The Absorption and Accumulation Characteristics of Ethyl Carbamate in Human HepG2 Cells Revealed by UPLC-TOF-MS

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Abstract: Ethyl carbamate (EC), a toxic contaminant occurring in food processing, may possess a risk to human health. To facilitate the basic study on its metabolism and applied study on its monitoring, we first established a rapid determination method of EC in human cells (HepG2) based on UPLC-TOF-MS. This method showed good linearity over a range of 60-6000 ng/mL (R2＞0.99) in both extra/intra-cellular matrixes. The limits of detection (LOD) and the limits of quantification (LOQ) for both matrixes were 31 ng/mL and 40 ng/mL, 133 ng/mL and 103 ng/mL, respectively. Recoveries and stabilities (RSD) of this method for both extra/intra-matrices were over 76% and smaller than 13%, respectively. Then, using the established method, we determined the absorption and accumulation characteristics of EC in both matrixes, which, for the first time, showed that EC could be absorbed and accumulated by HepG2 cells. Notably, the kinetics of EC accumulation in cell pellets was consistent with results of cell viability assay, demonstrating that exposure to EC had potential adverse effects on cell viability.
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Keywords: cell viability, food safety, human hepatoblastoma cell, MTT assay, naturally occurring toxic contaminant

1. Introduction

Ethyl carbamate (EC), also known as urethane, was used as hypnotics and anti-tumor drugs in human, or anesthetics for laboratory animals such as mouse (Tang ASP, et al, 2009). But it was found to be toxic and carcinogenic to mouse and other rodents in 1940s (Schmahl D, et al, 1977). Subsequent studies found that EC, together with its metabolites, is genotoxic and can cause gene mutations in multiple sites (Josef Schlatter and Beland FA, et al, 2010). Public health concern about EC in alcoholic beverages began in 1985 when relatively high levels were detected by Canadian authorities[6]. Subsequently, Canada set an upper limit of 0.4 mg/L EC for fruit spirits and it was adopted by many other countries (Conacher H B S, et al, 2005). In 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the daily intake of EC in human diet and found it was about 1μg/person/day; with additional alcohol consumption, the daily intake can increase to 5μg/person/day (JECFA, 2005). In 2007, World Health Organization's International Agency for Research on Cancer (IARC) classified it as a group 2A carcinogen that "probably carcinogenic to humans" (EFSA, 2007).

EC is generally found in fermented foods and alcoholic beverages with variable levels, ranging from several ng/L to several hundreds μg/L(Zhu H and Liu Y P, et al, 2013), and the highest EC concentrations are also found in spirits derived from stone fruits (e.g. cherries, plums, mirabelles, apricots) (Battaglia R and B Zimmerli, et al, 1990) Notably, higher levels of EC are also reported in breads and acidified milks (Weber J V, et al, 2009). Today's improved living standard has brought more attention to food safety and healthy dietary, and the probable health risk of EC in food products and alcoholic beverages has increasingly raised public concerns.

To investigate probable hazardous effect of EC to human, efficient detection methods of EC need to be established. Various methods have been developed to qualitative and quantitative determine EC in foods and beverages such as wine and flavorings (Jiao Z H and Ajtony Z, et al, 2013), which provided powerful tools for the monitoring of EC in those foods and drinks, and indicated that each EC determination method has to be adapted to the level of EC and to the studied materials (Weber J V and Jiao Z H, et al, 2009). However, detection method based on UPLC-MS has not yet established in human cells, particularly in HepG2 cells. A rapid determination of

EC in human cells is indispensably required for studies on the risk assessment of EC.

In this study, to facilitate the basic study on its metabolism and applied study on its monitoring, we first developed a simple, fast and economical method to detect EC in human cells, HepG2, based on UPLC-TOF-MS system combined with external standard method. The kinetics of EC in both extracellular and intracellular matrices was then determined by established method, which, for the first time, revealed the EC absorption and accumulation properties in HepG2 cells.

