Simultaneous Quantification of 8 Bioactive Polyphenolic Compounds in EDTA Rat Plasma by a High Throughput LC-MS/MS Method

David Gingrich[3], Xiaochuan Li[3], Hong Zhou[3], Kim Plath[3], Uwe Christians[1,3], Isaac Cohen[2], Yan Ling Zhang[1,3*]

[1]Clinical Research and Development, Department of Anesthesiology, University of Colorado Denver, Aurora, CO 80045

[2]Bionovo Inc., Emeryville, CA 94608, [3]Bionovo Inc., Aurora, CO 80045

*Correspondence to: yanling@bionovo.com

ASMS-2011

INTRODUCTION

It is estimated that 40 million women in The U.S. are currently transitioning through menopause, with aging baby boomers expecting to add 20 million to the total over the next decade. Menerba, an oral botanical drug, was designed for the safe and effective treatment of vasomotor symptoms (hot flashes) associated with menopause by targeting the estrogen receptor beta pathway. Unlike hormone therapy, Menerba does not activate estrogen receptor alpha pathway, known to be implicated in both breast and uterine cancer formation.

Menerba is extracted from 22 species and the extracts include flavones, isoflavones, chalcones, and norligans. Many of these polyphenolic compounds have been previously studied for biological activities and medicinal properties such as anti-inflammatory, anti-oxidant, anti-cancer, anti-bacterial, anti-viral, anti-mutagenic and anti-carcinogenic effects. Our goal was to develop and validate a high-throughput sensitive LC-MS/MS method for simultaneous quantification of multiple polyphenolic compounds in Sprague Dawley EDTA rat plasma.

OVERVIEW

NOVEL ASPECT

Simultaneous quantification of 8 bioactive polyphenolic compounds in EDTA rat plasma with a high throughput LC-MS/MS method.

PURPOSE

To develop a simple and fast liquid chromatography-tandem mass spectrometry platform for the simultaneous quantification of bioactive polyphenolic compounds in Menerba.

To validate the method following FDA guidelines, using LC-MS/MS.

To quantify multiple actives in EDTA rat plasma for pre-clinical study samples.

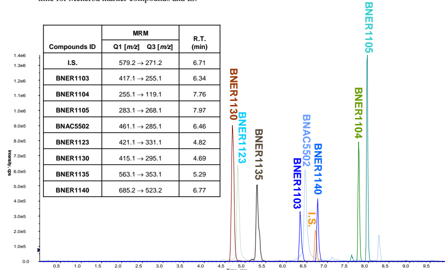
METHODS

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

RESULTS

The simultaneous quantification of 8 bioactive polyphenolic compounds extracted from EDTA rat plasma using a LC-MS/MS method was developed and validated. Figure 1 is an LC-MS/MS chromatogram of all actives and an internal standard. No ion suppression interfered with any of the signals for the compounds of interest. All accuracy, precision, selectivity, sensitivity, reproducibility and stability criteria were acceptable. Extraction recovery was optimal for all compounds.

Table 1. Monitored ion transitions and typical retention time for Menerba marker compounds and IS.



Compounds ID	MRM Q1 [m/z] Q3 [m/z]	R.T. (min)
I.S.	579.2 → 271.2	6.71
BNER1103	417.1 → 255.1	6.34
BNER1104	255.1 → 119.1	7.76
BNER1105	283.1 → 268.1	7.97
BNAC5502	461.1 → 285.1	6.46
BNER1123	421.1 → 331.1	4.82
BNER1130	415.1 → 295.1	4.69
BNER1135	563.1 → 353.1	5.29
BNER1140	685.2 → 523.2	6.77

Figure 1. Representative ion chromatogram of a standard mixture containing all marker compounds and internal standard (I.S.).

Table 2. Analysis linearity range, regression coefficient (r), and LLOQ for 8 actives in EDTA rat plasma.

Compound ID	Linearity Range (ng/mL) LLOQ	Linearity Range (ng/mL) ULOQ	r Day 1	r Day 2	r Day 3	LLOQ (ng/mL)	SN at LLOQ
BNER1103	0.195	25	0.9979	0.9999	0.9975	0.195	8.3
BNER1104	0.195	25	0.9989	0.9995	0.9991	0.195	13.4
BNER1105	0.156	20	0.9992	0.9993	0.9990	0.156	10.4
BNAC5502	3.93	402	0.9994	1.000	0.9994	3.93	5.7
BNER1123	1.56	200	0.9969	0.9995	0.9991	1.56	20.7
BNER1130	1.17	120	0.9993	0.9994	0.9998	1.17	26.6
BNER1135	1.56	200	0.9989	0.9996	0.9971	1.56	18.5
BNER1140	1.95	200	0.9983	0.9997	0.9996	1.95	15.1

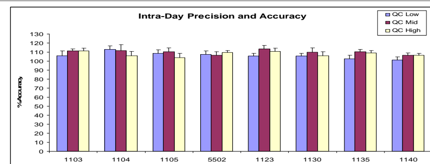


Figure 2. Intra-day precision and accuracy of quality controls at low, middle and high concentrations (n=6)

