**Cholesterol and oxysterol sulfates: Physiological roles and analytical challenges**

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**Abstract**

Cholesterol (Chol) and oxysterol sulfates are important regulators of lipid metabolism, inflammation, cell apoptosis, and cell survival. Among the sulfate-based lipids, cholesterol sulfate (CS) is the most studied lipid both quantitatively and functionally. Despite the importance, very few studies have analysed and linked the actions of oxysterol sulfates to their physiological roles. Over expression of sulfotransferases confirmed the formation of a range of oxysterol sulfates and their antagonistic effects on liver X receptors (LXRs). It is therefore important to understand how further changes to oxysterol/oxysterol sulfate homeostasis can contribute to LXR activity in the physiological milieu. Here, we aim to bring together evidences for novel roles of oxysterol sulfates, the available techniques and the challenges for analysing them. Understanding the oxysterol/oxysterol sulfate levels and their physiological mechanisms could lead to new therapeutic targets for metabolic diseases.

**Introduction**

Sulfate-based lipids (SL) have been proposed as players in inflammation, immunity and infection prompting a deeper investigation into the human sulfateome in health and disease (Hu et al., 2007; Merten, 2001; Suzuki et al., 2003). The biotransformation of lipids by sulfation and desulfation reactions are fundamental to many cellular pathways. SL represent a diverse class of lipids including sulfate-, sulfonate- and thiol- or thioether- based lipids (Dias et al., 2019). In humans, steroid sulfates represent a highly abundant and mostly studied lipid class among the other glycerol-, sphingosine- or taurine-derived lipids. Steroid sulfates were traditionally viewed as inactive precursors as they require active transport into cells via organic anion transporters. However, recent research suggests that these derivatives have active roles. For example, pregnenolone sulfate (PregS) and dehydroepiandrosterone sulfate (DHEAS) are neuroactive and more membrane transporters are uncovered for cellular uptake of sulfated sterols (Fietz et al., 2013). In fact, research groups who have focused their attention on oxysterol sulfates found that these molecules are key mediators in the cellular processes, such as resolution of inflammation (L. Xu et al., 2012) and the regulation of lipid metabolism via SREBP (Sterol Regulatory Element-Binding Protein-1) (Bai et al., 2012; Ma et al., 2008; Ren et al., 2007). Oxysterol sulfates show dynamic ways of activating, inhibiting or shuttling of Chol in biological systems. This review brings together current understanding of sulfated Chol and oxysterols and analytical challenges in measuring their biological levels.

**Biosynthesis of sterol sulfates**

The biological activities of sterol sulfates are regulated by the balanced activity between steroid sulfotransferases and steroid sulfatases that catalyse the formation and hydrolysis of steroid sulfates, respectively (Purohit, Potter, Parker, & Reed, 1998). The biosynthesis of sulfated lipids is mediated by a large family of sulfotransferases (SULTs) that catalyse the transfer of sulfate groups from a 3´-phosphoadenosine-5´-phosphosulfate (PAPS) donor compound to an acceptor molecule with aromatic or aliphatic hydroxyls functional groups (Falany, 1997). The transfer of the sulfate group by SULTs at 3-position of the main sterols results into mono-sulfated sterols such as CS, (PregS) and (DHEAS) (**Figure 1**). The cytochrome P450 (CYP) enzymes catalyse addition of hydroxyl group to the side chain of Chol generating oxysterols which can be further sulfated at 3-position resulting in 24(*S*)-hydroxycholesterol-3-sulfate (24HC3S), 25-hydroxycholesterol-3-sulfate (25HC3S), (25*R*)-26-hydroxycholesterol-3-sulfate (26HC3S), 20(*S*)-hydroxycholesterol-3-sulfate (20HC3S) and 22(*R*)-hydroxycholesterol-3-sulfate (22HC3S) (Cook, Duniec-Dmuchowski, Kocarek, Runge-Morris, & Falany, 2009; Javitt, Lee, Shimizu, Fuda, & Strott, 2001). The additional hydroxyl group acquired by these oxysterols allows formation of disulfated derivatives, such as 24(*S*)-hydroxycholesterol-3,24-disulfate (24HCDS), 25-hydroxycholesterol-3,25-sulfate (25HCDS) and (25*R*)-26-hydroxycholesterol-3,26-disulfate (26HCDS). Oxysterols that are formed by free radical attack, namely 7α-hydroxycholesterol (7αHC), 7β-hydroxycholesterol (7βHC), 7-ketocholesterol (7KC), epoxy cholesterols [5β,6β-epoxycholesterol (5,6βEC) and 5α,6α-epoxy cholesterol (5,6αEC)] can also be converted into the corresponding sulfated derivatives (**Figure 1**).

**Figure 1: Structures of sterol and oxysterol sulfates**



The family of SULTs consist of membrane-related enzymes, mainly localised in the Golgi apparatus, and cytosolic enzymes (Falany, 1997). The SULTs cytosolic enzymes have been associated with metabolism of endo- and xenobiotics while the membrane-bound enzymes primarily with sulfation of tyrosyl protein residues (Nowell & Falany, 2006). So far, four families of human cytosolic SULTs have been identified: SULT1, SULT2, SULT4, and SULT6. As enzymes of the SULT2 family have been associated with the sulfation of oxysterols, this review will focus on the members of this group (Lindsay, Wang, Li, & Zhou, 2008). In particular, members of the SULT2 family are divided into two subfamilies, SULT2A and SULT2B, based on their amino acid sequence and encoded by the two corresponding genes, *SULT2A1* and *SULT2B1* (Gamage et al., 2006).

