** =O	$tr_1 > tr_0$	SIM

In Figure 14 the main metabolism in bile for each new semi-synthetic BA is shown.

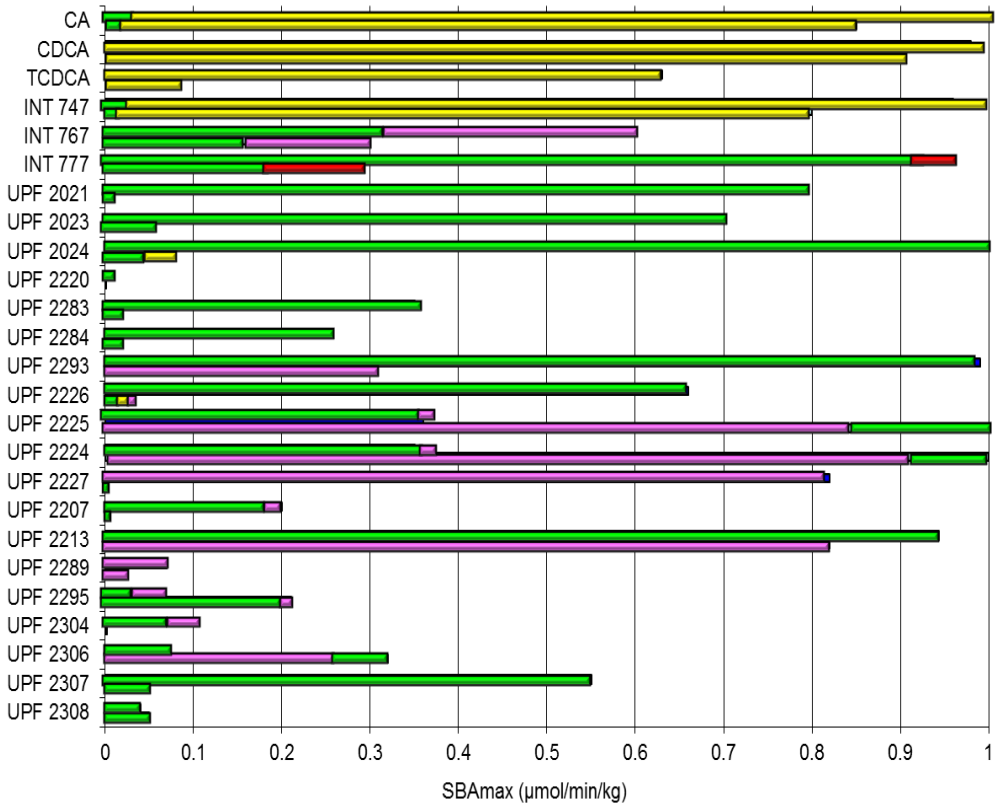


Figure 14: main metabolism of the new semi-synthetic BAs in bile

These data demonstrate that the 6α-ethyl analogues with a carboxyl group in the side chain have a quite similar metabolism of the natural BAs, thus they undergo to taurine conjugation (i.e. INT 747), with the only exception that the ethyl in 6α position prevents the 7α-dehydroxylation and therefore increases the biological half-life time of the molecule by preventing this pathway by intestinal bacteria.

Indeed, the presence of side chain substituents in C-23β position (fluorine or methyl) hinders the taurine conjugation process usually mediated by a CoA activation of the carboxyl which cannot be obtained by the presence of these C-23 substituents. This is the case of INT 777, UPF 2226, UPF 2227, UPF 2213, UPF 2207 and UPF 2304).

Moreover, for 23β mono-substituted it could be hypothesized an epimerization, that allow the taurine conjugation (INT 777 and its epimer with 23α methyl, UPF 2226 and its epimer UPF 2227).

The alternative pathway that increases their polarity and hydrophilicity is the conjugation with glucuronic acid, in order to be excreted with stools. The glucuronidation occurs probably in 3α position, because of 6α ethyl group reduces the reactivity of the 7α-OH not only for oxidation or dehydroxylation but also for conjugation with glucuronic acid.

About the new analogues containing a sulphate/sulphonate group as a side chain, they are mostly secreted as such in bile and eliminated by urine or stools. The conjugation is not observed with the only exception of a low 3α-glucuronidation for more lipophilic analogues. Moreover In plasma, all semi-synthetic BAs are almost present only as such.