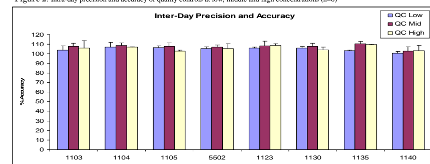


Figure 3. Inter-day precision and accuracy of quality controls at low, middle and high concentrations

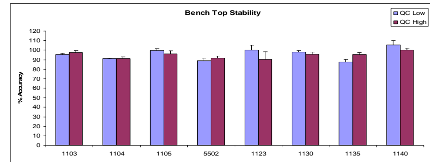


Figure 4. Bench top stability precision and accuracy of quality controls at low, and high concentrations (n=3)

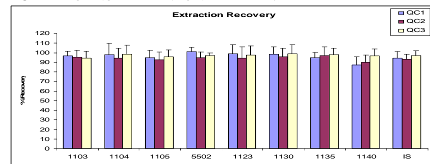


Figure 5. Extraction recoveries from plasma at low, medium, and high concentration levels (n = 4).

EXPERIMENTAL

Sample Preparation and Instrumentation:

The calibrators and QCs were prepared by serial dilution of the standard working solution in blank plasma, which contained 8 Menerba marker compounds (BNER1103, BNER1104, BNER1105, BNAC5502, BNER1123, BNER1130, BNER1135, and BNER1140).

0.1mL of plasma samples were immediately mixed with 0.8mL 0.5% formic acid in water and 0.1mL of the internal standard working solution after the sample was thawed. Samples were extracted using 30mg/mL strata-X solid phase extraction column (Phenomenex) under a slight vacuum. The columns were first conditioned with 1mL methanol and then equilibrated with 1mL water. Loaded columns were then washed with 1mL of water, followed by sample extraction with 1.2mL methanol/acetonitrile (50/50, v/v). After sample extraction, the eluted solution was evaporated under 30ºC (CentriVap). Samples were reconstituted with 100µL methanol/water/formic acid (80/19.9/0.1, v/v), and then transferred to clean inserts for LC-MS/MS analysis.

The reconstituted sample (20 uL) was injected and analyzed by an Agilent 1260 2D-HPLC system in combination with an API5000 system (LC-MS/MS) in ESI negative MRM mode. All analytes were separated on an Agilent Zorbax Eclipse XDB-C8 column (250 mm x 4.6 mm, 5 um). Mobile phases were composed of aqueous formic acid (0.1%, v/v) and methanol. The linear gradient was started with 30% methanol and ramped to 98% methanol in 7 min and the total run time was 10 min. An automated online sample extraction was achieved by loading samples on an Agilent Zorbax Eclipse XDB-C8 guard column (12.5 mm x 4.6 mm, 5 um) and washing with 1% methanol for 0.5 min at a flow rate of 1.5 mL/min. The monitored ion transitions and typical retention times are show in Table 1.

RESULTS & CONCLUSIONS

The extraction procedure was systematically developed and the conditions described above gave the best results. The extraction recoveries of all compounds from plasma were above 87.1% (Figure 5). No interfering peaks were found at the retention times of any analytes.

The method had a range of reliable response for all analytes. The lower limits of quantitation (LLOQ) ranged from 0.156 to 3.93 ng/mL, and 1/x linear regression was used with a regression coefficients (r) value greater than 0.9969 for all analytes (Table 2). The intra-day accuracies for this method ranged from 99.8-107.7% with a precision of less than 13.3% for all analytes (Figure 2). The inter-day accuracies for the method ranged from 100.5-110.5% with the precision less than 6.8%, coefficients of variance as assessed over 3 days at 3 different concentrations (Figure 3).

Bench top stability proved to be a challenge, as previous literature demonstrated that BNER1140 was not stable in plasma. Bench top stability was established only when QC were pre-treated with 0.5% aqueous formic acid. Study samples were thawed on ice and immediately pre-treated with 0.5% aqueous formic acid. The accuracies for bench top stability were 87.6-105.6% with the precision ≤ 8.2% for low and high QC levels. Freeze/thaw stability was established with three freeze/thaw cycles with an accuracy range of 82.1-117.8%. The post preparative stability was established for 4 days at -80ºC with an accuracy range of 86.9-106.8%. The long term storage at -80ºC was established for 35 days with an accuracy range of 84.2-114.7%, and working stock solution stability passed the criteria with an accuracy range of 98.2-101.9%.

Matrix effect was investigated in 6 lots of Sprague Dawley EDTA rat plasma at a middle QC concentration. The % matrix effect ranged from 85.6-109.0%. Matrix effect was also investigated by direct infusion of analytes during a blank injection, and no observable ion suppression or enhancement was observed at the retention times of any analytes.

In summary, the development of an LC/LC-MS/MS assay for the quantification of BNER1103, BNER1104, BNER1105, BNAC5502, BNER1123, BNER1130, and BNER1135 in EDTA rat plasma was validated, and was used in analysis of pre-clinical trial samples and PKPD samples.