PTB Reports, 2016; 2(3): 52-55
A Multifaceted Peer Reviewed Journal in the field of Pharmacology, Toxicology and Biomedical Reports
www.ptbreports.org | www.phcog.net

Original Article

Simple and Effective HPLC Method Development and its Validation for Ursolic acid in Drug Free plasma: Application to bioanalytical studies

Selvadurai Muralidharan[1], Jayaraja kumar[2] and Venugopal Vijayan[2]

[1]Unit of Pharmaceutical Chemistry, Faculty of Pharmacy, AIMST University, MALAYSIA.
[2]Unit of Pharmaceutical Technology, Faculty of Pharmacy, AIMST University, MALAYSIA.

ABSTRACT

Objective: Simple and effective high performance liquid chromatographic (HPLC) method was developed for estimation of ursolic acid in drug free human drug free blank plasma. **Material and Methods:** The current method was used protein precipitating extraction of ursolic acid from blank plasma. Separation was achieved on reversed-phase C18 column (250×4.6 mm, 5µ) and the detection was monitored by UV detector at 220 nm. The optimized mobile phase was used acetonitrile: 0.5% triethyl amine (pH 4.0), in the ratio of 70:30 % v/v at a flow rate of 1.0 mL/min. This linearity was achieved in this method range of 25.0–150.0 ng/ml with regression coefficient range is 0.99. **Results:** The present method is suitable in terms of precise, accurate and specific during the study. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as GC–MS/MS or LC–MS/MS that are costly, time consuming and complicated rather than a simple HPLC–UV method. The present method was successfully applied for bioanalytical studies.

Key words: Bioanalytical studies, HPLC, Ursolic acid, Method Development and Validation.

Correspondence:
Dr.Selvadurai Muralidharan, Senior Lecturer, Faculty of Pharmacy, Semeling-08100, Bedong, Kedah, MALAYSIA.

Phone: +60164953594; Fax: +6044298009
Email: murali23pharm@hotmail.com
DOI: 10.5530/PTB.2016.2.8

INTRODUCTION

Ursolic acid has shown many important pharmacological activities, analgesic, anti-inflammatory, anti-tumor, anti-bacterial, anti-diabetic, hepatoprotective, anti-HIV and anti-cancer against various human cancer cell lines.[1] The computer aided prediction of biological and, toxicological activity showed ursolic acid has insulin promoting, chemoprotecting effects and not showing any predicted toxic effect for carcinogenicity and mutagenicity.[2] Because of its biological activities, ursolic acid has been the subject of interest for preclinical anti-cancer research. The pharmacokinetic properties of ursolic acid in unclear because of availability of limited number of analytical and bioanalytical methods have been reported for the quantification of ursolic acid in biological sample using analytical instruments such as High-Performance Liquid Chromatography (HPLC).[3-7] To the best of our knowledge no reports were found for the validation of ursolic acid in drug free human plasma. The objective of this study was to develop and validate for the ursolic acid using RP-HPLC.

Experimental

Materials and reagents

Acetonitrile and Triethylamine (both HPLC grade) were obtained from Merck, Darmstadt, and West Germany. Triethyl amine was obtained from systerm, Malaysia. Potassium dihydrogen phosphate was obtained from HmBG. Methanol obtained from QREC. Water HPLC grade was obtained from a Milli-QRO water purification system. A reference standard of ursolic acid and [U6753] was procured from Sigma chemicals, USA. Milli-Q water purification system supplied by Millipore was used for the preparation of the aqueous mobile phase.

Equipment

HPLC chromatographic separation was achieved on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50 µL loop volume. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan). Princeton SPHER C_{18} (250 mm x 4.6 mm i.d., 5 µ) was used for the present analysis.

Preparation of the calibration standards and quality control (QC) samples

The stock solution of ursolic acid was prepared using water and acetonitrile mixture 1:1 at a concentration of 1.0 mg/mL each. Ursolic acid working solution was used to prepare the spiking stock solutions for construction of six-point calibration curve (25.0 -150.0 ng/mL) and QC samples at three different levels (25.0, 50.0, 150.0 ng/mL). All the stock solutions were refrigerated (2-8°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 25.0 µL of respective spiking stock solutions. These were stored at -70°C until analysis.

Sample preparation for analysis

Calibration standards, validation QC samples and healthy volunteer plasma samples were prepared by adding 0.5 ml plasma to 2.0 mL centrifuge tube and added 0.5 ml (10 µg/mL) of internal standard and 0.5 mL of precipitating agent (10% v/v Trichloro acetic acid) vortexed for 2 min. The resulting solution was centrifuged at 4000 rpm for 7 min. The supernatant layer was separated and estimated by HPLC.

