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Analytical strategies for characterization of bile acid and oxysterol metabolomes

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ABSTRACT

Cholesterol is the precursor of many compounds with functions in the physiology and metabolism of the organism. Methods for the multicomponent analysis of these compounds and their metabolites (metabolomics) are needed to improve our understanding of their roles in different species, organs, cells and metabolic situations and to clarify structure/activity relationships. This review discusses methods based on combinations of ion exchange and reversed-phase separations for sample preparation with derivatization and "charge-tagging" for chromatography-mass spectrometry in qualitative and quantitative characterizations of oxysterol, bile alcohol, bile acid, and steroid hormone metabolomes. Advantages, disadvantages and potential improvements for high-throughput applications are briefly discussed.

1. Introduction

Cholesterol is the precursor of several families of compounds with important regulatory functions in physiology and metabolism. These include oxysterols, bile alcohols, bile acids and hormonal steroids. The biosynthesis of biologically active end products in these families takes place via chains of reactions in which the intermediates may also be metabolized to side products with or without known biological functions. The end products can be metabolized by hydroxylations, oxidoreductions, and conjugation with neutral or acidic sugars, sulfuric acid, amino acids, etc. Thus, the transformation of cholesterol into biologically important compounds will potentially lead to a complex pattern of compounds present at widely differing levels. The formation of major metabolites directly influences cholesterol balance, while less abundant metabolites may have regulatory functions via interactions with receptors and signaling systems. The common origin of the regulatory molecules, their potential interactions and inactivation, raises a need for simultaneous analysis of all components, biologically active or inactive, i.e. a metabolomic approach. The only available method providing sufficient sensitivity and specificity is mass spectrometry (MS). The complexity of the metabolomes, including isomerism of isobaric components, makes a combination with high-resolution chromatography necessary. Appropriate preparation of the samples to be analyzed is a third requirement. This review discusses strategies that have been applied in our laboratories for multicomponent analysis of oxysterols, bile acids, bile alcohols and hormonal steroids.

2. Metabolome analysis

2.1. Oxysterol metabolome

Oxysterols can be regarded as oxygenated forms of cholesterol (and plant sterols), although this definition can be extended to also include oxygenated forms of precursors of cholesterol [1]. The first step of all cholesterol metabolism leads to the formation of an oxysterol, and most of the enzymes responsible for such reactions are members of the cytochromes P450 (CYP) superfamily [2]. It is important to emphasize that the generic term oxysterol covers many distinct chemical entities, and although many oxysterols are isomers of one another they may have widely different biological roles. As well as being formed by enzyme-catalyzed reactions oxysterols can also be formed by autoxidation of cholesterol in air [3]. It is virtually impossible to prevent formation of oxysterols by autoxidation and the only way to distinguish analytical artifacts from endogenous oxysterols is to add pure isotope-labeled cholesterol to the sample and then calculate percentage of autoxidation product from the isotope excess in the oxysterols [3]. This is an important analytical consideration when oxysterols are being studied in biological systems which usually contain a 10^3 fold excess of cholesterol. Oxysterols with specific structural motifs have been shown in vitro to inhibit cholesterol synthesis by interacting with proteins involved in regulation of transcription of genes encoding enzymes of the cholesterol synthesis pathway [4] and to be ligands of the liver X receptors (LXRs) [5]. LXRs are usually thought of as regulators of the expression of genes important for

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lipid homeostasis, but recent results indicate that LXR activation by oxysterols regulates cell division, neurogenesis and neuron development in embryonic brain [6], while other reports show LXRs to be important regulators of hedgehog signaling [7]. It is possible that different oxysterols act as LXR ligands in different cell types, but as yet, there have been few studies of the comprehensive profile of oxysterols in different cells, tissues or body fluids.

Early analytical procedures were based on Folch-like extractions and gas chromatography (GC)–MS, and involved hydrolysis of fatty acid esters followed by derivatization of oxysterols and GC–MS utilizing selected ion monitoring (SIM) [8–10]. However, the use of SIM is only of value for the identification of expected compounds and precludes the identification of unexpected molecules. In recent years the authors have developed an alternative approach for multicomponent oxysterol analysis, including analysis of conjugates, based on liquid chromatography-electrospray ionization (LC-ESI)-MS and –tandem mass spectrometry (–MS/MS or –MS^n).