*SULT2A1*

In humans, SULT2A1 has been primarily linked to sulfation of DHEA; however, it is also responsible for the sulfation of other steroid substrates such as pregnenolone (Preg), androgens and bile acids (Gamage et al., 2006; Kong, Yang, Ma, Tao, & Bjornsson, 1992; Otterness et al., 1992). The SULT2A1 isoform is highly expressed in human liver, foetal adrenal glands, adult adrenal cortex and small intestine (Nowell & Falany, 2006; Thomae, Eckloff, Freimuth, Wieben, & Weinshilboum, 2002). As result, endogenous and orally administered steroids undergo sulfation by SULT2A1 as part of their metabolism. In particular, DHEAS obtained from DHEA by SULT2A1, serves as a precursor in the synthesis of androgens and oestrogens in human peripheral tissues (Mortola & Yen, 1990). The circulating endogenous levels of DHEAS is known to decrease with age and therefore associated with age-related diseases such as, osteoporosis, muscle loss, vaginal atrophy, fat accumulation, hot flashes, skin atrophy, type 2 diabetes and cognitive deficits (Orentreich, Brind, Vogelman, Andres, & Baldwin, 1992). In 2002, observations by Thomae *et al*. suggested an ethnic-specific variation in the expression and activity of SULT2A1 among Caucasian and African American individuals (Thomae et al., 2002), explaining inter-individual variability of DHEAS.

*SULT2B1a and SULT2B1b*

The subfamily of SULT2B, including its two splice variants, namely SULT2B1a and SULT2B1b, are widely distributed in human tissues and are able to metabolise sterol-like structures (Javitt et al., 2001). Both isoforms are encoded by *SULT2B1* gene and are originated by alternative splicing of the gene localised to chromosome band 19q13.3, approximately 500 kb telomeric to the location of SULT2A1 (Her et al., 1998). In the gene for SULT2B1, exon 1A encodes a unique amino-terminal end for the B1a isoform and additional 48 amino acids, while exon 1B encodes for the unique amino terminal region of B1b spliced variant (H. Fuda, Lee, Shimizu, Javitt, & Strott, 2002). In 2001, Javitt *et al*. reported that SULT2B1b is expressed in tissues responsive to hormones in a higher fashion than SULT2B1a (Javitt et al., 2001). In fact, the B1b isoform preferentially acts on Chol, whereas the B1a isoform catalyses the sulfation of Preg, but not Chol (H. Fuda et al., 2002). The expression of the isoform B1b is usually several-fold higher that the isoform B1a (Falany, He, Dumas, Frost, & Falany, 2006) and it has been identified in human prostate, placenta, breast, lungs, platelets and kidney (Falany et al., 2006; Geese & Raftogianis, 2001; He, Meloche, Dumas, Frost, & Falany, 2004).

**Metabolism of sterol sulfates**

The cleavage of the sulfate moiety of 3β-hydroxysteroid sulfate is catalysed by membrane-bound microsomal steroid sulfatases (STSs) (Conary, Nauerth, Burns, Hasilik, & von Figura, 1986). Mammalian STS superfamily consists of 12 different enzymes (Reed, Purohit, Woo, Newman, & Potter, 2005). The gene encoding the human STSs is located on the distal short arm of the X-chromosome (Yen et al., 1988) and ubiquitously expressed in many human tissues including placenta, breast, skin, lungs, ovaries, adrenal glands and brain (Reed et al., 2005). STSs have been associated with high intra-tumoral oestrogen and androgen levels and therefore, linked to steroid hormone-dependent tumours growth (Nardi et al., 2009). Studies by Zaichuk *et al*. in 2007 showed that oestrogen regulates the transcription of STSs in breast carcinoma (Zaichuk, Ivancic, Scholtens, Schiller, & Khan, 2007).

Deficiency in STSs was observed in X-linked ichthyosis, a disease characterised by skin peeling localised in the anterior and posterior areas of upper and lower extremities. In healthy epidermis, CS is produced by the action of SULT2B1b and desulfated in the outer epidermis thus contributing to epidermal differentiation, maintenance of barrier function and desquamation. As consequence of STSs deficiency, CS levels could exceed 10% of the total lipid mass in epidermal cells (Rizner, 2016).

**Cholesterol-3-sulfate and its receptors**

Besides being the most abundant steroidal sulfoconjugate present in human plasma, in an average concentration of 2 μM (Meng, Griffiths, Nazer, Yang, & Sjövall, 1997), CS is also highly abundant in cell membranes and widely expressed in all the tissues, especially in the skin. Even though CS is typically considered the hydrophilic excretion form of Chol, CS also represents a biosynthetic precursor of several bioactive steroids. In this scenario, the sulfoconjugation reaction may represent a key event in the formation of a readily available hydrophilic form of Chol. Indeed, CS can be subjected to several enzymatic transformations carried out by microsomal cytochromes (e.g. CYP11A1, also referred to as cholesterol side-chain cleavage enzyme) in order to obtain sulfated precursors of sex hormones. During the last decades, the role of CS as a signalling molecule has been enlightened, although many questions are still opened and unanswered.

As described above, recessive X-linked ichthyosis has been related to a deficiency in cholesterol sulfatase expression with a subsequent accumulation of CS. In 1998, Sato *et al.* correlated this pathologic condition with the ability of CS to inhibit serine proteases involved in cell dissociation, a key feature in skin development (Sato, Denda, Nakanishi, Nomura, & Koyama, 1998). As a matter of fact, Ito *et al*. demonstrated the direct inhibition of several hydrolytic enzymes by CS (e.g. pancreatic elastase, trypsin, chymotrypsin, thrombin, plasmin and DNAse I) in the latest nineties (Ito, Iwamori, Hanaoka, & Iwamori, 1998; Iwamori, Iwamori, & Ito, 1997; Iwamori, Suzuki, Kimura, & Iwamori, 2000). The inhibitory behaviour of CS towards these pancreatic enzymes has been related to its protective role at the gastrointestinal mucosa level. In addition, it is noteworthy to underline that the inhibition of these enzymes occurred in a non-specific fashion. In other words, the interaction between the two molecular partners is based only on the physico-chemical properties of CS and the presence of an anion binding region on the tertiary structure of the target protein.

