

A Rapid Bio-Analytical Method for Simultaneous Quantification of Boeravinone B and Eupalitin-3-O- β -D-Galactopyranoside from *Boerhaavia Diffusa* Using LC-MS/MS

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ABSTRACT

Today people are more dependent on herbal drugs with over 80 percent of the world population relying on the traditional systems of medicine, largely plant based, for treatment of several acute diseases. The current research is based on the plant *Boerhaavia diffusa* Linn. which has been widely used for treating acute diseases and possess various biological activities. An array of pharmacodynamic studies have been conducted on animals using this plant. In order to bridge the gap between pharmacokinetics and pharmacodynamics, an understanding of the pharmacokinetic profile of the active markers in the plant is necessary for improved drug therapy. In the current research a rapid and sensitive Bio-analytical LC-MS/MS method has been developed and validated for simultaneous quantification of active markers Boeravinone B and Eupalitin-3-O- β -D-galactopyranoside in human plasma. The analytes were extracted from human plasma by SPE. Sildenafil citrate was used as the internal standard. A RP₁₈ column enabled chromatographic separation of the analytes. The method involves simple isocratic chromatography and MS detection in positive-ionization mode. Validation of the method showed response was a linear function of concentration in the range 5.0–500.0 ng mL⁻¹ for both Boeravinone B and Eupalitin-3-O- β -D-galactopyranoside. The method was suitably validated and was found to be precise and robust, with recoveries for both the analytes being consistent. The method can be successfully applied for the analysis of actual samples from dosed human and animals from pharmacokinetic studies.

Keywords: Bio-Analytical, Boeravinone B, Eupalitin-3-O- β -D-Galactopyranoside etc.

INTRODUCTION

Many drugs that are thought to be of potential value in treating human disease, are introduced into development, based on knowledge of invitro receptor binding properties, identified pharmacodynamic effects and the pharmacokinetic profile of the active ingredients. *Boerhaavia diffusa* Linn is one such plant on which a number of pharmacodynamic studies have been performed. *Boerhaavia diffusa* belongs to the family Nyctaginaceae. The *Boerhaavia diffusa* plant contains a large number of compounds such as flavonoids, rotanoids, alkaloids, steroids, triterpenoids (Gupta et al., 2011; Jain and Khanna, 1989; Kadota et al. 1989; Lami et al. 1990; Lami et al., 1992). *Boerhaavia diffusa* has been widely used for treatment of edema, dermatopathies, heart disorders, anaemia, renal disorders, hepatic disorders and inflammatory disorders (Gupta et al., 2011). The plant has drawn lot of attention due to following biological activities it posses. The leaves of *Boerhaavia diffusa* have been found to possess anti-diabetic (Pari and Amarnath, 2004), analgesic and anti-inflammatory activity (Hiruma-Lima and Gracioso, 2000), whilst diuretic (Misra and Tiwari, 1971), immunomodulatory (Mehrotra and Mishra 2002a; Mungantiwar and Nair,

1999), anti-lymphoproliferative (Mehrotra and Singh, 2002b) and hepatoprotective properties (Gulati et al., 1991) have been attributed to the roots. Using the whole plant anti-fibrinolytic activity (Barthwal and Srivastava, 1990), anti-stress and adaptogenic activity (Sumantha and Mustafa, 2007) and chemopreventive action (Rupjyoti and Mohammed, 2003) have been proven.

Inspite of a large number of pharmacodynamic studies conducted in animals using this plant, there has not been a single study to understand the pharmacokinetic profile of the active markers that have been reported to be present in the plant. By understanding the pharmacokinetic profile, drug developers would be successful at establishing the relationship of pharmacodynamic response to blood concentrations. Thus providing a basis for adjustment of dose, dosing interval, monitoring procedures and dosage form to develop, thereby ensuring improved drug therapy.

There is hence an urgent need for developing a Bio-analytical method for quantification of the pharmacologically active markers in *Boerhaavia diffusa*. The current research involves using an LC-MS/MS to quantify the pharmacologically active markers

Boeravinone B and Eupalitin-3-O- -D-galactopyranoside from human plasma.

MATERIALS AND METHODS

Chemical and reagents: The working standards of Boeravinone B (97.30%) and Eupalitin-3-O- -D-galactopyranoside (97.10%) were obtained from Natural Remedies Pvt Ltd., India. Internal standard Sildenafil citrate (97.50%) was obtained from Sigma. High purity water was prepared in house using Milli-Q water purification system. HPLC grade methanol, acetonitrile and formic acid were used. HPLC grade ammonium formate was procured from Fluka. Phenomenex Strata-X (30 mg/ml) solid phase extraction cartridges were employed. Drug free human plasma were stored at -80°C prior to use.

