Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols

Hanne Roberg-Larsen,* Kaja Lund,†,§ Tore Vehus,§ Nina Solberg,† Caroline Vesterdal,† Dorna Misaghian,§ Petter Angell Olsen, † Stefan Krauss, † Steven Ray Wilson, †,* and Elsa Lundanes*  
Department of Chemistry, † University of Oslo, Oslo, Norway; Unit for Cell Signaling, Cancer Stem Cell Innovation Centre (SFI-CAST), † Oslo University Hospital, Rikshospitalet, Oslo, Norway; and Affitech Research AS, § Oslo, Norway

Abstract  Iso-octyl chain-hydroxylated oxysterols were determined in attomoles per 10,000 cells concentrations in 10,000–80,000 cultured pancreatic adenocarcinoma cells, using a sensitive, highly automated nano-LC-ESI-MS-based method. Identified oxysterols included 24S hydroxycholesterol (24S-OHC), 25 hydroxycholesterol (25-OHC), and 27 hydroxycholesterol (27-OHC), while 20S hydroxycholesterol and 22S hydroxycholesterol were not detected. Lower mass limit of quantification was 23 fg (65 amol) for 25-OHC and 27-OHC (100 times lower than our previous method) and 54 fg (135 amol) for 24S-OHC, after derivatization into Girard T hydrazones and online sample cleanup using simplified and robust automatic filtration and filter back flushing solid phase extraction LC/MS/MS. The instrument configuration was easily installed using a commercial nano-LC/MS system. Recoveries in spiked sample were 96, 97, and 77% for 24S-OHC, 25-OHC, and 27-OHC, with within- and between-day repeatabilities of 1–21% and 2–20% relative SD, respectively. The study demonstrates the potential of nano-LC in lipidomics/sterolomics.–Roberg-Larsen, H., K. Lund, T. Vehus, N. Solberg, C. Vesterdal, D. Misaghian, P. A. Olsen, S. Krauss, S. R. Wilson, and E. Lundanes. Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols. J. Lipid Res. 2014. 55: 1531–1536.

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Oxysterols, which are hydroxylated derivatives of cholesterol, have been implied as activators of the Hedgehog (Hh) signaling pathway through binding to the Smoothed (SMO) receptor (1, 2). The degree of oxysterol activation of SMO is isomer dependant, with iso-octyl chain hydroxylated oxysterols (Fig. 1A) clearly being the most active (3, 4). In addition, the –OH position on the octyl chain is also of great importance for activity [e.g., 25 hydroxycholesterol (25-OHC) is a stronger agonist than 24S hydroxycholesterol (24S-OHC)] (3, 4). Methods are called for to quantify endogenous oxysterol isomers (which are often very low abundance) to fully understand their roles and regulation in Hh signaling (1, 5). In this context, it is advantageous that a method is compatible with small samples (e.g., aggressive tumor cell side populations that are associated with altered signal pathway activity) (6).

In recent years, LC/MS methods have gained popularity for measuring oxysterols. Due to their neutral nature, oxysterols are not easily ionized with ESI, the most common ionization source in LC/MS. The ionization issue has been solved by, for example, charge tagging of the oxysterols into, for example, picolinyl esters (7), Girard derivatives (8–10), and N,N-dimethylglycine esters (11), which enhances sensitivity and selectivity. However, most methods based on the above-mentioned approaches are not optimal for limited cell/tissue samples, as they typically include extensive manual preparation steps and/or analyte-diluting conventional bore LC.

We have developed a nano-LC-based method enabling quantification of Hh active oxysterols in small samples (10,000–80,000 cells). Features of this method include robust, automatic sample purification, one-vial sample preparation, MS-based monitoring for autoxidation, high chromatographic selectivity, targeted mass spectrometric methods

Abbreviations: ACN, acetonitrile; AFFL, automatic filtration and filter back flushing; cLOD, concentration limit of detection; Hh, Hedgehog; ID, inner diameter; RP, reversed phase; RSD, relative SD; SPE, solid phase extraction; 20S-OHC, 20S hydroxycholesterol; 22S-OHC, 22S hydroxycholesterol; 24S-OHC, 24S hydroxycholesterol; 25-OHC, 25 hydroxycholesterol; 27-OHC, 27 hydroxycholesterol.

*To whom correspondence should be addressed.

e-mail: stevenw@kjemi.uio.no

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Calibration solutions for quantification. Calibration solutions were prepared from 1 nM working solutions and covered the concentration range 13–108 pM of each oxysterol. New calibration solutions were made for each assay.