In 1999, the ability of CS to inhibit serine proteases was extended by Iwamori *et al*. to thrombin and plasmin (Iwamori, Iwamori, & Ito, 1999). As these two proteases are involved in blood clotting and fibrinolysis, respectively, CS, can be considered an endogenous modulator of haemostasis by a presumably non-specific irreversible mechanism. Moreover, CS has been found to promote divalent cation-independent adhesion of both activated and inactivated platelets, although the exact role of this compound in the control of haemostasis still remains unclear (Merten, 2001).

More recently, CS has been shown to play a significant role in the control of inflammation by modulating key targets. Inflammation is a complex multistep biological response of body tissues to harmful stimulations which stereotypically involves a multitude of mediators and many different cell types. 5-Lipoxigenase (5-LO) is involved in the production of leukotrienes, soluble mediators of the inflammatory state and immune system functionality. In particular, leukotrienes play a pivotal role in asthma and bronchitis. When a Ca2+ influx takes place, 5-LO binds the nuclear membrane where it can convert arachidonic acid into the bioactive leukotrienes. As a constituent of cell membranes, CS can modulate the function of several proteins, including 5-LO, directly interacting with them at membrane level. Aleksandrov *et* al. (Aleksandrov et al., 2006), indeed, demonstrated the inhibitory behaviour of CStowards 5-LO in a cell-free assay. Moreover, CS has been found to decrease 5-LO interaction with the nuclear membrane in a cell-based assay upon stimulation, thus decreasing leukotrienes biosynthesis.

In 2016, Wang *et al.* demonstrated the relevance of CS as modulator of T-cell receptor (TCR) functionality (Wang, Beck-García, Zorzin, Schamel, & Davis, 2016). The TCR is a multisubunit membrane receptor which includes an antigen-recognition domain composed of the TCRα and β (or γ and δ) heterodimer and a signalling domain, typically three CD3 dimers. Although TCR binds its corresponding peptide-MHC ligands with extremely weak affinity, it is well-known that a single molecule of its ligand is able to activate the T cell. The low affinity and the high sensitivity of this receptor has been related to the nanoclustering of several TCRs. Chol is able to interact with TCRβ thus promoting TCR nanoclustering. Conversely, CS can disrupt TCR clusters by interfering in the Chol-TCRβ interaction. Interestingly, the Chol /CS ratio is a variable parameter during T cell development and differentiation (Wang et al., 2016).

Dedicator of cytokinesis protein 2 (DOCK2) is a guanine nucleotide exchange factor which plays a key role in immune surveillance and immune responses by regulating the chemotaxis and the activation of leukocytes. In some districts (e.g. eye) immune evasion is a characteristic hallmark. Furthermore, immune evasion is a desirable goal to achieve in the case of organ transplant. Interestingly, DOCK2 deficiency is related to a decreased rejection. In 2018, Sakurai *et al.* demonstrated that CS, whose content was high in the eye, was able to inhibit DOCK2 (Sakurai et al., 2018). In particular, the direct interaction between CS and DOCK2 has been confirmed by a cell-free surface plasmon resonance binding assay (Sakurai et al., 2018).

CS has been also reported as an endogenous ligand of macrophage inducible Ca2+-dependent lectin receptor (Mincle), an innate immune receptor involved in skin allergic inflammation (Kostarnoy et al., 2017).

In the studies above reported, the specific interaction of CS with the corresponding target protein has never been proved and in most cases the observed activity of CS has been related to its amphiphilic nature without identifying a proper binding pocket/site on the polipeptidic counterpart. Otherwise, in 2004, Kallen *et al.* reported the crystal structure of CS with the nuclear receptor retinoic acid-related orphan receptor α (RORα) (Kallen, Schlaeppi, Bitsch, Delhon, & Fournier, 2004). Since RORα could be implicated in the control of Chol homeostasis, the Authors set up crystallization trials both with Chol and CS. Both lipids co-crystallized with the ligand-binding domain of the receptor interacting at the same level. Remarkably, CS showed an increased affinity due to the interaction of the sulphate group with key polar residues of the ligand binding pocket (Gln289, Tyr290 and Arg370) with the consequent displacing of several water molecules which were instead present in the interaction with Chol. Anyway, even though the crystal unambiguously pointed out the interaction of this orphan nuclear receptor with CS there are still lacks of evidences of this interaction *in vivo*. Indeed, even if the activation of this nuclear receptor occurs upon stimulation with CS, the latter is considered so far only a putative RORα endogenous ligand (Han et al., 2014; Kim et al., 2008; Zenri et al., 2012).

In any case, CS is able to influence the Chol homeostasis by negative regulation of 3-hydroxy 3-methylglutaryl-CoA reductase (HMG-CoA reductase) and lecithin-cholesterol acyltransferase, in an indirect and direct manner, respectively (Nakagawa & Kojima, 1976; Williams, Hughes-Fulford, & Elias, 1985).

Finally, CS has been also found to have an important role in the substrate specificity of phosphatidylinositol 3-kinase (PtdIns-3K) (Woscholski, Kodaki, Palmer, Waterfield, & Parker, 1995) Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), produced by PtdIns-3K’ s activity, is associated with the signalling pathway of several growth factors and it is considered a secondary messenger. Phosphatidyinositol diphosphate (PIP2) is the preferred substrate of PtdIns-3K *in vivo*, inside the cell. Conversely, phosphatidyinositol monophosphate and phosphatidylinositol are the preferred substrates of PtdIns-3K in cell-free systems. In 1995, Woscholski *et al.* demonstrated that the characteristic substrate specificity of this enzyme *in vivo* could be restored in the presence of CS pointing out its potential relevance as an interacting partner inside the cell (Woscholski et al., 1995).

