

**High-throughput Quantification of isoflavones, Biochanin A and Genistein, and  
their conjugates in female rat plasma using LC-ESI-MS/MS: application in  
pharmacokinetic study<sup>§</sup>**

Sheelendra Pratap Singh<sup>#1</sup>, Wahajuddin<sup>#\*1</sup>, Mushir M. Ali<sup>2</sup>, Girish Kumar Jain<sup>1</sup>

<sup>1</sup>Pharmacokinetics and Metabolism Division,  
Central Drug Research Institute, CSIR, Lucknow-226001, Uttar Pradesh, India

<sup>2</sup>Department of Pharmaceutics, Faculty of Pharmacy,  
Hamdard University, New Delhi, India

\*Corresponding author:

Tel.: +91-522-2612411-18 (Ext-4377); fax: +91-522-2623405

*E-mail address:* wahajuddin@cdri.res.in, wahajuddin@gmail.com

<sup>§</sup>CDRI communication no. 7804

<sup>#</sup> Authors contributed equally to this work

## ABSTRACT:

Isoflavones containing foods and dietary supplements are widely consumed for putative health benefits (e.g., cancer chemoprevention, beneficial effects on serum lipids associated with cardiovascular health, reduction of osteoporosis, relief of menopausal symptoms). This paper describes the development and validation of a sensitive high throughput LC-ESI-MS/MS method for quantifying biochanin A (BCA) and genistein (GEN), and their conjugates in rat plasma. The analytes were separated on a Supelco Discovery C18 (4.6 × 50 mm, 5.0 μm) column under isocratic condition using acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) as a mobile phase. The intra- and inter-day assay precision ranged from 2.66 to 8.34% and 4.40 to 8.10% (R.S.D. %), respectively, and intra- and inter-day assay accuracy was between 90.67 to 109.25% and 95.86 to 106.32%, respectively for both the analytes. The lowest quantitation limit for BCA and GEN was 0.5 ng/mL in 0.1mL of rat plasma. The method was successfully applied to the estimation of BCA, GEN and their conjugates in rat plasma following oral administration of BCA. Circulating conjugates (glucuronides/sulfates) of BCA and GEN were quantified using enzymatic hydrolysis of plasma samples. The levels of isoflavones glucuronides/sulfates were found to be much greater than the corresponding aglycones.

**KEY WORDS:** Biochanin; genistein; rat plasma; validation; LC–MS/MS; pharmacokinetics

## 1. INTRODUCTION

High intake of isoflavones has been associated with a variety of human health benefits, including prevention of cancer, cardiovascular diseases, and osteoporosis. The biochemical and pharmacological properties of isoflavones that may be related to these health benefits are multifaceted and may involve antioxidative, antiestrogenic (as well as estrogenic), and antiangiogenic activities, as well as their inhibition of procarcinogen metabolic activation and inhibition of cell growth [1-5]. Soy bean and soy products are known as the richest food sources of isoflavones, a group of phytoestrogens among which GEN is most studied. However, the available literature suggests that another leguminous plant, red clover (*Trifolium pratense*) also contains significantly higher concentration of isoflavones. BCA, a 4-O-methyl derivative of GEN, is the major isoflavone in red clover but is not present in soy foods. BCA has been shown to inhibit chemical-induced tumor carcinogenesis and prevent tumor growth after implantation in animal models [6-7]. Red clover extracts with BCA as the major component have been shown to reduce the loss of lumbar spine bone mineral content and bone mineral density in women and to lower the LDL cholesterol level in men [8-9]. Red clover isoflavones have been extracted and commercialized as nutraceutical agent (e.g. Promensil from Novogen). These are also available in the market as dietary supplements for relieving postmenopausal symptoms such as hot flashes and bone loss and for maintaining men's prostate health. Various *in vitro* studies shown BCA to be potent inhibitor of the efflux transporters, P-glycoprotein and breast cancer resistance protein, which are important

molecular mechanisms for both multidrug resistance and drug disposition, indicating the potential of this compound for drug interactions [10-11]. BCA is reported to be metabolized into isoflavone GEN *in vitro* and *in vivo* [12-13]. In order to probe more efficiently the potential benefits, or adverse effects, and drug interaction potential of BCA consumption, it is necessary to develop analytical methodologies which are capable of the sensitive and accurate quantification of analytes in low volumes of biometrics. Several analytical methods have been reported to quantify isoflavones in food supplements and in various biometrics e.g. plasma, serum and urine [14-23]. Validated methods are essential for the determination of plasma concentrations in pre-clinical (for pharmacokinetics, protein binding, drug-drug & drug-food interaction and toxicokinetic studies) and clinical studies. Till date, only two studies embodying pharmacokinetics of BCA has been reported [24-25]. To the best of our knowledge, no validated LC-MS/MS method is reported in literature for quantification of BCA and GEN in female rat plasma. Further, pharmacokinetics in female rats has also not been reported yet, in spite of isoflavones popular use in osteoporosis and menopause. Therefore, we have developed and validated a LC-ESI-MS/MS method for quantification of BCA and GEN in female rat plasma and the method was successfully applied for pharmacokinetic study in female rats for the first time.