Cell samples. Cells (10,000–80,000) were lysed in 300 µl absolute ethanol (Kemetyl) containing 120 pmol cholesterol-25,26,27-13C. The samples were stored at –80°C before derivatization of oxysterols into Girard T derivates.

Internal standard. Aliquots of 50 µl of 1.5 nM internal standard working solution were added to all standard, calibration, and validation solutions and cell samples before evaporation into dryness and derivatization with Girard T as described subsequently.

Charge tagging of oxysterol

Standard and calibration solutions, as well as cell and validation samples, were charge tagged with Girard T reagent as described previously (9) with small changes to adapt to the change in sample size; after evaporation into dryness, the residue was redissolved in 20 µl 2-propanol. Aliquots of 200 µl of 30 µg/ml cholesterol oxidase dissolved in 50 mM phosphate buffer pH 7 were added to all solutions and samples to convert the 3β-hydroxy-5-ene to a 3-oxo-4-ene (Fig. 1B, step 1), performed at 37°C for 1 h using a Grant-Bio PHMT thermoshaker (Grant Instruments, Cambridge, UK) set to 300 rpm. To attach the Girard T reagent (Fig. 1B, step 2), 500 µl of a mixture consisting of 15 µg Girard T reagent, 15 µl glacial acetic acid, and 485 µl methanol was added to the sample. This resulted in a final volume of 720 µl of all solutions. The reaction was completed in the dark at room temperature overnight. Schematic view of the sample preparation is shown.

**Fig. 1.** A: The structures of the Hh active oxysterols 24S-OHC, 25-OHC, and 27-OHC. B: Charge tagging of 24S-OHC with Girard T reagent. In step 1, the 3β-hydroxy-5-ene is enzymatically oxidized to 3-oxo-4-ene with cholesterol oxidase (1 h, 37°C). In step 2, the Girard T reagent reacts with the 3-oxo group to form Girard T derivates of oxysterol. See Materials and Methods for more details.
in supplementary Section II. Derivatized samples were stored at 4°C and analyzed within a week.

**Automatic filtration and filter back flushing/solid phase extraction/nano-LC**

Sample cleanup was performed online using automatic filtration and filter back flushing (AFFL) and online solid phase extraction (SPE), coupled upstream to nano-LC-ESI-Q Exactive MS. With the AFFL setup (Fig. 2 and supplementary Animation 1) (12, 13), sample with excess reagent was injected (5 µl) by an Agilent G1377A micro well autosampler (Agilent Technologies, Santa Clara, CA) (Fig. 2, step 1). An Agilent 1100 series pump was used as loading pump and filter back-flush pump [15 µl/min of 0.1% formic acid (aq)]. Cell debris and particulate matter from cells were trapped online on a 1 µm Valco (Houston, TX) stainless steel filter (1/16″, 1 µm screen) fitted in a Valco union (1/16″, 0.25 mm bore), while a reversed phase (RP) HotSep Tracy C8 trap column [0.3 mm inner diameter (ID) × 5 mm] from G and T Septech (Ytre Enebakk, Norway) trapped derivatized oxysterols (Fig. 2, step 2). Excess reagent, not sufficiently hydrophobic to be trapped by the RP trap column, was flushed to waste. The valve was subsequently switched after 3.5 min, and the loading pump automatically redirected to back flush the filter unit with 0.1% formic acid (aq) to clean the filter prior to the next injection (Fig. 2, step 3). Simultaneously, an Agilent 1200 series pump eluted the oxysterols from the trap column onto the ACE 3 C18 (0.1 mm ID × 150 mm) RP analytical column (Advanced Chromatography Technologies, Aberdeen, UK) for isoocratic separation using a mobile phase consisting of formic acid-water-methanol (0.1:5:95 v/v/v %) (Fig. 2, step 3) at a flow rate of 500 nL/min. Cholesterol residues in the column might accumulate and make a pseudostationary phase (14) and hence disturb the chromatographic separation. To remove cholesterol residues from column, the column was washed with formic acid-water-acetonitrile (ACN) (0.1:5:95 v/v/v %) (40 column volumes) after approximately six injections of cell samples to avoid changing the separation properties of the column. The column switching was performed with a 10-port two-position switching valve from Valco (1/16″, 0.25 mm bore) controlled by the LC pump’s Chemstation software.