**Oxysterols sulfates and their receptors**

Oxysterols are bioactive lipids which share the 27-carbons skeleton with Chol and differ from the latter by the presence of extra oxygenated functionalities in respect to the 3β-hydroxyl group. In addition to being biosynthetic precursors of bile acids and sex hormones, they serve as selective ligands towards several targets (e.g. G protein-coupled receptors, enzymes, nuclear receptors and other membrane and cytosolic proteins). Similarly, their sulfoconjugates have been found to act as modulators of different targets. Traditionally, oxysterol sulfates have been viewed as detoxification derivatives of oxysterols that are synthesized for excretion. However, recent work proposed that oxysterol sulfates were bioactive molecules that acted as selective ligands with biological outcomes **(Table 1)**.

**Table 1**: Cellular activities of oxysterol sulfates.

|  |  |  |  |
| --- | --- | --- | --- |
| Oxysterol | Cell type | Outcome | References |
| 5,6αECS | Colorectal Cancer cell line, Caco-2 | Accumulation sensitise cells to apoptosis | (Segala et al., 2013) |
| Neuroblastoma cell line, SHSY-5Y cells | No effect on cell viability | (Warns, Marwarha, Freking, & Ghribi, 2018) |
| Human embryonic kidney 293 cells | Attenuates the 26HC-induced increase in α-synuclein expression | (Marwarha, Rhen, Schommer, & Ghribi, 2011) |
| Human embryonic kidney 293 cells | Inhibit transactivation of reporter genes by LXR | (Song, Hiipakka, & Liao, 2001) |
| 7KCS | Human retinal pigment epithelial cell line, ARPE-19 | Reduce cytotoxicity induced by 7KC | (Hirotoshi Fuda, Javitt, Mitamura, Ikegawa, & Strott, 2007) |
| Human embryonic kidney 293 cells | Attenuates ABCA1 and VEGF inductions by 7KC | (Moreira, Larrayoz, Lee, & Rodríguez, 2009) |
| Cell-free system | Inhibit transactivation of reporter genes by LXR | (Song et al., 2001) |
| 24HC3S/  24HCDS | Hepatocytes | LXR antagonists | (Cook et al., 2009) |
| 25HC3S | Human monocytic cell line, THP-1 | Inhibits the LXR/SREBP signaling pathway, regulates lipid metabolism, inflammatory responses, and cell proliferation | (Ma et al., 2008), (Ren et al., 2014; Ren et al., 2007), (Ren & Ning, 2014) |
|  | Attenuates inflammatory response via PPARγ signaling | (L. Xu et al., 2012) |

Oxysterol sulfoconjugation occurs mainly by the cytosolic PAPS-dependent enzyme SULT2B1b, also referred to as hydroxysteroid sulfotransferase. This metabolic transformation is generally reversible as the enzymatic activity of STS is able to afford the parent oxysterol in its active form. In 2001, Song *et al.* demonstrated that 5α,6α-epoxycholesterol-3-sulfate (5,6αECS) and 7-ketocholesterol-3-sulfate (7KCS) were able to bind both nuclear receptors LXRα and LXRβ inhibiting their activation acting as antagonists. It is noteworthy that in addition to a cell-based gene transactivation assay, the authors also performed a cell-free coactivator peptide recruitment binding assay in order to demonstrate the direct interaction of 5,6α-ECS and 7KCSwith the receptors. Moreover, a structure-dependant ligand recognition mechanism was sought out by testing two closely related sulfated oxysterols, 5β,6β-epoxycholesterol-3-sulfate (5,6βECS) and 6-ketocholestanol-3-sulfate, in the same assays. As both the latter compounds failed in modulating LXRs activation, the Authors speculated that the antagonistic behaviour of 5,6α-ECS and 7KCS towards LXRs was independent of their physiochemical properties (e.g. amphiphilicity) (Song et al., 2001).

In 2009, Cook *et al.* reported that the LXRs endogenous agonist 24(*S*)-hydroxycholesterol (24HC) could be sulfated by three different sulfotransferases, namely SULT1E1, SULT2A1 and SULT2B1b at 3-OH or 24-OH positions with different rates and affinities affording 24HC3S, 24(*S*)-hydroxycholesterol-24-sulfate and 24HCDS. Surprisingly, 24HC3S and 25HC24Sshowed a remarkable antagonistic behaviour in a TR-FRET LXRα coactivator recruitment assay suggesting a dramatic switching in ligand properties as the sulfate moiety was introduced in the structure of the parent compounds. Interestingly, *SULT2B1b* is a LXRs target gene whose expression increases in presence of agonists. Accordingly, the sulfation of LXRs endogenous agonists can be considered a negative feedback mechanism able to control LXRs activation (Cook et al., 2009).