Chromatographic conditions

Standardization of ursolic acid by RP-HPLC method was carried out using the optimized chromatographic conditions. The mobile phase used was acetonitrile: 0.5% triethyl amine (pH 4.0) in the ratio of 70:30 % v/v at a flow rate of 1.0 mL/min. Potassium dihydrogen ortho phosphate used was 0.5 % solution in water with pH being adjusted to 4.0 with orthophosphoric acid solution. The injection volume was 25.0 µL. The UV-visible detector was set at 220 nm.

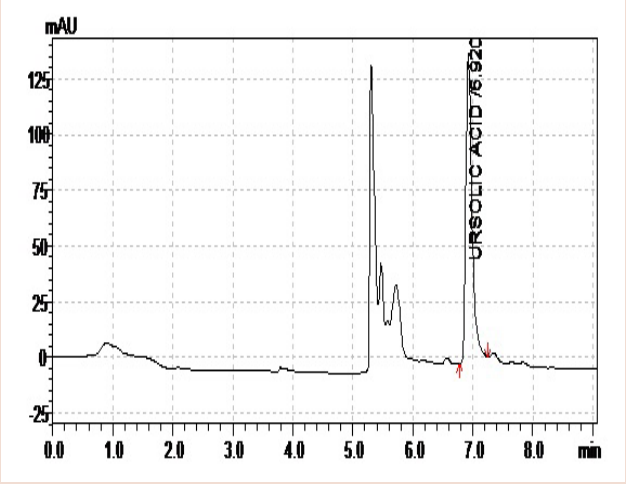


Figure 1: Typical Chromatogram of Ursolic acid Sample.

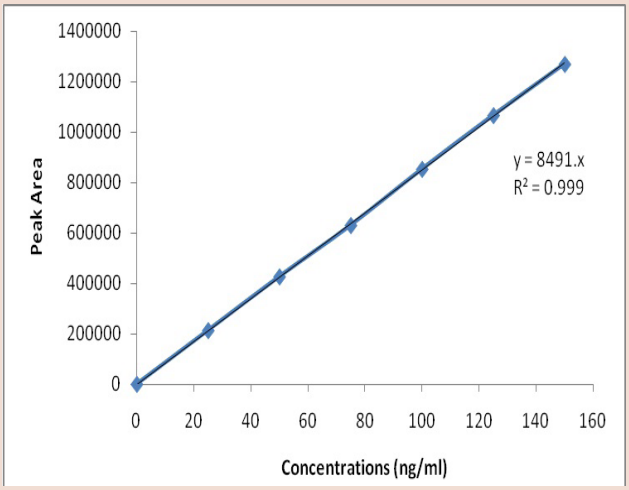


Figure 2: Calibration Curve of Ursolic acid.

Table 1: Inter-run accuracy and precision of plasma calibration standards for Ursolic acid

Standard concentration (ng/mL)	Average calculated Concentration (ng/mL)	SD
25	24.68	1.01
50	48.24	0.82
75	74.92	0.95
100	99.87	1.10
125	124.89	0.98
150	149.83	1.12

SD=Standard deviation

Table 2: Intraday and Interday accuracy and precision of Ursolic acid in plasma

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD
Inter-day (n=3)		
25	24.85	0.68
100	99.34	1.13
150	149.68	0.87
Intra-day (n=3)		
25	24.76	0.95
100	99.87	0.62
150	148.96	1.28

SD=Standard deviation

Table 3: Stability Study of Ursolic acid

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD
Bench top (n=5)		
100	98.93	1.24
150	149.18	0.82
Freeze thaw Stability (n=5)		
100	99.13	0.53
150	148.36	0.98
Long term Stability (n=5)		
100	99.68	1.10
150	148.93	1.34

SD=Standard deviation

Validation

The method was validated for selectivity, sensitivity, recovery, linearity, precision, accuracy and stability.[8]

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing ursolic acid and the internal standard with those obtained from blank samples.

Sensitivity

Sensitivity was achieved in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

Linearity

Different concentrations of standard solutions were prepared from 25.0 ng/mL to 150.0 ng/mL of ursolic acid. These solutions were analysed and the peak areas and response factors were calculated. The calibration

curve was plotted using response factor vs concentration of the standard solutions.

Precision and Accuracy

The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for five replicates at each Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs.

Stability Studies

Various stability studies were carried out. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of ursolic acid equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of ursolic acid and internal standard at 3 and 8 hours was carried out against the zero hour value. Refrigerated stock solution stability was determined at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with ursolic acid were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 h and long term stability at–70° C over four weeks. In addition, stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with ursolic acid was investigated at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for long term stability were stored in the freezer at -70° C until the time of analysis.