## 2. EXPERIMENTAL

### 2.1. Chemicals and reagents.

BCA and GEN were purchased from Indofine Chemical Co. Inc. (Hillsborough, USA). 4-hydroxymephenytoin (IS) was purchased from Sigma Aldrich Ltd (St Louis, USA). Chemical structure of BCA, GEN and IS are shown in Fig. 1. HPLC grade acetonitrile and methanol were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India).  $\beta$ -glucuronidase (from *Helix pomatia*, type H-1; 577,900 units/g) was purchased from Sigma Aldrich Ltd. (St. Louis, USA). Auto-sampler carry-over was determined by injecting the highest calibration standard then a blank sample. Dimethyl sulfoxide was purchased from Thomas baker chemicals Pvt. Limited (Mumbai, India). Diethyl ether was purchased from TKM Pharma (Hyderabad, India). Glacial acetic acid (GAA) AR was purchased from E Merck Limited (Mumbai, India). Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd. (New Delhi, India). Heparin sodium injection I.P. (1000 IU/mL) was purchased from Gland Pharma (Hyderabad, India). Blank, drug free plasma samples were collected from adult, healthy female *Sprague–Dawley* rats at Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood (25 IU/mL) at 15450×g on Sigma 1-16K (Frankfurt, Germany) for 10 min. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

### 2.2. Instrumentation and chromatographic conditions.

HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin- Elmer instruments, Norwalk, USA) was used to inject 10  $\mu$ L aliquots of the processed samples on a Supelco Discovery C18 column (4.6  $\times$  50 mm, 5.0  $\mu$ m). The system was run in isocratic mode with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) at a flow rate of 0.7 mL/min. Mobile phase was duly filtered through 0.22  $\mu$ m Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min prior to use. Separations were performed at room temperature. Auto-sampler carry-over was determined by injecting

the highest calibration standard then a blank sample. No carry-over was observed, as indicated by the lack of BCA, GEN and IS peaks in the blank sample.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at -4500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 30, 10, 30 and 10, respectively. Compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were -92, -30, -8, -10 V, -93, -42, -8, -10 V and -65, -40, -8, -10 V for BCA, GEN and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI negative ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of  $m/z$  283 precursor ion  $[M-H]^-$  to the  $m/z$  268 product ion for biochanin,  $m/z$  269 precursor ion  $[M-H]^-$  to the  $m/z$  133 product ion for genistein and  $m/z$  233.1 precursor ion  $[M-H]^-$  to the  $m/z$  161 product ion for IS. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

### 2.3. Preparation of stock and standard solutions.

Primary stock solutions of BCA, GEN and IS were prepared by dissolving the compounds in DMSO followed by diluting the solution with methanol to achieve desired concentration of 1 mg/mL. Working standard solutions of BCA and GEN were prepared by combining the aliquots of each primary stock solution and diluting with methanol. A working stock solution of IS (1  $\mu$ g/mL) was prepared by diluting an aliquot of primary stock solution with methanol. Calibration standards of BCA and GEN (0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL) were prepared by spiking 90  $\mu$ L of pooled drug free rat plasma with the appropriate working standard solution of the analytes (10  $\mu$ L) and IS (10  $\mu$ L from 1  $\mu$ g/mL) on the day of analysis. All the stock solutions were stored at 4 °C until analysis. Quality control (QC) samples were prepared by individually spiking control rat plasma at four concentration levels [0.5 ng/mL (lower limit of quantitation, LLOQ), 1.5 ng/mL (QC low), 60 ng/mL (QC medium) and 160 ng/mL (QC high)] and stored at -70  $\pm$  10°C until analysis.