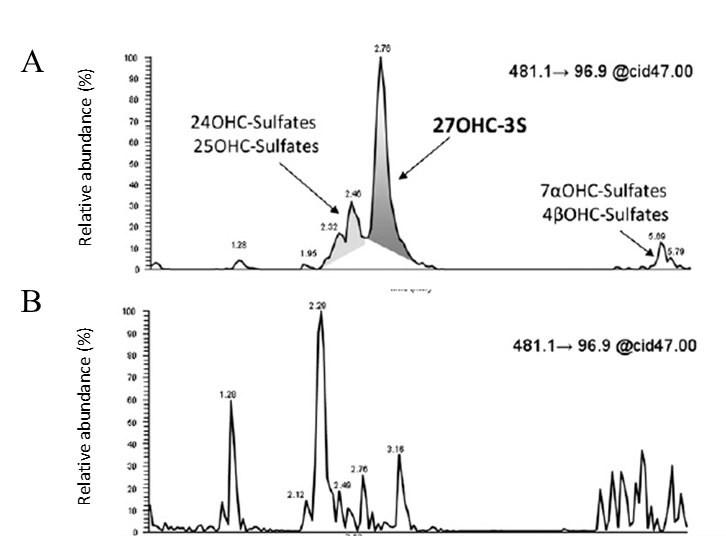
Also 25-hydroxycholesterol (25HC), another endogenous LXRs agonist, can be converted into an antagonist when sulfated at 3β-OH. 25HC3S was identified by Ren *et al*. in 2007 first in hepatocytes nuclei. In particular, 25HC3S has been found to decrease the expression of SREBP-1 target genes (e.g. HMG-CoA reductase) with a consequent overall decrease of Chol levels. Moreover, its administration to human hepatocytes resulted in reduced SREBPs, in particular SREBP-1, expression and maturation. Accordingly, in a preliminary stage the Authors suggested a direct inhibition of SREBP-1 operated by 25HC3Salthough their first results showed a putative impaired LXR signalling (Ren et al., 2007). Starting from these promising results, Ren *et al*. decided to deepen into the physiological relevance and potential therapeutic application of 25HC3Sand several papers were published by Ren’ s research group since 2007. Hence, 25HC3S was found to decrease NF-κB nuclear levels by increasing cytosolic levels of its inhibitor IκBα, thus repressing TNFα-induced inflammatory response in HepG2 cells. Interestingly, its parent compound, namely 25HC, elicited the opposite activity (Leyuan Xu et al., 2010). In the same paper, the antagonistic behaviour of 25HC3S towards LXRs was enlightened. Indeed, 25HC3Swas able to decrease the expression of LXR target genes involved in Chol biosynthesis and lipogenesis (e.g. Fatty acid synthase and Acetyl-CoA carboxylase-1) (Leyuan Xu et al., 2010). By contrast, Zhang *et al.* demonstrated that 25HC3S up-regulated several genes involved in hepatic cells proliferation (Zhang et al., 2012). According to its biological profile, as LXR and SREBP-1c signalling pathways inhibitor (Bai et al., 2012) as well as to its anti-inflammatory properties (Leyuan Xu et al., 2010; L. Xu et al., 2012), 25HC3S is currently evaluated in phase II clinical trial for its potential application in liver diseases (e.g. NAFLD) by Durect corporation. In 2012, 25HC3Shas been also found to act as a PPARγ agonist in THP-1 macrophages, where it can suppress inflammatory responses by increasing IκBα amount at transcriptional level. Indeed, IκBα bears a PPRE sequence on its promoter (L. Xu et al., 2012). Although no co-crystallized structures are available, recently the binding mechanism of 25HC3Sto PPARγ was simulated *in silico* by Yang *et al.*, showing the selection of a partial-agonistic conformation of the receptor by the ligand (Yang et al., 2019).

One of Ren’s group discoveries has been the identification of the sulfolipid 25HCDS. Like 25HC3S, 25HCDS is able to reduce Chol levels and to negatively regulate immune responses at transcriptional level probably interfering with LXRs, SREBPs and PPARγ (Ren et al., 2014). However, since no proof of concept regarding the exact mechanism of action of 25HCDShas yet been reported in literature, the latter hypothesis still remains elusive.

**Analytical strategies in the analysis of plasma oxysterol sulfates: current challenges**

Most of the findings reported on oxysterol sulfates in cells and tissues have been carried out using the commercially available 25HC3S standards (Bai et al., 2012; Ma et al., 2008; Ren et al., 2007; Leyuan Xu et al., 2010; L. Xu et al., 2012; Y. Xu et al., 2013) but exploratory studies have shown that the panel of oxysterol sulfates in circulation is broader.

One of the first studies focused on the screening of oxysterol sulfates in biological fluids described the presence of elevated levels of a compound compatible with compatible with the presence of a glucuronidated cholestenediol sulfate in serum and urine samples of children with severe cholestatic liver disease (Meng et al., 1997). The authors were able, after extensive sample handling and derivatisation steps, to identify and characterize it as the glucuronidated form of the 24HC3S by fast atom bombardment mass spectrometry using glycerol as a matrix compound (Meng et al., 1997). The authors also reported the occurrence of oxysterol glycine and taurine conjugates though sulfation seemed to be the main detoxification route in cholestatic liver disease and with potential prognostic value during clinical evaluation (Meng et al., 1997). Later, Acimovic *et al*. suggested that sulfation could act as a protective mechanism against the accumulation of oxysterols in circulation (Acimovic et al., 2013). A glimpse into the panel of oxysterol sulfates was expanded by Sanchez-Guijo *et al.* 2015 who reported the presence of the 27-hydroxycholesterol sulfate (27HCS, otherwise known as (25R)-26-hydroxycholesterol-3-sulfate) and found that 27HCS was not the only sulfated steroid derivative that was consistently elevated in serum samples of RLXI patients (Sánchez-Guijo et al., 2015), but was one among a wider panel of oxysterol sulfates including isomers containing the hydroxyl group at the 25-, 4-, and 7-position of cholesterol moiety and even 27HCDS (otherwise known as (25*R*)-26-hydroxycholesterol-26-sulfate) **(Figure 2)**.



**Figure 2**. Chromatographic separation of oxysterol sulfates in serum samples from RLXI patient (A) and healthy control subject (B) using targeted multiple reaction monitoring (MRM) detection mode. This data was originally published in the Journal of Lipid Research. Sánchez-Guijo A, et. al. High levels of oxysterol sulfates in serum of patients with steroid sulfatase deficiency. J Lipid Res. 2015;56(2):403–412. © the American Society for Biochemistry and Molecular Biology.

