

Development of a High Throughput LC-MS/MS Method for the Simultaneously Quantification of Multiple Bioactive Polyphenolic Compounds in Various Matrices

Yan-Ling Zhang^{1,2)}, James Garcia²⁾, Jim Tallman²⁾, Jessica Krank²⁾, Christine Casey²⁾, Richard Staub²⁾, Scott Bagett²⁾, Isaac Cohen²⁾, Uwe Christians¹⁾

¹⁾ Clinical Research and Development, Department of Anesthesiology, University of Colorado Denver, Aurora CO 80045;

²⁾ Bionovo, Inc. Emeryville, CA 94608

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OVERVIEW

NOVEL ASPECT

Simultaneously quantification of multiple bioactive polyphenolic compounds in various matrices with a high throughput LC-MS/MS method.

PURPOSE

To develop an automated, simple and fast liquid chromatography-tandem mass spectrometry platform for the simultaneous quantification of natural bioactive polyphenolic compounds in Chinese herbal medicine and biological samples. To validate the methods in various biological matrices and different solvent combinations of MeOH/water following FDA (GLP) guidelines, using LC-MS/MS. To quantify multiple actives in biological samples and herbal extracts for pharmacokinetic (PK), toxicity studies and production samples.

METHODS

Using LC-MS/MS in combination with automated online sample preparation (LC/LC-MS/MS) to quantify trace amounts of bioactive polyphenolic compounds by LC-MS/MS.

RESULTS

An LC/LC-MS/MS method for simultaneous quantification of thirteen bioactives in various samples was developed. The method is high through-put and sensitive, and it has been fully validated in several biological matrices (human plasma, dog plasma, rat plasma, mouse plasma, human urine and rat liver), and various solvent combinations of MeOH/water (80/20 MeOH/H₂O, 50/50 MeOH/H₂O and 100% H₂O). The linear ranges were broad in the range of 0.05 to 50 ng/mL for most of actives with the regression coefficient value (r) always greater than 0.995. In human plasma, the LLOQ on column were between 1 to 10 pg. The accuracies and precisions for both intra-day (n ≥ 5) and inter-day (n ≥ 15) analyses were between 95.8 -116.3% and 3.3 -14.3% for all levels.

The results demonstrated that the assay met all predefined acceptance criteria for FDA guidelines and is suitable for extensive pharmacokinetic studies.

INTRODUCTION

Herbs have been used as medicinal remedies in China for thousands of years. From herbal extracts a large number of polyphenolic compounds have structurally been identified, including flavones, isoflavones, chalcones, and nortigans. Many of these polyphenolic compounds have been studied and were shown to possess documented biological activities and medicinal properties such as antioxidant activity, anticancer, digestive stimulation, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic effects. Many analytical methods have been developed and published, however, those either focused on only a few compounds or lacked sensitivity. Our goal was to develop a high throughput and sensitive LC-MS/MS method for the simultaneously quantification of multiple polyphenolic compounds in herbal extracts commonly used in traditional Chinese medicine and various biological matrices.

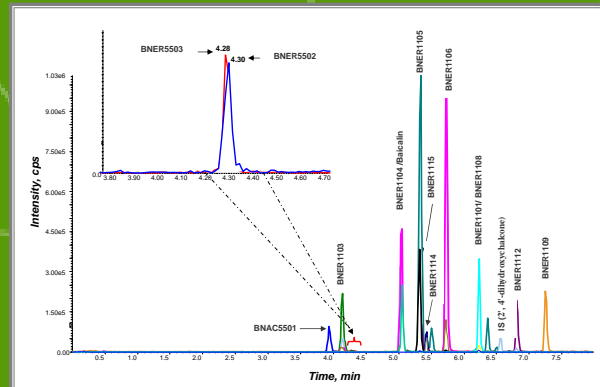


Figure 1. Chromatogram of standard mixture for all actives and internal standard.

