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Analysis of acetylcholine, choline and butyrobetaine in human liver tissues by hydrophilic interaction liquid chromatography-tandem mass spectrometry

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ABSTRACT

The strong polar quaternary ammoniums, acetylcholine (ACh), choline (Ch) and butyrobetaine (BB, (3-carboxypropyl)trimethylammonium), are believed playing important roles in liver metabolism. These metabolites are at low levels and are weakly retained on reversed-phase liquid chromatographic (RP-LC) columns. Several hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC–MS/MS) methods have been reported to analyze these compounds from different samples. However, no application to human liver tissues has been published. In this study, HILIC–MS/MS method was developed to simultaneously determine these three metabolites in human liver tissues. They were simply extracted from tissue, separated on a HILIC column, and detected by tandem MS in the mode of multiple reaction monitoring (MRM). Further studies on the recovery and repeatability based on real samples indicated the method was accurate and reliable. This method was successfully applied to measure the levels of ACh, Ch and BB in 61 human liver tissue samples including normal, hepatocellular carcinoma (HCC) and matched non-cancerous liver tissues. By comparison of Ch and ACh contents in 29 HCC with their matched non-cancerous liver tissues, it was found that ACh content increased in 11/29 HCC cases and decreased in 13/29 cases. Furthermore, the ACh/Ch ratio increased in 16/29 HCC cases, while it decreased in 8/29 cases. These results strongly indicated that there exist different patterns of ACh content in cancer tissues among HCC patients, thus highlighting the understanding of ACh and its relevant signal pathways in hepatic carcinogenesis and HCC progression.

1. Introduction

Liver is one of the most important organs in human body. It is the main location of carbohydrate, lipid and protein metabolism. The small molecular metabolites in the liver play very important roles in physiological functions and the interactions between liver and the other organs.

Acetylcholine (ACh), a well known cholinergic neurotransmitter, plays crucial roles in transmitting important biological information both in the central and autonomic nervous system and neuromuscular junctions [1]. It has been detected in heart, muscle, kidney, plasma, liver and red blood cells [2]. Choline (Ch) is not only the precursor of ACh, but also extensively involved in phospholipids or other metabolism pathways, and it is present in almost all tissues. Butyrobetaine (BB, (3-carboxypropyl)trimethylammonium), the isomer of ACh, is the precursor of carnitine [3] in metabolic pathway of lysine degradation [4], while carnitine is an essential metabolite in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, and this biosynthesis takes place mainly in kidney and liver for human. These essential metabolites are strong polar quaternary ammonium and at a low level of content in liver, which make their detection and quantification relatively difficult.

For determination of ACh and Ch, gas chromatography followed by mass spectrometry (GC-MS) or high performance liquid chromatography with electrochemical detection (HPLC-ECD) was frequently used. The analytes were derived into the tertiary amine analogs before injection on GC-MS [1], making the whole procedure time-consuming and leading to inadequate sensitivity or specificity. By comparison, HPLC-ECD provided the advantages of simplicity, sensitivity and specificity [5–10], but a rapid drop from the initial sensitivity of a newly installed electrode was frequently observed. This was a serious disadvantage of HPLC-ECD method. As a sensitive, specific and non-discriminating

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analytical technique, mass spectrometry (MS) has been applied more and more frequently in the identification and quantification of molecules of biological origin [11,12]. Recently, the contents of ACh and/or Ch were measured by HPLC-MS in brain microdialysates or corneal epithelium cells [13–15]. BB was also analyzed from rat brain microdialysate, or plasma [12,15,16]. To separate these hydrophilic quaternary ammoniums, reversed-phase chromatography with high aqueous mobile phase [14], ion-pair [12,16] or ion exchange [13,15] chromatography was used. At these conditions, the sensitivity of MS was poor because of the high aqueous mobile phase or high concentration of salt used.