**MS of oxysterols**

MS detection was performed in MS2 mode using a Q Exactive Orbitrap with nanospray flex ion source (Thermo Scientific, Waltham, MA). However, other mass spectrometers (e.g., triple quadrupole) would also be suitable. Ionization was performed in positive mode using a capillary voltage of 2.0 kV. The monitored MS/MS transitions were based on the fragmentation of the Girard T group (see supplementary Section III for illustration of the MS/MS fragmentation) and were 514.44→455.36 (analytes), 520.40→461.36 (internal standard), and 517.40→458.36 (autodetection monitoring). Collection of data and processing were performed using Xcalibur 2.2 software from Thermo Scientific. More MS instrument parameters (mostly based on recommended settings from the manufacturer) are found in supplementary Section IV.

**RESULTS AND DISCUSSION**

Our goal was 2-fold: both enabling sensitive, simple, highly automated analysis of small samples (10,000 cell scale) and quantification of 27-carbon iso-octyl hydroxylated oxysterol analytes possibly present in cells. Candidates were 20S-, 22S-, 24S-, 25-, and 27-OHC, which are structural isomers with very similar LC/MS properties.

High sensitivity was achieved by using nano-LC and online sample preparation. Nano-LC columns (50–100 µm ID) allow for compounds to elute in substantially smaller volumes compared with that in more conventional columns (e.g., 1 mm ID or larger). Therefore, analytes enter the ESI-MS in larger concentrations, resulting in enhanced signal. A nanobore column packed with the same stationary phase and particles as used in our previously published microbore LC-based method (9) was used, as the material has good oxysterol separation properties and durability. When replacing our previous microbore LC-ion trap MS methodology with nano-LC coupled with a Q Exactive MS, the mass limit of detection (and quantification) was reduced by a factor of 100 (from 2.5 pg to 23 fg). Separation properties (retention and selectivity) in the nano- and microbore format were similar, as expected.

The oxysterols were charge tagged with Girard T reagent (Fig. 1B, step 2). This established approach was chosen as
the entire procedure can be performed in one tube, mini-
mizing sample loss (9). In addition, it is compatible with
automated SPE enrichment and cleanup (9).

The Girard T reagent reacts with the keto group as shown
in Fig. 1B, step 2. A potential pitfall was that the LC/MS
method would not distinguish between species with an al-
ready existing keto group and those with keto groups
formed during sample preparation. However, analyte anal-
logs with preexisting keto groups were not detected in the
analyzed cell samples, investigated by performing Girard T
tagging with and without step 1 in Fig. 1B (see supplemen-
tary Section V for chromatograms).

The 23 fg mass detection limit of this method corre-
sponds to 13 pM in the vial (720 µl). It is important to no-
tice that the in-vial concentration will be affected by the
available number of cells because the resulting total volume
is the same for all samples and standard solutions after de-
rivatization. Using more cells per sample will therefore give
higher analyte amounts and concentrations, and hence a
lower limit of detection per cell. For calculation example,
see supplementary Section VI.

A 0.5 mm ID SPE column was coupled online with the
nano-LC column. This avoids the overloading effects asso-
ciated with direct injection of microliter amounts onto
nano-LC columns (which can usually only handle nanoli-
ter injections). Such overloading effects may call for ex-
tensive column purification routines; in the only previous
nano-LC study of oxysterols (to the authors’ knowledge),
Karut et al. (10) had to inject a washing solvent between
each sample injection to avoid carryover effects. In SPE-
nano-LC mode, however, 5 µl injections could easily be
SPE trapped, and concentrated analyte bands were subse-
quently transferred to the LC column in appropriate vol-
umes for satisfactory chromatographic resolution without
carryover effects. In addition to allowing rather large in-
jection volumes, online SPE greatly simplifies analysis of
our 10,000 cell-scale samples, as excess Girard reagent re-
moval is performed online. This reduces difficult, manual
handling of minute samples/fractions, which can be a source
of analysis variance (15).

Conventional online SPE-nano-LC can be especially
prone to pressure buildups due to the small, relatively eas-
ily clogged connections. To avoid this, an AFFL setup was
installed upstream to the SPE step. This allows crude sam-
ple to be injected directly (here: unfiltered lysates) as re-
mainin cell debris and so forth is trapped on a stainless
steel filter and washed out of the system after each sample
injection. This reduces off-line sample preparation steps
(hence possible loss of analyte). The AFFL-SPE-nano-
LC/MS methodology could handle thousand injections of
relatively unprepared samples, representing an unprece-
dented robustness regarding nano-LC-based instrumenta-
tion. Our AFFL-SPE-nano-LC system can be assembled
using a commercial SPE-LC system [see (16) for various
systems], only requiring replacement of one valve (10 port
instead of a 6 port) (13), compared with our original sys-
tem, which required an additional pump (12).

