Medicinal Chemistry Research, 19(suppl.1), S79-S79

LC-MS/MS METHOD FOR SIMULTANEOUS ANALYSIS OF CLADRIN AND EQUOL IN RAT PLASMA AND ITS APPLICATION IN PHARMACOKINETICS STUDY OF CLADRIN

Lakshmi Manickavasagam[1], Sonal Gupta[1], Smriti Mishra[1], Rakesh Maurya[2], Naibedya Chattopadhyay[3] and Girish Kumar Jain[1]

[1]Pharmacokinetics and Metabolism Division,

[2]Medicinal and Process Chemistry Division,
[3]Endocrinology Division,
Central Drug Research Institute, Lucknow226001, India.

Acknowledgement

The authors LM and SM are thankful to Council of Scientific and Industrial Research (CSIR)

and Indian Council of Medical Research (ICMR) respectively for their fellowship grants.

Abstract

A rapid and selective LC-MS/MS method for simultaneous analysis of cladrin and equol in female rat plasma has been developed and validated. The chromatographic separation was carried out on RP18 column and MS/MS analysis was performed in triple quadrupole mass spectrometer with electrospray ionization (ESI). The method was linear for the concentration range from 7.8ng/ml to 1000ng/ml for cladrin and 3.9ng/ml to 1000ng/ml for equol. The intra-day and inter-day accuracy and precision of the method were within the acceptable limits. The validated LC-MS/MS method was successfully applied for the pharmacokinetics study of cladrin at 10mg/kg in female S.D. rats.

Keywords: Cladrin; Equol; rat plasma; LC-MS/MS.

Corresponding author:
Girish Kumar Jain
email address: gk_jain@cdri.res.in
Central Drug Research Institute, Lucknow 226001, India.
Tel.: +91-522-2612411-18 (Ext-4277); fax: +91-522-2623405

Introduction

Osteoporosis is one of the common problems in postmenopausal women due to deficiency of ovarian hormone estrogen. Hormonal replacement therapy (HRT), selective estrogen receptor modulators (SERM), bisphosphonates, calcitonin are the commonly used therapeutic agents for the prevention and treatment of fractures occuring due to osteoporosis. These thereapeutic agents prevent bone loss in osteoporosis but they provide little help as far as restoration of bone mass is concerned. The search for an effective therapy to prevent bone loss and also to restore bone mass led to the identification of naturally occuring compound isoflavones for the management of osteoporosis (Arjmandi 1996, Draper 1997, Setchell 2003, Lee 2004). Daidzein, genistein and glycitein are some of the isoflavone compounds extensively studied for their positive effect on bone metabolism and prevention of bone loss (Setchell 1999, Ishimi 1999, Song 1999, Nakajima 2001).

Among natural sources, *Butea monosperma*, Family Fabaceae, is found to be rich in isoflavones and cladrin, a dimethoxyl derivative of daidzen, is one of the the active osteogenic constituents isolated from the stem bark of *Butea monosperma* (Maurya 2009). Both the daidzein and cladrin are isoflavones but they differ in their degree of substitution and O-methylation, and therefore exhibit differences in their biological activities. Daidzein exerts its activity selectively on estrogen receptors of bone while cladrin does not show any such interaction with estrogen receptors. Moreover, daidzein gets biotransformed to equol which contributes to the enhancement of estrogenic action of daidzein (Kulling 2001, Hwang 2006).

Pharmacokinetics studies of a drug helps in relating the pharmacological and toxicological action to the level of drug circulating in the body and to understand the fate of drug once it enters into the biological system (Setchell 2001). To the best of our knowledge, there is no report on the development and validation of a bioanalytical method for cladrin. Therefore, the objective of our present work is to develop and validate a selective, sensitive, accurate and precise LC-MS/MS method for simultaneous analysis of cladrin and equol in female rat plasma so as to apply it for the pharmacokinetics study of cladrin and to demonstrate whether cladrin is biotransformed to equol after its oral administration in female *S. D.* rats.