2. Material and Methods

Apparatus and Chemicals: The Agilent's Infinity 1290 UHPLC system was used in this study. The chromatographic separation was carried out with a C18 column (2.1×50 mm, 1.8 μm) with and an additional C18 pre-column (2.1×5 mm, 2.7 μm) at 35℃. The elution was performed with a gradient program with the mobile phases including solvent A (0.2% formic acid in H_2O) and solvent B (0.2% formic acid in methanol) as follows: 5%-35% (0-3 min), 35% (3.01-4 min), 35%-90% (4.01-5 min), 90%-5% (5.01-7 min). The elution rate was 0.2 mL/min, while the injection volume was 2μL.

Methanol (MeOH) and acetonitrile, gradient grade for MS, were purchased from CNW Technologies GmbH (Germany); DMEM cell culture media were purchased from Invitrogen (San Diego, CA, USA); fetal bovine serum (FBS) was purchased from Biowest (Loire valley, France). Penicillin-streptomycin solution and 3-(4,5-dimetrhylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Solarbio (Beijing, China); dimethyl sulfoxide (DMSO), formic acid, trypan blue, and ethyl carbamate (EC) were purchased from Sigma (St. Louis, MO, USA).

Chromatographic Procedure: The mass spectrometer used in this study was Agilent's Time of Flight (TOF) with an ESI source (Agilent 6230). MS was operated in positive ionization mode (ESI+) with following settings: gas temperature was 325℃; sheath gas temperature 350°C; speed 11L/min; capillary voltage 3.5KV; spray voltage 200V. Data were acquired and processed by both Mass Hunter workstation software and Origin Pro 8.5. The [M+H]+ ion of 90.055 (m/z) was selected for EC determination with errors less than 5 ppm. Extracts from both intracellular and extracellular were analyzed (Figure 1).

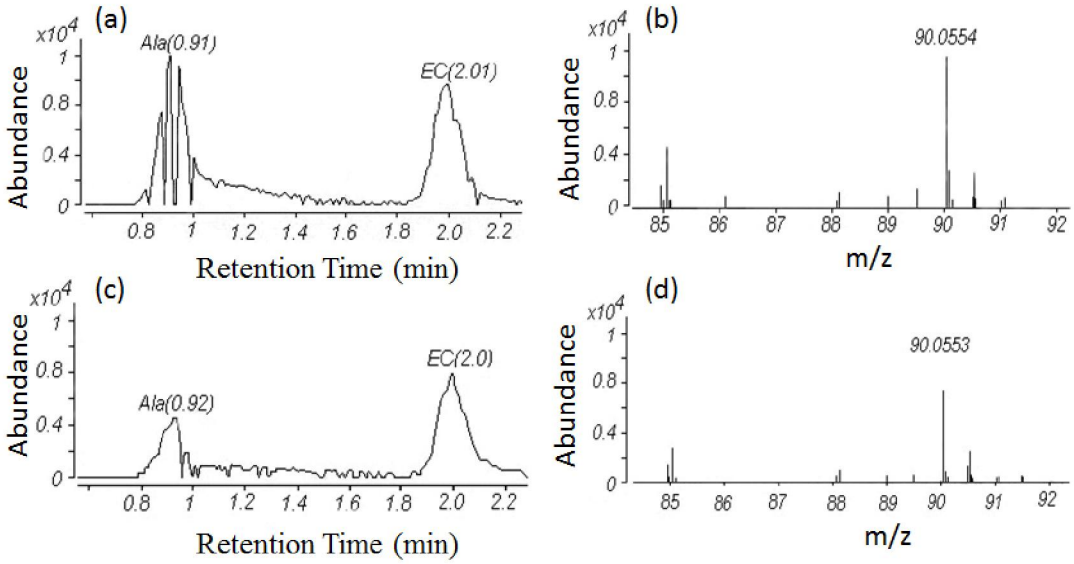

Figure 1. Identification of EC in both Extracellular and Intracellular Matrices: (a) and (b) are the extracted chromatogram and accurate mass for the extracellular matrix; (c) and (d) are the extracted chromatogram and accurate mass for the intracellular matrix.

