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Determination of total plasma oxysterols by enzymatic hydrolysis, solid phase extraction and liquid chromatography coupled to mass-spectrometry

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ABSTRACT

The potential use of cholesterol esterases was tested to avoid alkaline hydrolysis for cleavage of plasma esterified oxysterols. The enzymatic hydrolysis was optimized by testing two sources of enzyme—*Pseudomonas* and bovine pancreas, presence of surfactants, incubation time and amount of enzyme. Free forms of 4β-, 7-, 24-, 25- and 27-hydroxycholesterol (HC) as well 7-ketocholesterol (7-KC) were analyzed by liquid chromatography and mass-spectrometry using the deuterated internal standard, 25-HC(d6). Enzymatic hydrolysis was more effective using the *Pseudomonas* enzyme and in presence of surfactants. Compared to alkaline hydrolysis, it generated a cleaner chromatographic baseline and better recovery of the internal standard. Oxysterols were assayed with detection limits between 7 and 31 pg/mL. Interassay coefficients of variation were lower than 10% and extraction recovery efficiencies, higher than 90%. The procedure was used to characterize plasma levels of *Cyp7b1*-deficient rat, where it showed increased plasma levels of 7, 24 and 25-HC. Due to the low volume of sample required, it may be used in other animal models, particularly rodents, as well as in pediatric samples where sample amount is always a problem. Thus, the proposed new method offers mild enzymatic processing that greatly facilitates oxysterol determinations to delineate their role in physiopathology.

1. Introduction

Oxysterols, particularly those presenting the hydroxyl radical at positions 25 and 27 (Fig. 1), are enzymatically derived oxidation products of cholesterol that are lately being associated with highly prevalent diseases [1]. In this regard, as oxysterols are metabolites of cholesterol, they have mainly been studied in cardiovascular diseases [2]. When they accumulate, they have toxic effects on several cell types. For instance, 25-hydroxycholesterol (25-HC) and 7-ketocholesterol (7-KC) were shown to induce apoptosis of smooth muscle cells via the mitochondrial pathway and reactive oxygen species generation [3]. In macrophages, incubation with 25-HC reportedly activates stress response genes and reprograms protein translation, independently of liver X receptors and sterol-response element-binding proteins [4]. It has also been shown that the production of 25-HC promotes the transformation of macrophages into foam cells in atherosclerotic plaques [5]. Moreover, 25-HC has been proposed to exert antiviral activity [6]. Likewise, oxysterols have been a matter of intense research in the nervous system, where their accumulation has been found to modify oxidative stress [7], apoptosis [8] and cell death in several types of neural cells such as PC12 cells [9,10] and sympathetic [11], retinal [12] and cerebellar [13] neurons. 25-HC has also been proposed as a marker of Alzheimer's disease progression due to its ability to modulate choline acetyltransferase [14] and an etiological agent in amyotrophic lateral sclerosis [15]. Finally, 25-HC has been found to regulate the activity of estrogen receptors alpha and beta [16], which could cause reproductive disorders. Likewise, it has been reported to be involved in breast and ovarian cancer [2], as well as other types of cancer, and even in metastasis, through oxysterol binding protein [17,18]. Plasma 24-HC and 27-HC seem to be sensitive biomarkers for the evaluation of mild cognitive impairment and Alzheimer's disease [19]. Brain 7-KC, 7-HC and 4beta-HC levels have also been found associated with progression of this disease [20]. Furthermore, plasma 7-HC and 27-HC have been considered po-

Abbreviation: 4-βHC, 4β-hydroxycholesterol; 7-HC, 7-hydroxycholesterol; 7-KC, 7-ketocholesterol; 24-HC, 24-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; LC–MS, liquid chromatography-mass spectrometry; SIR, single ion recording; SPE, solid phase extraction

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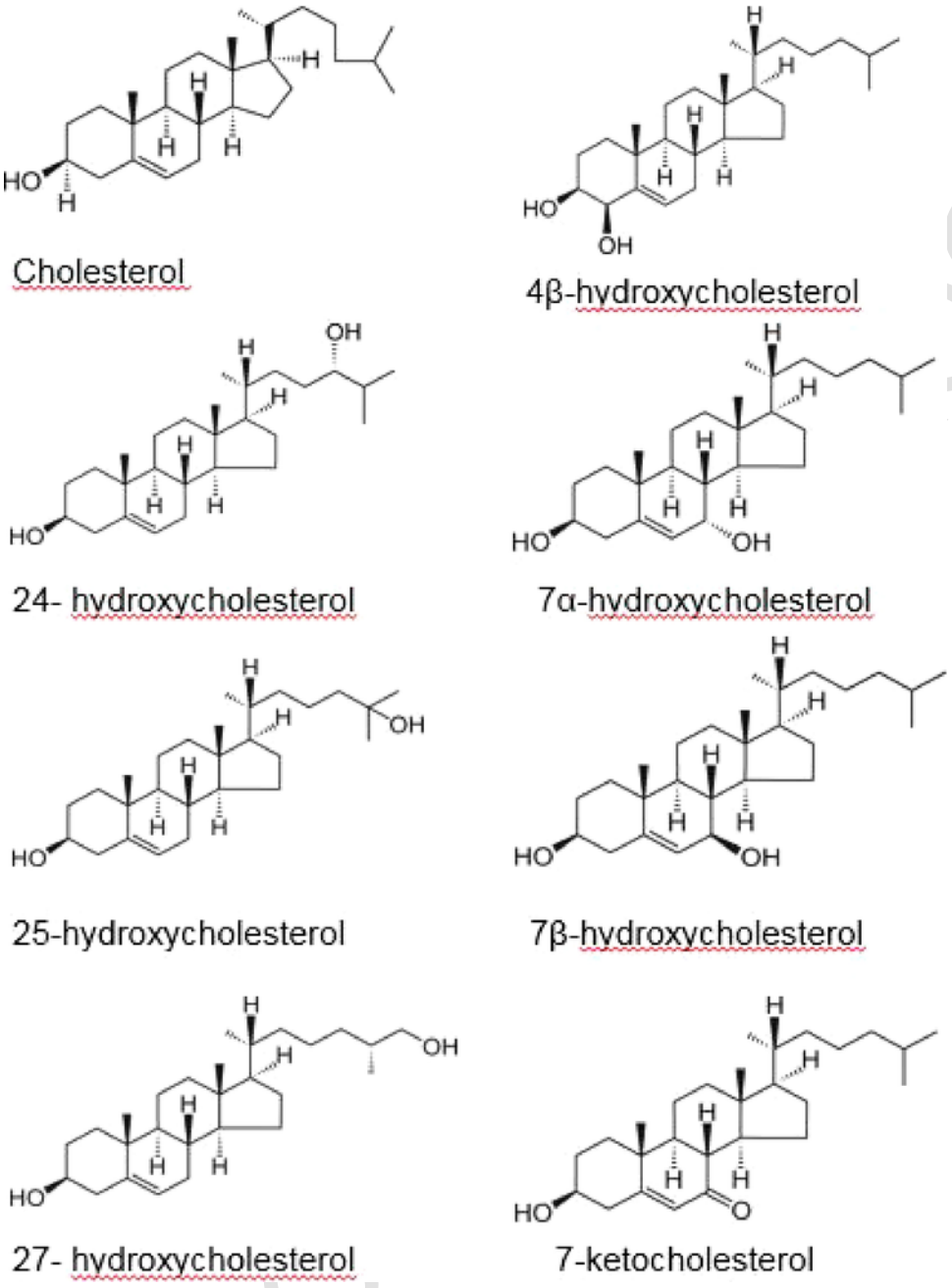

