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Studies on the oxysterols 4α- and 4β-hydroxycholesterol

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Abstract

Cytochrome P450 (CYP) enzymes catalyze the metabolism of both foreign and endogenous substances such as drugs and steroids. Enzymes in subfamily CYP3A sum up to 30% of the total CYP content in the liver and metabolize about 50% of all drugs. There is a large inter-individual variability in expression and activity of hepatic CYP3A, mainly due to ethnic, age- and gender-related factors. The CYP3A activity is induced by certain drugs, such as the anti-epileptic drug carbamazepine (CBZ).

Plasma midazolam clearance and the 6β-hydroxycortisol to cortisol ratio in urine have been proposed as clinical markers of CYP3A activity. The suitability of these markers has been discussed as they are difficult to use in for example children and pregnant women due to technical and ethical issues.

The plasma level of the endogenous oxysterol 4β-hydroxycholesterol (4b-OHC) has shown to be a marker of CYP3A activity. In the present project we have studied whether 4b-OHC can be used as a marker of drug induced CYP3A activity in pediatric patients after initiation of treatment with CBZ (Paper 2) and in mothers and neonates at time of birth (Paper 3). In order to increase sample throughput the sample preparation method was optimized (Paper 1). The similar oxysterol 4α-hydroxycholesterol, 4a-OHC, not formed by CYP3A, was determined in parallel (Papers 2-3).

When the sample preparation method was optimized for analysis of 4b-OHC the sample throughput increased about three times by scaling down the sample volume and using solid phase extraction instead of liquid-liquid extraction followed by rotary evaporation. The linear correlation between the two methods was y=1.0x-2.1, r2 = 0.99 (y=new method, n=90).
The plasma level of 4b-OHC was successfully used as a marker of drug induced CYP3A activity in the study of CBZ treatment in children with epilepsy. The CBZ treatment resulted in increased plasma levels of 4b-OHC until at least 8 weeks of treatment. According to the steady plasma levels of CBZ and CBZ-epoxide there was a complete induction of CYP3A within 1-2 weeks and the continued increase of 4b-OHC levels in circulation may be due to slow equilibriums between different compartments.
4b-OHC proved useful also in the study on CYP3A activity in mothers and neonates. Mothers had higher 4b-OHC to cholesterol ratio at delivery as compared to non-pregnant women,

indicating increased CYP3A activity during pregnancy. Also the plasma levels of cholesterol and 4b-OHC were higher in the mothers than in the cohort of non-pregnant women. Neonates had lower levels of plasma 4b-OHC and cholesterol at birth as compared to the levels in a cohort of 125 healthy adults. However, the 4b-OHC to cholesterol ratios were similar, indicating similar total CYP3A enzyme activity in neonates as in adults.

In conclusion, 4b-OHC is a non-invasive marker of CYP3A enzyme activity that is easy to use in neonates, children and vulnerable patient groups where probe drugs are difficult or unethical to administer or urine collections are difficult to perform. The blood sample can be taken any time of the day irrespective of food intake which is beneficial in a clinical setting.

List of publications

1. Diczfalusy U, Nylén H, Elander P, Bertilsson L. 4β-Hydroxycholesterol, an endogenous marker of CYP3A4/5 activity in humans. *Br J Clin Pharmacol*. **71**, 183-189 (2011).

2. Wide K, Larsson H, Bertilsson L, Diczfalusy U. Time course of the increase in 4β-hydroxycholesterol concentration during carbamazepine treatment of paediatric patients with epilepsy. *Br J Clin Pharmacol*. **65**, 708-715 (2008).

3. Nylén H, Sergel, S, Forsberg, L, Lindemalm, S, Bertilsson, L, Wide, K, Diczfalusy, U. Cytochrome P450 3A activity in mothers and their neonates as determined by plasma 4β-hydroxycholesterol. *Eur J Clin Pharmacol*. doi: 10.1007/s00228-010-0984-1 (2011).

Abbreviations

ABCG1	ATP-binding cassette, subfamily G, member 1
BHT	butylated hydroxytoluene
CAR	constitutive androstane receptor
CBZ	carbamazepine
CBZ-E	carbamazepine-10,11-epoxide
CH25H	cholesterol 25-hydroxylase
CS	caesarean section
CSF	cerebrospinal fluid
CYP	cytochrome P450
CYP2C19	limonene-6-monooxygenase
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4
CYP7A1	cholesterol 7α-hydroxylase
CYP27A1	sterol 27-hydroxylase
CYP46A1	cholesterol 24-hydroxylase
DHEA	dehydroepiandrosterone
GC	gas chromatography
HDL	high density lipoprotein
LC	liquid chromatography
LXR	liver X receptor
MS	mass spectrometry
4a-OHC	4α-hydroxycholesterol
4b-OHC	4β-hydroxycholesterol
7a-OHC	7α-hydroxycholesterol
7b-OHC	7β-hydroxycholesterol
24-OHC	24S-hydroxycholesterol
27-OHC	27-hydroxycholesterol
PXR	pregnane X receptor
SI	silica
SIM	single ion monitoring
TBDMS	tert-butyldimethylsilylether
TMS	trimethylsilylether

Contents

Introduction

The liver is a vital organ with multiple functions such as synthesis and breakdown of hormones, carbohydrates, proteins and lipids, as well as storage of vitamins and minerals. Production of bile, detoxification and drug metabolism also takes place in the liver. During drug metabolism lipophilic substances are converted into more readily excreted polar products. Drug metabolism is divided into two steps, Phase I (oxidation, reduction and hydrolysis) and Phase II (conjugation) handled by various enzymes, such as cytochrome P450 enzymes (CYP) and glucoronosyltransferases. These enzymes are abundant in the smooth endoplasmic reticulum or mitochondria in liver cells. The pharmacological action of a drug is determined by the speed of the Phase I and II reactions, which is subject to individual variation due to genetic, environmental and physiological factors.

Drug metabolism takes place in other organs and tissues as well, such as the gastrointestinal tract, lungs, kidneys and skin. In fact, all biological tissue has the ability to metabolize drugs to some extent.

Cytochrome P450 and CYP3A

Humans have around 60 different cytochrome P450 (CYP) enzymes with a wide array of substrates and functions. The CYP enzymes are classified into different families and subfamilies based on the amino acid sequence similarity. In general, the enzyme families CYP1, CYP2 and subfamily CYP3A are important in Phase I metabolism of drugs, but these enzymes are able to metabolize several endogenous substances such as steroids and bile acids as well. Enzymes participating in drug metabolism generally have wide substrate specificity and are predominately localized to the liver and intestine, which serve as the main detoxification site and the main barrier against ingested substances, respectively. Since increased hydrophilicity targets a compound for excretion the CYP-mediated Phase I hydroxylation and the subsequent Phase II conjugation serve as a key defense against foreign and potentially harmful substances. A few CYP enzyme families are mainly specialized in biosynthesis of steroid hormones and bile acids and have narrower substrate specificity, e.g. CYP7, CYP11 and CYP21.

Nearly all prescribed drugs are oxidized by the enzymes in subfamilies CYP3A (50% [1]), CYP2C or CYP2D (20 and 25%, respectively [2]), making these enzymes the quantitatively most important in drug metabolism.

There are four CYP3A enzymes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43. These are similar and have overlapping, but not similar, substrate specificities. CYP3A enzymes have a large active site that can accommodate several structurally different molecules, possibly simultaneously and the substrate specificity is thus very broad.

CYP3A4 is the most abundant CYP3A enzyme and is the dominating enzyme in drug metabolism. It is predominantly expressed in liver and intestine, but is present also in other organs and tissues.

CYP3A5 has an 84% amino acid sequence similarity with CYP3A4 and is also predominantly expressed in liver and intestine. CYP3A5 is subject to a marked genetic variation (polymorphism) and its expression is abundant in some individuals and virtually non-existent in others.

CYP3A7 has an 88% amino acid sequence similarity with CYP3A4. CYP3A7 is the most abundant CYP3A enzyme in human fetal and neonatal liver and placenta [3-5]. A transition from expression of CYP3A7 to expression of CYP3A4 seems to take place during the first years of life [5-6]. However, there are reports on substantial contribution to total CYP3A activity from CYP3A7 also in adults [7-9].

CYP3A43 has a 76% amino acid sequence similarity with CYP3A4 [10]. CYP3A43 is expressed at very low levels in the liver and intestine and has therefore been regarded to be less important in drug clearance. However, the expression of CYP3A43 has shown to be high in the brain and a recent report suggests that polymorphism of this enzyme can explain differences in olanzapine clearance in the brain [11]. Olanzapine is an anti-psychotic drug that has a high rate of inefficacy and/or adverse effects.

