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In situ absorption study of Picroside-I and Kutkoside (active principles of Picroliv) using LC-MS-MS

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ABSTRACT

In situ absorption study was carried out to predict the intestinal absorbability of picroliv, in terms of its active moieties (Picroside I and kutkoside). Picroside I and kutkoside were found to be absorbed slowly through the intestine, which may be due to the saturation of the receptors responsible for the absorption by the other constituents present in the picroliv preparation.A rapid, sensitive and selective LC-MS-MS method for the simultaneous quantitation of picroside-I and kutkoside (active constituents of herbal hepatoprotectant picroliv) was revalidated. Analysis was performed on Spheri-5, RP-18 column (10 µm, 100 x 4.6 mm i.d.) coupled with guard column using acetonitrile: triple distilled water (50:50, %v/v) as mobile phase at a flow rate of 1 ml/min. The quantitation was carried out using an API-4000 LC-MS-MS with negative electro spray ionization in multiple reaction monitoring (MRM) mode. The method was successfully applied to determine concentrations of picroside-I and kutkoside.

Key words: LC-MS-MS, hepatoprotectant, Picroside-I, Kutkoside, MRM.

INTRODUCTION

Picroliv is a herbal hepatoprotectant, from the roots and rhizomes of *Picrorhiza kurroa* Royle (Scrophulariaceae)[1]. It forms a major ingredient of many Ayurvedic preparations prescribed in the treatment of several ailments of liver and spleen, fever and asthma[2-6]. It has shown excellent hepatoprotective activity and immunomodulatory action in number of laboratory studies[7-10]. Picroside I and kutkoside are the two active constituents of picroliv (Fig. 1)[11-12]. The preparation is standardized on the basis of these components[13-17].

The amount of drug absorbed determines the pharmacological activity of the drug. Therefore, it was deemed necessary to determine the amount of drug absorbed. The absorption of drug has usually been estimated by *in situ* re-circulation studies and analysis using a sensitive and selective assay method for simultaneous quantitative estimation of picroside-I and kutkoside, which will be useful in establishing their pharmacokinetic profiles.

This paper presents for the first time, the in situ absorption data for picroside-I and kutkoside by LC-MS-MS using ESI in negative ion mode.

MATERIAL AND METHODS

Herbal Materials and Chemicals

Pure standards (>99%) of picroside-I and kutkoside were obtained from picroliv by preparative HPLC and characterized using mass and HPLC. Acetonitrile (HPLC-grade) was obtained from Thomas Bakers (Chemicals) Limited, Mumbai, India. Triple distilled water was obtained from All Quartz Distillation Unit. Urethane was procured from S.D's Lab –Chem Industry, Mumbai, India.

Liquid Chromatography

A Perkin-Elmer Series 200 HPLC system (Perkin-Elmer, USA) consisting of flow control valve, vacuum degasser, pump and auto injector was used to deliver a premixed mobile phase [acetonitrile: triple distilled water (50:50 %, v/v)] at a flow rate of 1 ml/min. The mobile phase was degassed for 20 min in an ultrasonic bath (Bransonic Cleaning

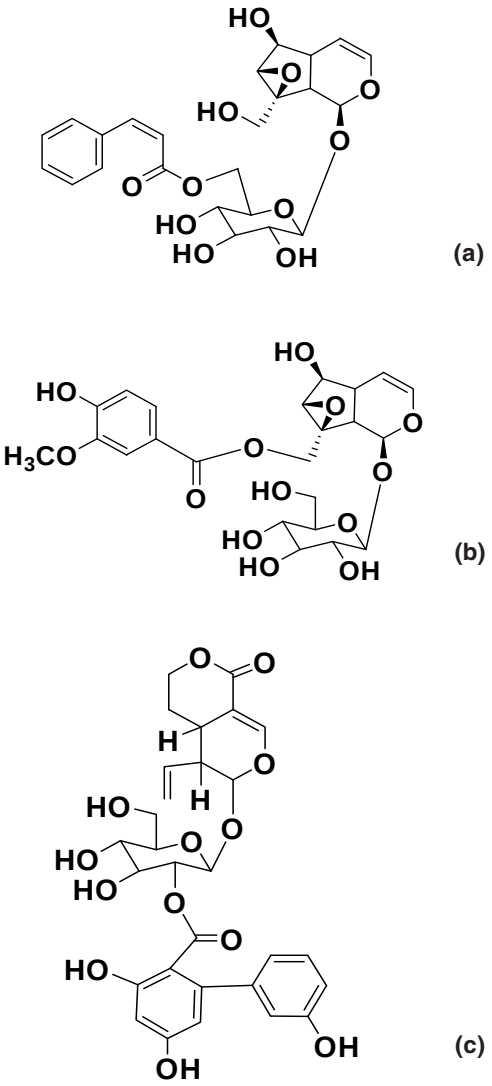

Fig. 1: Structures of (a) picroside-I, (b) kutkoside and (c) amarogentin (IS).

