Rapid Detection of Small Molecule Metabolites in Serum of Hepatocellular Carcinoma Patients Using Ultrafast Liquid Chromatography-Ion Trap-Time of Flight Tandem Mass Spectrometry

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A method was developed for analyzing broad spectrum small molecule metabolites in the serum of hepatocellular carcinoma (HCC) patients based on ultrafast liquid chromatography-ion trap-time of flight tandem mass spectrometry (UFLC-IT-TOF MS). Serum samples were collected from 80 HCC patients and healthy persons. After pretreatment process for protein precipitation, the supernatant was analyzed with the UFLC-IT-TOF MS to obtain information on the metabonomics of small molecules. The eight compounds of glycocholic acid, choline glycerophosphate, acetyl-L-phenylalanine, oleamide, tetradecanamide, acetyl carnitine, lyssolecithin and glycochenodeoxycholic acid in the HCC group were identified with significant differences from those in the health group (P <0.01). By using multidimensional analysis of variation coefficient and principal component analysis for the repeatability and 48 h stability, the method was demonstrated to have good repeatability, excellent precision, and high stability, which can satisfy the metabonomics research requirement. The high throughput and practical usability of the method further shows perspective for metabonomic analysis of large-batch serum samples.

Keywords UFLC-IT-TOF MS, hepatocellular carcinoma, serum, metabonomics

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in high-throughput screening of metabolites. At present, there are few reports on metabonomics research using IT-TOF MS technology.28 The detection of biological samples of liver cell carcinoma, liver cirrhosis and other diseases using IT-TOF MS has not been found yet. In this work, a UFLC-IT-TOF MS method was developed for the high-throughput screening of metabolites in large batches of hepatocellular carcinoma serum samples. The sample pretreatment procedure was also optimized to eliminate interference from proteins. This method provides a method for early screening of hepatocellular carcinoma, liver cirrhosis and other diseases using IT-TOF MS.

Experimental

Instruments and reagents

The instruments used in this study include: UFLC-IT-TOF MS (Shimadzu, Japan); Sigma 3K15 centrifuge (Sigma, German); VORTEX GENIUS3 Vortex (IKA, German) and −80°C refrigerator (Thermo, USA).

Acetonitrile, formic acid and methanol (HPLC grade) were obtained from Merck, German. Standard substances of 12 screening compound which were confirmed in this experiment, and internal standard substances were obtained from Sigma, USA. Experimental water was from a Mili-Q water purification system (Milipore, USA). Human serum samples were collected from Bethune International Peace Hospital, and were kept at −80°C before collection and centrifugation.

Chromatography–mass spectrometry conditions

Chromatography conditions. EndeavorPol™ C18 (50 × 2.1 mm, 1.8 μm) was used in the experiment; column temperature was room temperature; injection volume was 5 μL; flow rate was 0.30 mL/min; 0.1% formic acid aqueous solution (A)-acetonitrile (B) were used as mobile phase, gradient elution was adopted. The gradient elution is listed in Table 1.

Mass spectrometry conditions. The ESI source was operated both in positive and negative ionization mode. MS1 was scanned with m/z 100 - 1000 and MS2 was scanned with m/z 50 - 550; heating module temperature was set at 200°C; CDL temperature was 200°C; flow rate of atomizing gas was 1.5 L/min; flow rate of dry gas was 10 L/min; ion source voltage was +4.5 kV at positive ion mode and −3.5 kV at negative ion mode; detector voltage was 1.70 kV; CID parameter was collision energy 50%; automatic tuning was used to optimize voltage and calibration mass.

Standard solution and sample preparation

Standard solution preparation. The scan mode of analyte was confirmed according to the response both in positive and negative scan mode. Names of analytes, CAS number, scan mode and relative molecular weight are shown in Table 2. Each compound was prepared into 5.0 mg/mL standard solution using mobile phase, and 25 mg/L mixed standard working solution was made by diluting standard solution, which was used in comparison of different pretreatment methods and methodological investigation. 2-Chlorophenylalanine was prepared into 0.1 mg/mL as internal standard solution.