Despite the evidence for a wider panel of oxysterol sulfates in circulation provided by these exploratory studies (Meng et al., 1997; Sánchez-Guijo et al., 2015), very little is known about the predominant oxysterol sulfates circulating in fluids and tissues, their basal levels, and any variations introduced with age, gender and ethnicity in health and disease despite the common knowledge that SL gather at the surface of lipid-raft domains (Weerachatyanukul, Probodh, Kongmanas, Tanphaichitr, & Johnston, 2007) and contribute to cell-cell communication processes (Honke, 2017; Strott & Higashi, 2003). Oxysterols on the other hand, are widely studied and knowledge on the oxysterol signature in normolipidemia and normoglycemia conditions and their basal levels (Dias et al., 2018; Grayaa et al., 2018; McDonald, Smith, Stiles, & Russell, 2012; Murakami, Tamasawa, Matsui, Yasujima, & Suda, 2000; Narayanaswamy et al., 2015) is known. Oxysterols are predominantly found esterified to fatty acids (Dzeletovic, Breuer, Lund, & Diczfalusy, 1995) and are thought to be substrates for sulfotransferases (Hirotoshi Fuda et al., 2007) leading up to the formation of oxysterol sulfates.

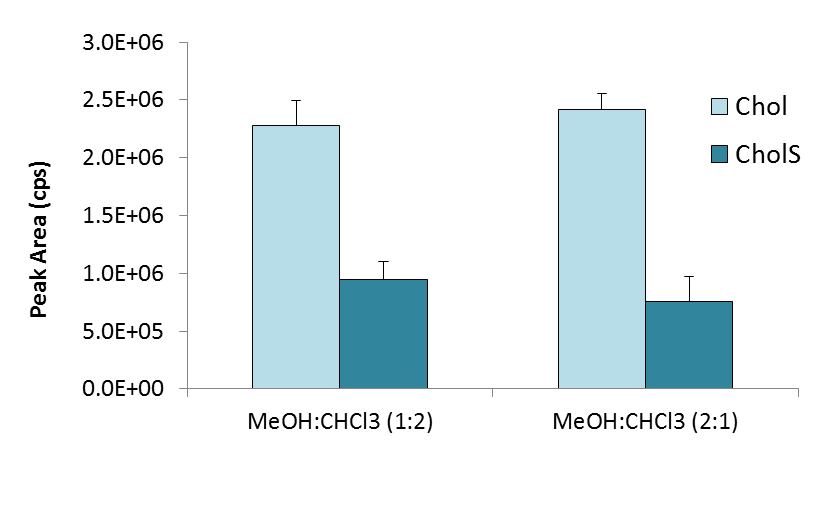
Regarding oxysterol sulfates, different data are available for 24HC3S where their levels range from 2-18 µM in cholestatic liver disease (Meng et al., 1997) and about 22.5-46 ng/mL (~46.7-95.4 nM) in steroid sulfatase deficiency (Sánchez-Guijo et al., 2015). The dispersity of values found could be attributed to differences in the characteristics of the individuals included in the study groups as well as to experimental and methodological conditions adopted, supporting the need for further investigation. Accurate knowledge on the basal levels of oxysterol sulfates in health and disease are intimately related to the experimental conditions chosen during the analysis pipeline including sample collection, storage, extraction, fractionation, separation, detection and quantification steps. Sample pre-treatment strategies are paramount in the discovery and validation of lipid-based markers in biological samples. Sample collection tubes, freeze-thaw cycles and storage conditions are often a major source of variability that affect not only the stability of samples but also the overall recovery and fingerprint of plasma lipids (Gonzalez-Covarrubias, 2013; Hammad et al., 2010; Lee, Kind, Yoon, Fiehn, & Liu, 2014; Sarafian et al., 2014). Work conducted on the analysis of structurally related-compounds such as CS and oxysterols (**Table 2**) reveals a diversity of sample pre-treatment strategies (e.g. anticoagulant), extraction solvent system used and analytical methodology has been largely overlooked.