Equipment Company, USA) prior to the analysis. The chromatography was performed on Spheri-5, RP-18 column (10 µm, 100 x 4.6 mm i.d.) preceded with guard column. The samples (10 µl) were injected through a Perkin-Elmer auto injector onto the mass spectrometer. The total effluent from the column was split such that half was injected onto the ESI.

Mass Spectrometry Analysis

The API-4000 (Applied Biosystems, Toronto, Canada) mass spectrometer was operated using a standard ESI source coupled with a LC separation system. Analyst 1.4 software (Applied Biosystems, Toronto, Canada) was used for the control of equipment, acquisition and data analysis. Each analyte were prepared in acetonitrile: triple distilled water (70:30 %, v/v). Zero air was used as nebulizing gas (GS 1, 25 psi) and nitrogen as curtain gas (20 psi). MS scan was performed in negative ion modes.

Standard and Working Solutions

Individual standard stock solutions of picroside-I (1000 µg/ml) and kutkoside (1000 µg/ml) were prepared by accurately weighing 5 mg of each compound in volumetric flasks and volume was made up to 5 ml with acetonitrile: triple distilled water (70:30 %, v/v). Stock solution of internal standard (IS) amarogentin (1000 µg/ml) was prepared by dissolving 5 mg of IS in 5 ml of acetonitrile. Mixed working stock solution (MWS) of picroside-I (2 µg/ml) and kutkoside (2 µg/ml) was prepared in acetonitrile: triple distilled water (70:30 %, v/v) and working stock for IS (2 µg/ml) was prepared in acetonitrile.

Sorenson buffer

Potassium dihydrogen phosphate solution (0.067M) was prepared by dissolving 9.112 gm of KH_2PO_4 in 1000 ml TDW. Disodium hydrogen phosphate solution (0.067M) was prepared by dissolving 11.93 gm of $Na_2HPO_4.2H_2O$ in 1000 ml TDW. Sorenson buffer (pH 7.4) was prepared by mixing 19.2 ml of 0.067M KH_2PO_4 and 80.8 ml of 0.067M $Na_2HPO_4.2H_2O$ and adjusted to the pH 7.4, then filtered through 0.22 µ filter [18].

Perfusion solution

Prepared by dissolving 8.48 gm of NaCl, 0.34 gm of KCl, 0.14 gm of $CaCl_2$ and 0.78 gm of $NaH_2PO_4.2H_2O$ in 1000 ml TDW. The solution was sonicated for 5 min. and then filtered through 0.22µ filter[18].

Method Revalidation

The method was revalidated in terms of linearity, specificity, limit of detection (LOD) and lowest limit of quantitation (LLOQ), accuracy and precision. Both picroside-I and kutkoside gave two prominent product ions during MS-MS experiments. The sum of the responses obtained for the two

intense transitions for picroside-I and kutkoside were considered in method revalidation. The method was revalidated in terms of establishing linearity, specificity, sensitivity, accuracy and precision. Linearity was observed over a concentration range of 1.56-200 ng/ml with a limit of detection (LOD) of 0.5 ng/ml for both analytes. Accuracy and precision of the revalidated method were within the acceptable limits.

Method Application to In Situ Absorption Study

Levels of picroside-I and kutkoside were determined at various time points during *in situ* absorption study in male wistar rat and stability study. The formulation of picroliv was prepared in Sorenson buffer for *in situ* absorption study. Samples were collected and analysis was carried out using the above validated LC-MS-MS method.

RESULTS AND DISCUSSION

Optimization of LC Conditions

Spheri-5, RP-18 column (10 mm, 100 x 4.6 mm i.d.) with ACN : TDW (50:50 %, v/v) at 1.0 ml/min elutes picroside-1, kutkoside and amarogentin at 1.39, 1.37 and 1.42 min respectively with a run time of 4 min.

