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# Development and Validation of Liquid Chromatography-Time of Flight Method of Bacopaside-I and Metabolites for Pharmacokinetic Study

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## Introduction

Bacopa monnieri (L.) Wettst. (Brahmi) is an Ayurvedic medicine plant used for centuries as a memory enhancer. There have been several studies on biological effects of this plant for a therapeutic potential in treatment and prevention of neurological diseases and improvement of cognitive processes. Bacopaside I ($C_{46}H_{74}O_{20}S$), a dammarane-type triterpenoid saponin, is one of the main active components of the plant and, therefore, it has been produced and used in biological and therapeutic studies. Various analytical method such as HPTLC, ELISA and HPLC methods were used to determine bacopasides I and other active components in the plant extracts as well as biological samples. However, so far LC/MS method for quantitative analysis of Bacopaside I in biological samples has not been reported. We report for the first time the development and validation of a high sensitivity LCMS (TOF) method, aiming for pharmacokinetic study of bacopasides I and identification of its metabolites produced in vitro and in vivo.

## Experimental

Rat urine and feces were used as the biological matrix for method development and validation of bacopaside I for quantitative analysis. Liquid-liquid extraction method was employed in sample extraction and purification. The LC-MS method used is based on high resolution TOF which is suitable for both quantitative analysis of Bacopaside I in biological samples and identification of metabolites formed. An LCMS-IT-TOF (Shimadzu Corporation, Japan) was used in this study. A gradient elution method using a Kinetex C18 column (1.7 um) was developed and optimized. Both target compound Bacopaside I and the internal standard Digitoxin showed very strong mass peaks (*m/z* 977.4387 and 763.4243) in negative ESI mode. A high resolution MS and MS/MS method were established to achieve high sensitivity and reliability of analysis.

Table 1 LCMS-IT-TOF conditions for analysis of Bacopaside-I and metabolites

| | |
|---|---|
| Column | KinetexC18, 1.7 um, 50 mm L x 2.1 mm ID |
| Mobile phase | A: Water (0.1% formic acid)<br>B: Acetonitrile (0.1% formic acid) |
| Elution mode | Gradient,16 min |
| Flow rate | 0.3 mL/min |
| Column temp. | 45°C |
| Injection vol. | 10 µL |

| | |
|---|---|
| Interface | ESI |
| MS mode | MS (-) and $MS^2$ (-)<br>*m/z* 200~1200<br>CID withAr gas |
| Block temp. | 200°C |
| CDL temp. | 250°C |
| Nebulizing gas | Nitrogen, 1.5 L/min |
| Drying gas | Nitrogen,10 L/min |

## Results and Discussion

This study was carried out in three stages, development of a LC-MS (TOF) method, validation trials and identification of metabolites of bacopaside I from in vitro enzymatic hydrolysis and faces samples using a MetID Solution program.

1. Method Development: Fig. 1 shows the chromatograms and MS, MS/MS spectra (TOF) of bacopaside I. The internal standard (IS) digitoxin eluted immediately

# Development and Validation of Liquid Chromatography-Time of Flight Method ofBacopaside-I and Metabolites for Pharmacokinetic Study

immediately after the target peak. Both target compound and IS were detected in negative ESI mode as $[M-H]^-$ ions. The LCMS-IT-TOF is a high resolution MS with mass error less than 5 ppm. The spectrum results in Fig. 1 are -3.8 ppm and -3.0 ppm for Bacopaside I and digitoxin, respectively. The advantage of high resolution MS is the superior mass selectivity of target peaks. Therefore, it is suitable for quantitation of trace level compounds in complex matrix. The fragmentation pathway of bacopasidel in negative mode (*m/z*977.4387→*m/z*845.3962) is shown in Fig. 2.

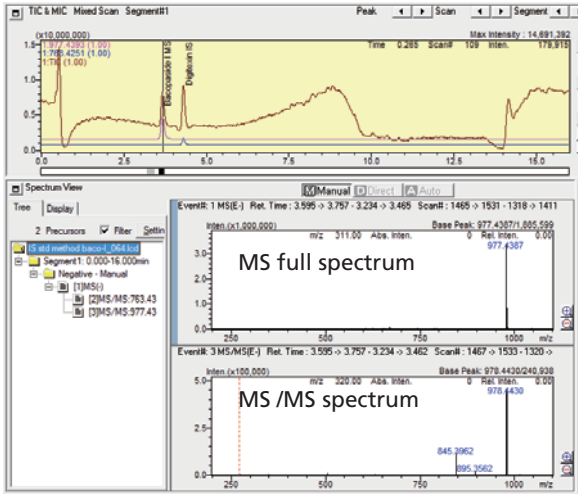



Fig 1. Chromatograms and mass spectra of bacopaside I and internal standard (digitoin, $C_{41}H_{64}O_{13}$) on high resolution LCMS-IT-TOF.

