Optimization and validation of cholesterol and oxysterols measurement in HepG2 cells using LC-MS/MS

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Introduction

Aim:

- To establish and optimize a method to quantify cholesterol and oxysterols in HepG2 cells using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Tested compounds

- Oxysterols are intermediates produced from cholesterol oxidation [1].
- Cholesterol, 7-ketocholesterol (7-KC) and 7α-hydroxy-4-cholesten-3-one (C4) were tested. These compounds are associated with diseases e.g. atherosclerosis and irritable bowel syndrome with diarrhea (IBS-D) [2, 3].

HepG2 cells

- Cancer cells originated from the liver of a 15-year-old Caucasian male [4].
- Cholesterol, 7-KC and C4 are synthesised or metabolised in the liver, so they are expected to be found in HepG2 cells [5-7].

Advantages of using LC-MS/MS

- More accurate cholesterol quantification than modified Abell-Kendall method or fluorometric-enzymatic assay [2]
- The sensitivity of LC-MS/MS exceeds GC-MS [8].
- Simultaneous analysis of free cholesterol and oxysterols within a short run time [9].

Experimental Design

LC-MS/MS method development

- Standard solutions of cholesterol, 7-KC and C4 at different concentrations were used for method establishment with cholesterol-d7 as the internal standard [10].
- LC system: Sciex X500R QTOF System (Sciex Applied Biosystems, MA, USA).
- The other settings followed a protocol from Arndt, Della Gatta and Bentley [11].
- An MS/MS spectrum, sample chromatogram and linear regression calibration curve was obtained for each analyte.

H_2O_2 challenge

- Mature HepG2 cells were challenged with H_2O_2 to produce oxysterols [12]. Different doses were used to find out the effective dose for HepG2 cells.
- MTT assay was carried out [12]. Absorbances were measured at 570 nm using Multiskan™ GO Microplate Spectrophotometer [13].
- $Cell\ viability\ (\%) = \frac{absorbance\ of\ experimental\ group}{absorbance\ of\ control\ group} \times 100\%$ [12].
- The H_2O_2 dose that killed about 50% of cells was selected.
- The selected H_2O_2 dose or complete medium was used to treat HepG2 cells in 6-well plates [12]. Both groups of cells were incubated, collected and then stored in freezer [12, 14].

Extraction of cholesterol and oxysterols for LC-MS/MS

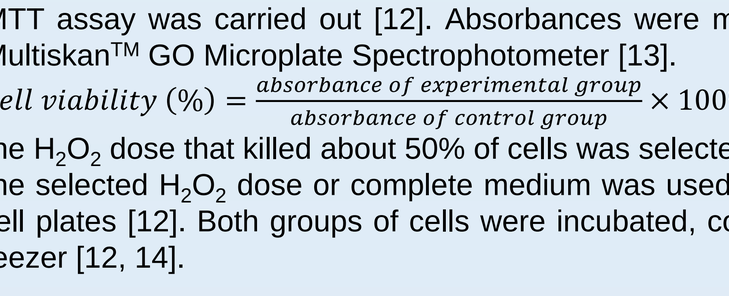
- Folch solution, i.e. chloroform and methanol (2:1, v/v) with 0.01% of BHT, was used [15, 16].
- Addition of 0.9% aqueous NaCl and centrifugation induced phase separation [16]. The lower phase (chloroform) was collected and dried under nitrogen [16].
- Alkaline hydrolysis by adding 1N methanolic KOH with 0.1 ng/μl internal standard [15].
- Cholesterol and oxysterols were extracted by SPE (MAX Waters, USA) [15, 17].
- The samples were analysed by our established LC-MS/MS method immediately for method validation.

Results

Sample chromatograms, MS/MS spectra and Calibration curves

Figure 1 is an example of the chromatograms and MS/MS spectra obtained. There were no interfering peaks in the chromatograms [18].

(a)



(b)

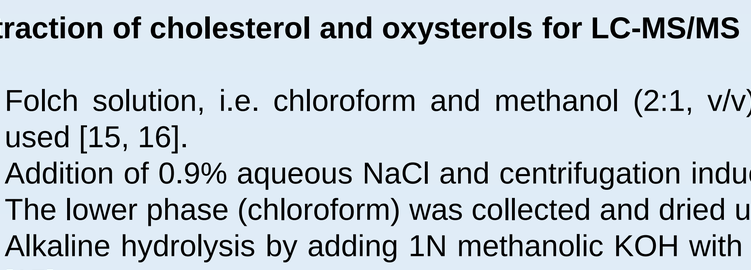


Figure 1a and 1b. (a) Chromatogram of C4 with m/z transition from 383.332 to 177.128. (b) MS/MS spectrum of C4.

Results (continued)

Table 1. Results from sample chromatograms and MS/MS spectra.