Optimization of LC-MS-MS Conditions

In negative mode of ionization, strong $[M-H]^-$ signals of picroside I, kutkoside as well as IS were observed at m/z, 491 511 and 585, respectively. The optimized declustering potentials for picroside I, kutkoside and IS in negative ion mode were found to be 55, 100 and 90 V respectively.

With the optimized MS conditions, MRM mode was explored for the two analytes. Product ions were generated through fragmentation of the molecular ions by collision-activated dissociation (CAD), using nitrogen as collision gas. The product ion spectra of components in negative ion mode are given in Fig. 2. Utilizing this information, an MRM method for quantitation was developed and the collision energy (CE) was optimized for different transitions. Nebulizing gas (GS 1), turbo gas (GS 2), curtain gas and temperature were set to 25, 40, 20 psi and 500°C, respectively.

The product ions for picroside-I (m/z 199 and 147) and kutkoside (m/z, 235 and 167) were used under the optimized conditions for quantitation with a single product ion for IS (m/z, 227). The selection of amarogentin, a seco-iridoid glycoside as IS was based on its structural similarity with picroside-I and Kutkoside. Hence, amarogentin was expected to behave closely in terms of ionization giving better results for linearity and quantitation.

The corresponding final MRM conditions for picroside-I, kutkoside and IS are summarized in Table 1. Analytical curves of picroside-I and kutkoside (1.56-200 ng/ml) showed that the peak area ratio of both components with IS varied linearly.

In situ Absorption Study

The method was successfully applied to determine levels of picroside-I and kutkoside for *in situ* absorption study. The results are shown in Table 2. Concentration-time profile of picroside-I and kutkoside at different time is shown in Fig. 3 and Fig. 4.

In situ absorption studies with male *wistar* rats using recirculation technique could evaluate the absorption characteristics of picroliv. There was no

Table 1: MRM conditions for picroside-I, kutkoside and IS.

Analyte	$[M-H]^-$	Product ion/s	Declustering Potential (V)	Collision energy (eV)
Picroside-I	491	199	55	14
		147		25
Kutkoside	511	235	100	30
		167		32
Amarogentin (IS)	585	227	90	35

Table 2: ***In situ*** **absorption study of picroliv (500 ng/ml) at pH 7.4**

Time (min)	Log Conc.			
	Kutkoside		Picroside-1	
	Mean	SD	Mean	SD
0	1.353	0.004	1.752	0.017
5	1.297	0.019	1.694	0.026
10	1.361	0.010	1.759	0.017
15	1.292	0.005	1.701	0.009
20	1.308	0.012	1.718	0.010
30	1.286	0.003	1.680	0.004
40	1.248	0.012	1.656	0.009
50	1.278	0.003	1.682	0.008
60	1.285	0.009	1.685	0.019
Mean K_a (hr^{-1})		0.164± 0.028	0.202± 0.011	
Mean Absorption $T_{1/2}$(hr)		4.313± 0.775	3.438±0.198	