Certain genetic variants of CYP3A4, CYP3A5 and CYP3A43 have been associated with the risk of prostate and breast cancer [12-13].

The activity of CYP3A4 is increased during pregnancy [14-16], possibly due to one or several of the physiological changes that occur, e.g. reduced intestinal movement, altered gastric pH, increased plasma volume, increased hormone levels and/or fetal enzyme activity [14]. The general advice during pregnancy is to avoid drugs, but certain conditions call for continuous

medication, e. g. epilepsy and HIV. The anti-epileptic drugs carbamazepine (CBZ), phenytoin and phenobarbital are all known to induce CYP3A. The same is true for the anti-retroviral agent efavirenz. However, anti-retroviral therapy with ritonavir-boosted atazanavir inhibits CYP3A.

Pregnancy has an effect also on other CYP enzymes. The activities of CYP2D6 and CYP2C9 are increased during pregnancy [14, 16-18], while the activity of CYP1A2 and CYP2C19 are decreased [16, 19-20].

Many drugs that are given to children and neonates are not licensed for pediatric use or are given by off-label prescription. Ethical aspects make it difficult to define pediatric pharmacokinetics in clinical trials. Children and neonates are often prescribed weight adjusted dosing of drugs based on the pharmacokinetics in adults, but precaution must be taken since the physiology of neonates is different compared to the physiology of adults and older children. As examples, neonates have higher gastric pH, lower gastrointestinal motility, lower serum proteins levels and larger extra cellular water volume than older children and adults, which may have an effect on the bioavailability of the drug [21-22]. For some drugs a lower than the weight adjusted dose is necessary in order to avoid toxic effects. For others, such as the anti-epileptic drug valproate a higher dose than the weight adjusted dose is needed in order to reach therapeutic concentrations [23].

Polymorphism is the occurrence of multiple variants of any given gene. Generally, at least 1% of a certain population must carry a certain genetic variant in order for it to be regarded as a true variant; otherwise it is referred to as a single mutation. The polymorphic trait of CYP2D6, CYP2C19 and CYP2C9 variants are well characterized. The enzyme function may be described as poor, intermediate, extensive or ultra-rapid. Prior to medication it is recommendable to determine the patients' genotype in order to avoid adverse drug reactions.

CYP3A5 has shown to be the most polymorphic of the CYP3A enzymes. We have found that 74% of Tanzanians, 13% of Swedes and 33% of Koreans express functional CYP3A5 [24-25]. CYP3A5 is expressed when there is at least one CYP3A5*1 allele. In these cases CYP3A5 is estimated to comprise 50% of the total CYP3A content in liver [26]. The CYP3A5 contribution to drug metabolism is thus very important for some individuals. The most common reasons for the loss of CYP3A5 activity are single nucleotide polymorphisms

(SNPs) yielding prematurely truncated and thus non-functional enzymes, as are the cases with the variants CYP3A5*3 and CYPA5*6 [26]. There are some reports on mutations in *CYP3A4*, but these mutations can not explain the variability in expression and activity of the enzyme [13].

There is large inter-individual variability in expression (≥50-fold) and activity (≥20-fold) of hepatic CYP3A, mainly due to ethnic and gender-related factors [1, 24, 27]. This together with physiological and environmental factors causes wide inter-individual differences in metabolism and response to drugs. Life style factors and diet also have a role in the drug response, e.g. St John's Worth induce and grapefruit juice inhibits CYP3A enzyme activity.
The wide inter-individual variability in expression of CYP3A4 may be a result of a very complex upstream regulatory region of *CYP3A4*, carrying multiple possible nuclear receptor binding sites for the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the Vitamin D receptor (VDR) and the glucocorticoid receptor [28-30]. These receptors control both the basal and inducible expression of CYP3A and ensure sensitivity to a number of structurally diverse substrates [30]. It may be so that not only the expression of CYP3A but also the induction of CYP3A shows large inter-individual variations and the possibility that polymorphisms in *PXR* are involved have been discussed [31].

A number of clinical markers to assess the activity of CYP3A enzymes have been proposed. The results are diverging, however, possibly due to the differences between the studied enzymes both concerning substrate specificity and product formation.
In vitro incubations with dehydroepiandrosterone (DHEA) and recombinant CYP3A4 produced the two major products 7β-hydroxy-DHEA and 16α-hydroxy-DHEA at a ratio 2:1. The same incubation with recombinant CYP3A7 produced almost exclusively 16α-hydroxy-DHEA [5]. CYP3A7 has been shown to catalyze the 16α-hydroxylation of DHEA and DHEA-sulfate also in vivo [8-9]. 6β-hydroxylation of testosterone is almost exclusively catalyzed by CYP3A4 and not by CYP3A5 or CYP3A7 in in vitro incubations with recombinant enzymes [32]. If the same is true also in vivo remains to be investigated.

The pharmaceutical industry is interested in studying the effect of candidate drugs on the CYP3A activity. Plasma midazolam clearance, erythromycin breath test and the 6β-hydroxycortisol to cortisol ratio in urine are generally used as markers of CYP3A activity.

In plasma midazolam clearance the 1-hydroxymidazolam to midazolam ratio is measured repeatedly in plasma after oral or intravenous administration of the drug. Midazolam is a benzodiazepine, and is used in treatment of acute seizures and as a sedative. In the erythromycin breath test radio-labeled N-methyl-erythromycin is given intravenously and the amount of exhaled radio-labeled carbon dioxide is measured. The 6β-hydroxycortisol to cortisol ratio in urine is an endogenous marker, but the diurnal variation of cortisol calls for at least 4h and preferably 24h collections of urine.

Accordingly, none of these markers can be recommended for use in children, neonates, the elderly, certain groups of vulnerable patients or pregnant women due to technical and/or ethical issues.

Cholesterol as a substrate for CYP3A

Cholesterol is of vital importance for the body and is found in all mammalian cells where it has several roles. It serves as a structural element in the cell membranes and lipid bilayers and is a precursor for many biologically active products such as steroid hormones and bile acids.

The homeostasis of cholesterol is strictly regulated as excess cholesterol in cells may be harmful and lead to atherosclerosis and cardiovascular disease.

Excess cholesterol may be stored as free cholesterol or cholesterol esters in the cells or may be transported as such from the cells to the liver by reverse cholesterol transport (RCT), which is central in whole body cholesterol balance. Once in the liver the cholesterol can be enzymatically processed into oxysterols and bile acids or be excreted as such. Cytochrome P450 enzymes are not only drug clearing enzymes, but play an important role in the maintenance of the whole body cholesterol balance as they oxygenate cholesterol to oxysterols and metabolize a number of other endogenous substances such as other steroids, fatty acids and vitamins. Generally, CYP enzymes in families 1–3 handle the metabolism of both drugs and endogenous substances, while CYP enzymes in families 4 and on mostly handle the metabolism of endogenous substances.