Calibration curves and quality control samples: Two separate stock solutions each of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside were prepared for bulk spiking of calibration curve and quality control samples respectively for the method validation exercise. For bulk spiking the screened blank plasma and from six sources with least interference at the retention time of analyte and the internal standard were pooled together and used.

The stock solutions of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and the internal standard Sildenafil citrate were prepared in Methanol:Water (60:40 v/v) and stored at 2-8°C. The stock concentration for Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and internal standard Sildenafil citrate were 1000, 1000 and 400 µg/ml respectively.

Subsequent dilutions of the stock solutions were prepared from stock solutions by dilution with Methanol:Water (60:40 v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as those for the primary dilutions. These working standard solutions thus prepared were used to prepare the calibration curve and quality control samples.

For Boeravinone B and Eupalitin-3-O- -D-galactopyranoside a nine-point standard curve was prepared by spiking screened blank plasma with appropriate amount of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside. The calibration curve ranged from 5.0-500.0 µg/mL for both Boeravinone B and Eupalitin-3-O- -D-galactopyranoside. Quality control samples were prepared at three concentrations i.e. at 15.0, 250.0 and 400.0 µg/ml for both of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside. The quality control samples were prepared in a manner similar to the preparation of the calibration curve. The spiked calibration curve and quality control samples were stored frozen at -80°C pending analysis.

Extraction procedure: All the frozen plasma samples (System suitability, blank plasma, zero standard, calibration curve standards and quality control samples) were thawed before analysis, followed by vortexing each plasma sample for 30 seconds. These plasma samples were extracted by solid phase extraction method as described below.

0.100mL plasma sample was pipetted out into previously labeled tubes, 25µL of IS concentration about (0.200 µg/mL for Sildenafil) was added in each tube (except blank plasma and zero standard). 0.100mL of 2mM Ammonium Formate (pH 3.00 ± 0.05) was added in each tube and vortexed for 30 seconds. The samples were centrifuged at 14000 RPM for 5 minutes. SPE cartridge Phenomenex Strata-X (30mg/1mL) were conditioned by passing 1mL of Methanol followed by 1mL of Water. Plasma samples were loaded on the cartridges. The plasma was drained by applying pressure. The cartridge was washed by passing 1mL of water followed by 1mL of 10% methanol in water. The samples were eluted with 1mL of mobile phase.

High performance liquid chromatography and mass spectrometric conditions: Chromatographic separation was carried out using a Waters Acquity UPLC with a Hypurity C8 (100 x 4.6mm), 5µ. A mobile phase consisting of 2mM Ammonium Formate (pH 3.00 ±

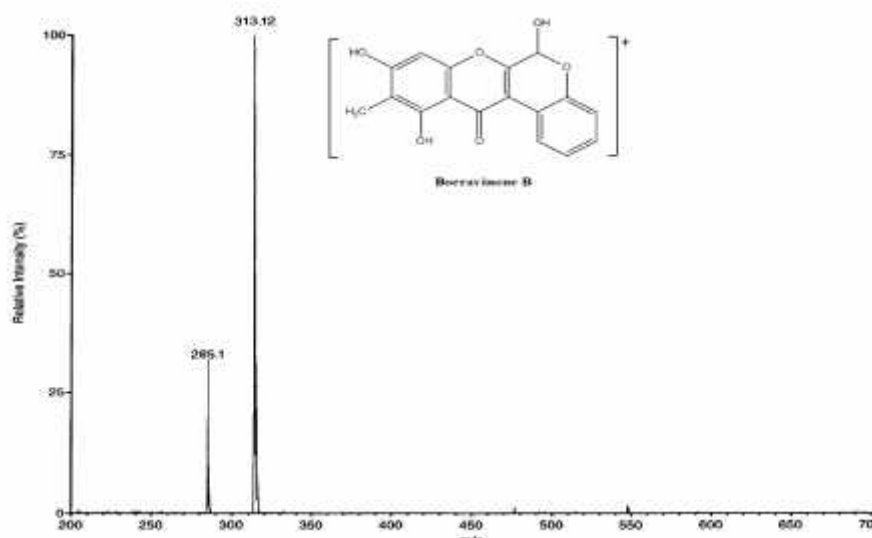


Figure 1: Mass spectrum of Boeravinone B in positive ion mode



Figure 2: Mass spectrum of Eupalitin-3-O-β-D-galactopyranoside in positive ion mode

