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A sensitive and selective LC–MS–MS method for simultaneous determination of picroside-I and kutkoside (active principles of herbal preparation picroliv) using solid phase extraction in rabbit plasma: Application to pharmacokinetic study☆

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Abstract

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 developed and validated in rabbit plasma. The analytes and internal standard (Amarogentin) were extracted using Oasis® HLB solid phase extraction cartridges. Analysis was performed on Spheri RP-18 column (10 μm, 100 mm × 4.6 mm i.d.) coupled with guard column using acetonitrile:MilliQ water (50:50, %v/v) as mobile phase at a flow rate of 1 ml/min with a retention time of 1.39, 1.33 and 1.42 min for picroside-I, kutkoside and amarogentin, respectively. The quantitation was carried out using an API-4000 LC–MS–MS with negative electro spray ionization in multiple reaction monitoring (MRM) mode. The precursor to product ion transitions for picroside-I, kutkoside and amarogentin were *m/z* 491 > 147, 199; 511 > 167, 235; 585 > 227, respectively. The method was validated in terms of establishing linearity, specificity, sensitivity, recovery, accuracy and precision (within- and between-assay variation), freeze–thaw (f–t), auto injector and dry residue stability. Linearity in plasma was observed over a concentration range of 1.56–400 ng/ml with a limit of detection (LOD) of 0.5 ng/ml for both analytes. The recoveries from spiked control samples were >60 and >70% for picroside-I and kutkoside, respectively. Accuracy and precision of the validated method were within the acceptable limits of <20% at low and <15% at other concentrations. The analytes were stable after three freeze–thaw cycles and their dry residues were stable at −60 °C for 15 days. The method was successfully applied to determine concentrations of picroside-I and kutkoside post i.v. bolus administration of picroliv in rabbit.

Keywords: LC–MS–MS; Hepatoprotectant; Picroside-I; Kutkoside; Rabbit plasma

1. Introduction

Picroliv is a herbal hepatoprotectant developed by Central Drug Research Institute, Lucknow, 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, 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].

A comprehensive method for fingerprinting and pattern profiling of picroliv is reported recently using LC–MS–MS [18]. However, there is no bioanalytical method available for simultaneous determination of picroside-I and kutkoside. An HPLC-UV method for determination of picroside-I alone is reported in rabbit plasma (0.5 ml) with 50 ng/ml as LLOQ [19]. Therefore, it was deemed necessary to develop

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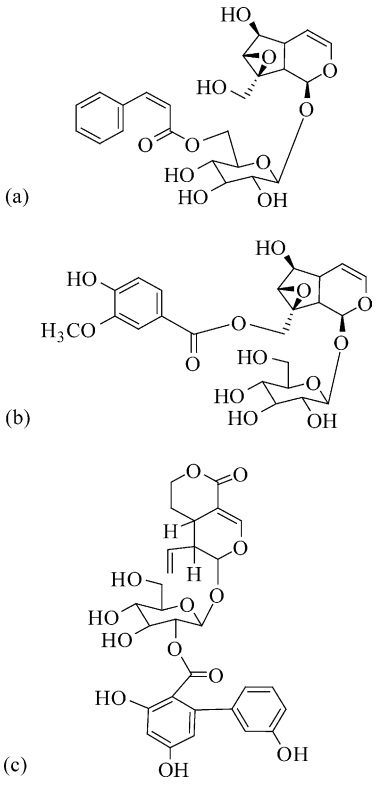

Fig. 1. Structures of (a) picroside-I, (b) kutkoside and (c) amarogentin (IS).

a sensitive and selective assay method for simultaneous quantitative estimation of picroside-I and kutkoside in biological fluids, which will be useful in establishing their pharmacokinetic profiles.

Mass spectrometry is renowned for its sensitivity and selectivity, which has many advantages in comparison to traditional HPLC-UV/flourimetry [20]. The development of the atmospheric pressure ionization (API) source is an important breakthrough, and the use of LC–MS–MS with an electro spray ionization (ESI)/atmospheric pressure chemical ionization (APCI) interface is the technique that is currently considered the method of choice for pharmacokinetic studies [21–25].