An additional simplifying aspect of the method was the
absence of a (off-line) cholesterol removal step, commonly
performed to avoid autoxidation effects that can nega-
tively affect method accuracy. Enzymatically formed side
chain-hydroxylated oxysterols (our analytes) are not typi-
cally associated with autoxidation; on the other hand, little
is known, for example, about the formation of 20S-OHC,
as no known enzyme is associated with the formation of
this compound. Nonetheless, heavy cholesterol (choles-
terol-25,26,27-13C) was added to all samples and calibra-
tion solutions. If autoxidation occurred during sample
preparation (turning cholesterol into our analytes), heavy
cholesterol would be converted to heavy oxysterols. This
mass transition was monitored and controlled for each
sample with MS. Autoxidation(-like) effects were in fact
observed for ≈1 in every 500 samples (these samples were
rejected).

The chromatographic resolution of our method re-
vealed that, presumably due to generation of syn/anti
forms during the derivatization process (7, 17), several of
the analytes appeared as two peaks. As these peaks were
well resolved, their areas could be combined for quantifi-
cation. By using methanol as the LC organic modifier in-
stead of ACN, we were able to quantitatively distinguish
24S-OHC and 27-OHC, which coeluted with ACN as modi-
fier. In spiked samples, 27-OHC and 20S-OHC coeluted
with methanol as the organic modifier, but it was possible
to resolve 20S-OHC from the other oxysterols when using
ACN (see supplementary Section VII for chromatograms).
In accordance with other recent studies (9, 18, 19), 20S-
OHC was not observed in the analyzed cell samples using
ACN as mobile phase, even when the sample amount was
increased 8-fold. Hence, methanol could be used as the
organic modifier for our method for reliable quantifica-
tion of 27-OHC.

In a pancreatic cell line (BxPC-3) and a derived clonal
mutant cell line (Δβcat BxPC-3), the sensitivity of the
method allowed detection of 24S-OHC, 25-OHC, and 27-
OHC in as little as 10,000 cultured cells (Fig. 3). In con-
trast, 20S-OHC and 22S-OHC were not detected in the
cells even when using larger samples. The compounds
24S-OHC, 25-OHC, and 27-OHC were quantified (38, 19,
and 35 fmol/10,000 cells, respectively). In support of these
oxysterols being generated in the cells was the within-cell
presence of corresponding enzymes responsible for for-
formation of these oxysterols, both at the transcriptional level
by RT-PCR and the protein level by Western blot and nano-
LC/MS/MS. Also, a consistent and significant upregulation
of 27-OHC and its corresponding enzyme (cholesterol
27-oxidase) as function of a cell perturbation (i.e., knock-
out of β catenin) was observed (for more details, see sup-
plementary Section IX).

Validation of the nano-AFFL-SPE-LC/MS/MS method

The concentration limit of detection (cLOD) was de-
finite as the lowest concentration that repeatedly produced
a chromatographic peak with signal-to-noise ratio >3. The
concentration limit of quantification (cLOQ) was defined
as the concentration that produced peak areas with a rela-
tive SD (RSD) ~20%. The cLODs were 13 pM derivatized
analyte for 24S-OHC, 25-OHC, and 27-OHC, corresponding
Recovery [or more correctly, the apparent recovery (20)] was examined by comparing samples spiked at three concentration levels with standard solutions of the same concentration levels, low (27 pM), medium (54 pM), and high (108 pM). The recovery was 96, 97, and 77% for 24S-OHC, 25-OHC, and 27-OHC, respectively. See supplementary Section VIII for more details (linearity curves, equations, etc.).

**CONCLUSIONS**

A highly sensitive, robust, and automated method for quantification of the natural iso-octyl chain hydroxylated oxysterols 24S-OHC, 25-OHC, and 27-OHC was successfully developed and validated and applied to measure oxysterols in small samples (≥10,000 cells). The study illustrates robust and successful use of nanoscale LC in targeted lipidomics/sterolomics, an approach that is usually “reserved” for proteomics. The identification/presence of the endogenous oxysterols in BxPC-3 cells was supported by identification of corresponding enzymes responsible for formation of these oxysterols within the cells. The method will be further used in studying endogenous side chain-hydroxylated oxysterols in the Hh pathway in the context of cancer.
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