Sample preparation. Fasting peripheral venous blood was collected and placed in a disposable blood sampling tube, and was centrifuged at 3500 r/min for 5 min to separate blood within 1 h after sampling. Then the blood was sealed in a 1.5-mL EP tube, marked, and frozen by keeping in a −80°C refrigerator.

The serum samples of 80 patients were thawed at room temperature before experiments, and the mixed serum sample was obtained by mixing the serum samples with each sample of 50 μL. The 100 μL mixed serum was collected as experimental sample, 20 μL 0.1 mg/mL 2-chlorophenylalanine solution was added as internal standard and 300 μL methanol/acetonitrile (3/1, v/v) solution (pre-frozen at 4°C for 0.5 h) was added and samples were mixed thoroughly for 2 min, ultrasonics for 1 min, frozen and kept at −20°C for 10 min, and centrifuged at 5000 r/min for 5 min at 4°C. The supernatant was filtered by a 0.22-μm filtration membrane and transferred into an autosampler vial for analysis. The serum samples of the healthy persons were also pretreated using the same method.

Data analysis

Shimadzu LCMS Solution (Ver. 3.60) software was adopted for data acquisition and processing, and Formula Predictor (Ver. 1.2) and Accurate Calculator software were used in the prediction of molecular formula and elemental composition. The 3D data were introduced into SIMCA-P software 12.0 (UmetricsAB, Sweden) to perform multidimensional statistical analysis to screen variables.

Results and Discussion

Selection of organic solvent system for protein precipitant

Protein should be precipitated and removed before
LC-IT-TOF MS analysis, because the large amount of protein macromolecular components in the serum could influence the detection. The organic solvent precipitation method was used in the experiment, and five different kinds of solvent systems were selected according to literature reports, including methanol, acetonitrile, methanol/acetonitrile (3/1, v/v), methanol/acetonitrile (1/1, v/v), and methanol/acetonitrile (1/3, v/v). Each method was performed with 3 parallel samples. The intensity of extract ion chromatography, separation resolution and detected peak number were examined to choose an optimum extraction solvent. The results showed that the methanol/acetonitrile (3/1, v/v) was the optimal solvent in positive ion mode, followed by methanol. The acetonitrile was the worst. The results are shown in Fig. 1 and Table 3.

Selection of the amount of organic solvent
The volume ratio of precipitant to serum in the organic solvent protein precipitation method was optimized. The protein precipitation efficiencies of the solvent systems respectively with methanol/acetonitrile (3/1) to serum volume ratio of 2:1, 3:1 and 4:1 were compared. The results showed that the column pressure would rise after continuous injection for 10 times when the samples were treated with organic solvent twice, which indicated that there were residual proteins. When the serum was treated by the organic solvents 3 - 5 times, there was no column pressure rise phenomenon even after continuous injection for 30 times (Fig. 2). In consideration of the environment protection, the 3:1 solvent system was adopted to precipitate protein in the serum pretreatment.

Selection of the temperature of organic solvent for protein precipitation
In order to improve the efficiency of protein precipitate and metabolite extraction, the effect of the temperature of the organic solvent was examined. Methanol/acetonitrile (3/1) of different temperatures was added to the samples. The tested temperatures were room temperature, cold storage temperature (with solvent placed in 4°C refrigerator for 0.5 h) and freezing temperature (with solvent placed in -20°C refrigerator for 0.5 h), respectively. Each method was performed with three parallel samples. As shown in Fig. 3, both cold storage and freezing had similar effects, which could improve the protein precipitation ability and compound extraction ability. The result was consistent with the conclusion in the literature, which confirm the effectiveness of cold-storage solvent for compound extraction in this method.