Hydrophilic interaction chromatography (HILIC) is a favorable alternative for retention of hydrophilic polar compounds [17]. It offers different selectivity from traditional RP HPLC. In HILIC, the elution is promoted by polar mobile phases as in normal phase (NP) HPLC, but frequently used mobile phases are water and water soluble modifiers. The presence of water in the mobile phase is crucial for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which solutes may selectively partition [18,19]. The highly volatile organic solvents in the mobile phases provide increased sensitivity in electrospray ionization-mass spectrometry (ESI-MS) [20]. HILIC has been applied in the separation of strong hydrophilic analytes, such as pharmaceuticals [21,22], peptides [23,24], and some small polar compounds in biosamples [25–33].

HILIC-tandem MS has been used to detect ACh or Ch from human plasma or serum [34], rat, mouse or monkey's brain microdialysates [35,36], and aritificial cerebral spinal fluid [37]. Simultaneous analysis of ACh, Ch, BB and several other Ch metabolites in rat or mouse's brain or liver by HILIC–MS has been published [38]. Part of the above applications have been summarized by Hemstrom and Irgum [17]. Thinking of their importance in understanding liver function and variation at different physiological conditions, we developed a HILIC-tandem MS method to simultaneously quantify the contents of ACh, Ch and BB in human liver tissues, hepatocellular carcinoma (HCC) and matched non-cancerous liver tissues. In the meantime, their contents in 61 human liver tissues were measured.

2. Experimental

2.1. Reagents and chemicals

Acetylcholine chloride, choline chloride and (3-carboxypropyl) trimethylammonium chloride were purchased from Sigma–Aldrich (St. Louis, NJ, USA). HPLC-grade acetonitrile (ACN), methanol (MeOH) and formic acid were from TEDIA (Fairfield, OH, USA). Distilled water was purified with a Milli-Q water purification system (Millipore, Billerica, MA, USA). Ammonium formate (A.R.) was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Stock solutions of 1 mg/ml of the analytes were prepared by dissolving the standard compounds in a reconstitution solvent (ACN/MeOH/formic acid, 750/250/2, V/V/V) [20] using a volumetric flask and stored at 4 °C. The calibration standards were prepared by diluting stock solutions with the reconstitution solvent to build the calibration curves.

2.2. Human HCC and liver tissue samples and their pretreatment

The human HCC and non-cancerous liver tissue samples were obtained from Qidong Liver Cancer Institute and Hospital (Shanghai, China). The non-cancerous liver tissues were the human liver tissue ≥2 cm outside the hepatic cancer nodules removed by surgical operation. The non-cancerous liver tissue has been verified by histopathological examination which excluded the presence of invading or microscopic metastatic cancer cells. Normal liver tissues were obtained from individuals with accident death. The utilization of human tissues was approved by the Ethical Review Committee of the World Health Organization Collaborating Center in Human Reproduction. All the samples were stored at −80 °C.

Before analysis, a block of tissue (30 mg typically) was weighted and pestled with 2% aqueous formic acid (10 μl/mg tissue) to make homogenate. The homogenate was further ultrasonically broken with 2 ml of the reconstitution solvent (ACN/MeOH/formic acid, 750/250/2, V/V/V), then de-proteinized by centrifugation (12,000 rpm, 16 min). The supernatant was lyophilized and redissolved with 300 μl reconstitution solvent and filtered by 0.22 μm filter membranes.

2.3. Liquid chromatographic conditions

The analysis was performed using an Agilent 1100 liquid chromatograph (Agilent Technologies, USA) consisting of a binary pump, an automatic sampler and a column compartment. PolyHYDROXYETHYL A™ particles (5 μm, 100 Å, PolyLC Inc., USA) were slurry packed into a stainless steel column (2.1 mm I.D. × 35 mm) to perform separation. The column temperature was 22 °C. The injection volume was 1 μl. The flow rate was 200 μl/min. Mobile phase A was 20 mM of aqueous ammonium formate with 0.1% formic acid (V/V); and mobile phase B was ACN. Both solvents were filtered by 0.22 μm filter membranes and degassed before use. Linear gradient was adopted in the separation. The mobile phase started at 3% A; linearly increased to 40% A in 5 min and kept for 3 min; then decreased to 3% A in 0.1 min and kept 3% for 8.9 min. The total separation time was 17.0 min.