Experimental

Chemicals and reagents

Cladrin was obtained from Medicinal and Process chemistry division, Central Drug Research Institute (C.D.R.I). Equol and the internal standard (IS) 7-hydroxy isoflavone were obtained from Indofine chemicals, New Jersey, United States. HPLC grade acetonitrile and glacial acetic acid were obtained from SRL, Mumbai, India and S.D.finechem limited, Mumbai, India respectively. Purified water was prepared in our division from Millipore Milli-Q system. Female S.D. rats were procured from Laboratory animals division, C.D.R.I.

Chromatographic and Mass spectrometric conditions

Shimadzu UFLC pump with autosampler and controller connected to API 4000 Q trap, Applied Biosystems mass spectrometer with electrospray ionization (ESI) and triple quadrupole was used for HPLC-MS/MS analysis of cladrin. The mobile phase consisted of acetonitrile and 0.1% acetic acid in water (v/v) in isocratic conditions of 60:40 at the flow rate of 0.5ml/min with run time of 7 minutes. For the separation of cladrin and IS, reverse

phase C18 Phenomenex (Ultramex), 150x4.6mm, 5µm column with Phenomenex guard cartridge was used.

Mass spectrometric analysis was performed in positive ion mode with multiple reactions monitoring (MRM) for both cladrin (m/z 299.3 to 283.1) and IS (m/z 238.9 to 137.2) while equol was analyzed in negative ion mode (m/z 240.8/119.0). The polarity switching approach was applied for the quantitative analysis of cladrin in positive mode and equol in negative mode in a single LC-MS/MS run. The other parameters optimized for MS/MS includes the curtain gas, nebuliser gas and collision gas at 20, 20 and 3.00 units respectively. The declustering potential (DP) and collision energy (CE) were used at 90 and 37 for cladrin; 81 and 44 for IS and -70 and -38 for equol. Data acquisition and analysis were performed in Analyst software.

Preparation of standard and quality control samples

The stock solution of cladrin (1mg) and equol (1mg) were prepared in 5 ml of acetonitrile (200µg/ml) separately. A composite working stock solution consisting of both cladrin and equol was prepared by spiking 100µl of their corresponding stock solutions in acetonitrile to get the concentration of 20µg/ml. This solution was serially diluted with acetonitrile to get the working stock solutions at the concentration ranging from 10µg/ml to 0.078µg/ml. These working stock solutions of cladrin and equol were used to prepare calibration standards (CS) and quality control (QC) samples in blank female rat plasma. The stock solution of IS (1mg/ml) was also prepared in acetonitrile and the working solution of IS (1µg/ml) was prepared from it in acetonitrile.

Sample preparation

5µl of composite working stock solution (20µg/ml to 0.078µg/ml) was added to 95µl of blank female rat plasma, and vortexed to get the final concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9ng/ml for calibration curve of both cladrin and equol. Quality control (QC) samples at the concentration of 1000ng/ml (quality control high-QCH), 62.5ng/ml (quality control medium-QCM) and 7.8ng/ml (quality control low-QCL) were prepared in blank plasma by spiking 5µl of composite working stock solutions of cladrin and equol at 20, 1.25 and 0.156µg/ml respectively in 95µl of blank female rat plasma. To each CS and QC, 5µl of IS working solution was added to get the concentration of 50ng/ml. The extraction of analyte and IS from rat plasma was carried out by liquid-liquid extraction (LLE) method using 3 mL of diethyl ether (2x1.5mL) after vortexing for a total of 10 minutes. The supernatant organic layer was separated after centrifuging at 3500 rpm for 15 minutes and evaporating it in vacuum evaporator. The dried residue was reconstituted in 200µl of mobile phase and 20µl of it was injected for analysis.

Validation of LC-MS/MS method

Calibration curve and Linearity

The sensitivity of the method was determined from the signal to noise ratio (S/N) of the response of analyte in calibration standard. The S/N ratio should be greater than three for limit of detection (LOD) and greater than ten for lower limit of quantitation (LLOQ). The method is considered to be linear for the concentration range of calibration standards when the regression coefficient (r) value is greater than 0.99

Selectivity and Specificity

The method was evaluated for the selectivity and specificity by processing the blank female plasma obtained from five different female rats similar to the processing of calibration standard and comparing the response obtained for blank plasma with that of cladrin and equol in LLOQ of calibration standard.