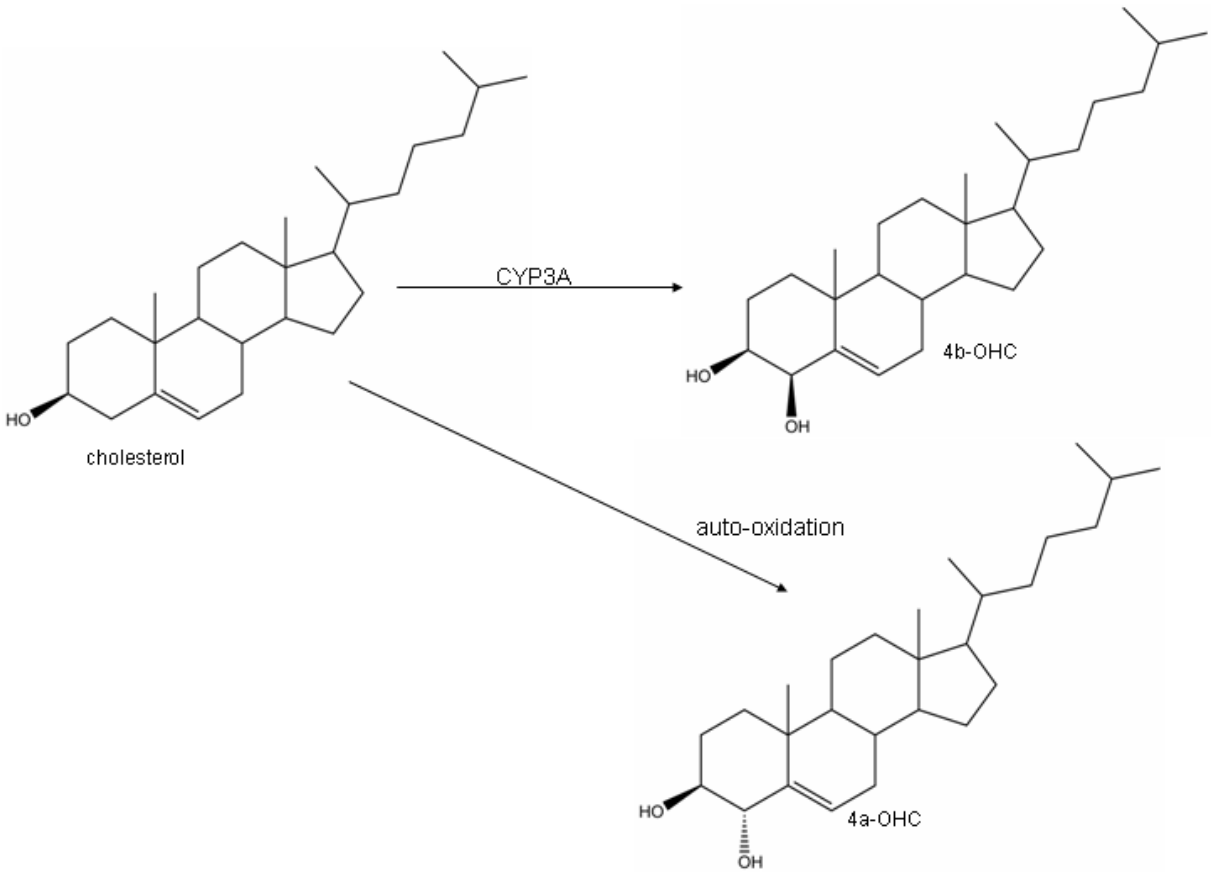

Figure 1: Formation of 4a-OHC and 4b-OHC from cholesterol.

CYP3A4 and CYP3A5 have been shown to stereo-selectively metabolize cholesterol to the oxysterol 4β-hydroxycholesterol (4b-OHC) in vivo (Figure 1) [24, 33-34]. CYP3A7 catalyses the in vitro conversion of cholesterol to 4b-OHC, but at a lower rate than CYP3A4 [35]. The in vivo 4β-hydroxylase activity of CYP3A7 is not known, neither is the in vitro nor in vivo 4β-hydroxylase activity of CYP3A43. The mechanism of formation of the stereoisomer 4α-hydroxycholesterol (4a-OHC) is still unknown, but it may be formed by auto-oxidation (Figure 1).

Oxysterols

Oxysterols are oxygenated derivatives of cholesterol which are formed either enzymatically or by auto-oxidation. 4b-OHC is abundant in human circulation in the nanomolar range and is primarily formed enzymatically by CYP3A [36]. Other enzymatically formed oxysterols abundant in the human circulation are 27-hydroxycholesterol (27-OHC) and 24-hydroxycholesterol (24-OHC) [37], both present in the nanomolar range. The formation of 27-OHC is catalyzed by CYP27A1, which is a mitochondrial enzyme present abundantly in

the liver where it takes part in the bile acid synthesis. CYP27A1 is expressed in most tissues in the body.

The enzyme CYP46A1 is exclusively expressed in neuronal cells in the brain where it catalyses the formation of 24-OHC. 24-OHC can cross the blood brain barrier and its formation contributes to the cholesterol homeostasis in the brain.

The plasma level of 24-OHC as well as the levels of 24-OHC and 27-OHC in cerebrospinal fluid (CSF) have been proposed as markers of neurodegenerative disease and blood brain barrier dysfunction in diseases such as Alzheimer's disease, Huntington's disease and multiple sclerosis (MS) (reviewed in [38]).

25-Hydroxycholesterol (25-OHC) is another side-chain oxidized oxysterol. This oxysterol is formed enzymatically by cholesterol-25-hydroxylase (CH25H), which is not a CYP enzyme but a dioxygenase. 25-OHC has been reported to have a possible role in inflammatory response [39].

Yet another oxysterol formed enzymatically from cholesterol is 7a-OHC, by the enzyme CYP7A1. The formation of 7a-OHC is the first and rate limiting step in the bile acid synthesis. 7a-OHC can also be formed by auto-oxidation, along with 7b-OHC and 7-ketocholesterol. The carbon at position 7 in the cholesterol molecule is highly susceptible to auto-oxidation. The carbon at position 4 is also susceptible to auto-oxidation, but to a much lesser extent [40].

4a-OHC may have auto-oxidative origin and the formation of this stereoisomer is not catalyzed by CYP3A [40], but possibly by other, still unknown, enzyme(s). The enzymatic formation of a few oxysterols as well as the numbering of the cholesterol molecule is shown in Figure 2.

The biological roles of oxysterols include being intermediates in the bile acid synthesis and mediators in the cholesterol transport. Some oxysterols have been proposed as specific ligands for liver X receptors (LXRs), which induce transcription of genes regulating the cholesterol efflux, absorption, transport and excretion.

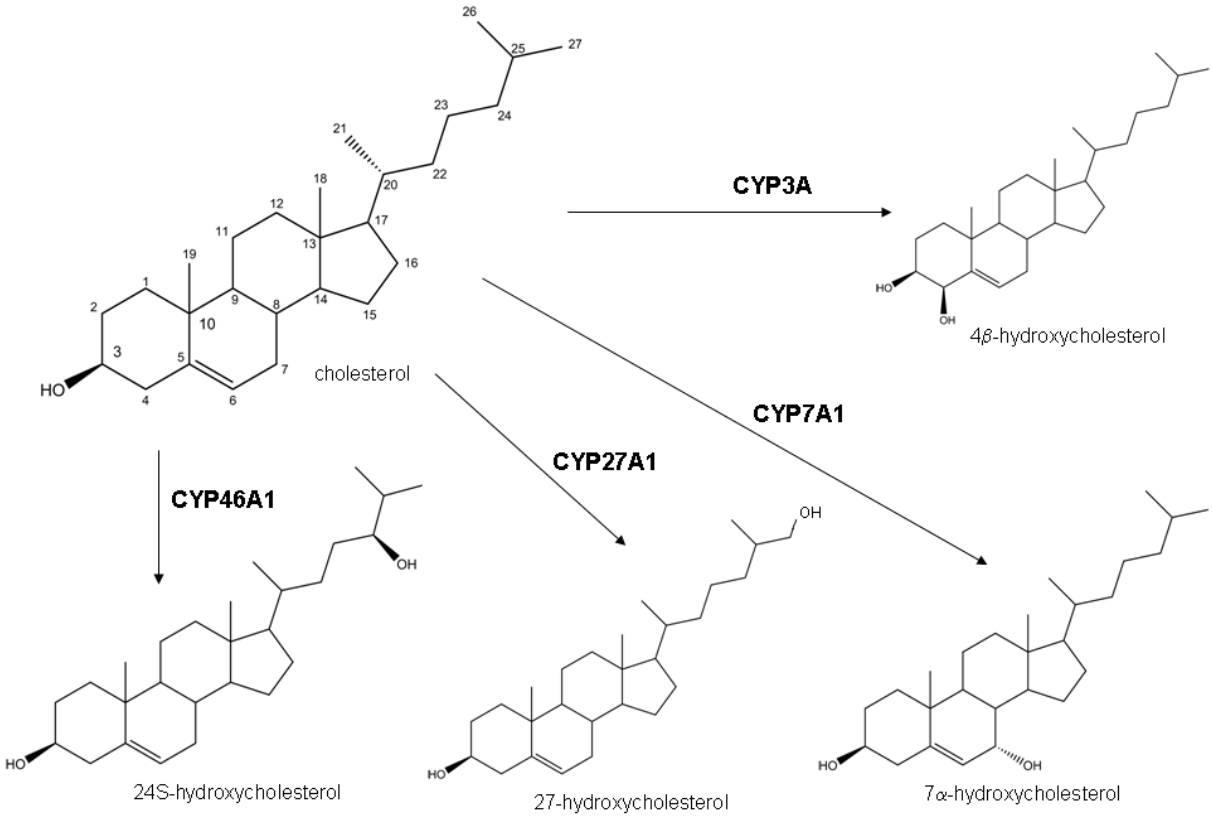

Figure 2: CYP enzymatic formation of oxysterols from cholesterol.