0.05): Acetonitrile (20:80 v/v) was delivered with a flow rate of 0.6 mL/min. The total run time for each sample analysis was 5.0 min. Mass spectra were obtained using

source dependant parameters such as Capillary Voltage, Desolvation Gas Pressure, Cone Gas Pressure, Source Temperature, Desolvation Temperature and Extractor

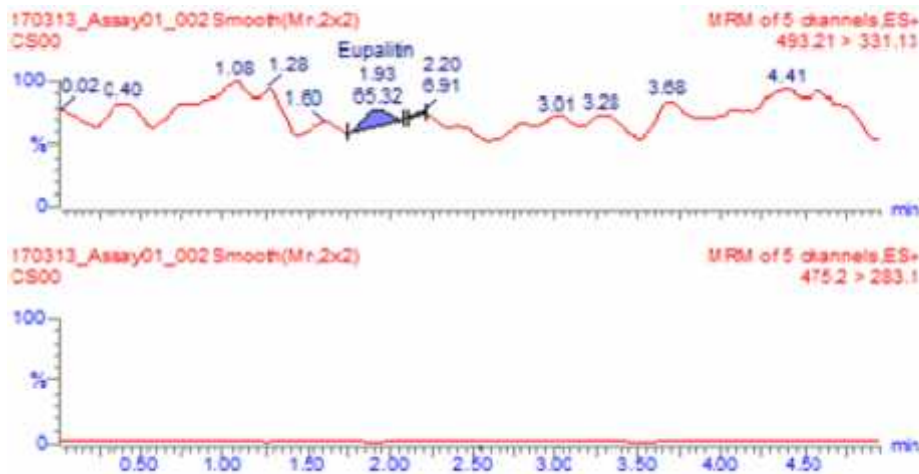


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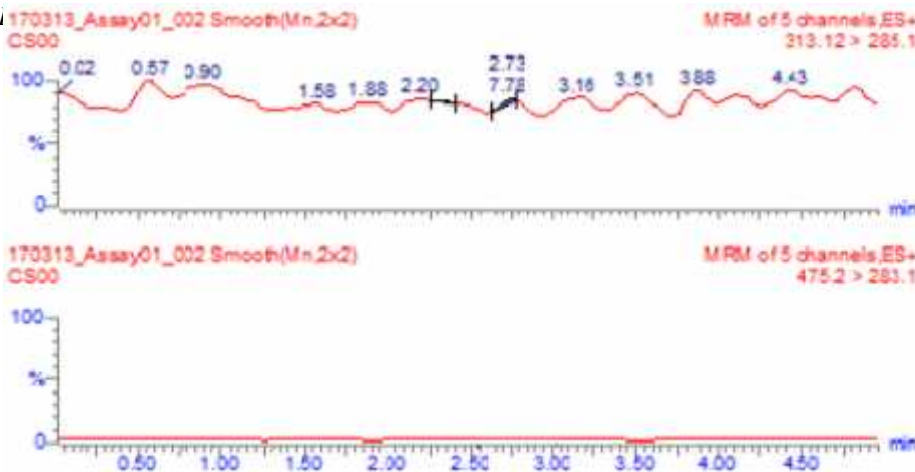


Figure 3: Representative Chromatogram of Plasma blank (Boeravinone B)

Waters XevoTQ equipped with electrospray ionization source operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization was electrospray ionization in the positive ion mode. The

Voltage were optimized to 4 V, 1100 psi, 150 psi, 150°C, 500°C, 3 V. The mass transition ion-pair selected were m/z 313.12amu 285.10amu for Boeravinone B,

493.21amu 331.13amu for Eupalitin-3-O- -D-galactopyranoside and 475.20amu 283.10amu for Sildenafil. The parent and product ion spectra for Boeravinone B and Eupalitin-3-O- -D-galactopyranoside are represented in Figure 1 and Figure 2 respectively. The data acquisition software used was MassLynx 4.1 SCN843. For quantification, the peak area ratios of the target ions of the drugs to those of the internal standard were compared with weighted ($1/x^2$) least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

RESULTS

Method Validation: The Bio-analytical method for quantification of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside from human plasma has been validated for selectivity, matrix effect, linearity, precision, accuracy, recovery and stability, following phase appropriate recommendations of the regulatory guidelines (CHMP 2011 & CDER 2001)

Selectivity: Selectivity was performed by analyzing the blank plasma from 6 different sources to test for interference at the retention time of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and the internal standard Sildenafil. No interfering peak of endogenous compounds was observed at the retention time of analytes or the internal standard in blank plasma containing K3EDTA as anti-coagulant. Representative chromatogram obtained from blank plasma is presented in Figure 3 and Figure 4.