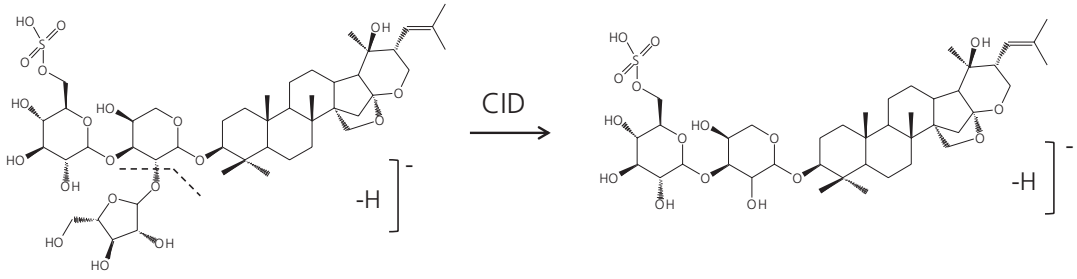



Fig. 2 Fragmentation scheme of bacopaside I (left) to main product ion under CID in negative mode

2. Validation of method: The performance of the quantitation method based on extract ion chromatogram (EIC) of MS spectrum was evaluated systemically using spiked samples prepared from rat urine and feces by L-L extraction. The calibration curve shows good linearity (r2=0.9985) for the testing range from 4.8 ng/mL to 380 ng/mL (Fig. 3). The specificity and matrix effect were studied and it was found that the method specificity was highly reliable due to the accurate mass (<5 ppm) (Fig. 4). Matrix effect was not obvious under the conditions. The LOQ of the method was lower than 4.8 ng/mL. The reproducibility at this level (spiked in urine) was 8.7% (RSD of concentration, n=6).

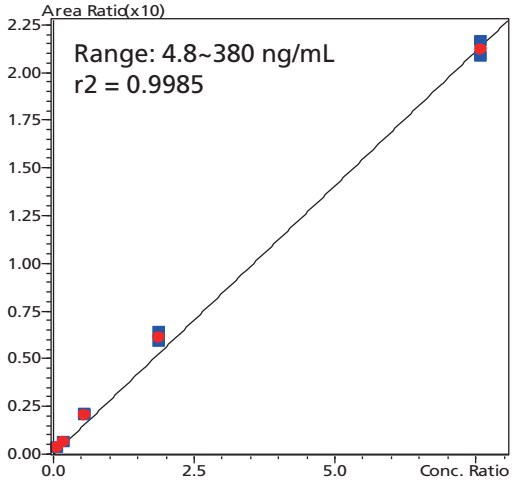



Fig. 3 Calibration curve of bacopaside I (IS method) based on EIC (*m/z* 977.4393) in negative mode.

The recoveries of bacopaside I spiked in urine were between 91.1% and 107.1% for three concentration levels (50, 80 and 100 ng/mL) as shown in Table 2. However, the recovery of feces extract samples was found at 40~70%. Further study to improve the recovery of bacopaside I in feces samples is undergoing.

# Development and Validation of Liquid Chromatography-Time of Flight Method ofBacopaside-I and Metabolites for Pharmacokinetic Study