2.4. Mass spectrometric conditions

Identification and quantification of the target analytes were performed on an API Q trap linear ion trap mass spectrometer with a Turbo ion spray source (Applied Biosystems/MDS SCIEX, USA). The ion spray voltage was 5000 V. The declustering voltages (DP) was 21 V for ACh and BB, and 33 V for Ch. Nitrogen was used as nebulising, anxilliary, curtain and collision gas. The pressure and temperature of nebulising gas were 40 psi and 350 °C, respectively.

Analysis of the human liver tissue samples was carried out in the positive ion mode using multiple reaction monitoring (MRM). The ion transitions of the analytes were investigated in enhanced product ion (EPI) mode by direct infusion of the standards using a syringe pump. Dwell time of each reaction was 200 ms. The collision energies (CE) was 19 eV for ACh and BB, and 48 eV for Ch; collision cell exit potentials (CXP) were 58 and 15 V, respectively. The data were collected and processed by Analyst 1.3 software. All the positive ESI parameters were tuned for the best sensitivity under HILIC conditions applied using the automatic tuning procedure recommended by the manufacturer.

3. Results and discussion

3.1. Analytical conditions

Since the target analytes were already charged in the solution due to the presence of quaternary ammonium groups, they could be easily detected by electrospray ionization-mass spectrometry without any modification or derivatization. Fig. 1 shows the EPI mass spectra of three analytes. As can be seen, the precursor ions were $[M]^+$, and the major product ions were $[M–N(CH_3)_3]^+$ or $[NH(CH_3)_3]^+$. For each analyte, the precursor ion and its major product ion (146 → 87 for ACh and BB, 104 → 60 for Ch) were used for quantification. Conceivable fragmentation patterns illustrated in Fig. 1a and b had been confirmed by a Q-TOF MS in the early study [35].