Matrix effect: The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. Matrix effect was performed using the post extraction addition method. In this experiment to the processed blank samples Boeravinone B and Eupalitin-3-O- -D-galactopyranoside at the LQC (low quality control) and HQC (High quality control) levels were added. The recovery of the analyte from the samples was

evaluated against a linearity using pure solutions. The recovery of the QCs added in extracted matrix blank after extraction was consistent for across lots and matrix. The CV of the IS-normalised MF calculated from the 6 lots of matrix was below 15 %

Linearity: The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 5.0 – 500 $\mu\text{g/ml}$ for Boeravinone B and 5.0 – 500 $\mu\text{g/mL}$ for Eupalitin-3-O- -D-galactopyranoside. The representative chromatograms obtained for LLQC (lower limit quality control) for Boeravinone B is presented in Figure 5 and for Eupalitin-3-O- -D-galactopyranoside is presented in Figure 6. The calibration curves appeared linear and were well described by least squares lines. A weighing factor of $1/\text{concentration}^2$ was chosen to achieve homogeneity of variance. The correlation coefficients were 0.9926 for Boeravinone B and 0.9845 for Eupalitin-3-O- -D-galactopyranoside. Across the points taken as calibration standards, the % R.S.D. obtained from 0.32 to 3.72 and 0.42 to 3.13 for Boeravinone B and Eupalitin-3-O- -D-galactopyranoside, respectively.

Precision and accuracy: The intra-run and inter-run accuracy were determined by replicate analysis of three quality control levels along with the LOQ (lower limit of quantitation). In each of the precision and accuracy batches, six replicates ($n = 6$) at each quality control level inclusive of the LOQ level were analysed. The precision was calculated in terms of coefficient of variation (%CV). Accuracy was calculated in terms of the degree of closeness of back calculated concentration value, calculated from calibration curve, to nominal concentration value. For accuracy study deviation from nominal value should not be more than 15%, i.e. accuracy should be within 85–115%.

The intra run precision was 3.07% for Boeravinone B and 4.26% for Eupalitin-3-O- -D-galactopyranoside. The intra run accuracy was within the range of 95.89% to 99.22% and 96.35% to 103.85% for Boeravinone B and Eupalitin-3-O- -D-galactopyranoside respectively. The inter-run run precision was 3.38% for Boeravinone B and 4.16% for Eupalitin-3-O- -D-galactopyranoside.

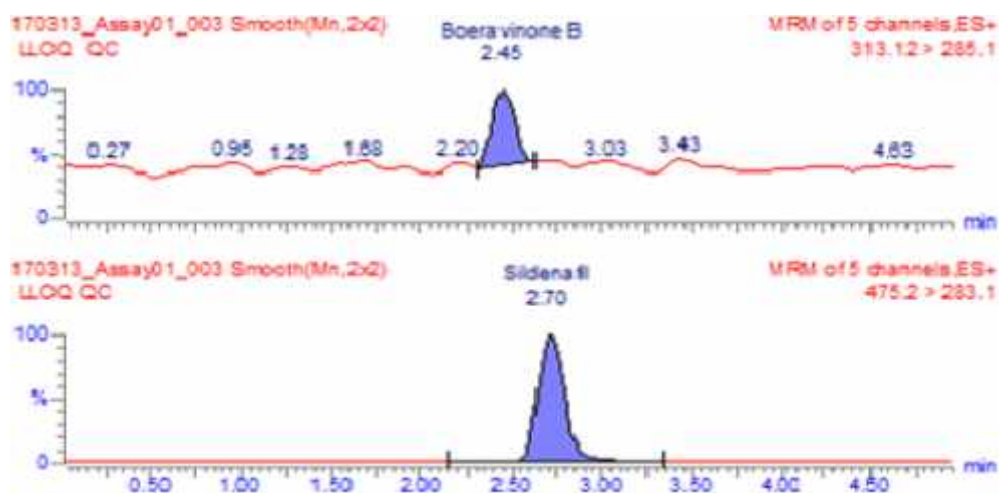


Figure 5: Representative Chromatogram of LLQC QC (Boeravinone B)