### 2.4. Recovery

The extraction recovery of analytes, through liquid-liquid extraction procedure, was determined by comparing the peak areas of extracted plasma (pre-spiked) standard QC samples ( $n = 6$ ) to those of the post-spiked standards at equivalent concentrations [26-28]. Recoveries of BCA and GEN were determined at three concentration levels QC low, QC medium and QC high concentrations viz., 1.5, 60, and 160 ng/mL, whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

### 2.5. Sample preparation

A simple liquid-liquid extraction method was followed for extraction of BCA and GEN from rat plasma. To 100  $\mu$ L of plasma in a tube, 10  $\mu$ L of IS solution (1  $\mu$ g/mL in methanol), was added and mixed for 15 sec on a cyclomixer (Spinix Tarsons, Kolkata, India). Then 2 mL of diethyl ether was added and the mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000 $\times$ g on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6 mL) was separated and evaporated to dryness under vacuum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 120  $\mu$ L of the mobile phase and 10  $\mu$ L was injected onto analytical

column. For determination of BCA and GEN conjugates the 0.1 mL plasma was incubated with 2000 unit of glucuronidase/sulfatase at 37°C for 4h [29] and then prepared as described above.

## **2.6. Validation procedures**

A full validation according to the FDA guidelines was performed for the assay in rat plasma [30].

### **2.6.1. Specificity and selectivity.**

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for analytes and IS using the proposed extraction procedure and chromatographic-MS conditions.

### **2.6.2. Matrix effect.**

The effect of rat plasma constituents over the ionization of BCA, GEN and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ( $n = 6$ ) with the response of analytes from neat standard samples at equivalent concentrations [26-28]. The matrix effect for BCA and GEN was determined at three concentration levels viz., 1.5, 60 and 160 ng/mL whereas the matrix effect over the IS was determined at a single concentration of 100 ng/mL. A value of >100% indicates ionization enhancement and a value of <100% indicates ionization suppression. The post-extracted samples were the drug-free control plasma spiked with working standard stock solutions after extraction.

### **2.6.3. Calibration curve.**

The calibration curve was acquired by plotting the peak area ratio of analyte to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/mL. The results were fitted to linear regression analysis using  $1/X^2$  as weighting factor. The calibration curve had to have a correlation coefficient ( $r$ ) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were  $\pm 15\%$  deviation from the nominal value except at LLOQ, which was set at  $\pm 20\%$  [30].

### **2.6.4. Precision and accuracy.**

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 0.5, 1.5, 60 and 160 ng/mL. The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within  $\pm 15\%$  standard deviation (S.D.) from the nominal values and a precision of within  $\pm 15\%$  relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed  $\pm 20\%$  of accuracy as well as precision [30].

### **2.6.5. Stability experiments.**

All stability studies were conducted at two concentration levels, i.e. QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 18 h to establish autosampler (AS) stability of analytes and IS. The peak areas of analytes and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of BCA and GEN in the biomatrix during 6 h exposure at room temperature in rat plasma (bench top, BT)

was determined at ambient temperature ( $25 \pm 5^\circ\text{C}$ ). Freeze/thaw (FT) stability was evaluated up to three cycles. In each cycle samples were frozen for at least 12 h at  $-70 \pm 10^\circ\text{C}$ . Freezer stability of BCA and GEN in rat plasma was assessed by analyzing the QC samples stored at  $-70 \pm 10^\circ\text{C}$  for at least 15 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e.,  $\pm 15\%$  S.D.) and precision (i.e.,  $\pm 15\%$  R.S.D.).

#### **2.6.6. Dilution integrity.**

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above the upper limit of quantification), which may be encountered during real subject samples analysis. Dilution integrity experiments were carried out by 20 times dilution of plasma samples containing 3200 ng/mL of BCA and GEN with blank plasma to obtain samples containing 160 ng/mL (HQC) of BCA and GEN.

#### **2.7. Application to a Pharmacokinetic study in rats.**

A pharmacokinetic study was performed to show the applicability of newly developed and validated bioanalytical method. Study was performed in female *Sprague–Dawley* rats ( $n = 4$ , weight range 200–220 g). BCA was administered orally at a dose of 50 mg/kg in 0.25% sodium carboxy methyl cellulose (CMC) suspension. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.25, 0.5, 0.75, 1, 3, 5, 7, 9, 24 and 28 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min and stored frozen at  $-70 \pm 10^\circ\text{C}$  until analysis. Plasma (100  $\mu\text{L}$ ) samples were spiked with IS, and processed as described above. Along with the plasma samples, QC samples were distributed among calibrators and unknown samples in the analytical run. The data was accepted based on performance of QCs prepared using rat blank plasma. Plasma concentration–time data of BCA and GEN was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, USA).

