



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 138-140
Received: 27-03-2019
Accepted: 29-04-2019

Shefali Thakkar
Medicinal and Natural Products
Laboratory, Department of
Pharmaceutical science and
Technology, Institute of
Chemical Technology, Matunga
East, Maharashtra, India

Kirti Laddha
Medicinal and Natural Products
Laboratory, Department of
Pharmaceutical science and
Technology, Institute of
Chemical Technology, Matunga
East, Maharashtra, India

Isolation and characterization of urolithin b from asphaltum

Shefali Thakkar and Kirti Laddha

Abstract

Asphaltum (vernacular name: Shilajit), is a traditional Ayurvedic medicine used as aphrodisiac. The present study was aimed for extraction, isolation, method development and validation of RP-HPLC method for bioactive compound called urolithin B (benzocoumarin) for Asphaltum. HPLC method was developed with the help of PDA detector for isolated compound. Method validation was performed as per International Conference of Harmonization guidelines for linearity, accuracy, precision, specificity, limit of detection and limit of quantification of urolithin B. Intra-day and Inter-day precisions for urolithin B was found precise as $\leq 2.0\%$ in intraday exercise & $\leq 3.0\%$ in inter day exercise. The concentration found in Asphaltum is 0.38% of urolithin B. The proposed method for estimation of urolithin B is found to be accurate, precise, reproducible and repeatable.

Keywords: Asphaltum, benzocoumarin, Shilajit, urolithin B

Introduction

Asphaltum an important Ayurvedic drug also known as *Shilajit*, is exudation found worldwide in sedimentary rocks of different formations, at altitudes between 1000 to 5000m^[1, 2]. The exudate when comes in contact with the rhizospheric metal ions or clay minerals is transformed into solidified complexes. This product is depicted as the prakrtik shilajit. *Shilajit* is called by different names in different languages. Asphalt and bitumen in English; *shilajitu*, *shilaras*, *adrija*, *girija* in Sanskrit; *shilajit* in Hindi and Bengali; hajar-ul-musa in Arabian; *momio* in Persian; *myemu* in Russian and *mumie* in German^[3]. Asphaltum was recommended for the treatment of diabetes, anemia, ulcers, wound healing, bronchial asthma, sexual dysfunction, prostate hypertrophy and in geriatric problems^[4, 5].

Shilajit can be broadly classified into non-humic and humic substances chemically that essentially originated from sedimentary rock matter^[6, 7]. The non humic organic compounds of *shilajit* comprised of low molecular weight benzocoumarin specifically urolithins^[8]. The micro flora human metabolites of dietary ellagic acid derivatives are urolithins. There are various known urolithins found in human urine^[9, 10, 11]. Urolithin A and B are found to be present in *Shilajit*; of which urolithin B was reported as a marker of Asphaltum. The medicinal importance of this mineral drug is due to urolithin B^[12].

Here isolation, identification and quantification of urolithin from Asphaltum are done. The objective of the current method was to develop inexpensive, easy and less time consuming process for isolation, identification and quantification of urolithin for laboratory as well as commercial use. Validation parameters such as system suitability, specificity, accuracy, precision, linearity, limit of detection, limit of quantification, and robustness are performed as per ICH^[13]. The established method can subsequently be used for formulation preparation as well as standardization of plant material or extract or herbal formulations containing urolithin B.

Materials and Methods

Raw Material: Asphaltum was procured from JKH exports (Mumbai) which was further authenticated and ICT/MNPRL/NV/4002 voucher specimen was deposited in Medicinal Natural products laboratory, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga, Mumbai.

Chemicals: All the chemicals used for extraction, identification were of Laboratory reagent grade and obtained from S.D. fine, India. All the solvents used for HPLC (High Performance Liquid Chromatography) analysis were of Analytical reagent grade and obtained from Rankem Limited, India.

Instrumentation: UV (Ultra-violet) spectrum was recorded on Jasco V-530 spectrophotometer. HPLC analysis was performed with a Jasco (C18 HiQ sil column-250

Correspondence
Shefali Thakkar
Medicinal and Natural Products
Laboratory, Department of
Pharmaceutical science and
Technology, Institute of
Chemical Technology, Matunga
East, Maharashtra, India

Mm X 4.6 mm i.d) Detector: Jasco MD-4010. Mass spectrum was recorded on Micro mass Q-TOF (Time of Flight) MS Mass spectrometer.