This paper presents for the first time, development and validation of an assay method for simultaneous estimation of picroside-I and kutkoside by LC–MS–MS using ESI in negative ion mode. The method was successfully applied to generate pharmacokinetic profile of picroside-I and kutkoside post i.v. administration of picroliv in rabbit.

2. Experimental

2.1. Herbal materials and chemicals

Pure standards (>99%) of picroside-I and kutkoside were obtained in house from picroliv by preparative HPLC and characterized using tandem mass and HPLC. Picroliv was obtained from Pharmaceutics Division and amarogentin (internal standard, purity > 99%) was obtained from Medicinal and Process Chemistry Division of the institute. Acetonitrile (HPLC-grade) and methanol (HPLC-grade) were obtained from Thomas Bakers (Chemicals) Limited, Mumbai, India. Ammonium acetate (GR-grade) and glacial acetic acid (HPLC-grade) was obtained from E Merck Ltd., Mumbai, India. Purified water was obtained from $MilliQ_{PLUS}$ system (Millipore, USA). Drug-free heparinised plasma was obtained from young, healthy male rabbits housed in the Laboratory Animal Services Division of the institute. All ethical guidelines for maintenance and experimental studies with rabbits were followed.

2.2. Liquid chromatography

A Perkin-Elmer Series 200 HPLC system (PerkinElmer, USA) consisting of flow control valve, vacuum degasser (Series 200 vacuum degasser), PerkinElmer, pump (Series 200 Pump, PerkinElmer) and autosampler (Series 200 Autosampler, PerkinElmer) were used to deliver a premixed mobile phase [acetonitrile:Milli-Q 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 Equipment Company, USA) prior to the analysis. The chromatography was performed on Spheri-5 RP-18 column (10 μm, 100 mm × 4.6 mm i.d., Pierce Chemical Company, Rockford, USA) 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.

2.3. Mass spectrometry analysis

The API-4000 LC–MS–MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer was operated using a standard ESI source coupled with a LC separation system. Analyst 1.4 software (Applied Biosystems/MDS SCIEX, Toronto) was used for the control of equipment, acquisition and data analysis. For optimization of MS parameters, approximately equimolar solutions of each analyte were prepared in acetonitrile:MilliQ water (70:30, %v/v). Zero air was used as nebulizing gas (GS 1, 25 psi) and nitrogen as curtain gas (20 psi). Declustering potential was optimized and ion spray voltage, nebulizing and curtain gas conditions were used in default mode. The dwell time and mass width were set at 0.2 s and ± 10 amu and MS scan was performed in both positive and negative ion modes.

For MS–MS optimization, the optimized declustering potentials were used with nitrogen as the collision gas to obtain prominent product ions. Collision energies for fragmentation of precursor to product ions were optimized by flow injection analysis, varying the collision energies for each of the analytes to obtain the most intense precursor to product ion transitions. The established operating conditions of MS–MS in MRM mode are summarized in Table 1.

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

2.4. 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:MilliQ 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:MilliQ water (70:30, %v/v) and working stock for IS (2 μg/ml) was prepared in acetonitrile.

MWS was used in the preparation of analytical and calibration standards. Analytical standards were prepared from MWS by diluting it with acetonitrile:MilliQ water (70:30, %v/v) to obtain a concentration range of 1.56–400 ng/ml for picroside-I and kutkoside. IS was spiked to each analytical standard to achieve a concentration of 50 ng/ml. All stock and mixed working stock solutions were prepared prior to start of validation and stored at 4 °C. These solutions were found to be stable over 4 months and used for the complete method validation programme.

2.5. Calibration and quality control samples

Calibration standards were prepared from MWS over a range of 1.56–400 ng/ml for picroside-I and kutkoside. IS (12.5 μl) was added to the plasma samples (0.1 ml) before solid phase extraction resulting in same concentration as in analytical standards. Quality control (QC) samples at four different concentration levels [L (3.125 ng/ml), M1 (12.5 ng/ml), M2 (25 ng/ml) and H (200 ng/ml)] were prepared in pentaplet once each day and were used to assess accuracy and precision of the assay method. The calibration standards and quality control samples were prepared fresh on each day of validation.