### **3. RESULTS**

#### **3.1. Liquid chromatography.**

Liquid–liquid extraction was chosen as the plasma preparation method since this extraction technique usually offers cleanest sample with good robustness. Several organic solvents including ethyl acetate, acetonitrile, methanol, n-hexane, tert butyl methyl ether and diethyl ether were investigated as the extraction solvent. Diethyl ether was chosen because of higher extraction efficiency for BCA and GEN, and cleaner samples than other solvents.

Several column types and chromatographic conditions were tested in order to develop a short, though robust and sensitive analytical method. A short (4.6  $\times$  50 mm, 5.0  $\mu\text{m}$ ) Supelco Discovery C18 column with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) at a flow rate of 0.7 mL/min provided the best compromise between selectivity and speed of analysis. The overall analysis time was only 3 min.

#### **3.2. Mass spectrometry.**

In order to optimize ESI conditions for BCA, GEN and IS, quadrupole full scans were carried out in negative ion detection mode. During a direct infusion experiment, the mass spectra for BCA, GEN and IS revealed peaks at  $m/z$  283, 269 and 233.1

respectively as deprotonated molecular ions  $[M-H]^-$ . Fig. 2 shows the product ion mass spectrum for BCA, GEN and IS. Following detailed optimization of mass spectrometry conditions (provided in instrumentation and chromatographic conditions section),  $m/z$  283 precursor ion  $[M-H]^-$  to the  $m/z$  268 product ion for BCA,  $m/z$  269 precursor ion  $[M-H]^-$  to the  $m/z$  133 product ion for GEN and  $m/z$  233.1 precursor ion  $[M-H]^-$  to the  $m/z$  161 product ion for IS was used for the quantitation purpose.

### 3.3. Validation procedures

#### 3.3.1. Specificity, recovery and matrix effect:

In the present study, the specificity and selectivity has been studied by using independent plasma samples from six different rats.

Fig. 3 shows a typical chromatogram for the drug-free plasma (Fig.3a), drug-free plasma spiked with BCA and GEN at LLOQ and IS (Fig.3b), and an *in vivo* rat plasma sample after oral administration of BCA (Fig.3c). As shown in Fig.3a, there is no significant interference from plasma found at retention time of either the analyte or the IS.

The extraction recovery was determined by comparing the peak areas of pre-spiked standards at 1.5, 60 and 160 ng/mL with those of post-extraction blank plasma standards spiked with corresponding concentrations. The extraction recoveries of the BCA and GEN ranged from 95.69 to 107.45%, and the extraction recovery of the internal standard was 92.83%.

The ion suppression or enhancement by plasma was less than 10% for the analytes and IS which demonstrated that the matrix effects do not cause quantitation bias. Therefore, matrix effect could be negligible under the experimental conditions.

#### 3.3.2. Calibration curve.

The plasma calibration curve was constructed using seven calibration standards (viz., 0.5–200 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte / peak area IS) versus concentration, and fitted to the  $y = mx + c$  using weighing factor ( $1/X^2$ ). The average regression ( $n = 3$ ) was found to be  $\geq 0.997$ . The lowest concentration with R.S.D. < 20% was taken as LLOQ and was found to be 0.5 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 91.40 to 113.00; while the % precision values ranged from 0.49 to 8.86 for both the analytes (Table 1).

#### 3.3.3. Accuracy and precision.

Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 2 and 3. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

#### **3.3.4. Stability.**

The predicted concentrations for BCA and GEN at 1.5 and 160 ng/mL samples deviated within the nominal concentrations in a battery of stability tests, viz., AS (18 h), BT (6 h), repeated three freeze/thaw cycles (FT-3) and at  $-70 \pm 10^\circ\text{C}$  for at least for 15 days (Table 4). The results were found to be within the assay variability limits during the entire process.

#### **3.3.5. Dilution integrity.**

Dilution integrity experiments carried out at six replicates by 20 times dilution with blank plasma and assay precision and accuracy were determined in a similar manner as described in Section 2.6.4. The % accuracy of diluted QCs was in the range of 95.18 to 106.67; while % precision values ranged from 4.2 to 8.01 for both the analytes. The results suggested that samples whose concentrations were greater than the upper limit of calibration curve could be re-analyzed by appropriate dilution.

#### **3.4. Application of the method.**

The rat plasma samples, generated following oral administration of BCA, were analyzed by the newly developed and validated method along with QC samples. During samples analysis it was found that some sample concentrations were falling above the calibration range, these samples were reanalyzed after dilution along with the diluted QCs. All the QCs met the acceptance criteria (data not shown). The mean plasma concentrations versus time profiles are shown in Figs. 4 and 5. The pharmacokinetic parameters of BCA and GEN in rats were determined by noncompartmental analysis and are presented in Table 5. The metabolite GEN, GEN and BCA conjugates (glucuronides and/or sulphates) were detected from first time point onward. These results suggest that BCA is rapidly O -demethylated to GEN and that both BCA and its metabolite GEN are rapidly conjugated. Upon enzymatic hydrolysis of the plasma samples, the AUC of BCA and GEN increased approximately 136 and 24 times, respectively. It suggests that the glucuronide/sulphate conjugates are major circulating metabolites in blood.