Extraction Procedure: About 100 g of *Shilajit* was subjected to solvent extraction using ethyl acetate (500ml) for 3 h in Soxhlet apparatus. The ethyl acetate extract was concentrated and precipitated using petroleum ether (60-80°C), and filtered. The precipitate (0.31 g) was further recrystallized using alcohol and petroleum ether (60-80°C) as solvent. Buff coloured crystals (0.28 g) of pure urolithin were obtained. The isolated compound was further characterized using TLC (Thin Layer Chromatography), UV spectroscopy, infrared (IR) spectroscopy and mass spectrometry. HPLC studies were carried out to quantify the same.

Standard Preparation for HPLC analysis: Stock solution of urolithin B (1 mg/ml) was prepared using methanol. From stock solution, 2 ml was transferred to a 10 ml volumetric flask and the volume was made up with methanol (200µg/ml).

Sample preparation: 5 g of *shilajit* was extracted with 50 ml methanol in Soxhlet apparatus. The extract obtained was concentrated, transferred to a 25 ml volumetric flask, and the volume made up with methanol.

Chromatographic conditions: HPLC analysis of the standard and extract was performed on HPLC Jasco, PU-1580, UV detector Jasco UV -1575, column C18 HiQ sil column-250 mm X4.6 mm i.d. Samples were injected by using a Rheodyne injector fitted with a 20 µL fixed loop. Solutions were filtered before injection. Acetonitrile: water (60: 40) was used as mobile phase and urolithin B was detected at wavelength 236 nm at a flow rate of 1.0 ml/min.

Method Validation

Validation of the developed HPLC method was done according to International Conference for Harmonisation (ICH) guidelines.

The calibration curves were constructed after triplicate determination of each standard by plotting peak area against concentration of the corresponding standard. Precision was determined as the intraday and inter day variation from three standards. Intraday precision was determined by triplicate analysis of the solutions in a single day. Inter day precision was determined by triplicate analysis of the solutions on three successive days.

The limits of detection and quantification were determined using ICH guidelines with signal to noise ratios of three and ten, respectively. The relative standard deviation (RSD) values for retention time and peak areas of the analyte for the PDA detector were employed to characterize the precision, repeatability, and stability.

Accuracy is the degree of agreement between measured value and accepted reference value. The accuracy of the method was determined by adding known amounts of the standards (80, 100, and 120%) to the Asphaltum methanolic extract and followed by analysis in duplicate. The total amount of each compound was calculated from the regression equation obtained from the corresponding calibration plot and the recovery of each compound was calculated by use of the Equation (1)

$$\text{Recovery (\%)} = \left[\frac{\text{(Amount found)}}{\text{(Amount contained + Amount added)}} \right] * 100 \quad \text{----- (1)}$$

Results and Discussion

Urolithin B was isolated from Asphaltum using simple solvent extraction process, which was found to be relatively

economical as well as less time consuming. Also, the yield obtained was about 0.38% of crude urolithin B. After recrystallization, the compound was found to be 95% pure. The isolated compound was identified by characterization using TLC, UV, IR, mass spectrometry. The retention time of urolithin B was obtained as 0.6 using chloroform: methanol (9:1) as mobile phase and derivatized using 10% alcoholic KOH solution under UV 366 nm as shown in fig 1. The UV/Viz maxima in methanol were found to be at 277 and 303 nm. IR spectrum of the isolated compound showed peaks at 1619 cm⁻¹ (C=C), 1712 cm⁻¹ (Carbonyl), 3356cm⁻¹ (Hydroxyl). Molecular ion peak at 211 *m/e* gave the molecular weight of the compound. The structure of the compound was confirmed by comparing the spectra with those in the literature [14].

A simple method was developed, optimized and validated by HPLC using PDA detector to quantify urolithin B in Asphaltum species. The composition of the mobile phase was optimized by varying the percentage of solvents in reverse phase column. Urolithin B showed maximum absorption at 236 nm. The linear regression equation was found to be $y = 16183x + 1006.2$ ($r^2 = 0.9995$). The result indicated a good linear relationship between the concentrations and peak areas. The intraday and inter day assay precision for urolithin B, expressed as the relative standard deviation, was less than 0.43%, as shown in Table 1. The accuracy of the developed method was calculated by recovery studies. Accordingly, it was clear that the method was accurate for the quantitative estimation of urolithin B as the value for relative standard deviation (RSD) was found to be within the acceptance criteria (i.e RSD < 3%). The percentage recovery of urolithin B in Asphaltum extract was found to be 98.74% respectively as shown in Table 2. The recovery was also calculated using an internal standard. The RSD was found to be < 5%. The percentage recovery of urolithin B in Asphaltum extract as compared to internal standard was found to be in the range of 96-101%.