Our methodology for the analysis of oxysterols and their immediate metabolites e.g. sulfate esters and C_{27} acids, involves the following steps. 1. extraction with ethanol; 2. delipidation by C_{18} solid phase extraction (SPE); 3. derivatization and "charge-tagging"; 4. analysis by LC-ESI-MS/MS or –MS^n [11,12]. This procedure does not include a saponification step and oxysterols esterified with fatty acids are partly lost in steps 1 and 2. A procedure for their analysis is suggested in Section 2.4. Delipidation is important to remove cholesterol, which is far more abundant than any oxysterol in a biological sample, and is prone to autoxidation. If analytical interest is only confined to oxysterols and not their acidic metabolites delipidation may alternatively be performed by normal-phase SPE [13–15]. Oxysterols give comparatively poor response in LC-ESI-MS, thus to improve sensitivity oxysterols are derivatized with a charged group i.e. "charge-tagging". "Charge-tagging" utilizing the Girard P (GP) hydrazine reagent improves sensitivity by two–three orders of magnitude. Rather than base our derivatization chemistry on the universal alcohol group we add specificity to the method by utilizing the enzymatic activity of cholesterol oxidase which converts the 3β-hydroxy-5-ene (and 3β-hydroxy-5α-hydrogen) group of oxysterols to a 3-oxo-4-ene (or 3-oxo) function which can then be derivatized with GP hydrazine. Derivatization in the absence of cholesterol oxidase allows the differentiation of oxysterols which naturally contain an oxo group from those oxidized to contain one. Using this methodology we have profiled the oxysterol content of plasma and cerebrospinal fluid (CSF). Sensitivity is such that pg quantities of oxysterols can be identified by LC-ESI-MS^n, and has allowed the identification of very low levels of oxysterol (e.g. 24S-hydroxycholesterol 18 pg/mL) and unexpected acidic metabolites such as 7α-hydroxy-3-oxocholest-4-enoic acid in CSF [12]. As with all derivatization reactions there are some side reactions. For instance the oxysterol metabolite 7α-hydroxy-3,24-*bis*oxocholest-4-enoic acid becomes decarboxylated to 7α-hydroxy-26-*nor*-cholest-4-ene-3,24-dione, while 24S,25-epoxycholesterol identified in rodent brain [15] suffers hydrolysis and methanolysis to the 24,25-diol or 24-ol, 25-methyl ether (or 24-methyl ether,25-ol) and 5,6-epoxycholesterols are hydrolyzed to 5α,6β-diols which subsequently eliminate water to give an enol or allyl alcohol.

Oxysterols conjugated at position 3 are not susceptible to oxidation with cholesterol oxidase and subsequent "charge-tagging" with GP hydrazine. However, those esterified with sulfuric acid at this position can be readily analyzed by LC-negative-ion-ESI-MS/MS. Sulfation may provide a route to inactivate oxysterol ligands of LXR, and SULT2B1, the enzyme which sulfates oxysterols, is induced during T cell activation, suggesting regulation of LXR activity by metabolism of its ligand [16]. Using the methods described above it is now possible to monitor active oxysterol ligands and their sulfated metabolites in parallel.

2.2. Bile acid metabolome

Bile acids and/or bile alcohols are end products of cholesterol metabolism in vertebrates [17]. The synthesis of bile acids in the liver involves 16 enzymes many of which have multiple roles in metabolism [18]. While the composition of bile acids in bile may appear simple, a detailed analysis reveals complex mixtures of molecular species found at different levels in different biological fluids, tissues and excreta and showing large species differences (for references see Hofmann et al. [17] and Sjövall et al. [19]). Bile acid synthesis is regulated by several mechanisms involving nuclear receptors and other factors [20] but knowledge of correlations between bile acid structure and regulatory efficiency is incomplete. Bile acids can also regulate a number of metabolic processes of pathophysiological importance [21]. Also in this case knowledge of structure/activity relationships is limited. It should also be pointed out that bile acids can be formed extrahepatically from oxysterols. Characterization of bile acid metabolomes should increase our understanding of how different bile acid structures might participate in the regulatory processes.