Fig. 1. Chemical structure of cholesterol and its oxysterol derivatives.

tential biomarkers of multiple sclerosis [21]. Plasma 4beta-HC has been proposed as a subrogate marker to assess hepatic CYP3A induction [22,23].

Plasma levels of 25- and 27-hydroxycholesterol were similarly elevated in mice lacking oxysterol 7alpha-hydroxylase CYP7B1 [24]. Thus, it was proposed that this enzyme could have a major role in their metabolism. In contrast, patients displaying low CYP7B1 activity showed dramatic increases in plasma 25-HC levels and only moderate in those of 27-HC [25]. These data indicate a preferential selectivity of the enzyme according to species. To further support this hypothesis, plasma oxysterol profile needs to be characterized in rats with inactivated C*yp7b1* gene.

Due to the above-mentioned findings, much interest has been devoted to improve the quantitation of oxysterols, which is complicated by their low plasma concentrations (in the range of ng mL^{-1}). In fact, cholesterol is approximately 1000 times more abundant than the next most abundant oxysterol [26]. Special efforts have been made to isolate the analytes from the plasma lipoproteins, their natural conveyance through the bloodstream. To this purpose, several extraction methods have been employed to remove interfering proteins [27,28]. Recently, the use of dichloromethane/methanol has been proposed to protect the sample and workers exposed to organic solvents [26]. Furthermore, their total quantification requires an alkaline hydrolysis step to transform oxysterol esters to free forms [29–33]. However, this alkaline hydrolysis not only has a serious drawback, since it re-

duces sample integrity due to the degradation of triglycerides and phospholipids [26]; it also generates unspecific and undesirable products from 7-ketocholesterol in a time-dependent manner, which may interfere during the chromatographic analysis as a source of high backgrounds [34]. The release of fatty acids and generation of 7-ketocholesterol degradation products complicate the analysis [26] so to avoid these byproducts, a final step of purification by solid phase extraction (SPE) is usually undertaken. Sometimes, to increase the signal level of these metabolites, derivatizations are performed [35,36], which add another step to sample processing. The present manuscript successfully explores the use of cholesterol esterases to free esterified oxysterols and avoid the described complications when alkaline hydrolysis is used. In addition, the proposed new method greatly improves sample handling.

2. Material and methods

2.1. Reagents

Standards used were 4β-HC (99% purity, CAS 17320-10-4), 7α-HC (99% purity, CAS 566-26-7), 7β-HC (99% purity, CAS 566-27-8), 7-keto cholesterol (99% purity, CAS 566-28-9), 24(S)-HC (99% purity, CAS 474-73-7), 25-HC (99% purity, CAS 2140-46-7), 27-HC (99%, CAS 20380-11-1), 25-HC(d6) (internal standard 99%, CAS 88247-69-2) and 27-HC(d6) (internal standard 99%, CAS 1246302-95-3), purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesteryl stearate (96%, CAS 35602-69-8), potassium hydroxide (85%, CAS 1310-58-3), butylated hydroxytoluene (BHT) (99%, CAS 128-37-0) and Triton X-100 (99%, CAS 9002-93-1) were purchased from Sigma-Aldrich (Madrid, Spain). Except cholesteryl stearate, which was dissolved in hexane, all standard solutions were prepared in methanol at a concentration of 1 mg mL^{-1} and stored at −20 °C. Cholesterol esterases from *Pseudomonas* sp. (CAS 9026-00-0) and from bovine pancreas (CAS 9026-00-0) were purchased from Invitrogen (Barcelona, Spain) and prepared at a concentration of 200 U/mL in 0.1 M potassium phosphate (KH_2PO_4), 0.05 M sodium chloride, 5 mM cholic acid and 0.1% Triton X-100. Solvents used, such as methanol (analytical grade LC–MS, CAS 67-56-1), ethanol (analytical HPLC grade CAS 67-63-0), chloroform (analytical HPLC grade CAS 67-66-3) and hexane (96%; CAS 110-54-3), were provided by Sharlab (Barcelona, Spain). Water was supplied by a Milli-Q Plus 186 (Millipore, Billerica, MS, USA) system.