4.3 OCA in cirrhosis treatment: biodistribution and metabolism *in vivo*

4.3.1 Induction of cirrhosis in rat

After chronic inhalation of CCl_4 according to the used protocol the rats show a moderate to severe cirrhosis with generalized fibrosis, periportal chronic inflammation, bile duct proliferation and destruction of normal liver architecture with the formation of regeneration nodules. In some rats it was associated to severe chronic hepatitis, i.e. the stage prior to the establishment of cirrhosis, in which there is considerable fibrosis and chronic inflammation, but without loss of the basic architecture to form nodules.

A main difference between the CCl_4 induced liver damage and human liver damage is that the former is not immuno-mediated, but it is hepatotoxicity induced, spanning over a few weeks period and not over years, with little lymphocytic activation, but rather macrophages activation. However the functional and hemodynamic pattern is similar [38].

The relative liver weight at the end of the induction period is increased in most of the animals, decreased in others, indicating in the latter group that the liver functional mass was greatly reduced. The great differences in liver weight (3, 6-16, 4 g) resemble the differences in humans. The liver weight increases by 50%, liver cells number increases and therefore it is possible that the different responses to Phenobarbital microsomal induction can be responsible for the highest liver weights observed.

About plasma biomarkers, the CCl_4 induced liver cirrhosis model provides a range of phenotypic expression of the liver disease, as it is the case with humans. Serum AST, ALT, and ALPK activities are changed significantly in rats; albumin is decreased from moderately to severely in about 75% of cases and plasma bilirubin is elevated in about 50% of cases. All the CCl_4 administered rats presented NH_3 elevated levels, encephalopathy [39] and ascites (fluid accumulation in peritoneal cavity), from mild to severe.

In addition, endogenous BA quali-quantitative composition in the studied biological fluids and organs have allowed to evaluate the consequence of

liver failure i.e. hepatic uptake and/or biliary secretion in their biodistribution and to compare data with OCA and its metabolites in cirrhotic and control animals (Figure 14).

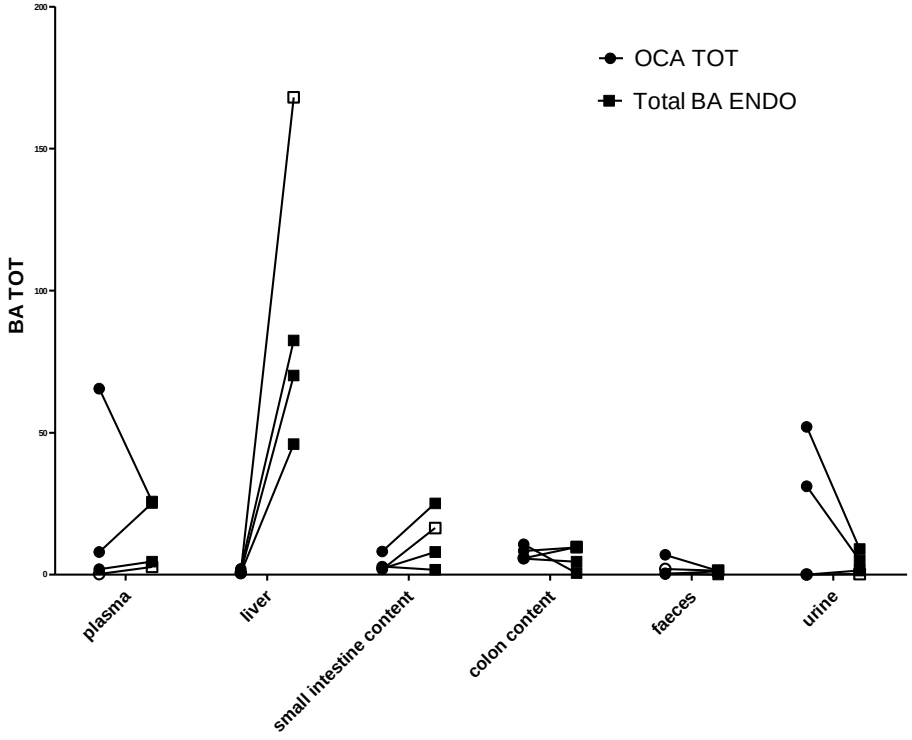


Figure 14: endogenous BAs compared with OCA and its metabolites in healthy (□) and cirrhotic (○) rats after 24h

Plasma total endogenous BAs are very high in comparison with controls: they were present free but also taurine conjugated.