Preparation of External EC Standard: Five mg of EC crystals was weighed and dissolved in 5mL ultra pure H_2O, the final concentration of this EC stock was 1 mg/mL. The stock solution was stored at 4°C. Working solutions of different EC concentrations were made by serial dilution from this stock and only made freshly when needed.

HepG2 Cell Culture, MTT Assay and Trypan Blue Staining: HepG2 cell was obtained from the Institute of Biochemistry and Cell Biology, Chinese

Academy of Science (Shanghai, China).Cell culture was done as previously reported(Jiao Z H et al, 2013). Essentially, cells were cultured at 37 ^{o}C in the presence of 5% CO_2 with 100% humidity, in DMEM media supplemented with 10% fetal bovine serum and 1% 100x penicillin-streptomycin. Culture media were replaced every two days, and cells were sub-cultured when the cells were about 80% confluent.

MTT assay was carried out as previous report (Zhang X N et al, 2011). HepG2 cells were trypsin digested for 3-5 min and resuspended into single cell suspension and the cell numbers were counted. Then, cells were plated in 96-well micro-well plates at a density of 6 x 10^3 in each well overnight at 37℃ with 100% humidity and the presence of 5% CO_2. Then the culture medium was replaced by serum medium containing different concentrations of EC (0, 30, 50, 75, and 100mM) and incubated for 6, 12 and 24 hours. After the addition of 15μL MTT (5 mg/mL) into each well, the cells were incubated for another 4 hours. Subsequently, the medium was sucked out, and 150μL DMSO was added to the cells. After additional 30 min incubation, the cultures were shaken, and stained with 150μL DMSO for 10 min. Cellular enzyme activity was measured using a Thermo Scientific Varioskan Flash Reader at 560 nm absorbance. Dose-response curves were plotted by converting the data to the percentages of the control response and the cell relative viability was calculated.

For trypan blue staining, HepG2 cells were trypsin digested for 3-5 min and resuspended into single cell suspension. Cell numbers were then counted and about 30000 cells were added into each of the wells in a 6-well cell culture plate. After the cells have attached to the plate surface, 100mM concentration of EC was added and the plates were incubated for 6, 9, 12 and 24 hours. The cells were then collected by trypsin digestion. Resuspended cells were stained with trypan blue at 9:1 ratio (9 cells: 1 trypan blue). The staining result was observed within 3-5 min under a microscope and the death cells were counted. The cells stained with Trypan blue staining dark blue were dead, while cells without staining were live. Also calculate the cell mortality.

Method Validation: The UPLC-TOF-MS based EC detection method was evaluated and validated for its linearity, sensitivity, quantification accuracy, and recovery. Standard curves were constructed with 7 points at concentrations of 60-6000 ng/mL for both extra- and intra-cellular matrices using detected EC peak areas. Limit of detection（LOD）was determined when S/N≥3, and limit of quantification (LOQ) was determined when S/N≥10. Inter-day and intra-day detection variations were evaluated for both extra/intra-cellular matrices. The 3 concentrations of EC (500, 1000, and 6000 ng/mL for extracellular matrix and 100, 1000, 6000 ng/mL for intracellular matrix) were measured 4 times within a day (intra-day) and measured once for 5 consecutive days (inter-day). Extract recovery was determined by comparing peak areas of EC from extra/intra-cellular matrices before extraction with those after extraction. All abovementioned validation measurements were repeated 4 times.

Absorption and Accumulation Characteristics Determination: Trypsin digested cells were transferred into 6-well culture plats at about 30000 cells per well in a 2mL volume with 5 replicates. When the cells were close to 80% confluent, the culture media were removed and the wells were washed with 1×PBS. Then, 2mL of fresh culture media containing 100mM EC was added to each well and the cells were incubated for 6, 9, 12, and 24 hours. Cells before EC addition and 6, 9, 12, and 24 hours after EC addition were collected for UPLC-TOF-MS analysis.