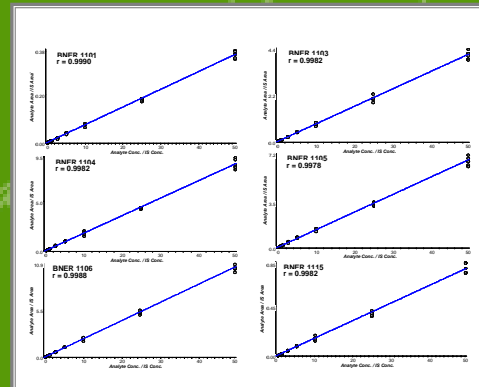


Figure 2. Typical standard curves for six major actives in human plasma.

Table 1. Analysis linearity range and regression coefficient (r) for six actives in six biological matrices.

Compounds/Matrix	LLOQ [ng/mL]	ULOQ [ng/mL]	Amount on column at LLOQ [pg]	[r]	Compounds/Matrix	LLOQ [ng/mL]	ULOQ [ng/mL]	Amount on column at LLOQ [pg]	[r]
BNER 1101	0.5	50	10	0.9990	BNER 1101	0.75	50	15	0.9993
BNER 1103	0.05	50	1	0.9982	BNER 1103	0.25	25	5	0.9986
BNER 1104	0.05	50	1	0.9982	BNER 1104	0.1	10	2	0.9988
BNER 1105	0.05	50	1	0.9978	BNER 1105	0.25	25	5	0.9970
BNER 1106	0.1	50	2	0.9988	BNER 1106	0.1	10	1	0.9973
BNER 1115	0.5	50	5	0.9982	BNER 1115	0.5	50	10	0.9993
BNER 1101	0.75	50	15	0.9988	BNER 1101	1	50	20	0.9989
BNER 1103	0.5	10	10	0.9978	BNER 1103	0.25	10	5	0.9969
BNER 1104	0.25	10	5	0.9984	BNER 1104	0.25	25	5	0.9984
BNER 1105	0.25	50	5	0.9985	BNER 1105	0.1	25	2	0.9978
BNER 1106	0.25	12.5	5	0.9978	BNER 1106	0.25	25	5	0.9968
BNER 1115	0.25	50	5	0.9987	BNER 1115	1	90	20	0.9987
BNER 1101	0.75	50	15	0.9974	BNER 1101	0.75	50	15	0.9960
BNER 1103	0.75	25	15	0.9960	BNER 1103	1.5	25	30	0.9971
BNER 1104	0.25	25	5	0.9978	BNER 1104	0.25	12.5	5	0.9966
BNER 1105	0.5	50	10	0.9977	BNER 1105	0.75	50	15	0.9966
BNER 1106	0.25	25	5	0.9966	BNER 1106	0.05	6	1	0.9974
BNER 1115	0.75	100	15	0.9983	BNER 1115	0.75	50	15	0.9959

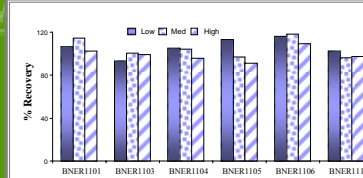


Figure 3. Stability of 6 actives in human plasma through three freeze-thaw cycles at lower (0.5 ng/mL), medium (20 ng/mL) and high (50 ng/mL) concentration levels.

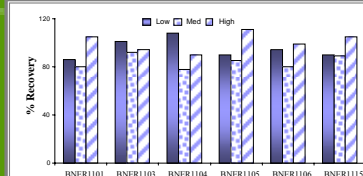


Figure 4. Recoveries from human plasma. Low (0.5 ng/mL) for BNER1103, BNER1104 and BNER1106, 1 ng/mL for BNER1101, BNER1105 and BNER1115, medium (10 ng/mL) and high (20 ng/mL).

EXPERIMENTAL

Sample Preparation and Instrumentation:

The protein precipitation/internal standard solution (2',4'-dihydroxychalcone, in 100% MeOH) was freshly prepared from stock solution (1 mg/mL) every month and stored at 4°C for use (should not be exposed to room temperature for more than 6 hours and must be stored at 4°C between extractions). It was added to the standards, QCs and samples (biological samples or herbal extracts) for protein precipitation. After samples were thawed at room temperature, tissue samples were homogenized in 0.1M phosphate buffer to yield a final concentration of 250 mg/mL. Samples were incubated for 30 min (plasma) or 2 hours (tissues) at 37°C. The protein precipitation reagent (600 μL) was added to sample (300 μL) and mixed on an orbital shaker for 2.5 minutes (30 min for tissue) at 500 RPM and room temperature. Samples were then centrifuged for 15 minutes at 13,000 RPM at 4°C. Dried herbal materials were ground to a powder and then extracted with methanol/water (80/20, v/v) for 30 min at room temperature on an orbital shaker for 30 minutes. Calibrators were prepared by spiking 6 polyphenolic compounds (BNER1101, BNER1103, BNER1104, BNER1105, BNER1106 and BNER1115) into all tested matrices. The supernatant (20 μL) was then injected onto an Agilent 1200 2D-HPLC system in combination with an API5000 MS/MS. Mobile phases used were: MeOH (100%) and 0.088% formic acid in water. The linear gradient was started with 40% MeOH and ramped to 100% MeOH in 4 min. The total run time was 8 min. The column temperature was set to 65°C. Ions were recorded in the negative SRM mode. The following ion transitions were monitored for major actives: BNER1101: m/z = 251 → 93; BNER1103: m/z = 417 → 255; BNER1104: m/z = 255 → 135 or 255 → 119; BNER1105: m/z = 283 → 268; BNER1106: m/z = 255 → 119; BNER1115: m/z = 253 → 117 and IS: m/z = 239 → 91.

RESULTS & CONCLUSIONS

The simultaneous quantification of multiple bioactive polyphenolic compounds in biological samples and extracts from Chinese herbal using a high-throughput and sensitive LC-MS/MS method was developed and validated in different matrices: human plasma, human urine, dog plasma, rat plasma, mouse plasma, rat liver and various ratios of aqueous/methanol solution. The assay was fully validated with human plasma. Figure 1 is a LC-MS/MS chromatogram of all actives and internal standard.

Extraction efficiency studies for six major active components demonstrated recoveries of greater than 80% (Figure 4). The method is high through-put, sensitive and had broad linearity for all actives. The limits of detection (LOD) were 0.025 ng/mL for BNER1103, BNER1104, BNER1105 and BNER1106, BNER1101 was 0.1 ng/mL, and BNER1115 was 0.25 ng/mL. All LODs were based on a signal to noise ratios greater than 3:1. The lower limits of quantitation (LLOQ) were 0.05 ng/mL for all actives with the exception of BNER1101 (0.5 ng/mL), BNER1106 (0.1 ng/mL) and BNER1115 (0.5 ng/mL). The LLOQ signal to noise ratios were greater than 8. The method calibration curves were linear from 0.05 to 50 ng/mL for BNER1103, BNER1104 and BNER1105; BNER1106 was linear from 0.1 to 50 ng/mL; BNER1115 and BNER1101 were linear from 0.5 to 50 ng/mL (all n = 6). The linear range was determined by regression coefficients (r) of greater than 0.995. The typical standard curves parameters for six major actives in six biological matrices show in Figure 2 and Table 1. Intra-day accuracy was better than ± 10% (91.8-108.9%) and intra-day precision was better than 14%. The assay has also validated with several biological matrices (dog plasma, rat plasma, mouse plasma, human urine and rat liver) and different solvent combinations of MeOH/water (80/20 MeOH/H₂O, 50/50 MeOH/H₂O and 100% H₂O). The validation was carried out by different analysts and different instruments (API5000 and API4000 QTrap).

Stock solutions consisting of the six analytes and internal standard, at room temperature are stable at least 6 hours. There was no degradation observed after three freeze-thaw cycles for all actives at all concentration levels (Figure 3). For most analytes, the recovery after five cycles was still high; the recovery can be obtained greater than 94%, except BNER1106, which had recovery of approximately 75% after five cycles. Six MF101 actives in human plasma were stable with no significant degradation observed within 24 hours stored at room temperature for both at lower and medium concentration levels.

In summary, the development and validation of a LC/LC-MS/MS assay for the quantification of BNER1101, BNER1103, BNER1104, BNER1105, BNER1106, BNER1115, and in human, mouse, rat and dog plasma, as well as in human urine, rat liver and in various MeOH/water combinations that met all pre-defined acceptance criteria.