They are also high when plasma bilirubin levels are normal and in presence of normal liver parameters, but always associated with histological evidence of end stage liver disease.

In the liver, endogenous BAs are present in concentrations similar to healthy rats, being both free and conjugated like in plasma.

In the small intestine they are present free and conjugated with taurine; on the contrary in the colon they are present almost in free form.

In faeces, BAs are present in unconjugated form similarly in healthy and cirrhotic rats; in urines BAs free and taurine conjugated are present in higher concentrations in cirrhotic than in healthy rats.

4.3.2 Identification of OCA metabolites

The main metabolites identified and quantified with HPLC-ES-MS/MS method previously described are reported in Table 14.

Table 14: parameters for identification and quantification of OCA and its main metabolites

Compound	**t_R (min.)**	**Cone (V)**	**Transition monitored (m/z)**
OCA	23.7	60	419→419
Tau-OC	22.5	90	526→107
Gly-OC	22.5	60	476→74
Glu-OC	4.6	40	595→595
3keto-OCA	25.3	60	418→418

4.3.3 Mass Balance in cirrhosis-induced rats

From the amount (µmoles) of OCA and its metabolites recovered in the different organs and biological fluids analysed, a mass balance is calculated. This has been done after 24 and 48 hours from OCA administration, for which stools and urine samples were quantitatively collected (thanks to metabolic cages) and analysed.

Mass balance is calculated in µmoles, extrapolating the total content in each organ/ fluid/ excrement (intestinal contents and faeces) from the concentration values and from the organ/ fluid/ excrement weight (or volume). Then the distribution (%) of OCA and its major metabolites is calculated and reported below.

Considering all the analysed metabolites, almost 94 % (Table 15) of the administered dose is recovered 24 hours after dosing and is distributed as reported in table 15 (numbers shown represent percentage of dose administered).

Table 15: distribution % of OCA and its metabolites in cirrhotic rat after 24 h
n.c.=not calculated (concentration less than LOQ)

Sample	OCA	Tau-OCA	Gly-OCA	Glu-OCA	3keto-OCA	OCA TOT
Plasma	0.13±0.01	0.5±0.1	0.008±0.001	0.6±0.1	n.c.	1.2±0.2
Liver	0.36±0.1	18±6	19±7	2.4±0.9	n.c.	40±14
Small intestine content	3±1	21±7	0.35±0.09	6.00±2	n.c.	31±10
Colon content	7±3	0.04±0.02	0.09±0.05	n.c.	1.58±0.2	9±3
Faeces	8±3	n.c.	n.c.	n.c.	0.75±0.3	9±3
Urine	0.04±0.01	n.c.	0.04±0.02	4.4±0.7	n.c.	4.5±0.7
Small intestine tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Colon tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Kidney	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Total	18±6	39±12	20±9	13±3	2.3±0.8	**95 ± 31**

Considering all measured metabolites, up to 98 % (Table 16) of the administered dose is recovered 48 hours after dosing and is distributed as reported in table 16 (numbers shown represent percentage of dose administered).

Table 16: distribution % of OCA and its metabolites in cirrhotic rat after 48 h

n.c.=not calculated (concentration less than LOQ)

Sample	OCA	Tau-OCA	Gly-OCA	Glu-OCA	3keto-CA	OCA TOT
Plasma	0.1±0.1	0.10±0.05	0.05±0.01	0.05±0.01	n.c.	0.3±0.1
Liver	0.3±0.1	8±1	12±3	1.4±0.4	n.c.	22±3
Small intestine content	2±1	25±3	0.10±0.02	1.1±0.3	n.c.	28±4
Colon content	7±1	0.10±0.05	0.20±0.03	n.c.	1.2±0.5	8±2
Faeces	38±3	n.c.	n.c.	n.c.	1.2±0.5	39±5
Urine	n.c.	0.01±	0.010±0.001	n.c.	n.c.	0.02±0.01
Small intestine tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Colon tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Kidney	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Total	47±5	33±4	12±3	3±1	2±1	**97±14**

The percent distribution of total OCA in the cirrhotic rats shows that OCA is more localized in the liver and to a less extent in the intestine in comparison with the one in control healthy rats (Tables 17 and 18).