2.2. Sample handling

Cyp7b1-deficient (knock-out or KO) rats were purchased from Transposagen BioPharmaceuticals (Lexington, KY, U.S.A). These rats were generated by transposon-mediated gene-trap insertional mutagenesis in the Fischer F344 strain [37] at the Medical College of Wisconsin under protocols approved by the Institutional Animal Care and Use Committee (IACUC). A single male offspring was identified harboring a gene-trap transposon insertion in the first intron of *Cyp7b1* that was predicted to truncate normal transcription. This male was backcrossed to the Fisher 344 strain and heterozygous carriers were intercrossed to generate homozygous animals for experiments. A three-primer assay using primers B3-306_F: 5′-AAACATCACCTTCTGCAGAGGAC-3′, B3-306_R: 5′-CCCTATTTGTATCTTGCTCAGCTTT-3′, and ITR-L: 5′-CCTAACTGACTTGCCAAAAC-3′ were used to confirm the gene-trap insertion and genotype the animals for zygosity. No message was observed by RT-PCR in any of the tissues studied (data not shown).

Blood from male wild-type (WT) Fisher 344 and homozygous *Cyp7b1*-deficient rats on chow diet was obtained by intracardiac puncture from left ventricle in CO_2-euthanized animals. Identical procedure was used to obtain blood from *Apoe*-deficient mice on chow diet as well. A volunteer subject consented to give his blood for the experiment once used for a medical revision. The protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza. Blood (1 mL) was immediately mixed with 6 μL of 0.5 M EDTA dissolved in phosphate-buffered saline (PBS). Blood tubes were kept on ice and centrifuged at 1150*g* for 10 min to separate plasma. The latter was aliquoted, deep-frozen in liquid nitrogen and stored at −80 °C until further processing. Samples, thawed on crushed ice, were processed without breaking the cold chain.

2.3. Determination of oxysterols

2.3.1. Free oxysterols

Three extraction methods were tested: those of Folch et al. [28] and Bligh and Dyer [27], and that described by McDonald et al. [26], being the Folch the most effective in terms of extraction recovery and quality. Briefly, 100 μL of rat plasma were placed in capped 10 mL glass tubes, to which 0.75 mL of chloroform/methanol (2:1, v/v) mixture containing 4.5 μL of 9.5 μg mL^{-1} 25-HC(d6) as internal standard and 6.5 μL of 1150 μg mL^{-1} BHT, both in methanol, were added. Then, 0.25 mL of chloroform were added and homogenized. Finally, 0.25 mL of Milli-Q water were mixed by shaking for 10 min. The mixture was centrifuged at 622*g* for 10 min and the lower organic phase was recovered. Two further cycles of extraction, homogenization and centrifugation were carried out. Combined organic phases were evaporated to dryness under N_2 stream at 40 °C. Solid extracts were dissolved in 1 mL of hexane with the aid of sonication for 5 min.

2.3.2. Total oxysterols: alkaline hydrolysis

To cleave oxysterol-fatty acid conjugates, two different alkaline hydrolysis procedures were applied to the combined organic phases, that described by DeBarber et al. [32] and the more sterol-specific alkaline hydrolysis method proposed by McDonald et al. [26]. A summary of steps in all procedures is shown in Fig. 2.

2.3.3. Total oxysterols: enzymatic hydrolysis

After optimization, the best experimental conditions were set up as follows: 100 μL of plasma were carefully mixed with 100 μL of potassium phosphate (pH 7.4, 0.5 M) containing 0.25 M NaCl, 25 mM cholic acid and 0.5% Triton® X-100 and 1.6 units of *Pseudomonas* cholesterol esterase in a 0.2 mL tube. Samples were incubated at 37 °C for 60 min in a Biorad thermal cycler (Hercules, CA, USA). After the enzymatic hydrolysis, samples underwent free oxysterol quantification.

2.4. Solid phase extraction

Samples were purified by solid phase extraction (SPE) MFE®-PAK Amino cartridges (500 mg/3 mL) (Análisis Vínicos, S.L, Tomelloso, Ciudad Real, Spain) using a manifold system from Waters (Milford, MA, USA). Vacuum was adjusted to maintain a flow of 1.0–1.5 mL/min. Solvents and conditions are described in supplemental Table 1. Eluted samples were evaporated to dryness under N_2 stream at 40 °C and then dissolved in 0.12 mL of methanol/Milli-Q water (9:1, v/v) with sonication for 5 min.