4β-hydroxycholesterol as a marker of CYP3A enzyme activity

The plasma level of 4b-OHC has been shown to be related to the induction and inhibition of the CYP3A enzyme activity. The plasma levels of 4b-OHC are markedly increased by treatment of patients with any of the anti-epileptic drugs CBZ, phenytoin and phenobarbital, all strong inducers of CYP3A [33]. The same is true for rifampicin, a drug used in tuberculosis therapy. Rifampicin treatment with three different doses resulted in dose dependent increases of 4b-OHC levels [41]. After termination of rifampicin treatment the plasma level of 4b-OHC decreased again [42]. Weak CYP3A inducers, such as ursodeoxycholic acid which is used in treatment of gallstone disease, increased 4b-OHC only moderately [33].

Moreover, there is a lowering effect on the plasma level of 4b-OHC by anti-retroviral therapy with the CYP3A4/5 inhibitor ritonavir-boosted atazanavir [43].

The plasma level of 4b-OHC and the 4b-OHC to cholesterol ratio have been shown to be correlated to the number functional CYP3A5 alleles (CYP3A5*1) in four populations

(Swedes, Tanzanians, Koreans and Ethiopians) [24, 34]. The mean basal level of 4b-OHC differs between these populations (Table 1) and it is noteworthy that the Tanzanian population had the lowest basal level of 4b-OHC while highest degree of functional CYP3A5. This is possibly due to lifestyle or unknown genetic factors [24].

Both 4b-OHC and cholesterol have half-lives of several days [42, 44] which makes the plasma level of 4b-OHC stable over time. This is useful in a clinical setting as the blood sample can be taken at any time of the day and irrespective of food intake. The long half-life of 4b-OHC is useful in situations where long time changes are studied, e. g. how long term treatment with an inducer affects the enzyme activity during the course of weeks or months rather than days. The plasma level of 4b-OHC reflects the average enzyme activity over a period of time rather than the momentaneous enzyme activity.

When medication or other factors, such as pregnancy cause changes in the plasma levels of cholesterol during the study period it is advisable to use the 4b-OHC to cholesterol ratio to correct for a possible change in substrate pool size [45-46].

Table 1: Mean basal level of 4b-OHC and allele frequency of CYP3A5*1 in Swedes, Tanzanians, Koreans and Ethiopians [24, 34].

	Swedes (n=161)	Tanzanians (n=138)	Koreans (n=149)	Ethiopians (n=150)
4b-OHC (ng/mL)	26.8 ± 9.1	21.9 ± 7.5	29.3 ± 8.6	35.4 ± 11.6
CYP3A5*1 (%)	7	51	17	20.5

Analysis of 4α- and 4β-hydroxycholesterol

4a-OHC and 4b-OHC are present in plasma in the nanomolar range and precautions during storage, handling and processing are needed in order to minimize artifact formation and cholesterol auto-oxidation.

Therefore, samples were taken in EDTA-containing tubes and after centrifugation plasma was stored at -70°. Immediately after thawing the anti-oxidant butylated hydroxytoluene (BHT) was added to the samples. 83% of 4b-OHC and 70% of 4a-OHC are present in conjugated form in plasma [33]. With analysis and detection by gas chromatography – mass spectrometry (GC-MS) oxysterols must be in the free form and alkaline hydrolysis was performed in order to be able to measure total oxysterols. For 4a-OHC and 4b-OHC the TBDMS-ethers has shown increased sensitivity as compared to the more commonly used derivate trimethylsilylesters (TMS) [36].

Aims of the study

Optimize the sample preparation method for 4b-OHC in order to increase sample throughput (Paper 1).

Determine if it is possible to use 4b-OHC as a marker CYP3A activity

- In children after initiation of treatment with the CYP3A inducer CBZ (Paper 2).
- In mothers and neonates at birth (Paper 3).

Materials and methods

The objective of papers 1-3 and the methods used are stated briefly here. Please refer to each paper for further details.

Paper 1

In Paper 1 we optimized the sample preparation for subsequent analysis of plasma levels of 4a-OHC and 4b-OHC. The original sample preparation procedure which is outlined below was developed for the simultaneous determination of nine oxysterols. It is accurate, but very laborious and time consuming. Some of the precautionary steps taken are not needed for analysis of 4a-OHC and 4b-OHC, but for analysis of oxysterols more prone for auto-oxidative formation.

In the original method hydrolysis was performed under argon by adding 0.35 M ethanolic potassium hydroxide (KOH) (10 mL) to a vial with a Teflon lined cap containing 1 mL plasma, 200 μg EDTA (10 mg/mL water), 50 μg BHT (5 mg/mL water) and 100 ng internal standard (d_6-4b-OHC, 2 μg/mL methanol). The samples were kept at room temperature for 2h with continuous stirring. The pH was adjusted to 7 with phosphoric acid and the reaction mixture was transferred to a separatory funnel. 18 mL of chloroform and 6 mL 0.15M NaCl were added. After separation the organic phase was transferred to a round bottom flask and rotary evaporated. Samples were then reconstituted in toluene (1 mL). Silica (SI) solid phase extraction columns (International Sorbent Technology, Mid Glanorgan, UK) were preconditioned with 2 mL hexane. The sample (1 mL in toluene) was put on the column, followed by 1 mL hexane. Cholesterol was eluted with 8 mL 0.5% 2-propanol in hexane. Oxysterols were eluted with 5 mL 30% 2-propanol in hexane. After evaporation, oxysterols were derivatized into tert-butyldimethylsilyl-ethers using tert-butyldimethylsilylimidazole-dimethylformamide (100 μL) at room temperature prior to addition of water (1 mL) and extraction twice with ethyl acetate (2x1 mL). After evaporation the samples were reconstituted in hexane and analyzed by GC-MS (Hewlett Packard 5890 Series II Plus gas chromatograph equipped with a HP-5MS capillary column, 30m x 0.25mm x 0.25 μm phase thickness, connected to a Hewlett Packard 5972 mass selective detector). Single ion monitoring (SIM) of m/z 573, (4a- and 4b-OHC) and 579 (internal standard, d_6-4b-OHC), was used for quantification.

For optimization of the sample preparation procedure different hydrolysis conditions (concentration, temperature and duration) were evaluated. For separation different solid phase extraction columns (Strata-X, Extrelut, SI) and elution conditions (% acetonitrile in water) were tested.

A seven-level calibration curve (0-200 ng/mL) made from appropriate amounts of 4b-OHC in methanol (Steraloids, Newport, RI) and a fixed amount of internal standard, d_6-4b-OHC, in methanol (synthesized as described in [36] and quality control samples were prepared along with the samples in each run.

Paper 2

In Paper 2 we monitored the plasma levels of 4b-OHC, 4a-OHC, CBZ, CBZ-epoxide (CBZ-E) and cholesterol in pediatric patients with newly diagnosed epilepsy treated with CBZ during 0-23 weeks. The CYP3A5 genotype was also determined. Ten consecutive patients were recruited whereof eight completed the study. Plasma 4b-OHC and 4a-OHC were analyzed by isotope dilution GC-MS using deuterium-labeled internal standard as described before [37]. CBZ and CBZ-E were determined in plasma by HPLC-UV as described previously [47]. Plasma cholesterol was determined by a commercial enzymatic method, Chod-PAPP (Roche Diagnostics, Mannheim, Germany). The single nucleotide polymorphism (SNP) A6986G (CYP3A5*3) was analyzed in all subjects as described previously [25]. When the CYP3A5*3 allele was not detected the allele was designated CYP3A5*1. Statistical calculations were done using Statistica version 7.1 (StatSoft Inc., Tulsa, OK, USA).

Paper 3

In Paper 3 we monitored the plasma levels of 4b-OHC, 4a-OHC and cholesterol in mothers and neonates at time of birth and four months post partum using the same methods as in Paper 2. The CYP3A5 genotype was also determined. 21 mothers and 22 neonates were recruited whereof 10 mothers and 11 neonates completed the study.

Blood sampling was as follows:

Vaginal delivery: Venous blood samples were collected from mother and neonate at the time of the neonatal screening and at the four month follow up.

Elective caesarean section (CS): Blood samples were collected from mother prior to the operation and from the cord blood immediately after birth. Venous blood samples were collected from mother and neonate at the four month follow up.

Statistical calculations were done using Statistica version 8. Plasma levels of 4b-OHC, 4a-OHC and cholesterol were also analyzed in one mother and one neonate after maternal anti-epileptic treatment with CBZ during pregnancy.

Results and discussion

Paper 1

In Paper 1 we developed an optimized sample preparation method for the subsequent analysis of 4a-OHC and 4b-OHC. The original method is still widely used for analysis of oxysterols, both in our and other laboratories [37]. With the original method a large number of oxysterols were measured simultaneously, i.e. 7a-OHC, 7b-OHC, 24-OHC, 25-OHC, 27-OHC, 7-ketocholesterol, the cholesterol-5,6-epoxides and 5α-cholestane-3β,5α,6β-triol (cholestane-triol). This wide array of oxysterols shows different chemical properties and different susceptibility towards auto-oxidation or pH-dependent decomposition. Some alterations from the original method had already been made in the sample preparation of 4a-OHC and 4b-OHC prior to the present work, and these are mentioned below or in the section "Analysis of 4α- and 4β-hydroxycholesterol".