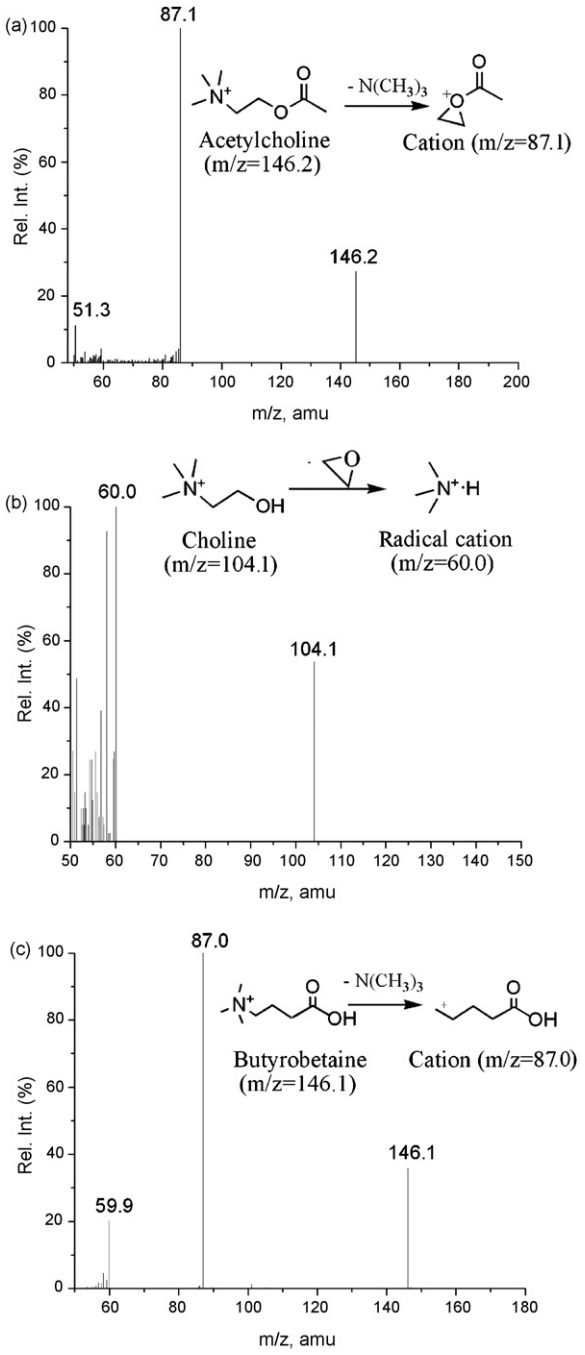

Fig. 1. Enhanced product ion spectra and corresponding fragmentation patterns of (a) ACh, (b) Ch and (c) BB.

Although mass spectrometry could detect and identify mixtures based on their mass differences, on-line HPLC separation played a critical role in the analysis of biological matrixes by adding another dimension of purification. It reduced the possibility of analog interferences and signal suppression from matrix components. For instance, BB has had the same ion transition as ACh (Fig. 1), they could not be distinguished by the direct-injection MS, but on the HILIC column they were easily separated with the retention times at 3.4 and 9.7 min, respectively (Fig. 2).

In the present study, a short column (35 mm long) and fast gradient were used for the whole separation, washing and re-equilibrium process. The volume of our static mixer was 420 μl, and the whole dead volume of the system was about 900 μl. It caused 4.5 min gradient delay at 200 μl/min of flow rate. To shorten the total analysis time and avoid the matrix disturbance to the target analytes, a fast gradient beginning from a higher ACN percentage was employed (see Section 2). On-column gradient was ended at about 12.5 min. Although the retention time of BB was at 9.7 min, it was actually eluted by high percentage of mobile phase A with a stable retention time.

3.2. Method validation

In this work the relative abundance of the two pairs of selected precursor → product ion transitions for each target compound was used to verify the selectivity, the used ion transitions were 146 → 87 and 146 → 51 for ACh, 146 → 87 and 146 → 60 for BB, 104 → 60 and 104 → 58 for Ch. The relative abundance data from the standard samples and real samples showed there was no noticeable interference from the co-existing other substances, the method has a good selectivity. After that, the precursor ions and their most abundant product ions in the EPI chromatograms (Fig. 1), 146 → 87 for ACh and BB, 104 → 60 for Ch, were chosen in the MRM mode for quantification of the target analytes.

The "matrix effect" was also an important issue in ESI-MS. The co-eluted compounds originating from the matrix might cause the suppression or enhancement of the analyte response. It could be diminished by adequate sample preparation and chromatographic separation. In this study, the matrix effect was checked in a similar mode as in literature [39] by post-column infusion of standard mixture at 20 μl/min. The concentrations of ACh, Ch and BB were 2,