The percent recovered in blood is slightly higher than in control animals. This accounts for an impaired BA hepatic uptake which involves also OCA and/or biliary secretion that results in an increase of hepatic concentration and reduced in intestinal content.

This distribution is similar to endogenous BAs, showing that OCA behaves very similar to common natural BAs.

The overall body clearance from OCA is faster in healthy rats than in cirrhosis: in healthy rat, after 48 hours, 75% OCA is in faeces, while only 42% is excreted in cirrhosis rats. OCA elimination by urines is present only in liver disease. The presence of OCA in plasma is associated with OCA higher elimination in urines.

Table 17: distribution % of OCA and its metabolites in healthy rat after 24 h n.c. =not calculated (concentration less than LOQ)

Sample	OCA	Tau-OCA	Gly-OCA	Glu-OCA	3keto-OCA	OCA TOT
Plasma	0.010 ±0.005	n.c.	n.c.	n.c.	n.c.	0.010 ±0.005
Liver	0.2±0.1	9±2	12±3	1.3±0.8	n.c.	22±4
Small intestine content	0.3±0.2	47±6	n.c.	n.c.	n.c.	48±9
Colon content	10±3	0.13 ±0.01	0.17 ±0.06	n.c.	1.6±0.2	11±2
Faeces	2±1	n.c.	n.c.	n.c.	0.13±0.02	2±1
Urine	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Small intestine tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Colon tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Kidney	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Total	13±4	57±8	12±3	1.3±0.8	1.8±0.2	**85±16**

Table 18: distribution % of OCA and its metabolites in healthy rat after 48 h n.c.=not calculated (concentration less than LOQ)

Sample	OCA	Tau-OCA	Gly-OCA	Glu-OCA	3keto-OCA	OCA TOT
Plasma	0.04 ±0.02	n.c.	n.c.	n.c.	n.c.	0.04 ±0.01
Liver	0.10 ±0.05	1.6±0.5	2.2±0.8	n.c.	n.c.	3.9±2
Small intestine content	3.0±1	9±5	0.03 ±0.01	0.35 ±0.06	n.c.	12.28±4
Colon content	4.0±3	0.09±0.03	n.c.	n.c.	1.3±0.5	5.41±3
Faeces	62.2±10	0.7±0.3	n.c.	n.c.	2.7±0.8	65.6±13
Urine	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Small intestine tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Colon tissue	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Kidney	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Total	69±14	11±6	2.2±0.8	0.35 ±0.06	4±1	**87±22**

5. Conclusions

Previous studies on endogenous and semi-synthetic BAs analogues have demonstrated that small modifications in the chemical structure are able to significantly modify the physico-chemical properties and consequently their pharmacokinetics, metabolism including also FXR receptor activity.

In particular the physico-chemical properties that properly define BA behavior in aqueous solutions and in biological fluids, allow to predict:

- Drug formulation strategy regarding solubility in the gastric content (at low pH): water solubility of protonated species that should be enhanced if administered as protonated form ; BAs with a poor solubility could be modified for their solid state crystalline structure trying strategy to improve the solubility such as micronization or amorphization

-Potential cytotoxicity due to modification of membrane structure by interaction and aggregation with lipids and proteins: detergency (critical micellar concentration, CMC);

- Efficiency and mechanism of intestinal absorption through the enterocyte membrane (passive or active-carrier mediate), body fluid distribution and toxicity: lipophilicity (partition coefficient, $LogP_{o/w}$):

- Blood transport and hepatic uptake: albumin binding affinity (%)

Analogues with a sulphate or sulphonate side chain head or carboxylate with a 23-substituent with electron withdrawing effect (fluorine or hydroxyl) are able to lower the pK_a down to 2. They are always present in the body as organic anions and therefore never absorbed by the intestine by passive diffusion, even if relatively lipophilic with a LogP>1 and at all the physiological pH.