#### **4. Conclusion.**

In this study, we have validated a LC-MS/MS method for simultaneous quantitation of BCA and its metabolite GEN after liquid-liquid extraction from rat plasma for the first time. Validation results show that there is no significant matrix effect on analytes and selected IS. This method utilizes a short run time of 3.0 min for each sample analysis. Due to good sensitivity (LLOQ-0.5 ng/mL) of the assay, it offers a suitable platform for the determination of BCA and GEN in pre-clinical studies. The applicability of the method in pre-clinical pharmacokinetic study has been demonstrated in female rats for the first time. Through rigorous method validations and actual sample analysis, method has been proven to be reproducible and robust.

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**Table 1.**

**Precision and accuracy data of back-calculated concentrations of calibration samples for BCA and GEN in rat plasma (n = 3)**

Nominal Conc. (ng/mL)	Biochanin				Genistein			
	Mean	SD	Precision <sup>a</sup> (%)	Accuracy <sup>b</sup> (%)	Mean	SD	Precision <sup>a</sup> (%)	Accuracy <sup>b</sup> (%)
0.5	0.52	0.04	7.01	103.07	0.51	0.02	4.70	101.40
1	1.00	0.01	1.44	100.17	0.97	0.09	8.86	96.53
2	2.06	0.12	6.02	103.17	2.05	0.12	5.83	102.33
5	4.98	0.38	7.68	99.53	4.95	0.13	2.72	99.00
10	10.43	0.12	1.11	104.33	10.04	0.11	1.09	100.37
20	22.60	1.57	6.95	113.00	21.40	1.04	4.88	107.00
50	50.27	2.05	4.08	100.53	45.70	1.25	2.74	91.40
100	96.10	1.80	1.88	96.10	94.20	2.61	2.77	94.20
200	188.00	4.36	2.32	94.00	204.00	1.00	0.49	102.00

<sup>a</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>b</sup> Calculated as (mean determined concentration/nominal concentration) × 100.

**Table 2.**

**Intra-day assay precision and accuracy for BCA and GEN in rat plasma (n = 6)**

	Biochanin (ng/mL)				Genistein (ng/mL)			
	0.5	1.5	60	160	0.5	1.5	60	160
<b>Day-1</b>								
Mean	0.52	1.55	65.17	158.50	0.48	1.58	65.55	173.83
SD	0.04	0.08	4.98	8.41	0.03	0.09	1.91	10.82
Precision <sup>a</sup> (%)	7.17	5.10	7.64	5.30	6.32	5.95	2.92	6.22
Accuracy <sup>b</sup> (%)	104.36	103.33	108.61	99.06	96.00	105.20	109.25	108.65
<b>Day-2</b>								
Mean	0.49	1.63	63.55	148.50	0.49	1.45	65.52	171.67
SD	0.02	0.04	3.88	7.29	0.03	0.11	2.22	6.95
Precision <sup>a</sup> (%)	3.87	2.66	6.11	4.91	7.08	7.54	3.39	4.05
Accuracy <sup>b</sup> (%)	98.44	108.44	105.92	94.04	98.13	96.53	109.19	102.60
<b>Day-3</b>								
Mean	0.45	1.60	55.72	151.17	0.50	1.45	60.67	172.33
SD	0.02	0.09	1.80	9.22	0.04	0.11	3.20	5.28
Precision <sup>a</sup> (%)	3.50	5.91	3.23	6.10	8.34	7.58	5.28	3.06
Accuracy <sup>b</sup> (%)	90.67	106.56	92.86	94.48	99.35	96.67	101.11	107.71

<sup>a</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>b</sup> Calculated as (mean determined concentration/nominal concentration) × 100.

**Table 3.**

**Inter-day assay precision and accuracy for BCA and GEN in rat plasma**

	Biochanin (ng/mL)				Genistein (ng/mL)			
	0.5	1.5	60	160	0.5	1.5	60	160
Mean <sup>a</sup>	0.49	1.59	60.82	152.72	0.49	1.50	63.71	172.61
SD	0.04	0.08	4.93	8.96	0.03	0.12	3.42	7.59
Precision <sup>b</sup> (%)	7.74	4.90	8.10	5.87	6.80	7.71	5.37	4.40
Accuracy <sup>c</sup> (%)	97.38	106.11	101.36	95.86	97.64	99.67	106.18	106.32

<sup>a</sup> n = 3 days with six replicates per day.