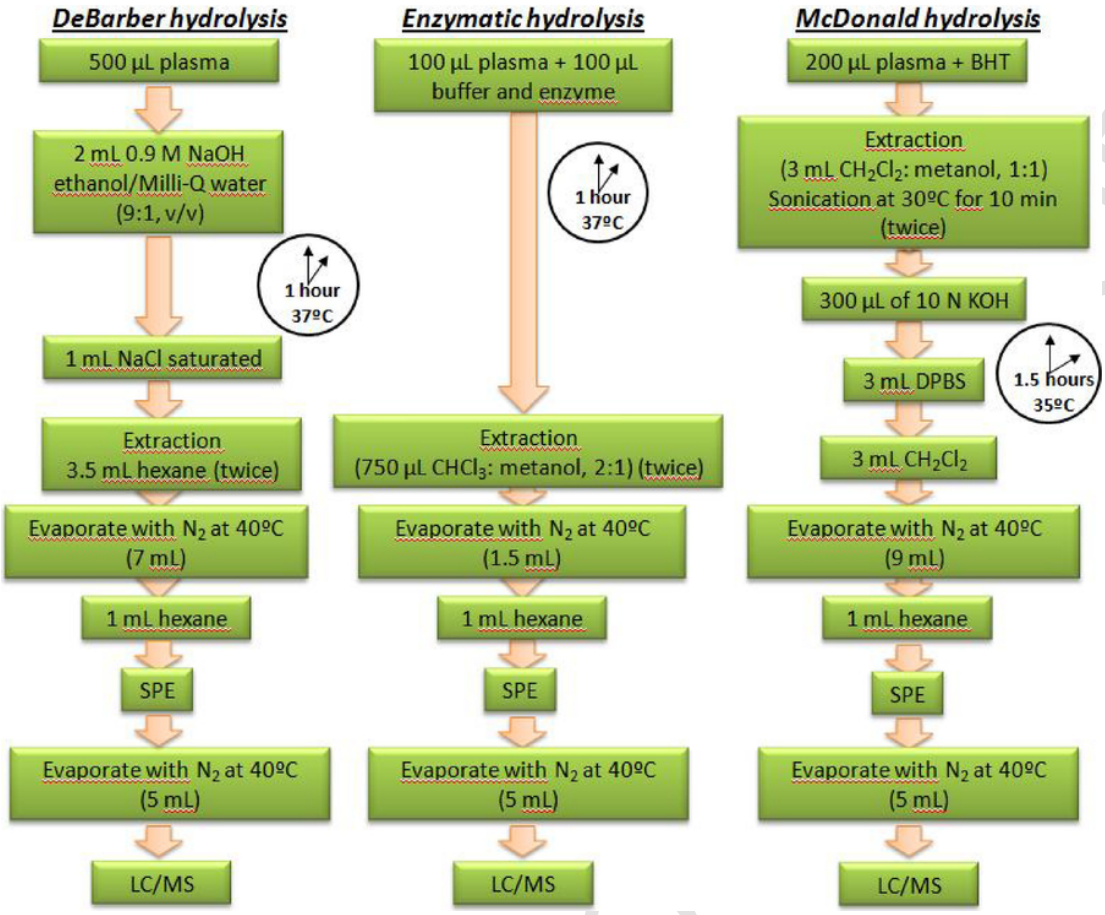

Fig. 2. Overview of compared analytical methods.

2.5. LC/MS analysis

An ultra-high resolution ACQUITY UPLC™ H-Class liquid chromatograph from Waters (Milford, MA, USA) equipped with an ACQUITY UPLC™ BEH C18 2.1 mm × 150 mm column and 1.7 µm particle diameter was used. Mobile phases were 0.1% formic acid in methanol (v/v) (A) and 0.1% formic acid in Milli-Q water (v/v) (B). The flow was set at 0.3 mL/min. Injection volume was 20 µL. Temperatures of column and samples were 40 and 10 °C, respectively. The chromatograph was coupled to an ACQUITY TQD™ tandem quadrupole detector mass spectrometer operating as a single analyzer from Waters. Gradient and detection conditions are shown in Table 1. The selected *m*/*z* values and used operational conditions for the different compounds are shown in supplemental Table 2. All data were acquired and processed using MassLynx v4.1 (Waters).

2.6. Analytical features

The stock solutions for 25-HC, 24(S)-HC, 27-HC, 7α-HC, 7β-HC, 4β-HC and 7-ketocholesterol were prepared in methanol at concentration of 1 mg mL^{-1}. The working internal standard (IS) solutions for 25-HC(d6) and 27-HC(d6) were prepared at the same concentration. All solutions were stored in glass tubes protected from light at −20 °C. Calibration curves were freshly prepared for each batch in methanol and MilliQ water (9:1, v/v), same solvent as final sample solvent. The final concentration for the calibration standards of each compound were 1, 2, 4, 20, 80, 150, 400 and 2000 ng·mL^{-1}. All of them were spiked with both IS at 150 ng·mL^{-1} each one. The linear dynamic range was determined from the triplicate response of oxysterol solutions at different concentrations. Limits of detection (LOD) = 3SD of blank/slope of calibrators and of quantification (LOQ) = 3.2 LOD were obtained. The determination of intra-day (n = 5) and inter-day (n = 15) precisions, the recovery and accuracy of the method were carried out by analyzing plasma spiked with 100 ng mL^{-1} of different oxysterols. Matrix effects were investigated

Table 1
Experimental conditions for the quantification of oxysterols in plasma.

LC	Time (min)	Solvent A (%, v/v)
Gradient	0.00	70
	2.00	70
	2.10	85
	4.00	85
	14.00	100
	19.00	100
	19.10	70
	22.00	70
MS		
Source	Electrospray	
Detection mode	SIR	
Ionization	positive	
Capillary voltage	3 kV	
Desolvation temperature	650 °C	
Desolvation gas flow	600 L/h	
Cone gas flow	40 L/h	
Source temperature	120 °C	

by a post-extraction addition procedure, as described by Trufelli et al. [38]. Basically, four sets of samples were analyzed: a standard solution with a known concentration (set A), plasma samples spiked with the same amount of analytes after the sample treatment (set B), plasma samples spiked with the same amount of analytes before the sample treatment (set C) and non-spiked plasma samples (set D), to subtract the endogenous concentration levels. The response of the analytes was used in the following equations to quantify the matrix effects (Eq. (1)), the extraction recovery (equation 2) and the process efficiency (Eq. (3)).

$$\text{Matrix effects (\%)} = 100 \times (\text{B-D})/\text{A} \quad (1)$$

$$\text{Extraction recovery (\%)} = 100 \times (\text{C-D})/(\text{B-D}) \quad (2)$$

$$\text{Process efficiency (\%)} = 100 \times (\text{C-D})/\text{A} \quad (3)$$

2.7. Statistical analyses

Results are shown as means ± standard deviation of the values. The data were analyzed using the Prism 5.02 software for Windows (GraphPad, S. Diego, CA, USA). Comparisons were carried out using one-way ANOVA and Bonferroni post-hoc test. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Initial chromatographic and mass-spectrometric settings