The large sample volume in the original method (1 mL) was needed because of the mass spectrometric detection limit for some of the oxysterols present in very low amounts, i.e. 25-OHC and 7b-OHC (~2 and ~3 ng/mL, respectively).
4b-OHC is present in plasma at ~30 ng/mL, and it was estimated that the sample volume could be reduced by at least a factor 4 without detection problems. Hence, a sample volume of 250 μL was chosen. The hydrolysis solution volume was reduced from 10 mL to 1 mL.
For hydrolysis two different KOH concentrations were tested, 0.7M and 0.35M at three different temperatures: 20°C (room temperature), 37°C and 54°C. The time allowed for hydrolysis with the different conditions ranged from 5 minutes to 2h. With the original method hydrolysis was performed with 0.35M ethanolic KOH during 2h at room temperature because there was a significant loss of 7-ketocholesterol if either temperature or pH was increased. In the original method this step was performed under argon in order to protect cholesterol from auto-oxidation. Such precaution was not needed when analysing only 4a-OHC and 4b-OHC.

The optimization of the hydrolysis conditions were done stepwise:
First, hydrolysis with 0.35M ethanolic KOH during 30 minutes, 1h or 2h was performed at 20°C, 37°C and 54°C. Samples were then processed according to the original method. The changes in hydrolysis conditions did not cause any differences in the analyzed 4a-OHC and 4b-OHC concentrations. This was true also when the hydrolysis was performed with either

0.35M or 0.7M ethanolic KOH during 30 minutes at room temperature. We concluded that 0.7M ethanolic KOH could be used for hydrolysis during 30 minutes at room temperature, hence reducing the hydrolysis time by at least 90 minutes while maintaining the efficiency. Shorter hydrolysis times were also investigated (5, 10 and 20 minutes) without major changes in the analyzed 4a-OHC and 4b-OHC concentrations. However, it was believed that a shorter hydrolysis time could have a negative impact the method variability (CV). The hydrolysis time was therefore set to 30 minutes.

In the original method, oxysterols were extracted from the 10 mL hydrolysis solution by liquid-liquid extraction with 18 mL chloroform. This large volume needed to be rotary evaporated, which was very time consuming. This was also the bottle neck in the sample processing procedure as the number of samples that could be processed simultaneously was limited to four, which was the number of available rotary evaporators.

The reduced hydrolysis volume used in the new method allowed faster extraction. Two solid phase extraction columns were tested for this purpose: Strata-X and Extrelut. The SI-column used in the original method was used as a reference. The recovery of the internal standard with the three columns was highest with the SI-column and lowest with the Extrelut-column (15-fold lower). Therefore, the Extrelut-column was omitted from further experiments.

A number of different elution concentrations were investigated for the Strata-X-column: 70, 80, 85 and 90% (v/v) acetonitrile in water. 85% (v/v) acetonitrile in water showed acceptable recovery of 4a-, 4b-OHC and the internal standard. However, cholesterol co-eluted with 4a-OHC and 4b-OHC. The Strata-X-column was washed with 10% (v/v) methanol in water prior to elution of the oxysterols and cholesterol.

To summarize, a faster sample preparation method for the subsequent analysis of 4b-OHC on GC-MS was developed. The differences in sample preparation with the original and new methods are highlighted in Table 2. Briefly, in the new method hydrolysis was performed by adding 0.7 M ethanolic potassium hydroxide (1 mL) to a glass centrifuge tube containing 250 µL plasma, 200 µg EDTA (10 mg/mL water), 50 µg BHT (5 mg/mL water) and 100 ng internal standard (d_6-4b-OHC, 2 µg/mL methanol). After vortex mixing (30 seconds) the samples were kept at room temperature for 30 minutes without stirring. Before centrifugation (10 min, 1000g) the pH was adjusted to 7 with 17% (v/v) phosphoric acid in water. For extraction the samples were put on preconditioned (methanol, 1 mL) and equilibrated (deionized water, 1 mL) Strata-X 30 mg/1mL SPE columns (Skandinaviska Genetec AB, Västra Frölunda, Sweden). The columns were washed with 10% (v/v) methanol in water. The

samples were eluted with 85% (v/v) acetonitrile in water (1 mL). The eluates were evaporated to dryness under argon before derivatization and analysis by GC-MS as described before [33].

Table 2: Differences between the new and original sample preparation methods.

	New method	Original method
Plasma	250 µL	1 mL
Hydrolysis	0.7 M ethanolic KOH (1 mL) 30 min no stirring, room temperature	0.35 M ethanolic KOH (10 mL) 2h stirring, room temperature
Extraction 1	Strata-X solid phase To evaporate: 85% (v/v) acetonitrile in water (1 mL)	Liquid-liquid extraction To evaporate: chloroform (18 mL)
Extraction 2		SI solid phase To evaporate: 30% (v/v) 2-propanol in hexane (5 mL)

Samples were analyzed in parallel with the two sample preparation methods and analysed by GC-MS. The correlation between 4b-OHC results with the two methods was high (y=1.0x-2.1, r2 = 0.99 (y=new method, n=90).

The within and between day variations for the two methods are given in Table 3. The between day variation was slightly higher for the new method when compared to the original method. This may be due to the differences in amount of plasma used for the analyses, 250 µL and 1 mL, respectively. Blood samples could be left at room temperature without centrifugation up to 21 hours without changes in the 4b-OHC concentration measured (data not shown). The linear range of the calibration curve is at least 0-600 ng/mL (data not shown).

Table 3: Within and between day variations for the new and original sample preparation methods at level ~25 ng/mL (4b-OHC).

	Within day variation (%)	Between day variation (%)
New method	4.5 (n=8, two occasions)	8.2 (n=59)
Original method	3.7 (n=12)	7.7 (n=78)

The new method has several advantages over the original method when it comes to sample preparation for analysis of 4b-OHC:

- It is faster and less labor intensive. The sample throughput is approximately three times higher compared to the original method.
- The use of disposable glassware makes the new method ideal for analysis of infectious samples.
- The use of less organic solvents is beneficial from an environmental and risk assessment point of view.

Paper 2

In paper 2 we studied the kinetics of formation of circulating 4b-OHC after CYP3A induction. Ten pediatric patients with newly diagnosed epilepsy subjected to monotherapy with CBZ were enrolled in the study. Eight children (patients A to H), ranging in age from 1 years 4 months to 17 years 8 months, completed the study in which we collected blood samples before and after 1, 2, 4, 8, 16 and 23 weeks of treatment with the CYP3A inducer CBZ. The plasma levels of 4b-OHC, 4a-OHC, CBZ, CBZ-E and cholesterol were monitored. CYP3A catalyses the conversion of CBZ to CBZ-E.

The CBZ treatment (5 mg/kg/day the first week, 10 mg/kg/day the following weeks) was found to at least double plasma levels of 4b-OHC in each patient within two weeks of treatment. The increase is shown for each patient individually in Figures 3a and 3b and collectively in Table 4. The continuous treatment further increased plasma levels of 4b-OHC and after eight weeks all patients had 5 to 10-fold higher plasma levels of 4b-OHC compared to their pre-treatment levels. However, the CBZ induction of CYP3A appeared to be complete within one week of treatment as indicated by the steady state plasma levels of CBZ and CBZ-E in Figures 3a and 3b and the stable CBZ-E to CBZ ratio in Table 4. This is in line with previous observations [48]. The continued increase of 4b-OHC levels in circulation may be due to slow equilibriums with different compartments, e. g. adipose tissue. It remains to be revealed if 4b-OHC is transported between different compartments by active or passive mechanisms. Drugs that induce CYP3A4 also induce transporters such as P-glycoprotein, ABCG1 and others [49-50], which may have a role in the compartmentalization of 4b-OHC. Presence of other enzymes may also be important, although in vitro experiments with CYP1A2, CYP2C9 and CYP2B6 failed to show conversion of cholesterol to 4b-OHC [33].

Accumulation of 4b-OHC in circulation due to CBZ inhibition of its further metabolism may also be possible. However, the apparent half-life of elimination of 4b-OHC from circulation has been reported to be approximately the same in controls (n=2) as in one CBZ treated patient [35].

Only one patient (G) expressed functional CYP3A5 (CYP3A5*1/*3) and no statistical calculations could be performed on the possible effect from CYP3A5.