Extraction recovery and matrix effect

The recovery of cladrin, equol and IS from female rat plasma by liquid-liquid extraction method using diethyl ether was calculated by comparing the peak area of the analyte in quality control samples in rat plasma with the peak area of analyte in standards prepared in mobile phase. The recovery of IS was also determined similarly at the concentration of 50ng/ml. The effect of matrix components on ionic suppression or enhancement was also evaluated by comparing the area of cladrin and equol obtained after spiking in blank plasma post extraction with the area obtained for standards prepared in mobile phase.

Accuracy and Precision

The intra-day and inter-day accuracy and precision of the method was determined for the quality control samples QCL, QCM and QCH prepared in triplicate and analyzed by LC-MS/MS. This procedure was repeated for three days. To determine accuracy, % bias was calculated and to determine precision, % C.V. was calculated by ANOVA.

Stability in plasma

The stability of cladrin and equol in female rat plasma was determined after subjecting the plasma spiked with cladrin and equol at three concentration levels of low (7.8ng/ml), medium (62.5ng/ml) and high (1000ng/ml) in triplicate, to three 24 hours cycles of freeze-thaw on storing at -80^0C. Long term stability test was also performed for both cladrin and equol in triplicate after storing the spiked plasma samples for two months at -80^0C.

Application of the method

The LC-MS/MS method developed for cladrin was applied to perform the pharmacokinetics study in female S.D. rats at the Laboratory animals division; C.D.R.I. 9 rats in the weight range of 200-225gm were grouped in to three with three rats in each group and kept for overnight fasting. Cladrin was administered orally at the dose of 10mg/kg using oral gastric needle and the blood samples (0.5ml) were collected in heparinised tubes at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 18, 24 and 30 hours by sparse sampling technique. Plasma was obtained from the blood samples by centrifuging at 2000 rpm for 10 minutes and stored at -80^0C till analysis by LC-MS/MS.

Results and discussions

Chromatography and Mass spectrometry

The LC method with 60:40 of acetonitrile and 0.1% acetic acid in MlliQ water was short with the retention time for both cladrin and equol being 4.2±0.1 minute, and IS being 4.8±0.1 minute. For quantitative analysis, the ionization of cladrin and equol was checked with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in both positive and negative modes. In APCI, the sensitivity is very less in positive and negative mode as well. For cladrin, the sensitivity and selectivity is better in ESI positive ion mode whereas for equol, negative mode is more sensitive. 7-Hydroxy isoflavone is structurally similar to cladrin and equol and it is sensitive in positive ion mode so it was selected as internal standard for the quantitative analysis of cladrin and equol.

Validation of LC-MS/MS method

Calibration curve and Linearity

The method is sensitive with 3.9ng/ml and 1.95ng/ml as the limit of detection (LOD) for cladrin and equol respectively. The LC-MS/MS method is linear over the concentration range of 7.8ng/ml to 1000ng/ml for cladrin and 3.9ng/ml to 1000ng/ml for equol with the regression coefficient (r) greater than 0.99 applying the weighting scheme of 1/x.

Selectivity and Specificity

The LC-MS/MS method developed for cladrin, equol and the IS (7-Hydroxy isoflavone) is selective and specific as there were no interferences observed in the chromatogram of the blank plasma for the MRMs selected when compared to the chromatogram of rat plasma spiked with cladrin and IS.

Recovery and matrix effects*:*

The extraction of cladrin, equol and IS by liquid-liquid extraction method using diethyl ether was effective and consistent with less matrix effects. The values for ionic suppression or enhancement were less than 15% (Table-1).

Accuracy and Precision*:*

To establish the intra-day and inter-day accuracy and precision of the method, % Bias and % C.V. were calculated for the quality control samples at the three concentration levels of high (1000ng/ml), intermediate (62.5ng/ml) and low (7.8ng/ml). The results showed that the method is accurate and precise with values of % bias and % C.V. being within the range of 20% for low concentration and 15% for other concentrations (Table-2 and 3).