When electrospray ionization is utilized, ammonium adduct formation of sterols is dependent on the mass spectrometer and, more importantly, the ionization source used [26,39,40]. To avoid operating problems involving the mass spectrometer, our equipment required temperatures higher than 50 °C. With the source temperature set at 120 °C, the spectra obtained in presence of ammonium salts are shown in supplemental Fig. 1. As can be observed, no ammonium adducts were formed (expected at *m*/*z* 420.3). At this point, fragments corresponding to *m*/*z* 385.3 and 367.3, also formed without adding ammonium acetate, were selected for alternative monitoring. To explore their potential use, several chromatographic conditions were tested (data not shown). In fact, as depicted in supplemental Fig. 2, the use of gradients containing acetonitrile and ammonium salts decreased the signals and retarded elution times of 25- and 27-HC when compared to 0.1% formic acid in methanol-Milli-Q water gradients. The use of ACQUITY UPLC™ BEH C18 2.1 mm × 150 mm column in our chromatographic conditions together with single ion recording allows rapid and specific determinations of 4β-, 7-, 24-, 25- and 27-HC as well as 7-KC without derivatization and even the use of 25-HC(d6) as internal standard (Supplemental Fig. 3 and Supplemental Table 2). In these chromatographic conditions, 22-HC was also separated (Supplemental Fig. 3).

3.2. Preliminary exploration of cholesterol esterases to hydrolyze hydroxycholesterol esters

As shown in supplemental Fig. 4, plasma incubation with the enzyme increased the levels of 25 and 27-HC in each group of rats compared to plasma without enzyme. The addition of Triton X-100 as surfactant further increased the concentrations. These results clearly indicate that the enzymatic approach is a promising method for hydroxycholesterol ester cleavage and that the presence of a surfactant facilitates the action of the enzyme.

Two sources of cholesterol esterase were tested to assess their ability to cleave hydroxycholesterol esters, one from *Pseudomonas* and the other from bovine pancreas. Despite the fact that both enzymes have been reported to have similar activity regarding cholesterol esters [41], a different behavior was observed with oxysterols. The use of *Pseudomonas* enzyme resulted in a stronger signal for both 25- and 27-HC (data not shown).

Representative chromatograms displaying the completeness of cholesterol ester hydrolysis under different experimental conditions are reproduced in Fig. 3. They show that increasing the amount of enzyme reduced the amount of cholesteryl esters present after a 120 min incubation time. In fact, 1.6 U of the *Pseudomonas* enzyme was able to hydrolyze all the cholesteryl esters in the sample. A similar result was obtained using the DeBarber et al. procedure [32]. In contrast, the alkaline hydrolysis proposed by Mcdonald et al. [26] did not achieve a complete hydrolysis of cholesterol esters. Overall, these results indicate that *Pseudomonas* enzyme is perfectly active on its proper substrate and that increasing amounts of enzyme favors complete hydrolysis.

3.3. Comparison of enzymatic and alkaline hydrolysis procedures

Once verified the feasibility of the use of cholesterol esterases to hydrolyze oxysterol esters, the enzymatic procedure was compared to conventional alkaline hydrolysis in terms of chromatographic background. Fig. 4 displays the chromatograms obtained following both hydrolysis procedures. It is noteworthy that the enzymatic hydrolysis resulted in a cleaner chromatogram with fewer unknown compounds than the alkaline hydrolysis. These methods were also compared in terms of recovery of the internal standard, 25-HC(d6), spiked before processing. As shown in Supplemental Fig. 5, both alkaline procedures, as proposed by DeBarber et al. [32] and McDonald et al. [26], resulted in similar recoveries and not significantly different from those obtained by the enzymatic treatment. The latter recovery was close to the values obtained for samples without hydrolysis. Thus, the enzymatic procedure induces fewer unknown compounds and results in similar recoveries compared to alkaline methods.

3.4. Analytical features

As shown in Table 2, using the enzymatic method, the LOD were less than 54 pg mL^{-1} and the LOQ was lower than 171 pg mL^{-1} for all the assayed oxysterols. Intra-day precision values were less than 5% except for 27-HC and 7-KC. Inter-day precisions were lower than 10% for the different oxysterols. The process efficiencies were around 90% except for 7-HC. Analytical features for alkaline hydrolysis using the DeBarber et al. method [32] are also included in Table 2. The LOD was never lower than 325 pg mL^{-1} and the LOQ was lower than 1000 pg mL^{-1}. Intra-day precision values were higher than 6%, and inter-day precision was higher than 10% for all assayed oxysterols. The process efficiencies were lower than 90% except for 25-HC and 7-HC. These results indicate that the enzymatic hydrolysis provides better sensitivity and accuracy than the alkaline hydrolysis.