The plasma level of 4b-OHC was successfully used as a marker of CYP3A induction by CBZ in treatment of children with epilepsy. The CBZ treatment resulted in increased plasma levels of 4b-OHC until at least 8 weeks of treatment. According to the steady plasma levels of CBZ and CBZ-epoxide there was a complete induction of CYP3A within 1-2 weeks and the continued increase of 4b-OHC levels in circulation may be due to slow equilibriums between different compartments.

Table 4: Mean levels of 4b-OHC, 4a-OHC and cholesterol in children before, after 7-9 weeks and 15-23 weeks of CBZ treatment.

	Treatment		
	Before (n=8)	7-9 weeks (n=8)	15-23 weeks (n=7)
4b-OHC (ng/mL)	43 ± 25	296 ± 104 **	321 ± 125 **
4a-OHC (ng/mL)	5.4 ± 2.1	7.4 ± 2.0 **	7.0 ± 1.9 a
cholesterol (mmol/L)	3.7 ± 0.8	4.4 ± 0.5 *	4.2 ± 0.8 a
HDL-cholesterol (mmol/L)	1.47 ± 0.48	1.50 ± 0.45 a	1.53 ± 0.42 a
CBZ-E / CBZ	0.14 ± 0.06 b	0.14 ± 0.04 a	0.17 ± 0.08 a

p-values from student's T-test for dependent samples. Reported by * ($p<0.05$), ** ($p<0.01$), a ($p>0.05$).
b = ratio given for 1 week of treatment.

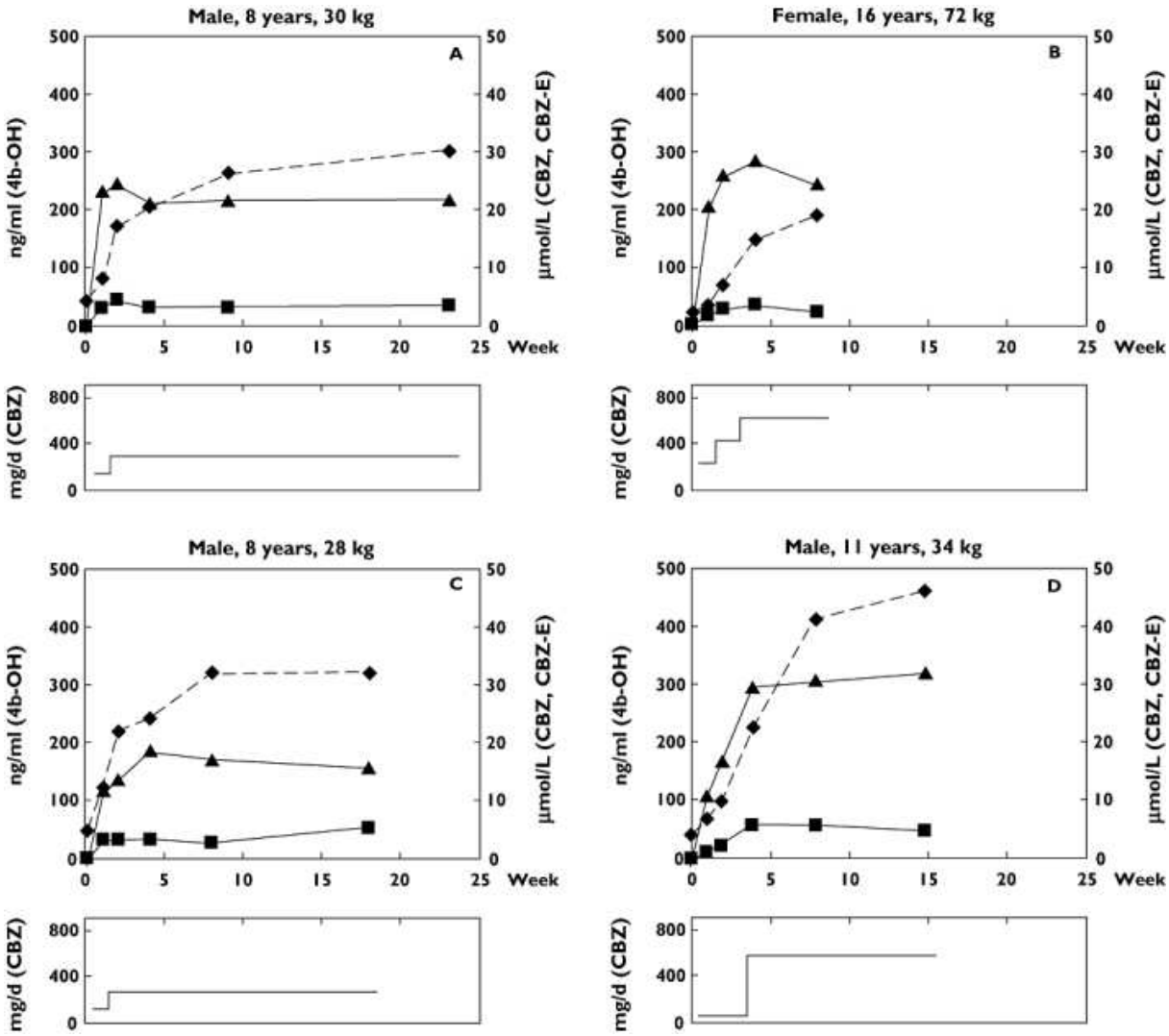

Figure 3a: Upper panels: CBZ induced increase of plasma levels of 4b-OHC (dashed line). Concentrations of CBZ (filled triangles) and CBZ-E (filled squares). Lower panels: Daily dose (mg/mL) CBZ for patients A-D.

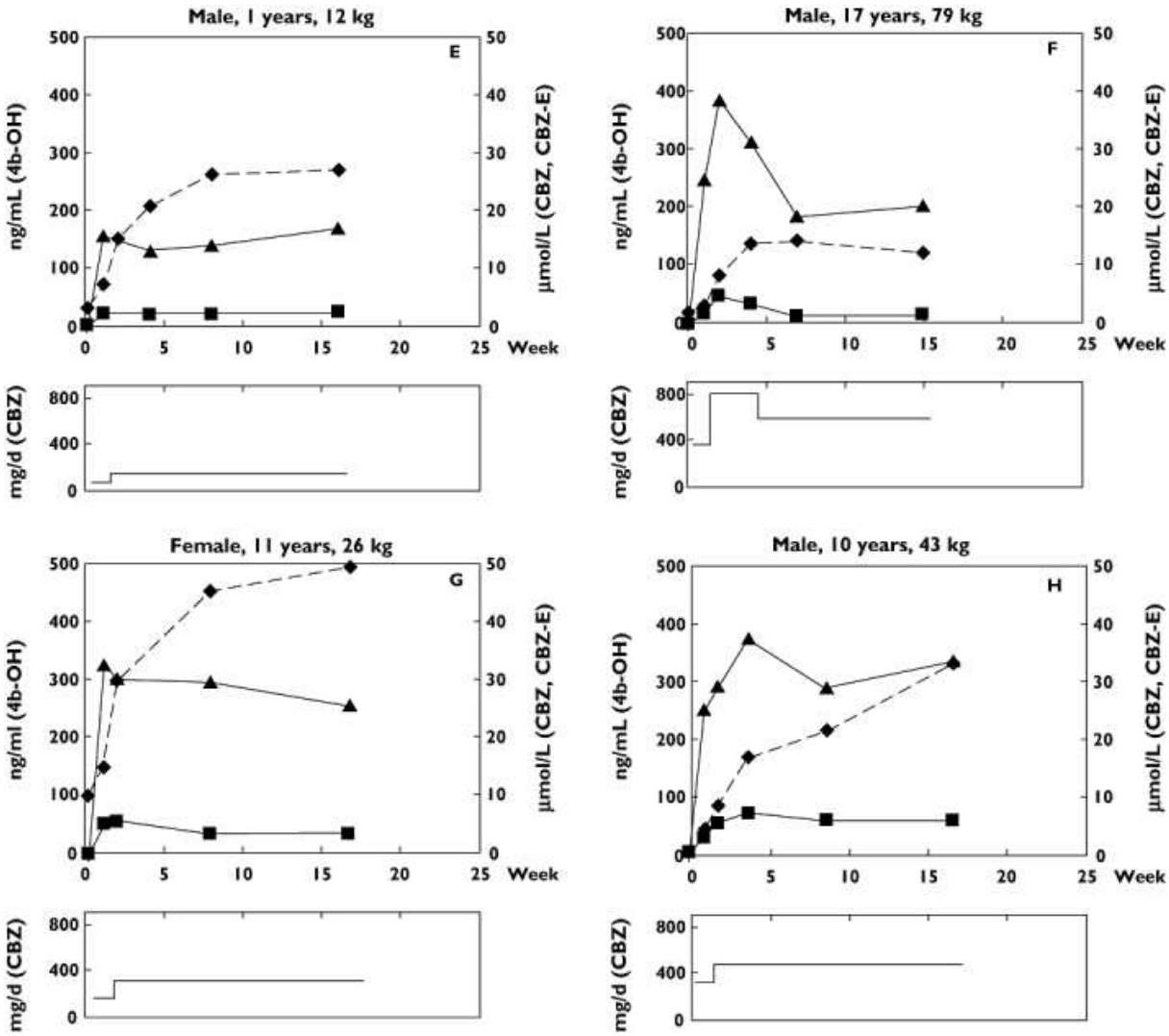

Figure 3b: Upper panels: CBZ induced increase of plasma levels of 4b-OHC (dashed line). Concentrations of CBZ (filled triangles) and CBZ-E (filled squares). Lower panels: Daily dose (mg/mL) CBZ for patients E-H.