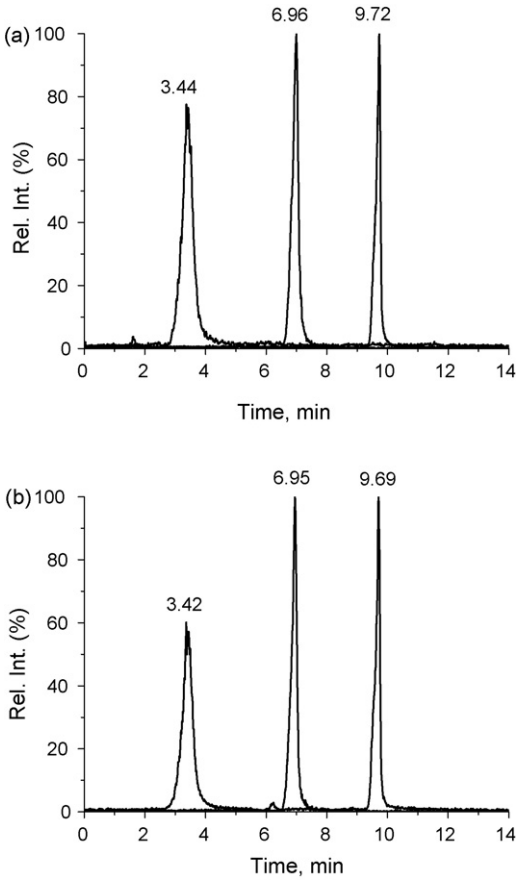

Fig. 2. MRM chromatograms of ACh (146 → 87, 3.44 min), Ch (104 → 60, 6.96 min) and BB (146 → 87, 9.72 min) in (a) standard mixture with 5.0 ng/ml of ACh, 3000 ng/ml of Ch and 1000 ng/ml of BB; (b) a HCC sample, in which the content of ACh was 21.4 pg/mg tissue, of Ch was 21.6 ng/mg tissue and of BB was 2.2 ng/mg tissue. The chromatographic and mass spectrometric conditions are shown in Section 2.

3000 and 50 ng/ml, respectively. Continuous infusion of the standard sample generated a stable baseline in each MRM channel. Real samples were then injected. It was found that no significant ion suppression or enhancement was observed in retention time windows of all the analytes.

Three analytes were endogenously present in human liver, and "blank" liver samples without them were un-obtainable. Because of a smaller matrix effect, the calibration standards were prepared in reconstitution solvents. The calibration curves for each analyte were constructed at eight levels by regression of nominal concentrations against average peak area of triplicate calibration standards. They were linear over 100-fold range of concentration, which were 1.0–200, 100–10,000, and 20–5000 ng/ml for ACh, Ch and BB, respectively. Their corresponding correlation coefficients (R) were larger than 0.999.

The limit of detection (LOD, S/N = 3) and of quantification (LOQ, S/N = 10) were measured by injecting low amounts of calibration standards at the experimental conditions. LODs of ACh, Ch and BB were 0.2, 30 and 2.0 ng/ml; LOQs of them were 0.6, 80 and 15.0 ng/ml, respectively.

The recoveries of the analytes for real samples were evaluated. A block of human liver tissue sample was divided into 12 parts and made homogenate separately. The standard analytes with known amounts at low, medium and high levels were spiked to 9 of 12 homogenate samples, respectively. For each spiked level, three parallel samples were prepared. 12 samples including 3 non-spiked samples were measured by the developed HILIC-tandem MS method. Both the non-spiked (endogenous) and spiked levels of the analytes were calculated from the calibration curve. Recoveries were calculated as the amount of spiked analyte found as a percentage to the theoretically spiked amount added, namely (observed value − endogenous value)/added value × 100. The results were listed in Table 1, the recoveries are from 94.0% to 105.7%. Intra-batch repeatability was calculated by the relative standard deviation (R.S.D.) of the three replicates at each level, ranged from 2.2% to 8.3% (Table 1). Inter-batch repeatability was investigated by analyzing non-spiked human liver tissue in three independent batches, and the R.S.D. was 11.0%, 4.8% and 8.0% for ACh, Ch and BB, respectively. These satisfactory results in recovery and repeatability obtained from real samples indicated accurate and reliable quantification of the three analytes in human liver could be achieved using the developed method.

Table 1
Recovery and repeatability for the measurement of ACh, Ch and BB in human liver tissue[a]

	ACh[b]	Ch[c]	BB[c]
Endogenous	75.8	48.6	5.1
Added			
Low	50.0	25.0	1.1
Medium	100.0	50.0	2.2
High	500.0	250.0	11.1
Measured (mean ± S.D.)			
Low	127.7 ± 2.5	72.2 ± 2.1	6.4 ± 0.1
Medium	173.6 ± 7.6	101.5 ± 1.9	7.2 ± 0.3
High	576.0 ± 22.6	298.9 ± 8.5	16.3 ± 1.3
Recovery (%)			
Low	103.8	94.0	101.8
Medium	97.8	105.7	98.3
High	100.0	100.1	100.2
Intra-batch repeatability (%)			
Low	2.3	2.8	1.8
Medium	4.6	2.2	3.7
High	4.0	2.7	8.3

[a] Three samples were measured at each level.
[b] pg/mg liver.
[c] ng/mg liver.