<sup>b</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>c</sup> Calculated as (mean determined concentration/nominal concentration) × 100.

**Table 4.**

**Stability of BCA and GEN in rat plasma**

	Biochanin				Genistein			
	Mean <sup>a</sup>	SD	Precision <sup>b</sup> (%)	Accuracy <sup>c</sup> (%)	Mean	SD	Precision <sup>b</sup> (%)	Accuracy <sup>c</sup> (%)
<b>1.5(ng/mL)</b>								
0 h (for all)	1.55	0.08	5.10	103.33	1.58	0.09	5.95	105.20
18 h (AS)	1.54	0.08	5.11	99.61	1.60	0.17	10.57	101.52
6 h (BT)	1.61	0.10	6.49	103.87	1.63	0.19	11.81	103.42

FT-3	1.52	0.12	8.16	98.28	1.56	0.17	10.90	99.07
15 day at -80°C	1.58	0.06	3.97	101.61	1.54	0.13	8.50	97.72
<b>160(ng/mL)</b>								
0 h (for all)	158.50	8.41	5.30	99.06	173.83	10.82	6.22	108.65
18 h (AS)	153.50	7.45	4.85	96.85	166.83	7.14	4.28	95.97
6 h (BT)	161.17	10.70	6.64	101.68	177.50	5.32	3.00	102.11
FT-3	150.33	6.19	4.11	94.85	157.67	6.56	4.16	90.70
15 day at -80°C	155.17	6.15	3.96	97.90	176.33	8.69	4.93	101.44

<sup>a</sup> Back calculated plasma concentrations (n=6)

<sup>b</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>c</sup> Calculated as (mean determined concentration/nominal concentration) × 100.

**Table 5.**

**Selected pharmacokinetics parameters of BCA and GEN in female SD rats following oral administration of BCA at 50 mg/kg (n=4)**

Parameters	Free BCA	Total BCA (free+conjugates)	Free GEN	Total GEN (free+conjugates)
AUC <sub>0-t</sub> (h*ng/mL)	220.29 ± 26.56	30062.62 ± 11082.02	94.91± 56.35	2236.86 ± 1171.71
C <sub>max</sub> (ng/mL)	33.47 ± 13.11	2700 ± 79.37	8.33 ± 1.78	136.20 ± 35.85
T <sub>max</sub> (h)	0.42 ± 0.14	0.50 ± 0.00	3.17 ± 5.05	11.17 ± 11.90
t <sub>1/2</sub> (h)	13.73 ± 1.40	13.42 ± 6.16	15.12 ± 11.58	11.35 ± 1.04
MRT <sub>Last</sub> (h)	8.59 ± 1.66	10.51 ± 2.01	9.92 ± 4.74	13.33 ± 2.79

## Legends to Figures

### Figure 1.

Structural representation of BCA, GEN and 4-hydroxymephenytoin.

### Figure 2.

MS/MS spectra of BCA, GEN and 4-hydroxymephenytoin showing prominent precursor to product ion transitions.

### Figure 3.

Typical MRM chromatograms of BCA and GEN in rat plasma (a) a drug free plasma, (b) drug free plasma spiked with BCA and GEN at LLOQ (0.5 ng/mL) and IS and (c) an *in vivo* rat plasma sample showing BCA and GEN peak obtained following oral administration of BCA.

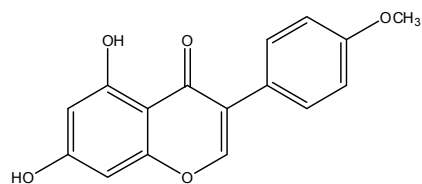
### Figure 4.

Mean plasma concentration-time profile of free BCA, GEN and their conjugates in rat plasma following oral administration of BCA (Mean  $\pm$  S.D., n = 4).

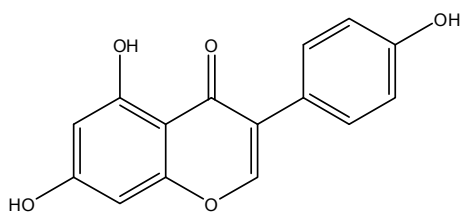
### Figure 5.

Mean plasma concentration-time profile of total (free + conjugates) BCA and GEN in rat plasma following oral administration of BCA (Mean  $\pm$  S.D., n = 4).