Paper 3

Our initial aim in Paper 3 was to define the effect of CBZ treatment during pregnancy on the CYP3A activity as measured by the plasma levels of 4b-OHC in mothers and neonates. CYP3A is induced by CBZ and the effect from maternal anti-epileptic treatment on fetal hepatic drug metabolism was studied. We aimed at identifying and enrolling CBZ treated pregnant women at their routine visits at the Department of Neurology, Karolinska University Hospital, Huddinge. Controls matched for gestational age, sex and weight were to be recruited at the delivery ward.

The women were to be supplied with information about the study to bring to the delivery ward at time of delivery, preferably at one of the delivery wards at Karolinska University Hospital, Huddinge or Södersjukhuset, Stockholm, where the staff was informed about the study. Blood samples were to be collected from the cord blood immediately after birth and from the mother at the time of the neonatal screening. The estimated number of women possible for enrolment in the study a year was fairly low, only 36. This number was based on the facts that only 0.3% of pregnant women have epilepsy and that there is a total of 12 000 deliveries a year at the delivery wards at Huddinge and Södersjukhuset. Since monotherapy with CBZ was the first hand choice of treatment we expected the majority of the women with epilepsy to be on this medication. However, the estimation proved to be wrong and we ended up with only one CBZ treated mother within the time of the study. We decided to start to enroll controls and we eventually canceled the initial project and focused our attention on the control group. Later we got to know that the pregnant women with epilepsy that were identified at the Department of Neurology were not on CBZ treatment and hence could not be included in the study.

In our untreated mothers we could see that the CYP3A activity was significantly higher at time of delivery, as measured by higher 4b-OHC to cholesterol ratios, compared to a cohort of healthy non-pregnant women (Table 5). The mothers had also higher plasma levels of 4b-OHC and cholesterol at delivery, but four months post partum there was no longer any difference in levels of 4b-OHC, cholesterol or 4b-OHC to cholesterol ratios between mothers and the cohort of non-pregnant women. These results are in line with previously published results indicating that the levels of cholesterol in circulation are increased during pregnancy [51-52] as well as the CYP3A activity. The latter has been shown using the markers midazolam [15] and 6β-hydroxycortisol to cortisol ratio in 4h urine [53]. Nakamura et al. has

reported no difference in the 6β-hydroxycortisol to cortisol ratios in spot urine between pregnant and non-pregnant women [54]. These inconsistencies may be due to different specificities of the markers and/or small data sets. Within the group of mothers one difference remained after four months: 4b-OHC in mothers giving birth by vaginal delivery was still increased as compared to the level in mothers giving birth by elective CS (Table 6).

Table 5: Median plasma levels of 4b-OHC, 4a-OHC and cholesterol and 4b- and 4a-OHC to cholesterol ratios in mothers and non-pregnant healthy women.

		mothers			women, non-pregnant			p-value
		median	QR	n	median	QR	n	
4b-OHC (ng/mL)	delivery	50.0	10.7	21	28.9	14.4	76	<0.00001
	4 months	32.6 **	11.9	10				n.s.
4a-OHC (ng/mL)	delivery	13.9	3.1	21	6.1	2.05	76	<0.00001
	4 months	10.8 **	3.9	10				<0.00001
4b-OHC/ chol($\cdot 10^4$)	delivery	0.19	0.06	21	0.15	0.07	76	0.0009
	4 months	0.15 [a]	0.09	10				n. s.
4a-OHC/ chol($\cdot 10^4$)	delivery	0.06	0.01	21	0.03	0.01	76	<0.00001
	4 months	0.05 *	0.01	10				0.001
cholesterol (mmol/L)	delivery	6.2	1.7	21	4.6	0.9	76	<0.00001
	4 months	5.2 *	2.5	10				n. s.

QR = quartile range. p-values from Mann Whitney U test. The change in 4b- and 4a-OHC / cholesterol ratios and cholesterol from delivery to four months later were statistically tested by Wilcoxon Matched Pairs. p-values are reported by * ($p<0.05$), ** ($p<0.01$) and [a] ($p>0.05$).

Neonates born by elective CS had 4b-OHC to cholesterol ratios comparable to the ratios in a cohort of healthy adults (Table 7), indicating that neonates had similar total CYP3A activity. No ratios could be calculated for neonates born by vaginal delivery due to limited sample material.

CYP3A7 is abundant in fetal and neonatal liver [3-5] and a postnatal decrease in CYP3A7 and a concomitant increase in CYP3A4 may result in unchanged total CYP3A activity. CYP3A7 catalyses the conversion of cholesterol to 4b-OHC to a small extent in vitro [35], but it is not known if this conversion takes place also in vivo.

Neonates had lower plasma levels of 4b-OHC and cholesterol at birth than the cohort of 125 healthy adults, but similar levels four months post partum.

Table 6: Median plasma levels of 4b-OHC and 4a-OHC in mothers and neonates, divided by mode of delivery.

		Elective CS			Vaginal delivery			p-value
		median	QR	n	median	QR	n	
Mothers								
4b-OHC	delivery	53.2	32.5	7	48.0	10.6	14	n.s.
(ng/mL)	4 months	28.6 *	3.7	5	40.5 a	7.8	5	0.032
4a-OHC	delivery	16.8	7.3	7	13.5	2.6	14	0.031
(ng/mL)	4 months	11.5 *	3.7	5	10.1 *	3.9	5	n.s.
Neonates								
4b-OHC	birth	12.0	5.4	8	20.2	4.7	14	0.003
(ng/mL)	4 months	43.0 *	16.0	6	25.3 *	16.0	5	n.s.
4a-OHC	birth	14.1	3.5	8	30.2	9.9	14	0.00001
(ng/mL)	4 months	16.4 a	6.4	6	15.1 *	1.6	5	n.s.

QR = quartile range. Vaginal delivery: Blood samples were taken at the neonatal screening two-three days after birth. p-values from Mann Whitney U test. The change in concentrations of 4a-OHC and 4b-OHC from birth to four months later were statistically tested by Wilcoxon Matched Pairs.
p-values are reported by * ($p<0,05$), a ($p>0,05$).

4a-OHC is a stereoisomer of 4b-OHC that is not formed by CYP3A. It is possibly formed by auto-oxidation during oxidative stress. Mothers had significantly higher plasma levels of 4a-OHC both at time of delivery and four months port partum compared to the cohort of non-pregnant women (Table 5). Also the 4a-OHC to cholesterol ratio in mothers was higher both at delivery and four months post partum when compared to the ratios in the cohort of non-pregnant women. Neonates born by vaginal delivery had twice the plasma levels of 4a-OHC at the time of birth compared to children born by elective CS (Table 6). Interestingly, all children born by vaginal delivery had higher 4a-OHC than their mother, while all children born by elective CS had lower 4a-OHC than their mother (Figure 4). Oxidative stress during vaginal delivery may be an explanation as to why neonates born by vaginal delivery had increased levels of 4a-OHC, almost double compared to neonates born by elective CS. Also 4b-OHC was increased in neonates born by vaginal delivery which may have a possible explanation in the increased oxidative stress associated with this mode of delivery. However, there are inconsistencies in the literature on the oxidative effect from vaginal delivery on both mothers and neonates [55-58], possibly due to the use of different markers.

The 4a-OHC to cholesterol ratio decreased significantly between birth and 4 months post partum in children born by elective CS. However, the ratio was still three times higher than in the cohort of healthy adults. No ratios were calculated for children born by vaginal delivery.