3.3. Detection of ACh, Ch and BB in human liver, HCC and matched non-cancerous liver tissues

By utilizing the established method, we quantitatively detected the contents of ACh, Ch and BB in tissue samples of normal human liver, HCC and matched non-cancerous liver. In cases where the ACh levels were extremely low, more liver tissues than 30 mg were used. While in cases where the Ch levels exceeded the calibration curve, dilution of samples was performed. The analytical results were illustrated in Table 2. As can be seen, the contents of ACh and Ch varied in a broad range per individual sample from normal liver, HCC and non-cancerous liver tissues. On the other hand, BB content difference was very small.

Ch is abundant in normal liver, non-cancerous liver and HCC tissues based on mean value comparison. Since Ch is involved in multiple biochemical pathways, including the lipid and amino acid metabolism, membrane phospholipids synthesis, which might be correlated with cell renewal. Also, a quite minute but functionally important proportion of Ch serves as the precursor of ACh, synthesized by choline acetyltransferase. But the ACh content is determined by the balance between ACh synthesis and its degradation mediated by acetylcholine esterase which converts ACh back to Ch. From Table 2, the Ch contents in both HCC and non-cancerous liver tissues were reduced as compared with normal liver tissues based on mean value. The alteration of Ch content in HCC samples will be subsequently presented in our analysis based on comparison of individual paired HCC and non-cancerous liver samples.

In addition, it was found that the ACh content in both HCC and non-cancerous liver was tremendously reduced to about 1/5 of normal liver. This phenomenon in fact is possibly the summation of the alteration of ACh synthesis from neural and non-neural cell origins. Since ACh in normal liver is predominantly derived from neural origin, i.e. mostly from terminals of vagus nerve innervation, the ACh decrease in non-cancerous liver and HCC could be attributed to the progressive degeneration and damage of autonomous nerve innervation. The other complex feature is that the level of ACh synthesis in hepatocyte is possibly altered along with the carcinogenesis process (to be published). Thus, the content of ACh detected at tissue level might reflect the net change originated from alterations of neural and non-neural systems occurred in the process of hepatocarcinogenesis. Furthermore, as illustrated in Table 2, the mean value of ACh/Ch ratio is higher in HCC as compared with non-cancerous liver tissues ($p = 0.028$), which might support the notion that ACh synthesis is quite active in HCC in which ACh may be predominantly derived from malignant hepatocytes and/or cancer-associated stromal cells.

Based on the presence of remarkable variation of ACh and Ch contents in individual samples from HCC and non-cancerous liver, we compared the difference of Ch and ACh contents per individual pair of HCC and matched non-cancerous liver tissues. Interestingly, we can categorize 29 HCC cases into three subgroups Group I, Group II, and Group III (Fig. 3). In Group I, the content of Ch and ACh in HCC was $\geqslant$20% lower than that in non-cancerous liver tissues. In Group III, the contents in HCC was more than 20% higher than those in non-cancerous counterparts; while in Group II, the difference was less than 20%, indicating no significant alteration. As shown in Fig. 3, Ch content in 20/29 HCC cases (68.9%) was reduced (Group I), while it was increased only in 5 HCC samples (Group III). In case of ACh, the content in 11/29 (37.9%) HCC samples was increased (Group III) and decreased in 13/29 (44.8%) HCC cases (Group I). If we had ACh/Ch ratio comparison, we surprisingly found that 16/29 HCC samples (55.1%) exhibited an increased ACh/Ch ratio, while only 8/29 HCC samples (27.5%) gave a decreased ratio as compared with their non-cancerous liver tissues. The remarkable change in ACh/Ch ratio in HCC might be attributed the concurrence of the increase of ACh and the decrease of Ch in some of these HCC cases. The ACh/Ch