Extra cellular matrix (culture media): 200μL cell culture media was transferred into a 1.5mL Eppendorf tube, spin down at 13,000rpm for 5min. 100μL supernatant was mixed with 1.9mL H_2O (1:19 dilution) by vortex. 100μL mixture was then added to 450μL methanol, and homogenized in a Geno-Grinder homogenizer (Spex Sample Prep, USA) at 680 rpm for 3 min. The homogenized cells were span again at 13,000 rpm for 10 min and 150μL supernatant was transferred into a sample vial for UPLC-TOF-MS analysis.

Intracellular matrix (cells): Cultured HepG2 cells were washed 2-3 times with 1×PBS and scrapped into 1mL 1×PBS in a 2mL Eppendorf tube. The culture flask was washed again with 500μL 1×PBS, which was combined with the previously collected 1mL cells. The cells were span at 1000 rpm for 2 min, resuspended in 1mL H_2O, span again and resuspended in 500μL H_2O. The cells were then mixed with glass beads and homogenized in a Geno-Grinder homogenizer at 1350 rpm for 4 min. After homogenization, the extracts were span at 13000 rpm for 10 min, and 100μL supernatant was added into 450μL methanol. This mixture was homogenized again at 680 rpm for 3 min, span at 13000 rpm for 10 min, and then 100μL supernatant was transferred into a sample vial for UPLC-TOF-MS analysis.

3. Results and Discussions

Optimization of UPLC: Optimization of mobile phases: Methanol and acetonitrile have been mainly used as organic solvents in LC detection of EC in fermented foods (Lim H S et al, 2011). Therefore, in this study, initially 0.1% formic acid in H_2O was used as mobile phase A, while 0.1% formic acid in either methanol or acetonitrile was used as

mobile phase B. During the setting up of the system, one problem hindered our progress was the elution of alanine (Ala), which is present in cells and cell culture media, and it has the identical molecular formula to EC. Under ESI+ mode, Ala has the identical [M+H]+ ion (m/z 90.055) to that of EC (Figure 2a,b). During UPLC separation, Ala had a shorter retention time (RT) than that of EC, therefore, Ala was eluted before EC. However, due to the abundant nature of Ala in the cell and culture media, its peak was usually much higher and broader than that of later eluted EC (Figure 2c-f), which often hid or covered the peak of EC if the difference between these two RT was small. Our optimization experiment discovered that mobile phase B with methanol as organic solvent could enlarge the RT difference between Ala and EC than mobile phase B with acetonitrile (Figure 2). In the case of acetonitrile, the RTs of Ala and EC were 0.9 min and 1.6 min, respectively; in the case of methanol, the RTs of alanine and EC were 0.9 min and 1.9 min, respectively. This increased 0.3 min RT difference rendered us to easily separate smaller EC peak from larger Ala peak (Figure 2c-f). In addition, as compared with acetonitrile, EC peak shape eluted with mobile phase B containing methanol was much better. Thus, methanol was chosen as the organic solvent in mobile phase B for further measurements in this study.