The first report of systematic multicomponent analysis of bile acids was presented in the mid 1970's. The method involved five separate steps: 1. SPE; 2. separation of groups of conjugates by ion exchange chromatography; 3. cleavage of conjugates by chemical or enzymatic methods; 4. conversion of the free bile acids into volatile nonpolar derivatives; 5. computerized GC–MS. These principles were applied in several laboratories, particularly at Karolinska Institutet and in Japan. The method combined separation of classes of bile acids (initially unconjugated, glycine, taurine, and sulfate conjugates) with separate analysis by GC–MS, monitoring ions representative of number and nature of nuclear and side chain substituents. With this method the urinary bile acid metabolome in cholestatic subjects could show presence of about 120 molecular species of about 30 bile acids with different nuclear or side chain substituents [22,23]. While this may seem complex, glucuronides were not analyzed and the existence of bile acid conjugates with glucose, galactose and *N*-acetylglucosamine was not known at the time. Further complexity arises from the presence of doubly or triply conjugated bile acids and from variations of the sites of conjugation.

With the advances in sorbent, capillary column, instrumentation, and computer technologies the original procedure has been and can still be greatly improved (see Sjövall et al. [19]). The lipophilic ion exchanger is of great value for separations of the neutral bile alcohols from bile acids [24] and in analyses of the variety of conjugated forms that result from bile alcohol and bile acid metabolism (see Sjövall et al. [19]) as exemplified by selected studies from Karolinska Institutet [25–28].

The lipophilic ion exchanger is also of great value for isolation of the naturally occurring unconjugated bile acids and those obtained after cleavage of the separated fractions of conjugates. When applied to studies of unconjugated bile acids in serum, three C_{27} bile acids were found [29]. The metabolomic approach was then used to evaluate the involvement of these acids in bile acid biosynthesis in vivo [30] and in hepatocytes and hepatoma cells [31,32]. This approach permitted simultaneous monitoring of 10 C_{27} bile acids (unconjugated) and nine C_{24} bile acids (unconjugated or conjugated with glycine/taurine or sulfated) and five hydroxysterols in the medium from the hepatoma cells.

While the combination of ion exchange separation and GC–MS analysis provides detailed information about bile acid and bile alcohol metabolomes, the method has several drawbacks. It is laborious and unfit for high-throughput analyses. The reactions for cleavage of conjugates and derivatization and the separations at high temperature can create artifacts. These limitations were largely eliminated with the introduction of fast atom bombard-

ment (FAB) and ESI. Depending on their concentrations, conjugated bile acids and bile alcohols in solid phase or solvent extracts of fluids and tissues can be directly analyzed by FAB- or ESI-MS. This led to discovery of inherited and acquired deficiencies of enzymes in bile acid biosynthesis and diagnostic use [33]. The spectra provide rapid information about the potential structures present, the nature of conjugates and metabolic blocks due to deficient enzymes. However, compounds of the same nominal mass are not separated unless high-resolution MS is used and high resolution does not resolve isobaric isomers commonly present. This structural ambiguity can be reduced or eliminated by high-resolution MS, collision-induced decomposition (CID) of selected ions or combination with LC. High-resolution MS permits calculation of elemental composition, CID determination of nature and position of conjugation with partial information on skeletal structure, and LC can separate isobaric isomers including ketones from unsaturated alcohols. Derivatization can also be used to modify specific functional groups for detection by MS.

A number of methods based on LC–MS (with positive or negative ion monitoring, with high or low mass resolution and with or without CID with multiple reaction monitoring (MRM) MS/MS have been published in the past few years (for references see Griffiths and Sjövall [34]). The methods based on ESI are more sensitive than those based on GC-electron impact ionization (EI)-MS. However, EI-MS provides much more structure information and possibilities to identify isomeric and other isobaric bile acids (see Sjövall et al. [19]). The LC–MS (and MS/MS) methods so far described in the literature are not truly metabolomic but rather targeted towards common bile acids, e.g. cholic, chenodeoxycholic, deoxycholic, lithocholic, ursodeoxycholic, muricholic acids, free or conjugated with glycine or taurine. Sulfated forms are sometimes included but glucuronides and other sugar conjugates known to be formed with selected isomers (e.g. glucosides and *N*-acetylglucosaminides) are usually not included. Several results obtained with LC–MS analyses are difficult to reconcile with previously published results obtained with the more comprehensive ion exchange-GC–MS methods (see Sjövall et al. [19]). The latter have shown much more complex metabolite mixtures in plasma, liver, urine and feces than those so far demonstrated with the LC–MS methods. Not only are there many more bile acid structures (exemplified for human urine in references above, for human feces in Ref. [35] and for rodents in Refs. [36,37], the conjugation differs with the bile acid structure so that the profiles of deconjugated bile acids differ greatly between different groups of conjugates (exemplified e.g. [26,27]). These differences have not yet been discussed in papers on LC–MS/MS and it will be important to compare results from analyses of the same samples from the same and different fluids and tissues with different methods. A comparative study of direct FAB- and ESI-MS with LC–MS of groups of conjugates separated by ion exchange detected about 150 bile alcohol and bile acid conjugates [38].