2.6. Sample preparation

Aliquots of 0.1 ml of plasma were vortex mixed with IS (12.5 μl). The samples were loaded on Oasis® HLB 1 cm^3, SPE cartridges (Waters Corporation, Milford, MA, USA) preconditioned with methanol (1 ml) and washing with MilliQ water (1 ml). The samples were passed through SPE under vacuum (10 psi). The SPE tubes were washed with 1 ml 25% methanol in ammonium acetate buffer (10 mM, pH 4) and finally analytes were eluted using 1 ml methanol. The organic phase was evaporated in savant speed vac concentrator and the dry residue was reconstituted in 100 μl of acetonitrile:MilliQ water (70:30, %v/v). The samples (10 μl) were injected onto the LC–MS–MS system. The calibration curve was obtained by weighted $1/x^2$ regression of the peak area ratios (picroside-I versus IS and kutkoside versus IS), versus nominal concentration using Analyst 1.4 quantitation wizard.

2.7. Method validation

The method was validated in terms of linearity, specificity, limit of detection (LOD) and lowest limit of quantitation (LLOQ), recovery, within run and between run accuracy and precision determination, freeze–thaw (f–t), auto injector and dry residue stability. The accuracy and precision studies and recoveries determination were carried out in pentaplet for 5 different days at low (L), medium (M1, M2) and High (H) concentration levels. 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 validation. The present method was extrapolated in rat plasma following partial validation.

2.7.1. Specificity

The specificity was defined as non-interference in the regions of interest with the endogenous substances, in the determination of the concentration. Six different lots of blank plasma were tested for interference or matrix effects.

2.7.2. LOD and LLOQ

The LOD of assay method for picroside-I and kutkoside was the quantity in plasma after sample clean-up corresponding to three times the baseline noise ($S/N > 3$). The LLOQ was defined as the concentration, which was quantified with less than 20% variation in precision.

2.7.3. Recovery

The recoveries of analytes were calculated using quality control samples at four concentration levels by comparing the observed concentration with the spiked concentration using

the following formula:

$$\%\text{Recovery} = \frac{\text{Observed concentration}}{\text{Spiked concentration}} \times 100$$

2.7.4. Accuracy and precision

The accuracy was determined by injection of calibration samples and four QC samples in pentaplet for 5 different days. The precision was determined by one-way ANOVA as within and between %R.S.D. The accuracy was expressed as %Bias:

$$\%\text{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

2.7.5. Stability studies

2.7.5.1. Freeze–thaw stability. QC samples at low (L) and high concentration (H) in pentaplet (three sets) were stored at −60 °C in glass tubes. One set of samples at each concentration was analyzed immediately after spiking which served as the reference concentration. The other samples were analyzed after 1, 2 and 3 f–t cycles over a period of 15 days. Thawing was achieved by keeping the stored samples undisturbed at ambient temperature for 30 min. The change in concentration during the f–t cycles were determined by comparing the concentrations after thawing with the reference concentration and was expressed as percent deviation from the reference concentration.

2.7.5.2. Dry residue stability. QC samples at low (L) and high concentration (H) in pentaplet (two sets) were processed and one set was stored at −60 °C in glass tubes. One set of samples at each concentration was analyzed immediately which served as the reference concentration. The other set was analyzed after 15 days. The change in concentration was determined by comparing the concentrations observed after 15 days with the reference concentration and was expressed as percent deviation.

2.7.5.3. Auto injector stability. Replicates ($n = 5$) of the spiked samples at low (L) and high concentration (H) in pentaplet were processed and reconstituted at the same time. The reconstituted samples were placed in the auto injector, and one set was injected immediately and the other after 12 h. The percent deviation for these two concentration levels was calculated.

2.8. Method application to pharmacokinetic study

The method was successfully applied to determine the levels of picroside-I and kutkoside following i.v. bolus dose of 30 mg/kg of picroliv in male rabbit ($n = 1$). The formulation of picroliv was prepared in saline and injected through marginal ear vein. Blood samples were collected at 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 post i.v. dose, plasma separated and stored at −60 °C pending analysis.