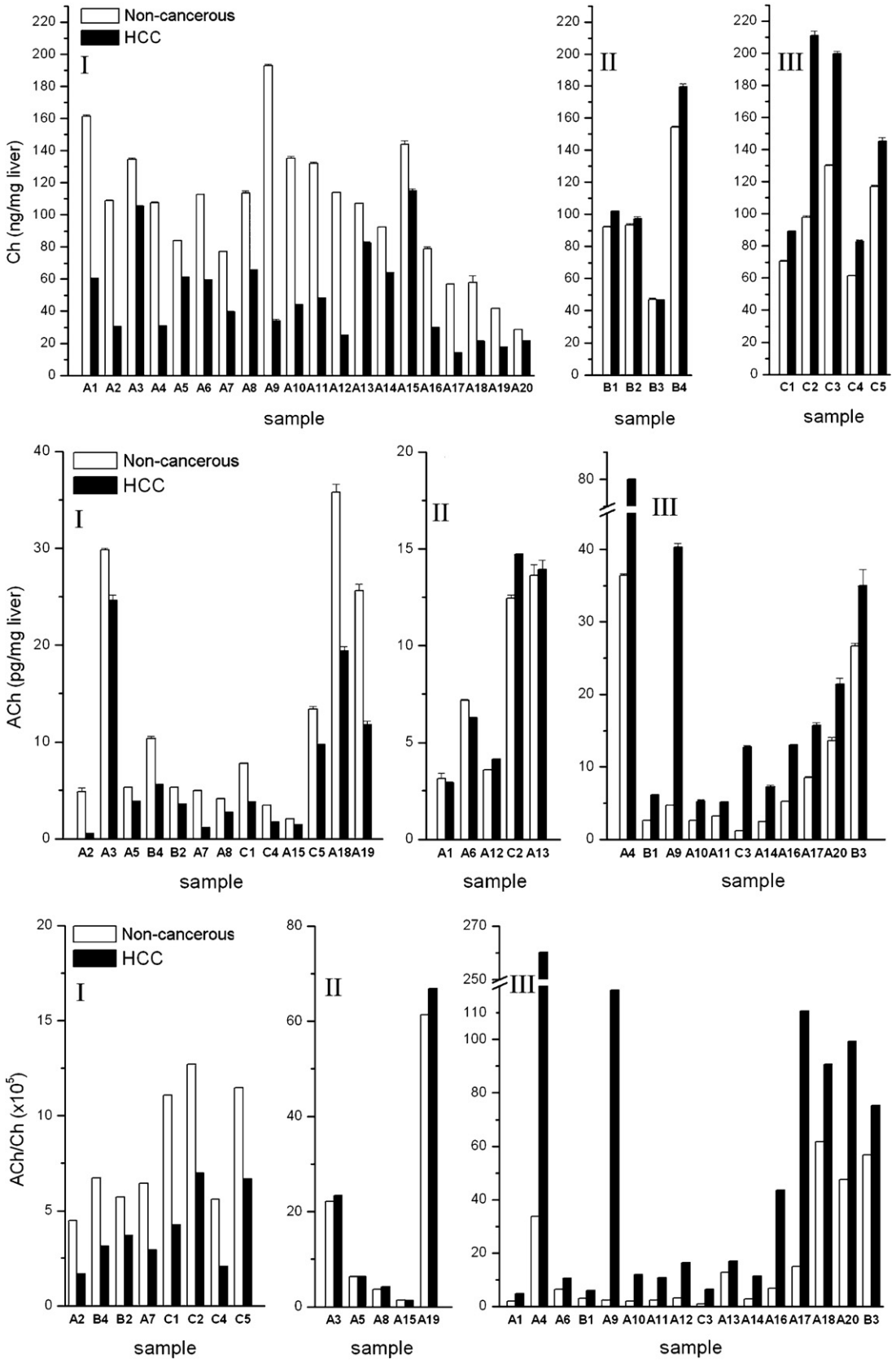

Fig. 3. Contents of (upper) Ch, (middle) ACh and (low) ratio of ACh to Ch in cancer vs. non-cancerous liver tissues of tested HCC patients. The abscissa was the codes of samples. They were subdivided into three groups: the value in HCC was 20% lower than that in non-cancerous liver tissues (I); >20% higher than that in non-cancerous liver tissues (III), and <20% in difference (II).

Table 2
Contents of ACh, Ch and BB in the different human liver tissue samples measured by the developed HILIC-tandem MS method

	ACh[a]		Ch[b]		ACh/Ch ($\times 10^5$)		BB[b]	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
Normal liver tissues (n = 3)	19.5–151.6	67.63 ± 72.98	38.8–103.5	78.57 ± 34.81	20.88–390.72	147.44 ± 210.74	1.1–10.0	4.16 ± 5.06
HCC (n = 29)	0.5–80.0	12.89 ± 16.24	14.2–210.9	73.20 ± 53.90	1.27–260.18	35.40 ± 56.59	0.4–6.5	2.32 ± 1.49
Matched non-cancerous liver tissues (n = 29)	1.2–36.4	10.34 ± 10.34	28.6–193.1	101.56 ± 38.42	0.92–61.79	14.41 ± 18.74	0.4–6.4	1.88 ± 1.40
p-Value[c]	>0.05		0.0094		0.028		>0.05	

[a] pg/mg liver.
[b] ng/mg liver.
[c] p-Value was calculated at 95% confidence interval from HCC and matched non-cancerous liver tissues of the same HCC patients, the p values less than 0.05 indicates significant differences of the measured compounds or their ratio between the tested HCC and matched non-cancerous liver tissues in the HCC patient.

ratio alteration could reflect the proportion of ACh derived from Ch pool in both cell membrane and cytosol, balanced by ACh synthesis and degradation.

In recent years, it has been revealed that most of cancer-related biomarkers or signaling molecules may not be ubiquitously present in all or most of cancer cases. Instead, it is quite common that the presence or absence of a biologically active molecule in different individuals may represent an important