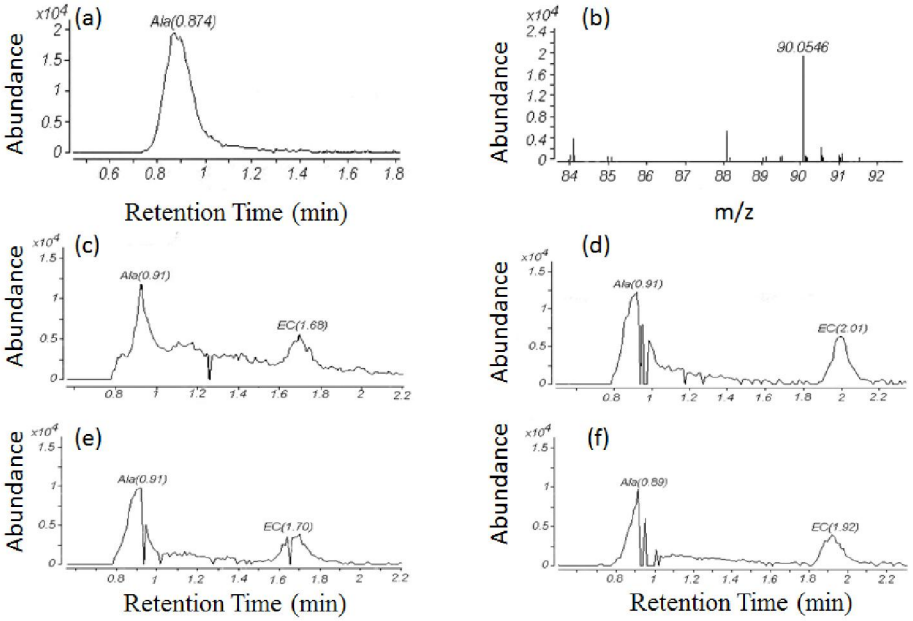

Figure 2. Identification of Ala and EC in H_2O and Extracts from both Extra/Intra-cellular Matrices: (a) and (b) are the extracted chromatogram and accurate mass for Ala; (c) and (d) are the extracted chromatogram for the extracellular matrix using acetonitrile and MeOH, respectively; (e) and (f) are the extracted chromatogram for the intracellular matrix using acetonitrile and MeOH, respectively.

Optimization of elution gradient: Two gradient elution programs for mobile phase B were tested. The first program was as follows: 30%-60% (0-1.7 min), 60%-85% (1.7-6.7 min), 85-30%(6.7-8.3 min) and 30% (8.3-10 min); and the second program was as follows: 5%-35%(0-3 min), 35% (3.01-4 min), 35%-90% (4.01-5 min) and 90%-5% (5.01-7 min). Different gradient elution program for mobile phase B had different effects on EC separation. As compared with the first elution gradient program, the second elution gradient program had larger RT difference between Ala and EC together with sharper EC peak (Figure 3). Therefore, second gradient elution was chosen in this study for further measurements.

Optimization of formic acid concentration: In addition to the optimization of and mobile phase and gradient elution, two concentrations of formic acid in the mobile phases were also optimized. From literatures, two different concentrations of formic acid (0.1% and 0.2% in the mobile phase) were tested. Results showed that EC peak extracted with mobile phase containing 0.2% formic acid was higher than that with mobile phase containing of 0.1% formic acid. Thus, 0.2% formic acid in the mobile phase was chosen for further measurements (Figure4).

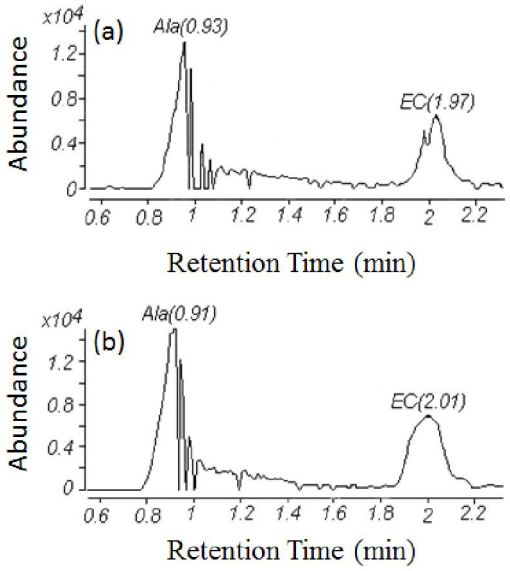

Figure 3. Optimization of the Gradient Elution Program of Mobile Phase B: (a) 30%-60% (0-1.7min), 60%-85% (1.7-6.7min), 85%-30% (6.7-8.3min), and 30% (8.3-10min); (b) 5%-35% (0-3min), 35% (3-4min), 35%-90% (4-5min), and 90%-5% (5.01-7min).