Only 3 mothers and 3 neonates expressed functional CYP3A5 (CYP3A5*1/*3) out of the 10 mothers and 11 neonates that were genotyped (scheduled at the four month follow up). No statistical analyses were done due to the small data set.

Table 7: Median plasma levels of 4b-OHC, 4a-OHC and cholesterol and 4b- and 4a-OHC to cholesterol ratios in neonates and healthy adults.

		neonates			adults			p-value
		median	QR	n	median	QR	n	
4b-OHC (ng/mL)	delivery	18.8	8.6	22	27.2	13.8	125	<0.00001
	4 months	39.6 **	21.2	11				0.03
4a-OHC (ng/mL)	delivery	26.2	21.1	22	6.0	2.8	125	<0.00001
	4 months	15.2 a	5.1	11				<0.00001
4b-OHC/chol ($\cdot 10^4$)	delivery	0.19	0.07	8	0.15	0.07	125	n.s.
	4 months	0.20 a	0.11	11				0.005
4a-OHC/chol ($\cdot 10^4$)	delivery	0.20	0.04	8	0.03	0.01	125	<0.00001
	4 months	0.09 a	0.02	11				<0.00001
cholesterol (mmol/L)	delivery	1.8	0.8	8	4.5	0.9	125	<0.00001
	4 months	4.2 *	0.8	11				n.s.

QR = quartile range. p-values from Mann Whitney U test. The change in 4b- and 4a-OHC to cholesterol ratios and cholesterol from delivery to four months later were statistically tested by Wilcoxon Matched Pairs. p-values are reported by * ($p<0.05$), ** ($p<0.01$) and a ($p>0.05$).

A mother treated with CBZ during the entire pregnancy had a very high plasma level of 4b-OHC two days after an elective CS (392 ng/mL, Table 8) when compared to untreated mothers (elective CS; 53.2 ng/mL). The 4b-OHC to cholesterol ratio was about nine-fold higher than in untreated mothers ($1.8 \cdot 10^{-4}$ and $0.19 \cdot 10^{-4}$, respectively). The neonate had a close to five-fold higher 4b-OHC to cholesterol ratio as compared to neonates of untreated mothers ($0.99 \cdot 10^{-4}$ and $0.19 \cdot 10^{-4}$, respectively). The level of 4b-OHC in cord blood plasma of the neonate was higher as compared to neonates born by elective CS of untreated mothers (76 ng/mL and 12.0 ng/mL, respectively). The CBZ treatment during pregnancy had no effect on the isomer 4a-OHC or cholesterol in neither mother nor child (Table 8) as their levels were comparable to the ones in untreated mothers and neonates, respectively.

We conclude that 4b-OHC is a useful marker in the study of CYP3A activity in mothers and neonates. Mothers had higher CYP3A activity as compared to non-pregnant women. Neonates had similar total CYP3A activity as adults, possibly due to neonatal CYP3A7

activity. CBZ treatment during pregnancy caused increased levels of 4b-OHC in one mother and her child, indicating increased CYP3A activity.

Table 8: Levels of 4b-OHC, 4a-OHC and cholesterol in CBZ treated mother and neonate at delivery/birth.

	4b-OHC (ng/mL)	4a-OHC (ng/mL)	cholesterol (mmol/L)
mother	392	18.9	5.5
neonate	76	19.1	1.9

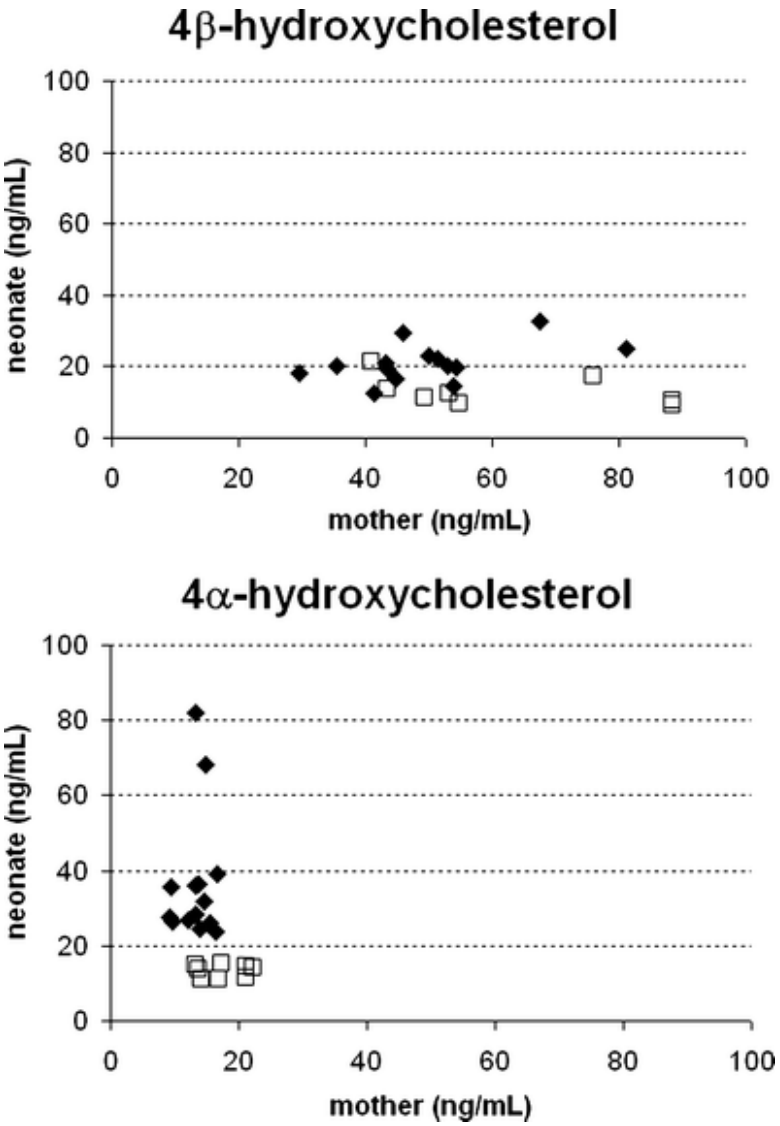

Figure 4: Plasma levels of 4b-OHC (upper panel) and 4a-OHC (lower panel) in mothers and neonates. Open squares = elective CS. Filled diamonds = vaginal delivery.

General summary

The sample preparation was optimized for the subsequent analysis of plasma 4b-OHC. The new sample preparation method increased sample throughput approximately three times and was less labor intensive than the original method. The use of less organic solvents was beneficial from an environmental and risk assessment point of view.

The time course of formation of 4b-OHC in children after initiation of treatment with CBZ was studied. We showed that CBZ increased CYP3A activity as measured by increased plasma levels (5-10-fold) of 4b-OHC within 8 weeks of treatment. Stable plasma levels of CBZ and CBZ-E within one week of treatment indicated complete induction of CYP3A within this time. The continued increase of 4b-OHC was possibly due to slow equilibriums between different compartments.

The CYP3A activity increased during pregnancy as measured by an increased 4b-OHC to cholesterol ratio in mothers at delivery. Neonates had similar total CYP3A enzyme activity as adults as indicated by similar 4b-OHC to cholesterol ratios. CBZ treatment during pregnancy caused increased levels of 4b-OHC in one mother and her child, indicating increased CYP3A enzyme activity. Children born by vaginal delivery had increased levels of 4a-OHC, possibly due to oxidative stress during delivery.

In our study we have shown that 4b-OHC is a suitable marker of CYP3A induction. 4b-OHC is a non-invasive marker that is easy to use in neonates, children and vulnerable patient groups where probe drugs are difficult or unethical to administer or urine collections are difficult to perform. Blood samples can be taken any time of the day and irrespective of food intake which is beneficial in a clinical setting.

Future perspectives

Further optimization and automatization of the sample preparation and analysis method: Transfer the sample preparation to the 96-well plate format and automate pipetting. Development of an LC-MS/MS method with increased resolution and sensitivity as compared to the currently used GC-MS method.

Perform further studies on the usefulness of 4b-OHC as a marker of CYP3A activity in other conditions, medications and age groups.

Investigate the effect on the CYP3A activity in mothers and children exposed to other treatments during pregnancy, possibly monitored the whole pregnancy.

Studies on the potential effect of plasma levels of 25-hydroxyvitamin D on CYP3A activity as measured by plasma levels of 4b-OHC. Observed seasonal changes in drug concentrations may be a result of Vitamin D induced CYP3A activity [59].

Studies on the possible 4β-hydroxylase activity of CYP3A7 and CYP3A43 both in vitro and in vivo.

Further studies on the formation and tissue distribution of 4a-OHC.

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