2.3. Hormonal steroid metabolome

Metabolic profiles of hormonal steroids were also analyzed by a combination of ion exchange chromatography and GC–MS [39,40]. The profile of conjugated steroids in urine from a healthy male included 77 different steroids many of which were conjugated in several ways to increase the number of molecular species to 116 [39]. At this time no steroid acids and only glucuronides, monosulfates and disulfates were included. Later modifications and improvements of the method have been described and discussed in detail [41,42]. Numerous studies of steroid profiles following initial hydrolysis of conjugates have been published. Large species and sex differences have been found. The profiles can yield important diagnostic information.

Profiles of unconjugated hormonal steroids in human plasma were analyzed by a sample preparation procedure using small beds of lipophilic cation and anion exchangers to remove charged and less polar interfering compounds [40]. Twenty-seven unconjugated steroids (many being isobaric) in the concentration range 1–300 ng/mL were identified in plasma from women in late pregnancy.

These early studies give indications of the demands put on an LC–MS/MS method that would cover the wide range of steroids and their different conjugates. At present it is difficult to envisage a method that would not require inclusion of sample preparation step(s) with capability to separate groups of steroids. The need for appropriate sample preparation is further emphasized by the wide range of steroid concentrations and the presence of orders of magnitude higher levels of interfering substances.

We have studied variations of the above procedures for application to the analysis of neurosteroids in rat brain by LC–MS/MS. The aim was to increase sensitivity, to widen the range of steroids to be analyzed, to enable direct analysis of intact steroid conjugates, and to simplify sample preparation [43]. While only some goals were reached, a target-limited method compatible with a general scheme for analysis of steroid metabolomes was achieved. Steroids containing an oxo group were targeted since the major neurosteroids contain this group and a simple "charge-tagging" method for their isolation as oximes was previously developed [42]. Conversion of oxo groups into oximes greatly increases sensitivity in positive ion ESI-MS.

The method consists of three major initial steps without any solvent evaporation: 1. ethanol extraction; 2. solid phase removal of nonpolar lipids and positively charged lipids; 3. separation of neutral steroids and groups of conjugates by anion exchange chromatography. The neutral steroid fraction is reacted with hydroxyammonium chloride and the "oxime-tagged" steroids are isolated by cation exchange in methanol. Steroid glucuronide and sulfate fractions are collected. Nano-LC–MS/MS is performed, in the positive mode for oximes, and in the negative mode for the sulfates. On the most sensitive MS/MS instruments narrow-bore or micro-bore columns may be used rather than nano-LC columns. Important features of the method are absence of evaporation steps prior to collection of the fractions for LC–MS/MS, use of SPE for delipidation rather than extraction of the steroids, and separate LC–MS/MS analysis of unconjugated and intact conjugated steroids. Early removal of cholesterol and separation of steroid sulfates by ion exchange rather than by polarity are essential. Depending on unreliable SPE procedures for steroid isolation and cholesterol removal, previous GC–MS methods could give erroneous results due to autoxidation of cholesterol to the neurosteroids pregnenolone and dehydroepiandrosterone [44]. The strategy used in Ref. [43] eliminates such errors.