The LOD and LOQ were determined using SD (Standard Deviation) of response and slope estimated from the calibration curve of a standard solution of urolithin B. The LOD and LOQ were 50.68 ng/ml and 153.59 ng/ml, respectively. The specificity was determined by comparing the chromatogram of the standard and the sample. The sample solution was spiked with standard solution in order to observe the interference. No interference was observed with the peak of urolithin B in the sample, therefore the method was found to be specific.

The results indicated that the method may be suitable for the estimation of urolithin B in different samples. The percentage content in Asphaltum was 0.38% respectively.

Conclusion

The developed method for the isolation of urolithin B from Asphaltum was found to be efficient on laboratory scale consuming minimum time and solvents. A simple and sensitive high performance liquid chromatographic method using PDA detector was developed for the determination of urolithin B. The validation results showed that the method provided rapid, sensitive, precise, and accurate analysis, demonstrating its application for quality control and authenticity.

Acknowledgement

We are grateful to the University Grants Commission- Basic Scientific Research (UGC-BSR) for financial support.

Conflict of Interest

None

Abbreviation

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

PDA: Photo Diode Array

**Fig 1:** TLC of isolated urolithin B under UV 366 nm after treatment with 10% alcoholic KOH**Table 1:** Precision data

Precision, relative standard deviation (%)				
Conc (µg/ml)	Intraday		Inter day	
	Retention time (min)	Peak area	Retention time (min)	Peak area
5	0.26	0.14	0.21	0.08
10	0.37	0.04	0.06	0.14
20	0.16	0.18	0.15	0.22

Table 2: Accuracy data

Recovery of urolithin B from Asphaltum extract (n=2)					
Initial Amount (µg)	Added Amount (µg)	Determined Amount (µg)	Recovery (%)	Mean (%)	Relative Standard Deviation (%)
380	304	659.1±2.46	96.35±0.25	98.74±0.46	0.47
380	380	765.23±1.19	100.65±1.12		
380	456	829.67±7.41	99.24±0.03		

References

- Ghosal S. Shilajit in perspective. Narosa Publishing house, New Delhi, 2006, 1-5.
- Ghosal S, Singh S, Kumar Y, Srivastava R, Goel R, Dey R *et al.* Anti-ulcerogenic Activity of Fulvic acids and 4'-Methoxy-6-carbomethoxybiphenyl isolated from Shilajit. *Phytotherapy Research.* 1988; 2(4):187-191.
- Wilson E, Rajamanickam V, Dubey P, Klose P, Musial F, Saha J *et al.* Review on shilajit used in traditional Indian medicine. *Journal of Ethno pharmacology.* 2011; 136:1-9.
- Khanna R, Witt M, Anwer K, Agarwal S, Koch B. Spectroscopic characterization of fulvic acids extracted from the rock exudate Shilajit. *Organic Geochemistry.* 2008; 39:1719-1724.
- Talbert R. Shilajit. *Amateria Medica Monograph,* California, 2004, 1-19.
- Agarwal S, Khanna R, Karmarkar R, Anwer K, Khar R. Shilajit: a review. *Phytotherapy research.* 2007; 21:401-405.
- Agarwal S, Khanna R, Karmarkar R, Anwer K, Khar R. Physico-chemical, spectral and thermal characterization of Shilajit, a humic substance with medicinal properties. *Asian Journal of Chemistry.* 2008; 20:209-217.
- Ghosal S. Mitochondria-targeted antioxidants. U. S. Patent. 8894993; 2007.
- Sreeram N, Henning S, Zhang Y, Suchard M, Li Z, Herber D. Pomegranate Juice Ellagitannin Metabolites Are Present in Human Plasma and Some Persist in Urine for Up to 48 Hours. *The Journal of Nutrition.* 2006; 136(10):2481-2485.
- Cerda B, Ceron J, Tomas-Barberan F, Aspin J. Repeated oral administration of high doses of pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *Journal of Agriculture and Food Chemistry.* 2003; 51:3493-3501.
- Cerda B, Espin J, Parra S, Martinez P, Tomas-Barberan F. The potent *in vitro* antioxidant ellagitannins from pomegranate juice are metabolized into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic micro flora of healthy humans. *European Journal of Nutrition.* 2004; 43:205-220.
- Ghosal S. Shilajit in perspective. Narosa Publishing house, New Delhi, 2006, 63-85.
- Guideline ICH. Validation of analytical procedures: text and methodology Q2 (R1). International Conference on Harmonization, Geneva, Switzerland, 2005, 11-12.
- Ghosal S. Isolation and characterization of its chemical constituents. In: Shilajit in Perspective. Narosa Publishing House, New Delhi, 2006, 64.