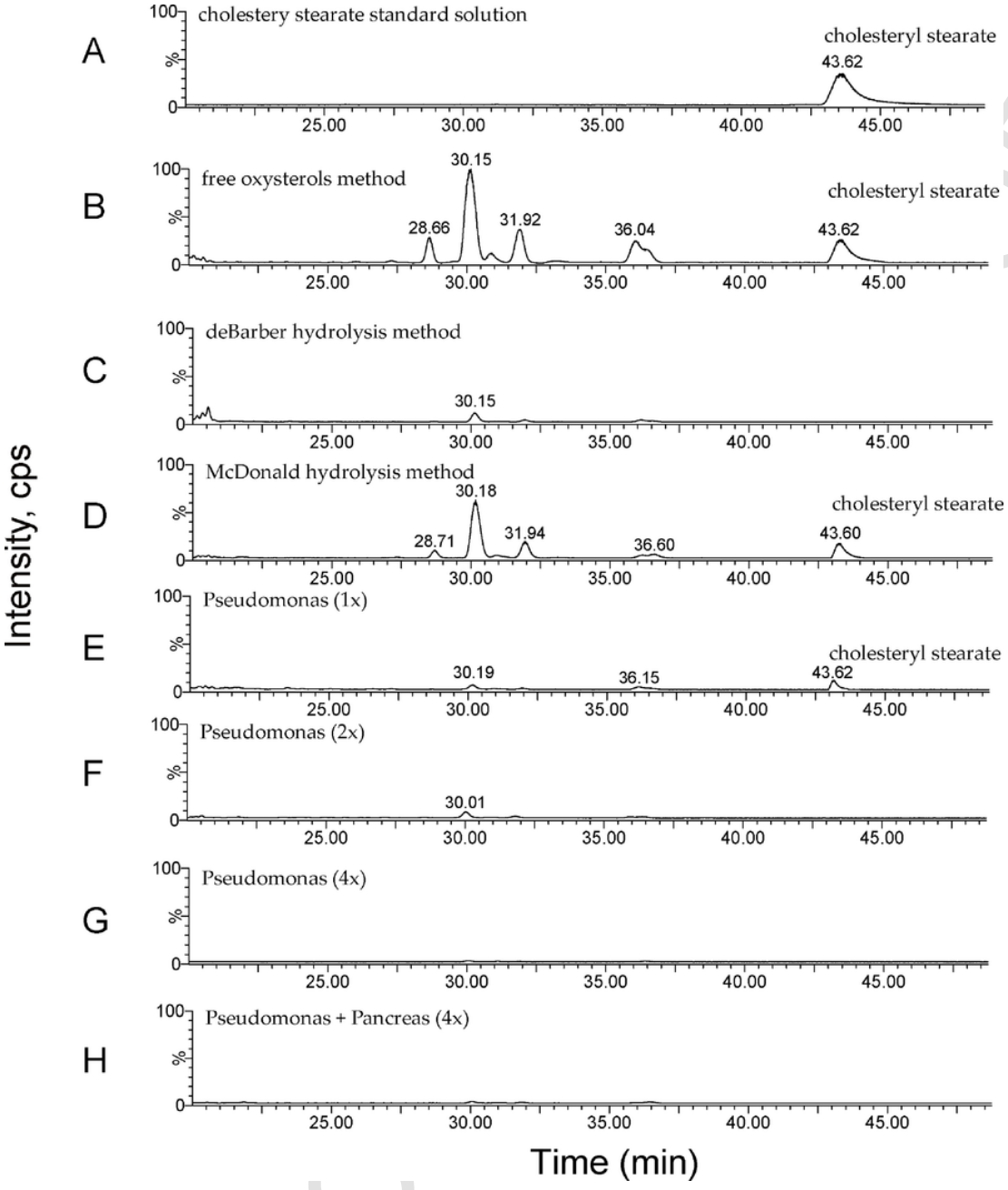

Fig. 3. Influence of analytical methods on chromatograms of plasma cholesterol esters from C*yp7b1*-deficient rats. Panel denoted A, standard solution of cholesteryl stearate; B, plasma without hydrolysis; C, alkaline hydrolysis following method of DeBarber et al.; D, alkaline hydrolysis following method of McDonald et al.; E, plasma treated with 0.4 units of *Pseudomonas* enzyme; F, with 0.8 units of the same enzyme; G, with 1.6 units of the same enzyme and H, with 0.8 units of *Pseudomonas* enzyme plus 0.8 units of pancreas enzyme. All enzymatic assays were carried out at 37 °C for 120 min.

3.5. Comparison of enzymatic and alkaline hydrolysis procedures for oxysterol quantification

The results obtained for 4β-, 7-, 24-, 25- and 27-HC as well as 7-KC from *Cyp7b1*-deficient rats are shown in Fig. 5. As can be observed, the concentrations of both unesterified 25- and 27-HC were similar and higher than that of 24-HC. Concentrations of unesterified 4β- and 7-HC were similar and higher than that of 7-KC.

Despite the fact that both alkaline hydrolysis methods gave similar contents of total 27-HC and 7-HC to the enzymatic, they only differed in the amount of 25-HC detected, the McDonald protocol resulted in lower levels of 25-HC than DeBarber. No 24-HC was detected with the alkaline hydrolysis procedures and the amount of 7-KC was particularly high and that of 4β-HC remarkably low using the DeBarber protocol. These results indicate that the amount of 24-HC was strongly influenced by the alkaline hydrolysis procedure used and that the profile of other oxysterols is also influenced by alkaline conditions.

Quantification of levels of oxysterols in different species is shown in Table 3. *Cyp7b1*-deficient rats showed increased plasma levels of 7-HC, 24-HC and 25-HC. Rodents showed decreased levels of

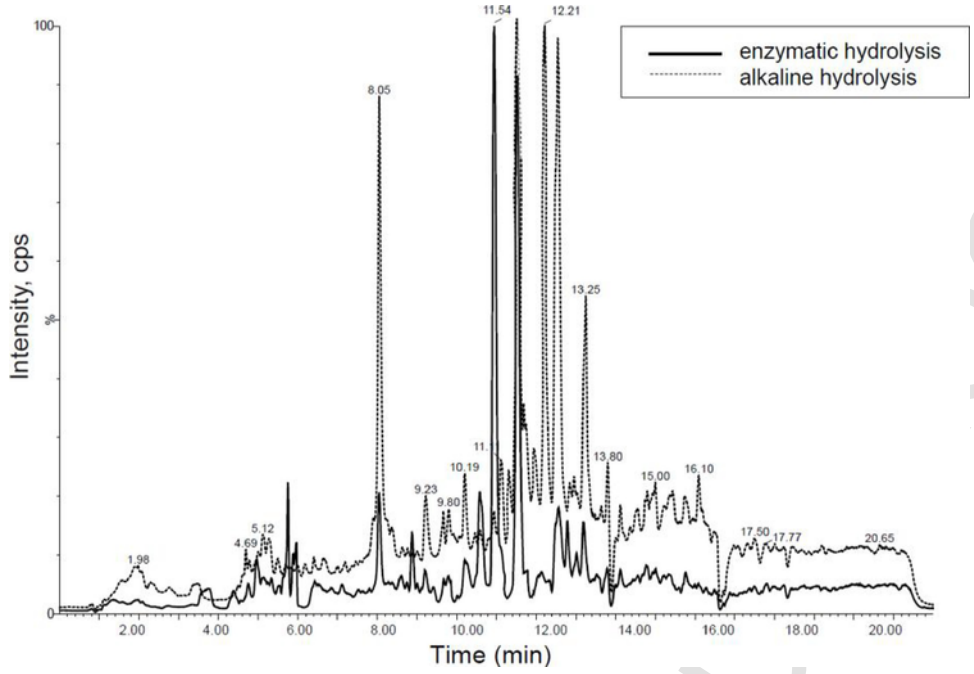

Fig. 4. Overlapping total ion chromatograms obtained by alkaline and enzymatic hydrolysis methods. The same amount of plasma was used in both procedures. Chromatograms were acquired using SIR mode.