2.4. Strategy for analysis of combined metabolomes

The design of a method for multicomponent analysis of neutral and acidic steroids obviously depends on the variations of structures to be included, the nature of the biological samples and the demands for sensitivity and dynamic range. In the simplest cases there will be no need for sample preparation other than extraction. The following potential analytical procedure for the "total steroid metabolome" assumes a much more complex mixture of steroids. It is also assumed that the mass spectrometric method is sufficiently sensitive to permit use of small sample sizes which can be desalted on the LC precolumn rather than in a prior step. Small sample sizes are also important for use of small sorbent beds and small solvent volumes to minimize solvent-derived background interference.

The steroid extract in ethanol is diluted to 70% ethanol, centrifuged and passed through a C_{18} SPE column. This will allow the

separation of the most hydrophobic sterols e.g. cholesterol, which are retained on the column, from more hydrophilic steroids which elute in the flow-through. This separation avoids the contamination of the "hydrophilic fraction" (70% ethanol flow-through) with potential cholesterol autoxidation products formed during further sample processing [44]. The "hydrophilic fraction" is also passed through a cation exchanger that traps positively charged steroids [43] e.g. the antimicrobial aminosterols [45] for separate isolation. The flow-through is finally passed through an anion exchanger to isolate neutral compounds and separate acidic steroids and conjugates. The latter can be sub-fractionated according to acidity as discussed in Sections 2.2 and 2.3 (Fig. 1). The elution protocol can be varied depending on the nature of the sample and the aim of the analysis. In the Karolinska laboratory the favored lipophilic anion exchangers have been Lipidex-DEAP and TEAP-LH-20 [42] but are no longer commercially available. The Japanese groups use PHP-LH-20 available from Shimadzu. An alternative may be provided by mixed mode anion exchange and reversed-phase sorbents. The neutral fraction from the anion exchanger can be dried and derivatized as described in Section 2.1 using a bacterial cholesterol oxidase (from *Brevibacterium)* active towards both C_{27} and C_{19}–C_{21} steroids. For analysis of steroids possessing an oxo group the cholesterol oxidase step can be omitted. The products are then analyzed by LC-ESI-MS/MS (or $-MS^n$). An alternative approach to derivatization is to directly modify an alcohol group on the oxysterol or steroid. For example Honda et al. derivatized oxysterols to picolinyl esters which ionize readily in ESI and can be analyzed with high sensitivity by LC-ESI-MS/MS with MRM [46]. The "charge-tagging" strategy also opens the possibility of purification by cation exchange. However, in all cases of derivatization incomplete reactions, or artefacts due to unexpected side reactions, are possible.