**Table 2**. Analytical strategies employed in the collection, extraction, and analytical approach in the detection and quantification of cholesterol sulfate and oxysterols in human plasma samples.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | ***Biological matrix (collection tube)*** | ***Extraction approach***  ***(method and solvent system)*** | ***Analytical approach*** | ***Reference*** |
| Cholesterol sulfate | Plasma  (EDTA tube) | LLE with MeOH followed by purification on Baker-10 quartenary amine column | GC-FID (TMS derivatives) | (Muskiet, Jansen, Wolthers, Marinkovic-Ilsen, & van Voorst Vader, 1983) |
| Sodium  (citrate) | LLE with MeOH (80%) | HPTLC coupled to densitometry | (Przybylska et al., 1995) |
| - | LLE with acetone/ethanol (1:1, v/v), followed by purification in silica column and elution with CHCl3/MeOH (1:1, v/v) | GC-MS (TMS derivatives) | (Tamasawa, Tamasawa, & Takebe, 1993) |
| Serum | LLE with acetone/ethanol (1:1, v/v), followed by purification in acidified NH2 Bond Elut cartridge and elution with CHCl3/MeOH (1:1, v/v) | GC-MS (acetylated derivatives) | (Delfino, Procaccini, Illiano, & Milone, 1998) |
| Plasma  (lithium heparin) | LLE with MeOH, followed by purification by C18 SPE and elution with CHCl3:MeOH (2:1, v/v) | LC-APCI-MS/MS detection (underivatized) and quantification by MRM in QTRAP 3200 | (Fong, Tam, & Leung, 2013) |
| Serum/plasma | Protein ppt ACN-ZnSO4 followed by fractionation by SPE (SepPak cartridge) | LC-MS/MS | (Sánchez-Guijo et al., 2015) |
| Serum  (-) | SPE extraction with Strata-X (33um), followed by | LC-(ESI)MS detection and quantification by SIM in QqQ | (Lee et al., 2014) |
| Oxysterols | Plasma (K2EDTA) | Saponification in ethanolic solution, followed by LLE with CHCl3 and purification in silica SPE and elution with 30% iso-propanol in hexane | GC-MS of TMS derivatives | (Dzeletovic et al., 1995) |
| Plasma  (heparin) | Liq-Liq extraction with CHCl3:MeOH (2:1, v/v) followed by fractionation in a packed silica column and eluted in ethyl acetate | GC-MS of TMS derivatives | (Murakami et al., 2000) |
| Plasma  (EDTA) | LLE with MeOH:iso-propanol (1:1, v/v) | LC-(APCI)MS detection and quantification by MRM in QqQ | (Helmschrodt et al., 2013) |
| Plasma  (EDTA) | LLE with ethanol, followed by alkaline hydrolysis and extraction with CHCl3:MeOH (2:1, v/v) and SPE fractionation in a silica column | GC-MS of TMS derivatives | (Grayaa et al., 2018) |
| Plasma  (EDTA) | LLE with MeOH followed by fractionation by SPE in a HLB Oasis PRIME column | LC-(ESI)MS detection and quantification by MRM in QTRAP 5600 | (Dias et al., 2018) |
| Plasma  (K2EDTA) | Saponification of plasma in ethanolic solution, followed by protein precipitation in ACN (1.5% formic acid) and purification by SPE 96-well plates | LC/ESI-HR-MS detection and quantification against cal curves built with deuterated standards | (Hautajärvi, Hukkanen, Turpeinen, Mattila, & Tolonen, 2018) |

As shown in Table 2, several different anticoagulants are typically used in the collection of blood samples. Even though there is a lack of studies on the effect of sample pre-treatment strategies in the levels of oxysterol sulfates, published results with oxysterols, reveal that plasma oxysterol levels collected with K2-EDTA and citrate collection tubes differed from those observed in serum samples (Hautajärvi et al., 2018; Reinicke, Schröter, Müller-Klieser, Helmschrodt, & Ceglarek, 2018) supporting the use of EDTA-collection tubes over citrate or heparin tubes, due to the complete and non-reversible chelation of Ca2+ and Mg2+ ions which supressed oxidative reactions (Reinicke et al., 2018). In the case serum samples were used, Helmschrodt *et al*. suggested the addition of antioxidant, Butylated hydroxytoluene (0.05%) to increase stability of oxysterols (Helmschrodt et al., 2013). Another aspect that is often ignored are the freeze-thaw cycles, often required for biochemical and chemical analysis appear to have no effect on the levels of oxysterols (Hautajärvi et al., 2018; Helmschrodt et al., 2013; Sánchez-Guijo et al., 2015) or of CS (Sánchez-Guijo et al., 2015). Storage up to 3 months led to the same conclusions (Hautajärvi et al., 2018; Helmschrodt et al., 2013; Sánchez-Guijo et al., 2015).

Extraction of steroid-related compounds is typically conducted by liquid-liquid extraction (LLE) protocols followed by fractionation in solid-phase extraction (SPE) cartridges (Table 1). In fact, LLE protocols remain the most popular method of choice due to their simplicity, cost, sample volume required, extraction efficiency, reproducibility, repeatability, lipidome coverage, and potential for automation, where the overall performance of LLE protocols is very similar in the extraction of predominant lipid classes (Reis et al., 2013). In the case of structurally similar compounds, the extraction performance of Chol and CS in two of the most popular LLE solvent mixtures is similar, though solvent systems with higher dielectric constant (ε) extracted higher amounts of CS compared to Chol (MeOH:CHCl3 (2:1, v/v)), whereas solvent mixtures of lower ε with more hydrophobic character were more efficient towards the extraction of Chol but not of CS (MeOH:CHCl3 (1:2, v/v)) (**Figure 3**).



CS

**Figure 3.** Evaluation of extractability of Chol and CS in two different solvent systems. Graphs depict Peak Area (counts per second, cps) in two solvent systems; bars represent standard deviation (±SD). This data was originally published in the Journal of Lipid Research. Reis et al., A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. J. Lipid Res. 2013 54:(7) 1812-1824 © the American Society for Biochemistry and Molecular Biology

However, based on our previous experience on the extraction of lipids from biological samples, it is clear that organic solvent mixtures have a major impact on the extraction performance (Reis et al., 2013) particularly on the less abundant lipids. Remarkably, the influence of solvent system in the extraction performance of oxysterol sulfates by LLE protocols has not yet been addressed. Despite this lack of knowledge, the sulfate group confers increased polarity to the oxysterol, though the position of the hydroxy group may also be responsible for changes in hydrophobicity to the oxysterol sulfate moiety and hence potentially have a strong influence on the extractability of oxysterol sulfates in organic solvents. To support this, it was previously shown that the elution of underivatized oxysterol positional isomers under reverse-phase HPLC conditions was very distinct. The 24HC and 25HC isomers eluted prior to the 7-ketocholesterol (7KC) and 4β-hydroxycholesterol oxysterols (Dias et al., 2018; Grayaa et al., 2018; Narayanaswamy et al., 2015; Reinicke et al., 2018) confirming the distinct hydrophobicity of oxysterol positional isomers. These slight differences in polarity facilitate the chromatographic separation under reverse-phase conditions but could also impact the extraction efficiency of oxysterol sulfates from aqueous biological matrices during the LLE when polar solvent mixtures are used. In the case of oxysterols sulfates, extraction by protein precipitation with ACN-ZnSO4 (4:1, v/v) followed by C18 SPE fractionation (Sánchez-Guijo et al., 2015) resulted in complete recovery (100.6%).