BAs with a conventional side chain carboxyl group can be protonated at the enterocyte micro-environment and therefore absorbed by passive diffusion only if they are lipophilic (with a LogP>1.5-2). They are soluble only at a pH > 7 and when protonated (pH < 3), their solubility falls down and they are practically insoluble.

These changes in physico-chemical properties could lead to modify transport through the cells lipophilic membranes and therefore the intestinal absorption, the hepatic uptake and in general biodistribution and metabolism.

Using , the in vivo experimental “bile fistula rat” model it was possible to evaluate by measuring the administered compound secreted in bile and simultaneously the plasma concentration the pharmacokinetic and metabolism of these compounds are therefore better definer the relationship between structure and pharmacokinetic and to classify these new compounds in three categories:

- ***Enterohepatic***: the analogues are efficiently absorbed when administered orally (id) either by active (through protein carrier) or passive (direct) mechanism and efficiently taken up by the liver from portal blood with a low spill-over in the systemic compartment. An efficient secretion in bile of the compounds or its hepatic metabolites (taurine conjugated) is associated with an efficient intestinal reabsorption and therefore an accumulation in the enterohepatic circulation with a minimal amount in the systemic circulation (less than 10%)
- ***Systemic***: the analogues are absorbed when orally administered (id) and will be preferentially distributed in peripheral blood due to a relatively low hepatic uptake by the portal vein resulting in a consistent spill-over in the systemic compartment. Additionally a back diffusion from the liver could be involved. Metabolites should be excreted in stools and urine and poorly reabsorbed.
- ***Intestinal restricted***: poor absorbed when administered orally (id) for low lipophilicity or lack of active transporters. Soluble in the intestinal content and poorly metabolized by intestinal bacteria.

As far as the CCl4 inhalation induced cirrhosis rat model this was developed to enter in more inside to the pharmacokinetic and metabolism of these molecules when used for the treatment of cholestatic liver disease where the liver functions is highly impaired. In the case of Obeticholic acid

(OCA), the animal model with hepatic decompensation due to cirrhosis induction is very useful to obtain more accurate and predictive informations, regarding its biodistribution and metabolism in human disease particularly excluding an excessive accumulation of the molecule in the liver.

Despite the high variability of the data derived from the decompensated cirrhosis model, OCA behaves in term of biodistribution in the different organs almost like endogenous analogue CDCA having similar structure and physicochemical properties.

The liver impairment caused by CCl_4 inhalation affect the endogenous BAs hepatic uptake and biliary secretion and on turn the hepatic metabolism of unconjugated BA to produce more hydrophilic taurine and glucuronide conjugates that facilitate their excretion from the liver in the bile or from blood to urine.

The relative high accumulation of OCA, like endogenous BAs, activates the conjugation metabolic pathway to form uncommon glycine (for rat) conjugate and glucuronide. This phenomenon is dose-response and different among animal species. In human this will be less relevant in respect to rat because the endogenous BA pool is more lipophilic and therefore more tolerant to OCA. In addition, the glycine conjugation metabolism is predominant in respect to rat, where the endogenous BAs are almost conjugated with taurine.

The percent recovery of the administered OCA and its main metabolites in the different organs is up to 95% after 24 hours and 97% after 48 hour from the oral administration.

Moreover, the high OCA levels reflect the overall distribution of endogenous BAs pool in the systemic compartment as a result of the liver impairment caused by the CCl_4. No selective or peculiar plasma levels have been observed. Similarly and expected behavior in the liver and small intestine.

The OCA administration could affect the endogenous BA pool qualitative composition since as expected the compound accumulate in the enterohepatic circulation like previous administered BAs (i.e. UDCA).

Based on these overall conclusions, the collaboration project with Servier, Intercept Pharmaceutical and University of Perugia, proposes to continue these kind of analysis adding new rapid screening methods and cell models, using bio-chemiluminescence, to know the activity for FXR and TGR5 receptors of new synthetic bile acid.

Furthermore an appropriate multivariate statistical analysis of the measured physico chemical properties and biodistribution parameters could be performed in order to create a predictive model for the physiological behavior of future semi-synthetic analogues.

All these proposals could lead to design other new candidate analogues in order to discover promising novel drugs for the treatment of hepatic and metabolic disorders as PBC, NASH, obesity and diabetes, increasingly frequent in western countries.

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