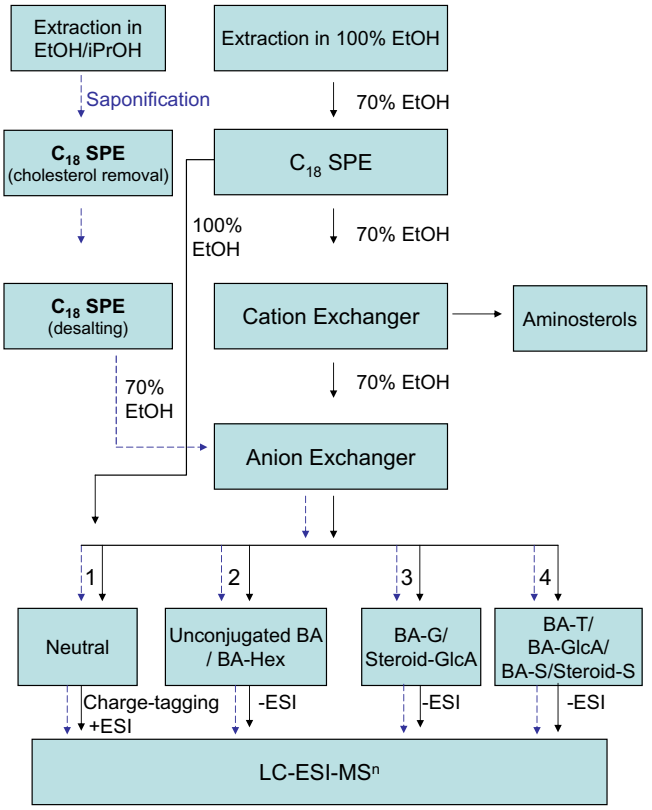

Fig. 1. Multicomponent analysis of neutral and acidic steroids. The group separation shown is an example using ammonium acetate buffers with increasing pH and/or ionic strength in 70% ethanol. Other sequences can be chosen depending on the needs and the nature of the steroid mixture. The lipophilic ion exchangers permit use of less polar solvent mixtures when needed to completely dissolve the material to be separated. Final analysis is by LC-ESI-MS/MS (or $-MS^n$). GC-EI-MS with derivatization may be required to glean additional structural information. *Abbreviations:* BA, bile acid; BA-Hex, hexose conjugated bile acid; BA-G, glycine conjugated bile acid; Steroid-GlcA, neutral steroid conjugated with glucuronic acid; BA-T, taurine conjugated bile acid; BA-GlcA, glucuronic acid conjugated bile acid; BA-S, sulfuric acid conjugated bile acid; Steroid-S, neutral steroid conjugated with sulfuric acid. Doubly or triply conjugated forms of the steroids will appear in the same fraction from the anion exchanger as the singly conjugated form when the additional conjugating moiety is neutral and in a later fraction if it is acidic. The last fraction from the anion exchanger is eluted with an ammonium acetate buffer at pH 9.5 that will elute even trisulfated bile acids i.e. all presently known forms of conjugates. If the carboxyl group of a bile acid is protected, by e.g. glycosylation, this conjugate will appear in a preceding fraction compared to the steroid with the free carboxyl group. Sample preparation utilizing saponification should be performed independently from the fraction containing non-esterified sterols and steroids. To emphasize this point the pathway for analysis of fatty acid esters of oxysterols and bile acids is indicated by blue broken arrows.

The unconjugated bile acids and their conjugates with neutral sugars in fraction 2 from the anion exchanger (Fig. 1) can be analyzed by negative ESI-MS/MS without or with LC, and so can fractions 3 (glycine conjugated bile acids and glucuronides of neutral steroids) and 4 (taurine conjugated bile acids and steroid sulfates and bile acid glucuronides and sulfates). Our preference for "metabolome" analysis is to always include an LC linked to ESI-MS/MS. Depending on the amount of material available and the sensitivity of the MS instrument this may be narrow bore or capillary-LC. Ultimate sensitivity is achieved with nano-LC linked to nano-ESI.

The strategy above does not include nonpolar fatty acid esters of oxysterols and bile acids. For example, monohydroxy bile acids esterified at C-3 are present in feces and oxysterol fatty acid esters are found in blood and tissues. These nonpolar compounds may be incompletely extracted with ethanol and/or appear in the nonpolar SPE fraction with cholesterol. They are better analyzed separately, e.g. by extraction of the sample with ethanol/2-propanol. Potassium hydroxide can then be added for saponification of the esters. Following acidification, water is added to a level that results in trapping of cholesterol and fatty acids in the SPE procedure. The flow-through containing the hydrolyzed oxysterols and bile acids can then be extracted/desalted in a second SPE using a recycling procedure [14,19]. The saponification procedure will lead to cholesterol autoxidation and thus should not be done with a fraction containing non-esterified sterols and steroids.

An important problem in any method is the presence of interfering compounds that will overload the column or affect the mass spectrometric specificity (e.g. polar hydroxy-lipids in neutral fractions, fatty acids, acidic phospholipids in steroid conjugate fractions). Therefore, depending on the needs, each fraction collected should have an individual scheme for clean-up. Since the fractions have already been separated by ion exchange, further purification can be based on polarity in an SPE mode or on derivatization. If "charge-tagging" of a specific functional group is used, steroids with this group can be isolated/purified by ion exchange as discussed in Section 2.3.

The method for analysis of the "total steroid metabolome" as described in Fig. 1 can be utilized in metabolomic studies, but also as a way to chemically characterize a biological activity or isolate an active component.

3. Conclusions

Renewed interest in metabolite profiling (now rechristened metabolomics) has stimulated major new research efforts. Most of these use MS as the ultimate detector with varying degrees of sample preparation prior to analysis, ranging from a simple protein precipitation to the more elaborate program described in Fig. 1. While simple preparation methods may be appropriate for profiling of the most abundant and readily ionized components they

only "scratch the surface" of the metabolome. To allow "deep-mining", and to observe low-level metabolites orthogonal sample preparation strategies are required as illustrated in Fig. 1.

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