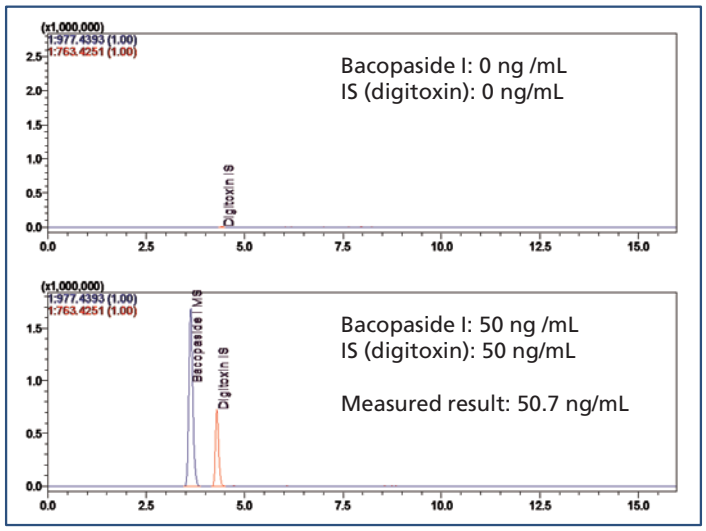
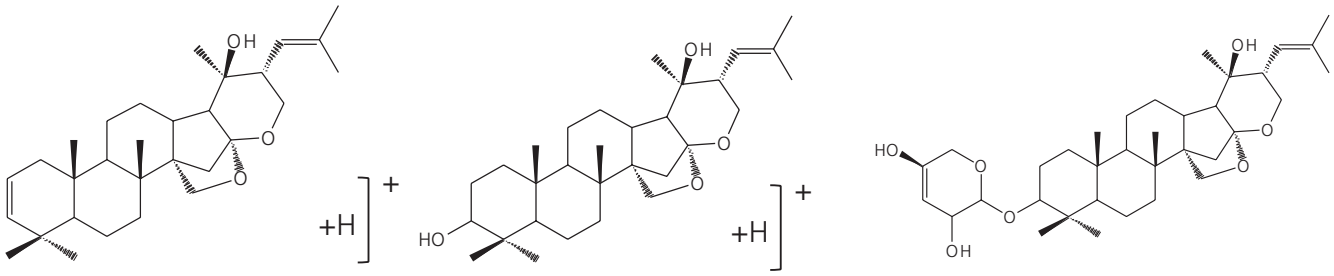



Table 2 Recovery of bacopaside I spiked in urine samples

| Sample Name | Spiked conc. (ng/mL) | Measure Conc (ng/mL) | Recovery (%) |
|---|---|---|---|
| 3/1 n1 | 50 | 45.6 | 91.1 |
| 3/2 n2 | 50 | 49.8 | 99.6 |
| 3/3 n3 | 50 | 53.8 | 107.5 |
| 3/4 n1 | 80 | 83.9 | 104.9 |
| 3/5 n2 | 80 | 80.5 | 100.6 |
| 3/6 n3 | 80 | 81.1 | 101.4 |
| 3/7 n1 | 100 | 98.7 | 98.7 |
| 3/8 n2 | 100 | 107.1 | 107.1 |
| 3/9 n3 | 100 | 105.5 | 105.5 |

Fig. 4 EICs of bacopaside I and IS in blank urine (top) and spiked sample (bottom).

3. Identification of metabolite: The LCMS-IT-TOF method established with its full spectrum (*m/z* 200~1200) and accurate mass measurement (<5 ppm) was applied to *in vitro* hydrolysis and faces samples to detect metabolites formed. The method was modified slightly to include MS positive mode (*m/z* 200~1200) in order to detect both positive and negative ions formed from metabolites. Three samples used are control, enzymatic hydrolyzed samples (20 mins) and rat feces collected after 12 hrs of oral administration of bacopaside I. In this preliminary study, we found seven metabolite candidates including $C_{30}H_{48}O_4$ (observed (+)*m/z*473.3594, RT3.98 min), $C_{30}H_{46}O_3$ (observed (+)*m/z*455.3493, RT3.76 min) and $C_{35}H_{54}O_7$ (observed (+)*m/z*587.3891, RT3.56 min). These three candidates are likely corresponding to hydrolysis of bacopaside I on the glycoside with leaving the pentacyclic structure intact (see Fig. 5).




M1 ($C_{30}H_{46}O_3$):
Predict *m/z* 455.3520

M2 ($C_{30}H_{48}O_4$):
Predict *m/z* 473.3625

M3 ($C_{35}H_{54}O_7$):
Predict *m/z* 587.3942

Fig. 5 Structures of metabolite candidates M1, M2 and M3 from bacopaside I

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## Conclusions

For the first time a high resolution LCMS-IT-TOF method for quantitative analysis of bacopaside I in biological samples has been established and validated using rat urine/faces spiked samples for pharmacokinetics study. The method with its full spectrum (*m/z* 200~1200) and accurate mass measurement ability (<5 ppm) was also used to detect and identify metabolites formed.

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