School of Pharmacy[1], University College Sedaya International, Cheras, Kuala Lumpur; School of Pharmaceutical Sciences[2], Universiti Sains Malaysia, Minden, Penang, Malaysia

Determination of betulinic acid from *Orthosiphon stamineus* leaf extract in rat plasma

G. A. Akowuah[1], I. Zhari[2]

Received April 30, 2008, accepted May 30, 2008

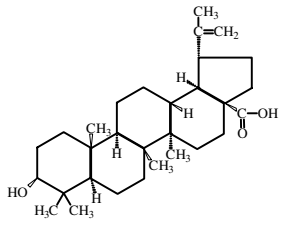
Dr. Gabriel A. Akowuah, School of Pharmacy, University College Sedaya International, 56000 Cheras, Kuala Lumpur, Malaysia
agabriel@ucsi.edu.my

Pharmazie 63: 788–790 (2008) *doi: 10.1691/ph.2008.8596*

A simple high-performance liquid chromatography (HPLC) method to determine the content of betulinic acid (BA) in rat plasma collected at different times (0–8 h) after oral administration of *Orthosiphon stamineus* leaf extract was developed. The features of the assay include protein precipitation using acetonitrile and isocratic elution using reverse phase C-18 column with ultraviolet (UV) detection. The recovery of BA from plasma varied from 98.4 to 102.5%. The R.S.D of intra- and inter-day precision from rat plasma ranged from 4.2 to 9.8%. The maximum concentration of BA in the plasma was 1.2 ± 0.3 μg/ml at 1 h after oral administration of the extract.

1. Introduction

Betulinic acid (BA) is useful for its pharmacological properties, especially the treatment of diarrhea, dysentery, and cholera. It is reported to slow down the activity of the human immunodeficiency virus (HIV), induce apoptosis and cell death in melanoma cells without impacting healthy tissue (Pisha et al. 1995; Melzig and Bormann, 1998; Fujioka et al. 1994). BA has several botanical sources. The compound was extracted from leaves of *Ziziphus mauritiana*, an African tree species and also analyzed in *Ziziphus fructus* (Bae et al. 1996), *Doliocarpus schottianus* (Oliveira et al. 2002), *Platanus acerifolia* and *Betula pendula* (Galgon et al. 1999). It can be chemically derived from betulin, a substance found in abundance in the outer bark of white birch trees (*Betula alba*) (Pisha et al. 1995; Herz et al. 1972). BA was quantified by HPLC method in cultivated plant samples of *Orthosiphon stamineus* leaves (Akowuah et al. 2004).



Betulinic acid

The chemical constituents in *O. stamineus* leaf extracts include highly oxygenated isopimarane-type diterpenoids, methoxylated flavones, triterpenoids including betulinic acid together with ursolic acid and oleanolic acid (Tezuka et al. 2000). Determination of BA in mouse blood, tumor and tissue homogenates by HPLC-electrospray Mass spectrometry after administration of BA in polyvinylpyrrolidone (PVP) by i.p. has been described (Shin et al. 1999). The present study described a simple validated HPLC method for the determination of BA in rat plasma after oral administration of *O. stamineus* leaf extract.

2. Investigations, results and discussion

The BA content was 0.26% in the freeze-dried extract determined by HPLC prior to oral administration (1 g/kg body weight) to the animals. BA was separated from potential interfering compounds in the plasma under the chromatographic conditions at the elution time of about 12.4 min. Chromatographic peak was identified with the aid of pure standard of BA based on retention time.

The linearity of detector response was assessed for extracted plasma samples. Calibration curve of BA was linear over the concentration range of 0.1–10 μg/ml with correlation coefficients greater than 0.9991 in all calibration curves. The limit of quantification (LOQ) of BA was 0.1 μg/ml in acetonitrile precipitated blank plasma. At the quality control (QC) concentrations (0.1 μg/ml, 1 μg/ml, 10 μg/ml) of BA the overall recoveries of 98.4–102.5% were obtained. The R.S.D. of recovery from plasma was less than 12% at the QC concentrations. Acceptable precision was achieved with the method as the R.S.D. of intra and inter-day precision did not exceed 10% of initial time response or concentration. The plasma and acetonitrile precipitated plasma samples were stable, at room temperature for 18 h, or stored in a refrigerator at 4 °C, −20 °C and −80 °C for 1 month.

The course of plasma concentrations of BA after oral administration of *O. stamineus* is shown in the Fig. An increase of the plasma concentration of BA was observed after oral administration of the extract which may indicate absorption of BA into the blood. The parameters estimated from the curve included, $t_{1/2} = 3.0 \pm 0.40$ h, $T_{max} = 1 \pm 0.3$ h, $C_{max} = 1.2 \pm 0.3$ μg/ml and $AUC_{0-8\,h} = 4.3 \pm 0.8$ μg/ml · h.