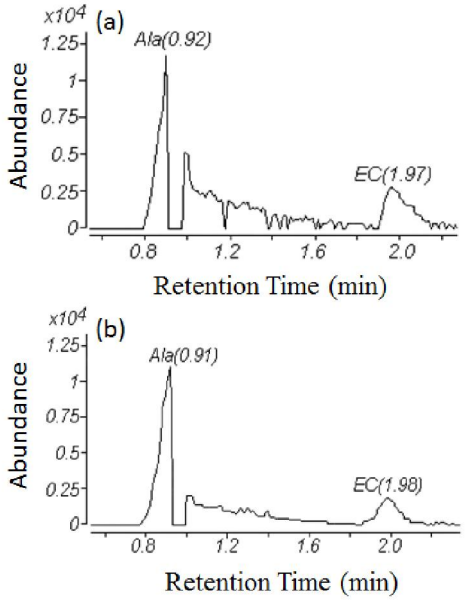

Figure 4. Optimization of Formic Acid Concentration in the Mobile Phases: (a) and (b) are the extracted chromatogram for the extracellular matrix. The figure (a) is the extracted chromatogram with 0.2% formic acid mobile phase, and the figure (b) is the extracted chromatogram with 0.1% formic acid mobile phase.

Linear Equation and Range of Linear Detection: The performances of this method such as linearity, limit of detection (LOD) and limit of quantification (LOQ)were in cell matrix with external standard (Table 1).Under optimized UPLC-TOF-MS conditions, its linearity was good, with $R^2 \geq 0.99$ at detection range of 60-6000 ng/mL for both intra/extra-cellular matrices. The sensitivity of detection was evaluated by calculating LOD and LOQ at S/N (the ratio of the signal measured to the background noise) ≥ 3 and S/N ≥ 10, respectively. They were 40 ng/mL and 31 ng/mL, and 133 ng/mL and 103 ng/mL for both extra/intra-cellular matrices, respectively.

Table 1. Calibration Curves of EC in Extracellular and Intracellular Matrices (n=4)

Chemical	Matrixes	Calibration equation	R^2	Range (ng/mL)	Sensitivity (ng/mL)	
					LOD	LOQ
EC	Extracellular	y=13.78x	0.9924	60-6000	40	133
	Intracellular	y=6.85x+517.7	0.9947	60-6000	31	103

Recovery and Accuracy: Recovery of EC was determined in both extra/intra-cellular matrices spiked with the target EC at three different concentrations (high, medium, and low), to evaluate the effectiveness of the extraction method. The extraction recoveries of EC at three tested concentrations for both extra/intra-cellular matrices were in the range of 85.58-90.46% and 76.67-92.42% (Table 2), indicating that extraction method in this experiment was acceptable for the quantitative detection of EC in human cells for both extra/intra-cellular matrices.

Table 2. Recovery Tests in Extracellular and Intracellular Matrices (n=4)

Matrices	Concentration (ng/mL)	Extraction recoveries (mean %)
Extracellular	100	87.1±4.55
	1000	85.58±6.15
	3000	90.46±3.76
Intracellular	100	88.94±8.27
	500	76.67±4.37
	3000	92.42±7.29

Table 3. The intra- and inter-day precision tests in extracellular and intracellular matrices (n=4)

Matrices	Concentration (ng/mL)	Intra-day (RSD %)	Inter-day (RSD %)
Extracellular	100	3.12	12.57
	1000	2.03	11.46
	6000	3.30	1.50
Intracellular	100	7.54	12.13
	1000	4.26	9.62
	6000	5.63	2.35

The precisions of the method in both matrices were measured for both intra/inter-day studies at three different EC concentrations. For extracellular matrix, RSDs of intra-day and inter-day variation were 2.03%-3.30% and 1.50%-12.57%, respectively; while those of intra-day and inter-day variation for intracellular matrix were 4.26%-7.54 and 2.35%-12.13% (Table 3), which were lower than 15%, the upper limitation for bio-analytical validation in precision tests in FDA guidance (2001). Lim and Lee (Lim H S et al, 2011) investigated EC recovery and accuracy in majority of fermented foods, in which the recovery was 76.9%-118.1%, the intra-day variation was 3.5% -34.2%, and the inter-day variation was 3.8%- 41.9% (Lim H S et al, 2011).