Selection of chromatography-mass spectrometry conditions
In order to obtain higher mass spectrometry detection sensitivity and reduce noise signals, different mobile phases
were systematically examined based on ionization efficiency and detection information integrity. Compared with methanol, the signal was better and the noise interference was lower when acetonitrile was used. So acetonitrile was selected as a mobile phase. The composition of the mobile phase could influence the retention time, peak pattern of analyte, as well as the ionization efficiency in mass spectrometry analysis and thus the detection sensitivity. Addition of formic acid or acetic acid to the mobile phase can reduce the peak tailing, while significantly improving the ionization efficiency of target objects. The influence of 0.2% formic acid and acetic acid added to the mobile phase on peak shape and ionization efficiency were compared in the experiment. Since better chromatograms were obtained by using formic acid, formic acid was used in the subsequent experiments. Different amounts of formic acid added to the mobile phase were also investigated, including 0.05, 0.1, 0.2 and 0.3%. It was found that the 0.1% formic acid mobile phase system had the best peak shape, sensitivity and separation degree. So 0.1% formic acid-acetonitrile was adopted as the mobile phase.

Both flow rate and column temperature could influence the retention time and peak shape. Increases in the flow rate and the column temperature could shorten the retention time and make the peak shape sharper. However, excessively high flow rate and temperature could damage the column and lead to peak overlap. Thus, appropriate flow rate and column temperature should be confirmed according to the experimental conditions. The column temperature was set at room temperature, and the flow rate was 0.30 mL/min. The obtained chromatograms of the mixed serum from the hepatocellular carcinoma group are shown in Figs. 4 and 5.

**Potential metabolic marker analysis**

Twenty mixed serum from the healthy group and 80 mixed serum from the hepatocellular carcinoma group were detected to obtain metabolic fingerprints. Many peaks were detected after injection, while 12 compounds (glycocholic acid, choline glycerophosphate, acetyl-1-phenylalanine, oleamide, tetradecanamide, phenylacetyl glutamine, 7-methylguanine, 7-((1S,2S)-2-(heptylamino)cyclohexyl) heptanoic acid, 2,6-dimethylheptanoylcarnitine, acetylcarnitine, lysolecithin and glycochenodeoxycholic acid) were confirmed through library search and document retrieval. Multivariate statistical analysis method was adopt to obtain the differences between the metabolic fingerprints of the healthy group and the hepatocellular carcinoma group. The results showed that contents of 8 compounds (glycocholic acid, choline glycerophosphate, acetyl-1-phenylalanine, oleamide, tetradecanamide, acetylcarnitine, lysolecithin and glycochenodeoxycholic acid) existed significant differences, which were potential metabolic biomarkers. The content and different analysis results of the compounds are shown in Table 4. The liver is the key to metabolism in the body, accounting for the majority of the synthesis, decomposition, transformation, excretion and other metabolic processes, and therefore the study of liver disease from the metabolism perspective is very important.31

![Fig. 4](image1.png) TIC chromatogram of HCC mixed serum.

![Fig. 5](image2.png) Mass spectra of 12 compounds in HCC mixed serum.
Table 4 Content and difference analysis of compounds in different groups

| Compound                  | Healthy group content/ mg kg⁻¹ | Hepatocellular carcinoma group/mg kg⁻¹ |
|---------------------------|-------------------------------|---------------------------------------|
|                           | Healthy                      | Hepatocellular                         |
|                           | content/ mg kg⁻¹              | carcinoma                               |
| Glycocholic acid          | 0                             | 2.148 P < 0.01                         |
| Choline glycerophosphate  | 0.263                         | 0 P < 0.01                             |
| Acetyl-t-phenylalanine    | 0                             | 0.573 P < 0.01                         |
| Oleamide                  | 2.591                         | 9.588 P < 0.01                         |
| Tetradecanamide          | 0.221                         | 0.266 P < 0.01                         |
| Phenylacetyl glutamine    | 3.409                         | 2.467 P < 0.05                         |
| 7-Methylguanine           | 1.245                         | 1.383 P > 0.05                         |
| 7-((1S,2S)-2-(heptylamino)cyclohexyl) heptanoic acid | 2.557 | 2.337 P > 0.05 |
| 2,6-Dimethylheptanoylcarnitine | 0.284 | 0.240 P > 0.05 |
| Acetylcarnitine           | 2.666                         | 5.762 P < 0.01                         |
| Lysolecithin              | 7.485                         | 5.063 P < 0.01                         |
| Glycochenodeoxycholic acid| 3.718                         | 4.745 P < 0.01                         |