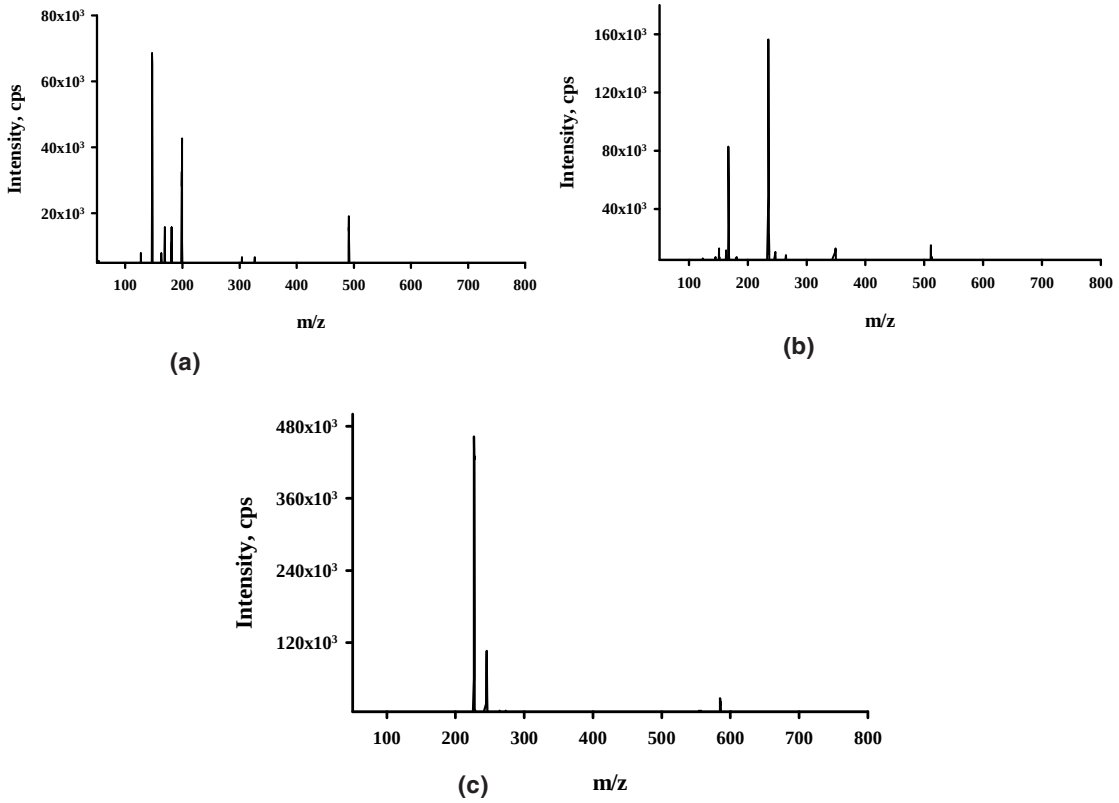

Fig. 2: MS-MS spectra of (a) picroside-I (b) kutkoside and (c) amarogentin (IS), showing prominent precursor to product ion transitions.

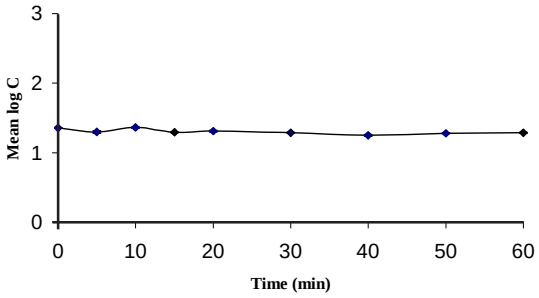

Fig. 3: Mean log C ± SD vs. time curve of Kutkoside

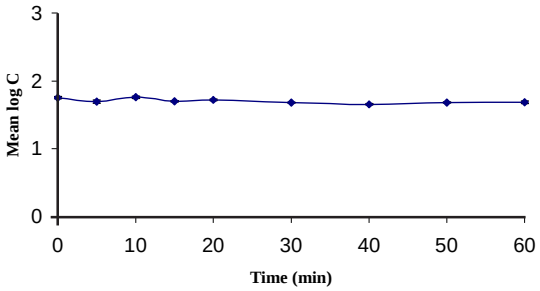

Fig. 4: Mean log C ± SD vs. time curve of Picroside-1

adsorption of Picroliv in the cannula used for the experiment. Picroliv (500 µg/ml) had shown an absorption rate of 0.164 ± 0.028/hr and 0.202 ± 0.011/hr of kutkoside and picroside-1 respectively, with a half life of 4.313 ± 0.775 hr and 3.438 ± 0.198 hr of kutkoside and picroside-1 respectively, which indicate the very slow absorption of the drug.

Conclusion

Analytical method validation is a tool, which ensures the reliability and authenticity of the assay data and pharmacokinetic studies. The LC-MS-MS was found to be rapid, sensitive, selective and linear for the analysis of picroside-1 and kutkoside with LOQ 1.56 ng/ml. Accuracy and precision of the method were within acceptable limits. The method is suitable for conducting stability and *in situ* absorption studies of picroliv.

In situ absorption studies with male *wistar* rats using recirculation technique could evaluate the absorption characteristics of picroliv. There was no adsorption of Picroliv in the cannula used for the experiment.

Picroliv (500 mg/ml) had shown an absorption rate of 0.164 ± 0.028/hr and 0.202 ± 0.011/hr of kutkoside and picroside-1 respectively, with a half life of 4.313 ± 0.775 hr and 3.438 ± 0.198 hr of kutkoside and picroside-1 respectively, which indicate the very slow absorption of the drug.

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