While the presence hydroxy group affects the hydrophobicity of the oxysterol moiety and may impact on the performance during extraction step, the presence of the sulfate and hydroxy groups in oxysterols sulfates also impacts on the detection approaches that can be used to detect and quantify oxysterol sulfates. Unlike oxysterols that are usually detected in the positive ion detection mode (Dias et al., 2018; Hautajärvi et al., 2018; Helmschrodt et al., 2013; Mendiara et al., 2018; Murakami et al., 2000) the presence of the sulfate group facilitates the detection of oxysterol sulfates in the negative ion mode through mass spectrometry-based approaches. Because oxysterols sulfates occur in residual levels in biological samples, detection of oxysterols sulfates is often achieved by targeted detection approaches such as MRM. Due to the specificity of the transitions in MRM approaches, these display an increased sensitivity in the detection step with the advantage of eliminating the contribution of the other sulfated metabolites that contribute to the overall plasma sulfometabolome and observed in targeted approaches like precursor ion scanning (Dias et al., 2019). Previous work by Sanchez-Guijo and colleagues established 1ng/mL as the limit of detection of oxysterol sulfates in MRM detection approaches (Sánchez-Guijo et al., 2015).

Contrarily, the presence of the hydroxy group has no influence on the efficacy of ionisation and hence on the detection step. As ionisation of oxysterol sulfate occurs by removal of hydrogen atom at the sulfate group, the ionisation efficiency is similar to that of CS. This was confirmed by the injection of an equimolar mixture of oxysterol sulfates and CS and detection under reverse-phase elution conditions in the negative ion mode (unpublished results).

Regardless of the collection, extraction, and analytical strategy adopted in the analysis of oxysterol sulfate, the values reported (Acimovic et al., 2013; Meng et al., 1997; Sánchez-Guijo et al., 2015) show that these are well below the micromolar range generally used in the biological assessment of oxysterol sulfates in cells and tissue (Ren et al., 2007; Leyuan Xu et al., 2010; L. Xu et al., 2012). Based on the literature reported, oxysterols which are structurally related compounds of oxysterol sulfates account for less than 1% of total Chol in hyperlipidemia (Björkhem et al., 2001; Dias et al., 2018; Reinicke et al., 2018) while oxysterol sulfates (24HC3S and 26HC3S) account for less than 15% of total oxysterols (Acimovic et al., 2013). This could explain why oxysterol sulfates have been largely overlooked by the scientific community.

**Concluding Remarks**

In summary the lack of a more complete panel of oxysterol sulfate standards commercially available and the poor knowledge on the optimal conditions for the extraction, detection and quantification of oxysterol sulfates from biological matrices has hampered the complete understanding on the role of oxysterol sulfates. New analytical methods are needed to improve our understanding of molecular interplay between oxysterols and oxysterol sulfates at cell and tissue levels that are at prime importance for Chol /oxysterol homeostasis. Moreover it would be desiderable a greater interest of synthetic organic chemist community in this research field potentially culminating in the commercial availability of novel oxysterol sulfates to be used as standards.

**Competing Interests' Statement**: None

Authors declare no conflicts of interest.

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**Abbreviations**

24HC: 24(*S*)-hydroxycholesterol

24HC3S: 24(*S*)-hydroxycholesterol-3-sulfate

24HCDS: 24(*S*)-hydroxycholesterol-3,24-disulfate

25HC: 25-hydroxycholesterol

25HC3S: 25-hydroxycholesterol-3-sulfate

25HCDS: 25-hydroxycholesterol-3,25-disulfate

26HC: (25R)-26-hydroxycholesterol

26HC26S: (25R)-26-hydroxycholesterol-26-sulfate

26HC3S: (25R)-26-hydroxycholesterol-3-sulfate

27HC: 27-hydroxycholesterol

5,6αECS: 5α,6α-epoxycholesterol-3-sulfate

5-LO: 5-Lipoxigenase

7KC: 7-ketocholesterol

7KCS: 7-ketocholesterol-3-sulfate

ACN: Acetonitrile

CS: Cholesterol sulfate

DHEA: Dehydroepiandrosterone

DHEAS: Dehydroepiandrosterone sulfate

DOCK2: Dedicator of cytokinesis protein 2

EDTA: Ethylenediaminetetraacetic acid

HMG-CoA reductase: 3-hydroxy 3-methylglutaryl-CoA reductase

HPLC: High Performance Liquid Chromatography

IκBα: NF-κB inhibitor

LLE: Liquid-liquid extraction

LXRα: Liver X receptor alpha

LXRβ: Liver X receptor beta

Mincle: Macrophage inducible Ca2+-dependent lectin receptor

MRM: Multiple Reaction Monitoring

NF-κB: Nuclear Factor-κB

PAPS: 3’-phosphoadenosine 5’-phosphosulfate

PIP2: Phosphatidyinositol diphosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

PPARγ: Peroxisome proliferator-activated receptor gamma

Preg: Pregnenolone

PregS: Pregnenolone sulfate

PtdIns-3K: Phosphatidylinositol 3-kinase

RLXI: Recessive X- linked ichthyosis

RORα: Retinoic acid-related orphan receptor α

SL: Sufate-based lipids

SPE: Solid Phase Extraction

SREBP-1: Sterol Regulatory Element-Binding Protein-1

STS: Steroid Sulfatases

SULTs: Sulfotransferases

TCR: T-cell receptor

TNFα: Tumor Necrosis Factor alpha

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