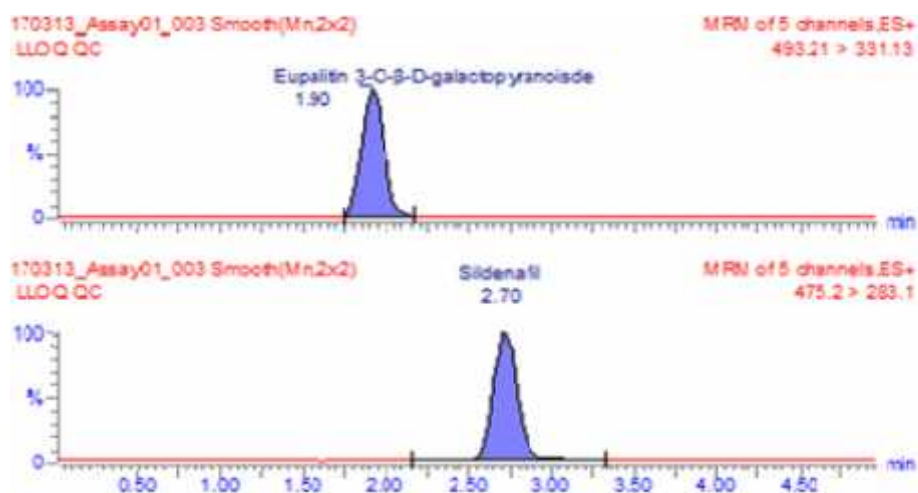


Figure 6: Representative Chromatogram of LLQC QC (Eupalitin-3-O- -D-galactopyranoside)

The inter-run accuracy was within the range 96.86% to 101.40% for Boeravinone B and 97.07% to 100.65% for Eupalitin-3-O- -D-galactopyranoside respectively.

Recovery: The extraction efficiency or recovery of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and the internal standard Sildenafil were determined by analysis of six replicates at low, medium and high quality control concentrations for Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and at the working concentration for the internal standard, Sildenafil. The percent recovery was evaluated by comparing the peak areas of extracted analytes to the peak areas of pure solutions of standards. The global recovery of Boeravinone B was 88.66% and for Eupalitin-3-O- -D-galactopyranoside was 97.01%.

Stabilities: As a part of the method validation, stability was evaluated. Analytes were considered stable if the recovery of the mean test responses were within 15% of freshly prepared standards. Stock solutions Boeravinone B and Eupalitin-3-O- -D-galactopyranoside and internal standard were stable at room temperature for seven days. The stability of spiked quality control plasma kept at room temperature of about 25 °C (bench-top stability) was evaluated for 20 hrs was found to be stable after comparing with fresh quality control samples. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 10 °C for 105 hrs and was found to be stable. The freeze-thaw stability was conducted by comparing the stability quality control samples that had been frozen and thawed for three cycles, with freshly spiked quality control samples, these samples were found to be stable after three freeze-thaw cycles.

Dilution integrity: The experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ (Upper limit of quantification)) which may be encountered during real subject samples analysis. The experiment was carried out at 2 times the concentration of the highest calibration level and diluted with blank plasma by dilution factors 5 and 3. Dilution factors 5 and 3 were selected to measure

dilution integrity of real subject samples having concentrations as high as five times more than the highest calibration level. The mean back calculated concentrations of 1/5th and 1/3rd diluted samples for Boeravinone B was 100.64% and 100.01% respectively to their nominal values and for Eupalitin-3-O- -D-galactopyranoside was 101.52% and 100.7% respectively to their nominal values. The coefficients of variance (%CV) were 2.76% and 3.18% for Boeravinone B and 6.25% and 7.06% for Eupalitin-3-O- -D-galactopyranoside.

DISCUSSION

The LC-MS/MS bio-analytical method has been developed and validated for quantification of Boeravinone B and Eupalitin-3-O- -D-galactopyranoside in human plasma. The validation data demonstrate good precision and accuracy of the method. The method was robust and did not encounter any significant lot-to-lot variation in matrix effect. This method can be applied to understand the pharmacokinetic profile of the pharmacologically active markers present in the plant *Boerhaavia diffusa*. By understanding the pharmacokinetic profile, drug developers would be successful at establishing the relationship of pharmacodynamic response to blood concentrations. Thus providing a basis for adjustment of dose, dosing interval, monitoring procedures and dosage form to develop, thereby ensuring improved drug therapy.

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