Stability in plasma:

The stability of both cladrin and equol in plasma samples was evaluated by comparing the concentration calculated for the stored samples with those of freshly prepared calibration standards. The results show that cladrin and equol were stable after three freeze-thaw cycles and for 2 months on storing at -80^0C (Table-4 and 5).

Application of the method*:*

The validated LC-MS/MS method was applied to obtain the plasma concentration-time data of cladrin after oral administration of 10mg/kg dose in female S.D. rats. The pharmacokinetic properties were calculated after fitting the data by non-compartmental analysis using WINNONLIN Ver 5.1 software (Table-6). The data shows that the absorption of cladrin is fast (T_{max}=0.5h) with a good systemic exposure ($AUC_{0-\infty}$=608.7 ng.hr/ml). The circulating cladrin level in plasma could be detected up to 24hrs. Equol was not observed in any of the samples of oral pharmacokinetics study and this shows that the metabolism of cladrin does not occur similar to that of daidzein.

Conclusion:

A selective, sensitive, accurate and precise method has been developed for the simultaneous analysis of cladrin and equol in female rat plasma. The method is linear for the concentration range of 7.8ng/ml to 1000ng/ml of cladrin and 3.9ng/ml to 1000ng/ml of equol. Recovery is high for both cladrin (83.5%) and equol (76.5%) with less matrix effects. Both cladrin and equol are found to be stable at storage condition of -80^0C in freeze thaw stability

study and in long term stability study. The validated method was successfully applied for single dose oral pharmacokinetics study of cladrin in female S.D. rats. The pharmacokinetics study shows rapid absorption of cladrin which could be detected in plasma till 24 hrs and has systemic exposure of 608.7ng.hr/ml. Equol was not detected in any of the plasma samples. This also supports the fact that cladrin is not associated with any estrogenic action indicating that the methoxylated isoflavone may have better therapeutic potential as a selective acting osteogenic agents.

References:

1. Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, Kukreja SC (1996) Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 126: 161-167
2. Draper CR, Edel MJ, Dick IM, Randall AG, Martin GB, Prince RL (1997) Phytoestrogens reduce bone loss and bone resorption in oophorectomized rats. J Nutr 127: 1795-1799
3. Hwang CS, Kwak HS, Lim HJ, Lee SH, Kang YS, Choe TB, Hur HG, Han KO (2006) Isoflavone metabolites and their *in vitro* dual functions: They can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J Steroid Biochem Mol Biol 101: 246–253
4. Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T, Ikegami S (1999) Selective effects of genistein, a soybean isoflavone,on B-lymphopoiesis and bone loss caused by estrogen deficiency. Endocrinology 140**:** 1893–1900
5. Kulling SE, Honig DM, Metzler M (2001) Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. J Agric Food Chem 49: 3024-3033
6. Lee YB, Lee HJ, Kim KS, Lee JY, Nam SY, Cheon SH, Sohn HS (2004) Evaluation of the preventive effect of isoflavone extract on bone loss in ovariectomizede rats. Biosci Biotechnol Biochem 68: 1040-1045
7. Maurya R, Yadav DK, Singh G, Bhargavan B, Murthy PSN, Sahai M, Singh MM (2009) Osteogenic activity of constituents from Butea monosperma. Biorg Med Chem Lett 19: 610-613
8. Nakajima D, Kim CS, Oh TW, Yang CY, Naka T, Igawa S, Ohta F (2001) Suppressive effects of genistein dosage and resistance exercise on bone loss in ovariectomized rats. J Physiol Antrhopol 20: 285-291
9. Setchell KDR, Brown NM, Desai P, Nechemias LZ, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A, Heubi JE (2001) Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr 131: 1362S-1375S
10. Setchell KDR, Cassidy A (1999) Dietary isoflavones: Biological effects and relevance to human health. J Nutr 129: 758S–767S
11. Setchell KDR, Eva LO (2003) Dietary phytoestrogens and their effect on bone: Evidence from in vitro and in vivo, human observational and dietary intervention studies. Am J Clin Nutr 78(suppl): 593S–609S
12. Song TT, Hendrich S, Murphy PA (1999) Estrogenic activity of glycitein, a soy isoflavone. J Agric Food Chem 47: 1607-1610