P denotes difference comparison between the healthy group and the hepatocellular carcinoma group: 0.01< P <0.05 = significant difference; P < 0.01 = extremely significant difference; and P > 0.05 = no significant difference.

Table 5 Precision results of compounds

| Compound                  | Method precision | 48 h stability |
|---------------------------|------------------|----------------|
| Glycocholic acid          | 3.6              | 5.9            |
| Choline glycerophosphate  | 3.2              | 4.7            |
| Acetyl-t-phenylalanine    | 8.6              | 11.2           |
| Oleamide                  | 4.8              | 7.9            |
| Tetradecanamide          | 3.2              | 2.8            |
| Phenylacetyl glutamine    | 1.9              | 6.2            |
| 7-Methylguanine           | 10.8             | 11.6           |
| 7-((1S,2S)-2-(heptylamino)cyclohexyl) heptanoic acid | 5.8 | 6.9 |
| 2,6-Dimethylheptanoylcarnitine | 13.0 | 12.4 |
| Acetylcarnitine           | 9.6              | 6.1            |
| Lysolecithin              | 12.5             | 12.8           |
| Glycochenodeoxycholic acid| 11.5             | 7.9            |

Method precision

Mixed standard solution was added to the mixed serum samples of the hepatocellular carcinoma group. The added amount was 25 mg/L for each substance. Samples were treated as above pretreatment method and detected in the positive mode. Each sample was analyzed with six parallel experiments. The RSD results showed that the method had excellent precision. The RSD values of 12 kinds of screened compounds were less than 13.0%. The method precision of the compounds are shown in Table 5.

48-h stability

The mixed serum samples of the hepatocellular carcinoma group were pretreated according to the above method, and were analyzed at 0, 2, 4, 8, 16, 24, 32, 40 and 48 h. Each sample was determined with three parallel experiments, and the average values were calculated to determine the stability. The results showed that the method had good 48-h stability in real serum samples, with all RSD values lower than 12.4% (see Table 5).

Reliability analysis

The 48-h stability data were obtained from quality control samples. The quality control samples were continuously measured six times to equilibrate the system. Then one quality control sample was detected after analyzing 10 serum samples, and all results of quality control samples were analyzed to acquire reliability data. The results from principal components analysis (PCA) of 13 quality control sample data in positive ion mode are shown in Fig. 6. All results focused together with deviation less than 2 times the standard deviation (2SD). It was shown that the method had good reliability, and the difference between single samples came from different metabolites of the samples, rather than generated from analytical method error.

Conclusions

Metabonomics has the advantages of the detection of hepatocellular carcinoma markers. The pretreatment of serum of hepatocellular carcinoma patients was studied and a UFLC-IT-TOF MS method was developed. The kind, mixing ratio and temperature of the organic solvent for protein precipitation were optimized to remove the matrix interference in serum samples. The chromatography-mass spectrometry conditions were also selected to acquire effective metabolic small molecule information. The methodology evaluation results showed that the method had high stability, good repeatability and excellent reliability, which can satisfy the high throughput requirement of metabonomics, and is suitable for metabonomics research with large batches of biosamples. The method could be used for screening of small molecule metabolites in serum of hepatocellular carcinoma in the situation of standard substance absence. Through the detection, 12 compounds were confirmed, and 8 compounds (glycocholic acid, choline glycerophosphate, acetyl-t-phenylalanine, oleamide, tetradecanamide, acetylcarnitine, lysolecithin, glycochenodeoxycholic acid) were indentified as potential metabolic biomarkers by PCA.

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