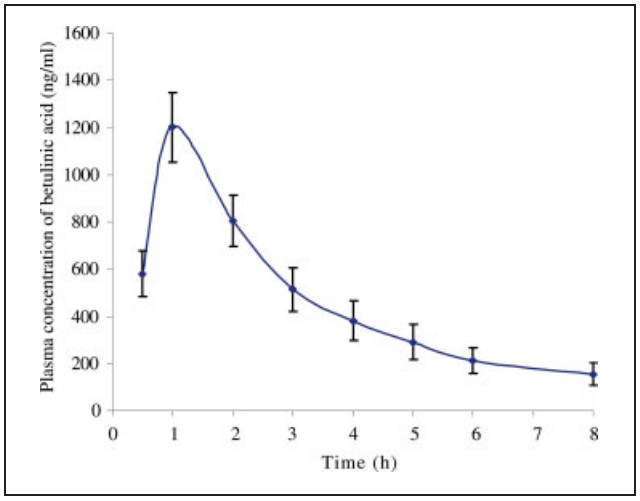


Fig.: Plasma concentration-time curve of betulinic acid after oral administration of *Orthosiphon stamineus* with a single dose of 1 g/kg in rats (mean ± sem, n = 6)

In conclusion, BA, a bioactive substance found in the leaf extract of *O. stamineus* was detectable in rat plasma after oral administration of the extract. The method is simple and reproducible and can be used for the determination of BA in biological matrix after intake of plant extracts or herbal medicinal products containing BA.

3. Experimental

3.1. Chemical and reagents

Betulinic acid (BA) was purchased from Sigma Chemical Company (St.Louis MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade was acidified to pH 3 with phosphoric acid) were obtained from Merck (Darmstadt, Germany). All other solvents were analytical grade or HPLC grade.

3.2. Preparation of extracts

Powdered *Orthosiphon stamineus* leaves from Kepala Batas (Pinang, Malaysia) were extracted with MeOH using a Soxhlet at 40 °C. The extract was concentrated and freeze-dried. The content of BA in freeze-dried extract was determined by HPLC before oral administration.

3.3. Animals and dosage

Experimental animals used were Sprague-Dawley rats, weighing 270–295 g, from the animal house of Universiti Sains Malaysia. The animals were kept in the animal room for 1 week prior to the start of the study. Animals were fed with standard diet prior to testing. Food and water were withheld overnight before administration of the extracts to rats orally with single dose of 1 g/kg body weight in 10 ml of the extract suspension.

3.4. Sampling of blood

Six overnight fasted rats received the suspension of the extract orally. The animals were put under anesthesia with sodium pentobarbital (50 mg/kg i.p.) and remained anesthetized throughout the experimental period. The jugular vein was cannulated with Pe-50 tubing to collect blood with an established heparin-lock using 100 U/ml heparin in saline. Blood samples (0.3 ml) were taken using a disposable syringe at 0 min (pre-dose) 30, 60, 120, 180, 240, 300, 360 and 480 min after dosing. Blood samples were immediately transferred to a heparinized microcentrifuge tube and centrifuged at 3000 rpm for 10 min at 5 °C. The resulting plasma samples (0.2 ml supernatant) were transferred into 1.5 ml tubes and stored at –80 °C until assayed.

3.5. Preparation of standard solutions of plasma

The stock solution 0.1 mg/ml of the reference BA was freshly prepared in acetonitrile. Solutions of BA in the range of 0.1–10 μg/ml were prepared in blank plasma samples for calibration curve. Acetonitrile was used for protein precipitation of the plasma. Spiked plasma samples were vortexed for 15 s and centrifuged at 3000 × g for 5 min at 5 °C. The resulting supernatants were used for injection. Quality control (QC) samples at low (0.1 μg/ml), medium (1 μg/ml) and high concentrations (10 μg/ml) were prepared by the same procedures as described.

3.6. HPLC conditions

The plasma (0.2 ml) was precipitated with (0.3 ml) acetonitrile and centrifuged at 3000 × g for 10 min at 5 °C. The organic layer was transferred into an empty tube and dried under a stream of nitrogen at 40 °C. The residue was reconstituted in acetonitrile to give a 0.5 ml solution. The solution was passed through a membrane filter (pore size 0.5 μm) prior to HPLC analysis. The chromatographic conditions used for analyzing plasma samples were; LiChrosorb RP-18 column (250 mm × 4.6 i.d. mm, 10 μm particle size) (Merck); flow-rate of 1 ml/min; UV detection at 210 nm; injection volume of 20 μl, ambient temperature. The mobile phase for elution of BA was acetonitrile : water (pH = 3) (8 : 2; v/v).
HPLC analysis was performed with a Gilson HPLC pump (Model 305), a Gilson UV/VIS detector (Gilson Medical Electronics, Villierss-le-Bel, France) connected to a Hitachi D-2500 Chromato-integrator (Hitachi, Tokyo, Japan) and a Rheodyne sample injector valve fitted with a 50 μl sample loop. The detector was operated using a sensitivity range of 0.005 AUFS, output of 15 mV.