RESULTS AND DISCUSSION

Selectivity

No interfering endogenous compound peak was observed at the retention time of drug and internal standard. Under chromatographic conditions, the retention time of ursolic acid was 5.5 min respectively. Representative chromatogram of Lower Limit of Quantitation (LLOQ) and one study sample containing ursolic acid is shown in (Figure 1) respectively.

Sensitivity

The sensitivity of the experiment was carried out at LLOQ level.

Linearity: The calibration curves correlation coefficient was > 0.999. Calibration curve data of ursolic acid result presented in Table 1 and Figure 2.

Precision and Accuracy

Intra-day and inter-day accuracy and precision of the method were determined by Analysis of the control rat plasma spiked with ursolic acid at LLOQ, LQC, MQC and HQC. All QCs concentration was calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage relative standard deviation; the results are given in Table 2.

Stability

Stock solution analysis was performed at 100.0 ng/mL. Proper storage after for 15 days at 2-8°C and at room temperature for 6h, more than 98% of ursolic acid remained unchanged, based on peak areas in comparison with freshly prepared solution of ursolic acid. This suggests that the ursolic acid in standard solution is stable for at least 15 days when stored at 2-8 °C and for 6h at room temperature. Bench top stability of ursolic acid in plasma was investigated at LQC and HQC levels. This revealed that the ursolic acid in plasma was stable for at least 6 h at room temperature. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with ursolic acid at LQC and HQC level did not affect the stability of ursolic acid Long term stability of the ursolic acid in plasma at -70°C was also performed after 30 days of storage at LQC, HQC levels. The results of the stability studies are shown in Table 3. The average long term stability was 96.82%. The above results indicated that the ursolic acid was stable in the studied conditions.

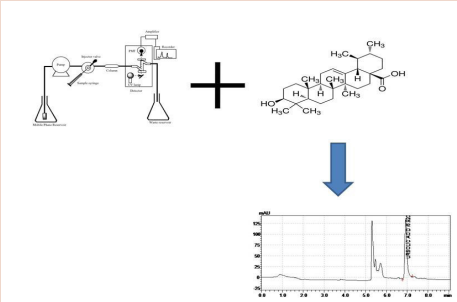
CONCLUSION

A simple and sensitive method for the determination of ursolic acid in plasma by HPLC was developed and validated. Adequate specificity, precision and accuracy of the proposed method were demonstrated over the concentration range of 25.0-150.0 ng/mL. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of ursolic acid and suitable for the bioanalytical study of Ursolic acid.

REFERENCES

1. Jesus Lago JH, Laurenti MD, Yamamoto ES, Passero LF. Antimicrobial activity of oleanolic and ursolic acids: an update. Evid Based Complement Alternat Med. 2015;620472.
2. Parasuraman S, Balamurugan S, Christapher PV, Petchi RR, Yeng WY, Sujithra J, Vijaya C. Evaluation of Antidiabetic and Antihyperlipidemic Effects of Hydroalcoholic Extract of Leaves of *Ocimum tenuiflorum* (Lamiaceae) and Prediction of Biological Activity of its Phytoconstituents. Pharmacognosy Res. 2015;7(2):156-65.
3. Janicsák G, Veres, Kállai, Máthé. Gas chromatographic method for routine determination of oleanolic and ursolic acids in medicinal plants. Chromatographia. 2003;58(5-6):295-9.
4. Wang H, Wang Z, Guo W. Comparative determination of ursolic acid and oleanolic acid of *Macrocarpium officinal* is (Sieb. et Zucc.) Nakai by RP-HPLC. Ind Crops Prod. 2008;28(3):328-32.
5. Tian S, Shi Y, Yu Q, Upur H. Determination of oleanolic acid and ursolic acid contents in *Ziziphora clinopodioides* Lam. by HPLC method. Pharmacogn Mag. 2010;6(22):116-9.
6. Zhou C, Chen K, Sun C, Chen Q, Zhang W, Li X. Determination of oleanolic acid, ursolic acid and amygdalin in the flower of *Eriobotrya japonica* Lindl. by HPLC. Biomed Chromatogr. 2007;21(7):755-6.
7. Zhang Y, Xue K, Zhao EY, Li Y, Yao L, Yang X, *et al.* Determination of oleanolic acid and ursolic acid in Chinese medicinal plants using HPLC with PAH polymeric C18. Pharmacogn Mag. 2013;9(1):S19-24.
8. FDA Guidance for Industry. Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, 2001.

PICTORIAL ABSTRACT



SUMMARY

- The developed and validated Ursolic acid HPLC method is high sensitive and its applicable to bio-analytical studies.

ABOUT AUTHOR

Dr. S. Muralidharan: Has been completed M.Pharm and Ph.D in the Department of Pharmaceutical Analysis in Dr. M.G.R. Medical university, India. Presently working as a senior lecturer in Asian Institute of medicine Science and Technology University (AIMST), Malaysia