pattern in cancer patients. Thus, each pattern may implicate some individual or personalized difference in metabolism or signaling pathways. The presence of ACh and ACh/Ch patterns in HCC patients is well consistent with this concept of personalized medicine, implicating the significance of ACh and relevant signaling pathways in the defined subgroup of HCC patients.

To the best of our knowledge, it was the first time that the contents of quaternary ammoniums, ACh, Ch and BB were compared in HCC and matched non-cancerous tissues in HCC patients. Based on our quantification results, content of BB remained a stable level but those of Ch and ratio of ACh to Ch showed variation after having cancer. To make clear the relationship of these variations with HCC, more physiological and pathological studies are necessary. Furthermore, the origin of ACh from nervous innervation and/or from non-neuronal cells needs to be clarified.

4. Conclusions

A HILIC-tandem MS method was developed to simultaneously determine the contents of ACh, Ch and BB in normal and carcinomatous human liver tissues. After simple pretreatment and hydrophilic interaction separation, the quaternary ammonium analytes were detected by tandem MS in the mode of MRM. The method validation was performed. The recoveries of the method for three target endogenous metabolites were 94.0–105.7%. The repeatability and linearity of the method were good enough for reliable quantification of the trace-level target analytes in human liver tissue samples. With this method, ACh, Ch and BB were simultaneously quantified in human liver tissues although they remarkably varied in concentrations. The contents of three analytes were compared between HCC and matched non-cancerous liver tissues. The developed method was demonstrated valuable in clinical applications.

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