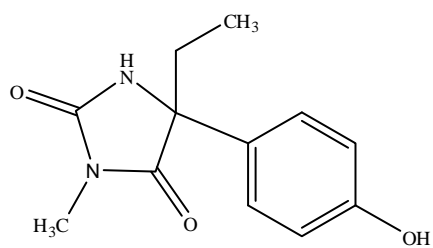
Figure-1



Biochanin A

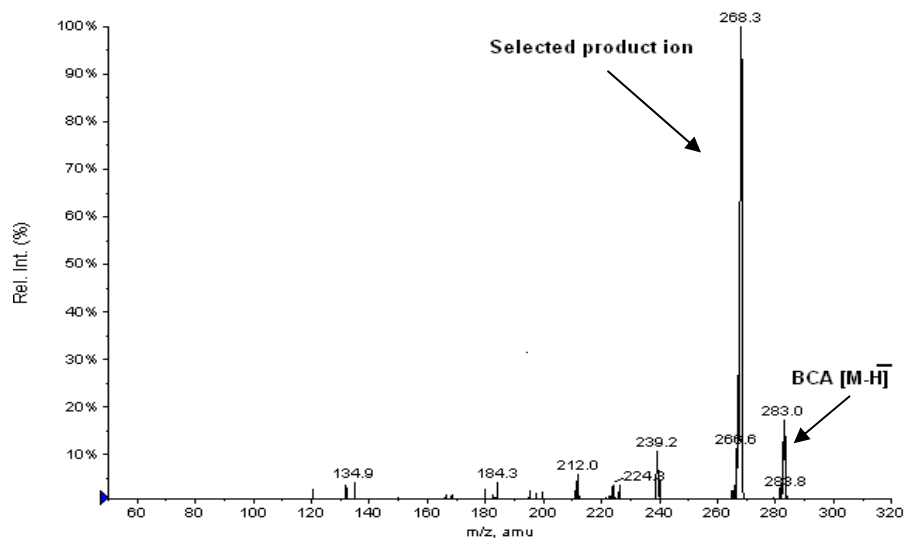


Genistein

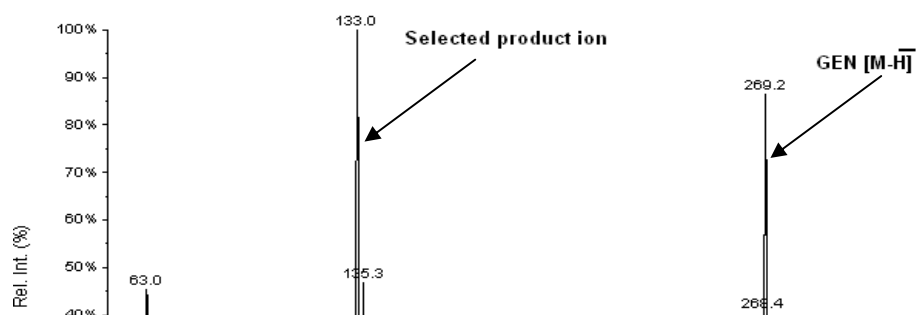


4-hydroxymephenytoin

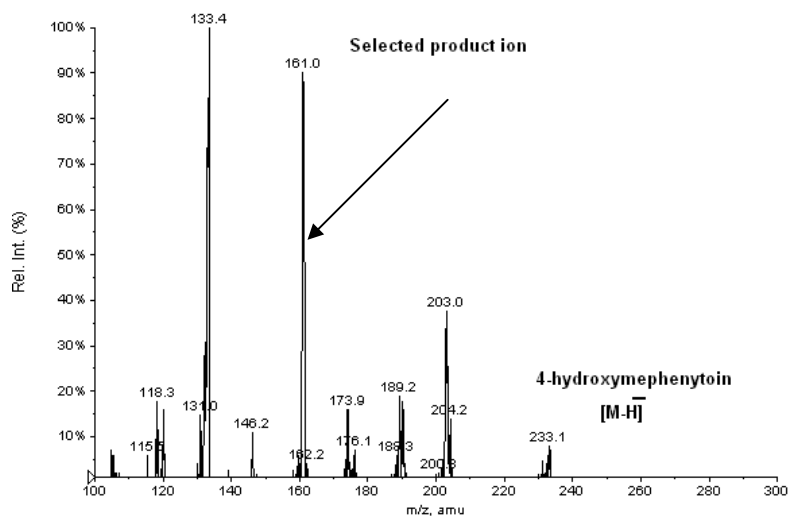
**Figure- 2**



BCA-Q3



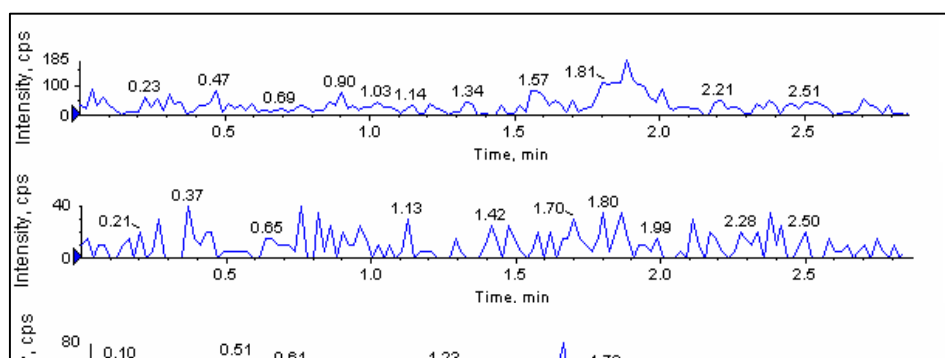