	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)
Cholesterol	7.495	369.353	147.118
7-KC	7.412	401.342	175.112
C4	4.011	383.332	177.128
Cholesterol-d7	3.834	376.397	147.117

Table 2. Linearity for cholesterol, 7-KC and C4.

	Linear equation	R^2	Calibration range (ng/mL)
Cholesterol	Y = 4.0839*X	0.99611	500 - 10000
7-KC	Y = 4.0079*X	0.9256	500 - 10000
C4	Y = 9.4658*X	0.973	500 - 10000

The results from sample chromatograms, MS/MS spectra and calibration curves are summarized in Table 1 and Table 2 [10]. Table 1 showed that cholesterol, 7-KC and C4 had different retention times. The m/z transitions for cholesterol-d7 and C4 in Table 1 were selected to be the quantifiers as these transitions showed the highest sensitivity [19]. For 7-KC, the m/z transition from 401.342 to 383.332 showed the highest sensitivity, but this transition was not selected because the precursor ion of C4 also had the m/z of 383.332. Furthermore, based on the experiment by Klinke et al. [10], product ions with similar m/z were monitored for cholesterol and its deuterated form. For cholesterol, the linearity was excellent in the range of 500 to 10000 ng/mL ($R^2 > 0.995$) [20].

H_2O_2 challenge results (MTT assay)

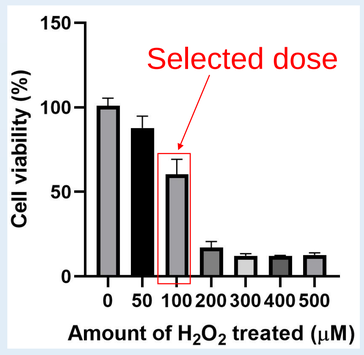


Figure 3. Results from MTT assay. The diagram showed that 100 μM H_2O_2 was the dose closest to kill 50% of the cells. This selected dose could generate oxidative stress without killing too many HepG2 cells in the sample.

Quantification of cholesterol, 7-KC and C4

(a) (b) (c)

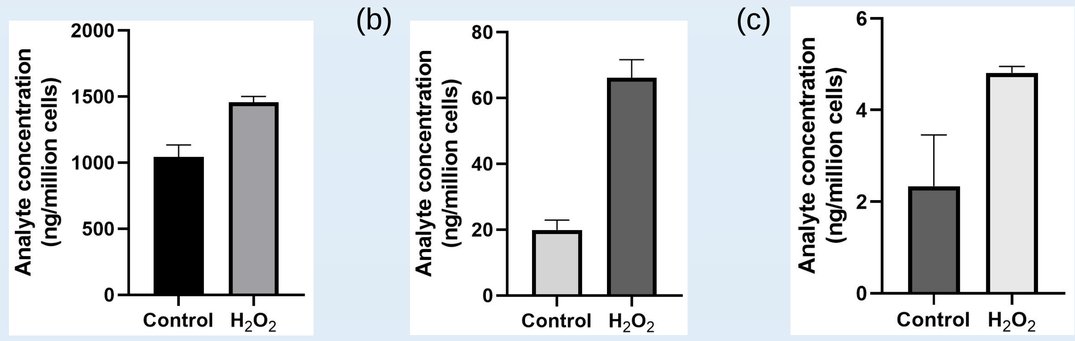


Figure 4a, 4b and 4c. (a) Analyte concentrations of cholesterol with and without H_2O_2 treatment. (b) Analyte concentrations of 7-KC with and without H_2O_2 treatment. (c) Analyte concentrations of C4 with and without H_2O_2 treatment. There was a significantly higher concentration of cholesterol ($p = 0.0021$), 7-KC ($p = 0.0002$) and C4 ($p = 0.0190$) in the H_2O_2-treated HepG2 cells than the control. The control and treatment group each had three replicates ($n = 3$).

Discussion

- The amount of cholesterol increased due to translocation of GLUT2 whereas the amounts of oxysterols increased due to cholesterol oxidation [1, 6, 21].
- Significance of this LC-MS/MS method
  - ❖ High level of cholesterol is associated with diseases (e.g. atherosclerosis) [2].
  - ❖ 7-KC has been considered as a biomarker for Niemann-Pick type C disease [1].
  - ❖ C4 may be used as a measurement of bile acid malabsorption [3].
- Limitations of this study
  - ❖ Only three compounds were measured.
  - ❖ Only three replicates for the control and H_2O_2 treatment group.
  - ❖ Linearity for 7-KC was suboptimal, which could be due to experimental errors.
  - ❖ Accuracy and precision of this method should be evaluated in the future.

Conclusion

Our optimised and validated LC-MS/MS method has been proven to be an accurate and reliable way to quantify cholesterol, 7-KC and C4 in HepG2 cells.

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