3.7. Validation of HPLC method

The linearity of the responses was determined for seven concentrations (in plasma as described above) by three injections. The content of BA was calculated using regression parameters obtained from the standard curve for blank plasma samples. The limit of quantification (LOQ) was established at signal to noise ratio (S/N) of 10.
The intra- and inter-assay relative standard deviation was used to validate the precision of the assay by determining standard samples of BA in plasma. Intra- and inter-day precision of the assay were determined five times on the same day and continuously for 5 days at the QC samples of BA.
Accuracy was determined by recovery studies. A recovery of BA from plasma was estimated by comparing the area obtained from injections of standard solutions (QC solutions) in blank plasma to those in acetonitrile. The mean recoveries were determined using the QC solutions in triplicates. The stability of BA was studied in the plasma and acetonitrile precipitated plasma with the QC samples. The samples were put aside at room temperature for 18 h, or stored in a refrigerator at 4 °C, −20 °C and −80 °C for 1 month. The content of BA was calculated. The compound was considered stable if the variation of the content was less than 10% of initial time concentration or response (Hu and Morris 2003).

3.8. Data analysis

Standard calibration curve for the analyte was prepared and the weight was calculated with the equation obtained from linear regression analysis:

$$y = mx + b \quad (1)$$

where y = relative peak area of analyte, m = slope of the line generated by a standard curve; x = concentration of analyte found (μg/ml); b = intercept of the line generated by the standard curve.
The amount of BA found in plasma (ng/ml) was calculated as follows:

$$A = C \times V_f / V_p \quad (2)$$

where A = ng/ml of the BA found in test sample; C = concentration in μg/ml of BA found in test samples from standard curve; V_p = test portion (0.2 ml, plasma); volume V_f = final volume of method (0.5 ml, plasma).
The following pharmacokinetic parameters were determined for the period of 0–8 h: the area under plasma concentration time curve (AUC_{0-inf}) from time zero to the last measurable BA sample time ($AUC_{0-8\,h}$); maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined directly from the data. The elimination rate constant Kel (h^{-1}) was calculated by log/linear regression using terminal phase of the plasma concentration time plot. The half-life, $t_{1/2}$, (h) was calculated by dividing 0.693 by Kel by 0.693.

Acknowledgement: Financial support by Ministry of Science, Technology and Environment of Malaysia (IRPA) is gratefully acknowledged.

References

Akowuah AG, Zhari I, Norhayati I, Sadikun A (2004) Quantification of betulinic acid, in leaf extracts of *Orthosiphon stamineus,* J Trop Med Plants 4: 225–228.

Bae KH, Lee SM, Lee JS, Lee ES, Kang JS (1996) Isolation and quantitative analysis of betulinic acid and alphitolic acid from *Zyziphi fructus*. Yakhak Hoechi 40: 558–562.

Fujioka T, Kashiwada Y, Kilkuskie RE, Cosentino LM, Balla LM, Jiang JB, Janzen WP, Chen IS, Lee KH (1994) Anti AIDS agents. Betulinic caid and platonic acid as anti HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. J Nat Prod 57: 243–247.

Galgon T, Höke D, Dräger B (1999) Identification and quantification of betulinic acid. Phytochem Anal 10: 187–190.

Herz W, Santhanam PS, Wahlberg I (1972) 3-Epibetulinic acid, a new triterpenoid from *Picramnia pentandra*. Phytochemistry 11: 3061–3063.

Hu, HE, Morris ME (2003) Determination of alpha-naphthyisothiocyanate in rat plasma and urine by high-performance liquid chromatography. J Chromatogr B 788: 17–28.

Melzig MF, Bormann H (1998) Betulinic acid inhibits amino-peptidase N activity. Planta Med 64: 655–657.

Oliveira BH, Santos CAM, Espindola DM (2002) Determination of the triterpenoid, betulinic acid, in *Doliocarpus schottianus* by HPLC. Phytchem Anal 13: 95–98.

Pisha E, Chai HB, Lee IS (1995) Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med 1: 1046–1051.

Shin YG, Kyung HC, Sang MC, Graham J, TKD, Gupta TKD, Pezzuto J (1999) Determination of betulinic acid in mouse blood, tumor and tissue homogenates by HPLC-electrospray Mass spectrometry. J Chromatogr B 732–331.

Tezuka Y, Stampoulis P, Banskota AH, Awale S, Tran KQ, Saiki I, Kadota S (2000) Constituents of the Vietnamese medicinal plant *Orthosiphon stamineus*. Chem Pharm Bull 48: 1711–1719.