Table 2
Analytical parameters of the enzymatic and alkaline (deBarber et al.) hydrolysis methods for total oxysterols.

	24-HC	25-HC	27-HC	7-HC	7-KC	4-βHC
Enzymatic						
LOD (pg/mL)	7	10	20	53	31	15
LOQ (pg/mL)	22.4	32	64	170	99	48
Intra-day precision (RSD%, n=5)	4.92	3.86	6.64	4.77	8.46	4.40
Inter-day precision (RSD%, n=15)	8.20	6.35	9.76	7.94	9.28	7.28
Process efficiency (%)	93.50	91.29	90.02	75.82	95.78	89.17
Alkaline						
LOD (pg/mL)	325	400	515	718	625	700
LOQ (pg/mL)	1040	1284	1647	2297	2000	2242
Intra-day precision (RSD%, n=5)	7.83	6.78	9.02	8.67	11.84	8.26
Inter-day precision (RSD%, n=15)	12.56	10.53	11.25	14.16	11.36	13.26
Process efficiency (%)	82.14	90.57	81.40	94.51	78.48	87.17

The determination of intra-day and inter-day precisions, the recovery and accuracy of the method were carried out by analyzing plasma spiked with 100 $ng \cdot mL^{-1}$ of different oxysterols.

24-HC compared to the human plasma. Notable difference was observed regarding 27-HC among rat strains.

4. Discussion

To reduce the unknown compounds generated during alkaline hydrolysis, due to the basic environment and high temperatures, and to shorten the sample processing time, a new procedure has been developed and optimized that makes use of the ability of cholesterol esterases to hydrolyze hydroxycholesterol esters. The proposed method may be considered as an improvement over previous approaches using chemical hydrolysis, as it offers the following advantages. First, it generates fewer unknown compounds since less sterol degradation was observed. Second, it shows a 90% recovery of the internal standard. Third, it allows the simultaneous determination of several oxysterols with acceptable analytical goals. The proposed method shows an improvement in the LOD and LOQ over conventional protocols involving alkaline hydrolysis. Indeed, it requires less sample volume what makes suitable for small animals or pediatrics medicine. Fourth, it requires less sample manipulation by eliminating the post-alkaline extraction step, its evaporation and subsequent dissolution. The latter aspects are translated into a reduction of sample processing time and, consequently, a higher number of samples processed per working session. Finally, it avoids the use of alkalis and reduces the environmental impact as it generates fewer chemical wastes.

Prior to undertaking enzymatic approaches, a considerable effort was made to adapt previously published analytical conditions to the mass spectrometer using underivatized samples [26,39,42]. Since the source temperature should be higher than 50 °C to avoid operating problems involving the mass spectrometer, no ammonium adducts were observed. Despite this circumstance, the mass spectra of 25-HC and 27-HC could be used to monitor these metabolites by single ion recording. Application of previously reported SPE and chromatographic conditions [43] resulted in gradients with higher sensitivities, suitable for working with 100 µL samples. Likewise, using this formic acid-methanol gradient, Narayanaswamy et al. were able to analyze 200 µL samples with no need for derivatization or the use of ammonium adducts [21]. These simplifications may facilitate clinical and experimental work.

The proposed enzymatic method shows an important reduction of chromatographic unknown compounds compared to alkaline hydrolysis. Widely used to cleave hydroxycholesterol esters, alkaline hydrolysis is the most controversial step and probably the major cause of their formation because of thermal degradation and the alkaline environment, which can degrade cholesterol and other products [44]. Regarding thermal degradation, it has been shown that the lower the hydrolysis temperature, the fewer the degradation products observed [34,45,46]. The presence of those unknown compounds

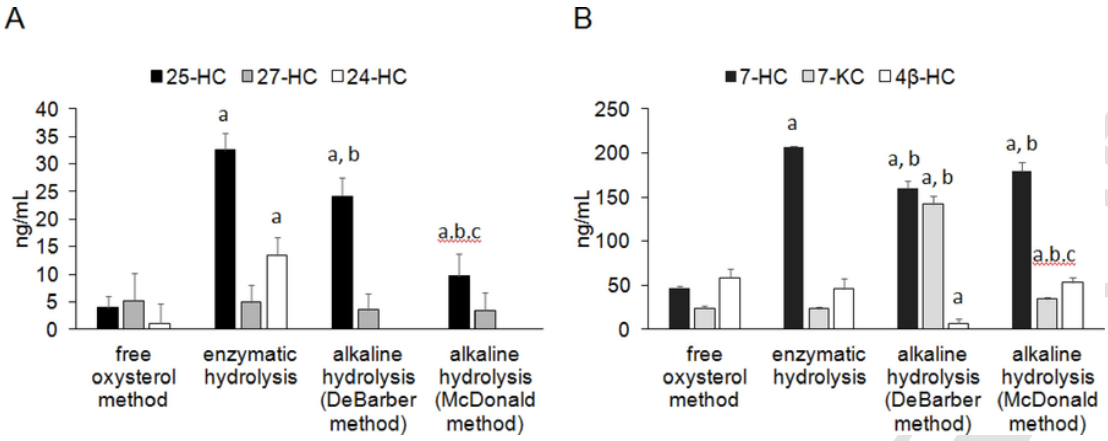

Fig. 5. Plasma oxycholesterol concentrations from Cyp7b1-deficient rats according to different methods. Results are mean and standard deviations obtained in plasma samples without hydrolysis (free oxysterol), subjected to alkaline saponification following DeBarber et al. or McDonald et al. and enzymatic hydrolysis using the Pseudomonas enzyme. Free oxysterols were extracted and determined as detailed in methods. Comparisons were carried out using one-way ANOVA and Bonferroni post-hoc test. a, $p < 0.001$ vs free oxysterol method; b, $p < 0.001$ vs free enzymatic hydrolysis and c $p < 0.001$ vs DeBarber alkaline hydrolysis.