MTT Assay and Trypan Blue Staining: Firstly, MTT was used to determine the effect of EC on the viability of HepG2 cells and to find the optimal concentration of EC to exam the kinetics of EC in HepG2 cells. After the treatment with different concentrations of EC (0, 30, 50, 75, and 100mM) for different treatment time (0, 6, 9, 12, and 24 hours), MTT was then added to the EC treated cells to evaluate cell viability. As shown in Figure 5(a), only did high levels of EC (more than 75mM) for long time (more than 12 h) treatment significantly reduce the cell viability. Therefore, 100mM was chosen for subsequent trypan blue staining, which showed a dose-dependent cell mortality upon EC treatment in HepG2 cells as shown in Figure 5(b).

Absorption and Accumulation Characteristics: The absorption and accumulation characteristics of EC in HepG2 cells was determined with HepG2 cells spiked with 100mM EC. During the 24 h incubation, samples of media and cell pellets were taken timely for EC determination, and the results were shown in Figure 6. Since the EC concentration used in this study was 100mM, equal to 1.8 mg/mL in the medium, which was over the upper detection limit, therefore, the changes of EC detected in the culture media (extracellular matrix) were very little (the media were diluted prior to be analyzed). In contrast, the EC levels detected in the cell pellets (intracellular matrix) started to increase after 6 h EC incubation, and peaked after 12 h EC incubation. Since then, the EC levels in the cells declined gradually and kept at a relative high level in samples after 24 h EC treatment. These results indicated that EC could be absorbed by HepG2 cells and accumulated in the cells, which lead to the decline of cell viability. Supportively, the kinetics of EC levels in the cell pellets was consistent with MTT assay and trypan blue staining results of the same samples (Figure 5).

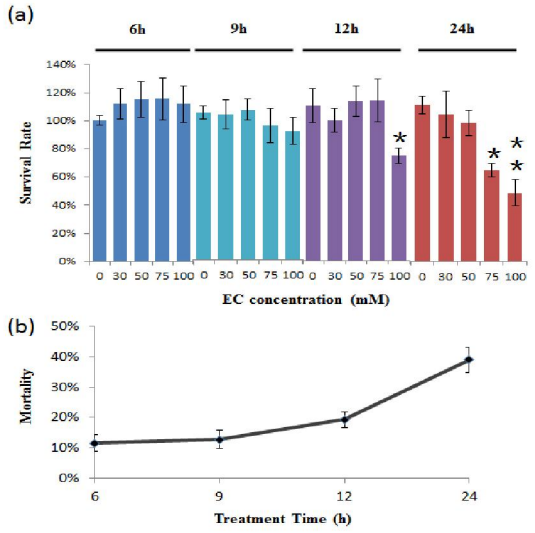

Figure 5. The Effects of EC on the Viability and Mortality of HepG2 Cells. (a): MTT assay (*p< 0.05 and **p< 0.01). (b): trypan blue staining.

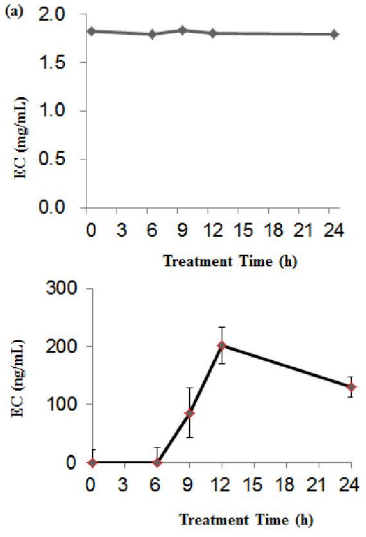

Figure 6. Real Sample Analysis by the UPLC-TOF-MS System: kinetic studies in both medium (a) and cells (b) after incubation with 100mM EC.

4. Conclusions

In this study, we first developed a simple, fast, and economical method to detect EC in human HepG2 cells based on UPLC-TOF-MS without the requirement of any sample clean processes. It gave good selectivity, sensitivity, calibration lines, accuracy and reproducibility. Then, using this developed method, we measured the EC spiked cells and revealed, for the first time, the absorption and accumulation properties of EC in HepG2 cells, indicating that EC could be accumulated in human cells, which paves the way for further EC safety assessment analysis.

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