**GEN- Q3**



4-hydroxymephenytoin- Q3

Figure- 3

(a) Rat blank plasma



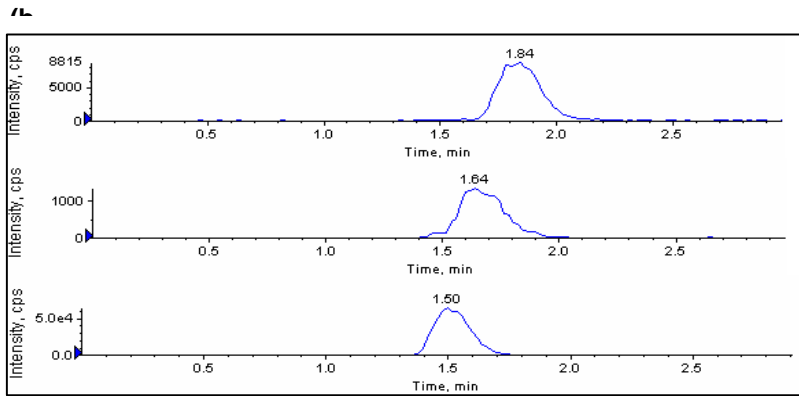
BCA

GEN

IS

LLOQ

BCA

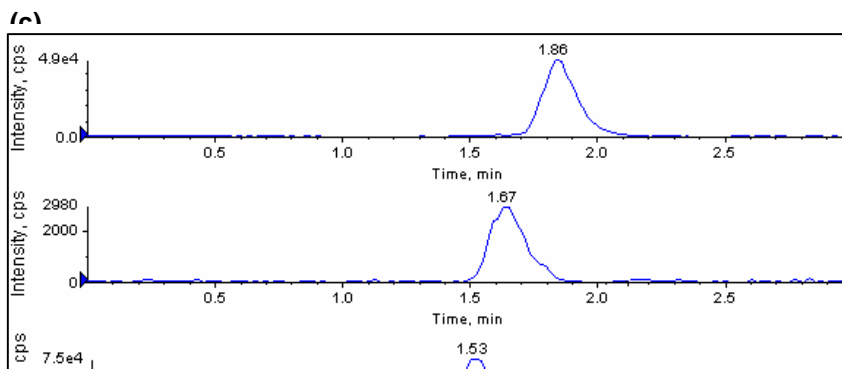


GEN

IS

In-vivo plasma sample

BCA



**GEN**

**IS**

**Figure- 4**

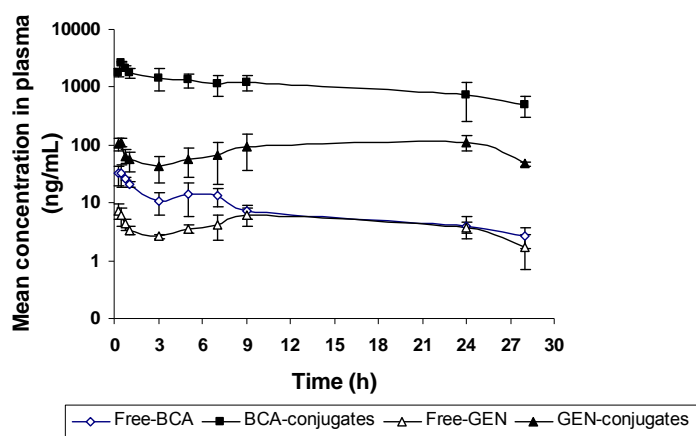


Figure- 5