Table 3
Plasma levels of total oxysterols from different species obtained by the enzymatic procedure.

Compound	Human (ng mL^{-1})	*Apoe*-deficient mice (ng mL^{-1})	Wistar rat (ng mL^{-1})	Wild-type Fisher rat (ng mL^{-1})	*Cyp7b1*-KO Fisher rat (ng mL^{-1})
24-HC	40.5	3.8	3.0	2.7	13.4
25-HC	6.8	3.3	3.4	2.3	32.5
27-HC	88.5	54.2	27.9	3.1	4.9
7-HC	36.8	75.1	10.0	12.6	206.2
7-KC	15.6	10.6	6.2	13.4	23.1
4-βHC	30.4	25.1	55.2	52.9	45.6

may influence the background and, thus, the chromatographic baseline used for calculations, therefore biasing analyte concentrations or even preventing their calculation. This is particularly relevant for oxysterols, which are found in very low concentrations. To overcome these problems, these compounds are derivatized [35,47–50], this approach gives low LOD and high sensitivity and also less background for quantitative measurement. However, the latter requires another step in sample processing, which further increases the time and cost of analysis. The use of cholesterol esterases to hydrolyze hydroxycholesterol esters, without requiring an alkaline environment or high temperatures, provides an optimal operational setting to overcome those drawbacks and their consequences. Therefore, a cleaner baseline that facilitates calculations was obtained.

An additional disadvantage of alkaline hydrolysis is the dependence on the protocol used. As shown in Fig. 3, with pathological samples, the method proposed by Mcdonald et al. [26] did not achieve complete hydrolysis of cholesterol esters. This could explain the lower levels of 25-HC obtained with the Mcdonald method (Fig. 5). However, it resulted in 27-HC levels similar to those obtained with the enzymatic procedure (Fig. 5). The other alkaline protocol proposed [32] yielded higher amounts of 7-KC. This discrepancy between the two protocols could be attributed to sample treatment under the specific conditions of this procedure, lending further support to the hypothesis of the possible generation of 7-KC during saponification in an alkaline medium, with high temperatures and high levels of cholesterol in the sample, as has been proposed [34].

The proposed enzymatic method requires incubation with surfactants to improve activities of the enzymes in the assay. This could be explained by its role in disassembling the plasma lipoproteins and facilitating enzyme access to their core, where esterified compounds are found, or blocking other hydroxycholesterol-protein interactions that might preclude access of cholesterol esterases. The enzymatic method for the determination of total oxysterols in plasma fulfills acceptable analytical goals for this kind of samples [51] in terms of linear range, limits of detection and quantification, repeatability, intermediate precision, overall efficiency and accuracy (Table 2). These parameters were satisfactory due to the use of the deuterated internal standard, 25-HC(d6), which compensates for the disturbances throughout sample treatment and instrumental analysis (especially during the ionization process) [43,52]. Regarding to 24-HC, 7-HC, 4β-HC and 7-KC, we did not use their deuterated internal standards as it should be because when we used 25-HC(d6) and 27-HC(d6) as internal standards consistent results and good recovery were obtained (Table 2).

A number of features of enzymatic hydrolysis are translated into the simplification of sample processing, and constitute further advantages over the alkaline methods. For example, the hydrolysis step of enzymatic processing was carried out directly on the plasma sample. Thus, an evaporation and dissolution step required in alkaline hydrolysis was eliminated. In addition, we found that incubations for periods longer than 120 min were clearly detrimental, even when they were carried out at 37 °C, a circumstance that defines a time frame for sample processing. This effect was particularly relevant for the 25-HC determination since, beyond that point, this compound may be degraded (data not shown). However, under the described conditions, 27-HC was quite stable in the interval from 120 to 240 min. The above experiment also suggests that, because of the need to avoid possible degradation, 25-HC determination is more demanding than that of 27-HC. It is interesting to note that *Cyp7b1*-deficient rats accumulate 25-HC but not 27-HC, a fact that would point to the former as the preferential substrate for this enzyme, in agreement with the data of Cui et al. obtained from the structural characterization of human CYP7B1 [53]. In patients having mutations in CYP7B1 [25], the plasma levels of 25-HC were increased about 100-fold while those of 27-HC were only elevated 6–9 times. However, mice lacking CYP7B1 showed a similar increase of both oxysterols [24]. The ratios of 27-HC to 25-HC obtained by the enzymatic method for human and mouse samples (Table 3) were similar to those reported by Quehenberger et al. [54] and Li-Hawkins et al. [24], respectively. These data indicate that the phenotypic level of oxysterol present in CYP7B1 mutants may be dependent on genetic background. In fact,

wild-type Fisher rat showed a low level of 27-HC compared to Wistar rats.

In conclusion, the proposed enzymatic method provides cleaner chromatograms, quantifies lower concentrations of oxysterols without using derivatization, and is as accurate as the methods currently used. It also represents a simplification in sample processing by avoiding high temperatures, alkaline hydrolysis and further purification steps. Thus, more samples may be processed within a given time period, without using hazardous alkalis, a circumstance that results in clear benefits for operators and in reduced costs. Moreover, an increase in the number of samples processed, make it suitable for large scale clinical samples and for the measurement of oxysterols in tissues. For all these reasons, the proposed simplification is expected to expand the determination of these compounds to investigate their role in several diseases.

Conflict of interest

The Medical College of Wisconsin receives royalties on sales of genetically modified rats from Transposagen BioPharmaceuticals, Inc.

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