3. Results and discussion

3.1. Optimization of LC conditions

For LC condition optimization, Spheri RP-18 column (10 μm, 100 mm × 4.6 mm i.d.) and Phenomenex Ultremex-Cyno (5 μm, 150 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) with guard column were tried. There was no appreciable difference in peak shapes and resolution pattern on both columns under similar chromatographic conditions, though it was expected that cyno might retain these components giving a better resolution due to their polar nature. Therefore, Spheri RP-18 column (10 μm, 100 mm × 4.6 mm i.d.) was tried with different mobile phase conditions. Reducing the acetonitrile content resulted in broadening of peaks without affecting the resolution. Hence, RP-18 column with acetonitrile:MilliQ water (50:50, %v/v) at 1.0 ml/min was selected. Under these conditions, picroside-I, kutkoside and amarogentin (IS) eluted at 1.39, 1.37 and 1.42 min, respectively with a run time of 4 min.

3.2. Optimization of LC–MS–MS conditions

In positive ion mode, picroside-I (MW-492) and kutkoside (MW-512) gave sodium adduct at *m*/*z* 515 and 535, respectively. Protonated and potassium adducts, though weak in intensity were also observed for picroside I and kutkoside at *m*/*z* 493, 531 and *m*/*z* 513, 551, respectively. Owing to division of signal between sodium, potassium and hydrogen ions, the sensitivity was compromised. Hence, possibility of ammonium adduct was explored but no adducts could be observed. 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. Thus, it was decided to utilize negative ion mode for detection and quantitation of picroside-I and kutkoside along with IS. 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. Initially, 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 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

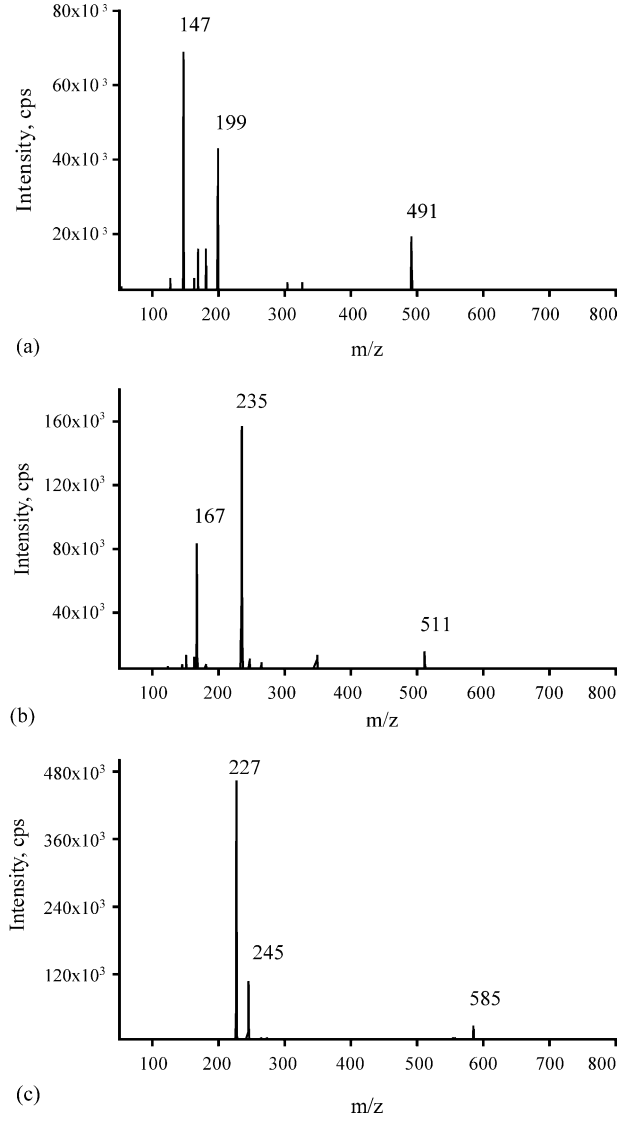

Fig. 2. MS–MS spectra of (a) picroside-I (b) kutkoside and (c) amarogentin (IS), showing prominent precursor to product ion transitions.

picroside-I, kutkoside and IS are summarized in Table 1. Analytical curves of picroside-I and kutkoside (1.56–400 ng/ml) showed that the peak area ratio of both components with IS varied linearly.

3.3. Extraction and matrix suppression

The next step was to develop an efficient sample clean up devoid of matrix suppression and interference from endogenous plasma components for estimation of the analytes in rabbit plasma. Protein precipitation using acetonitrile could not be followed due to severe matrix suppression (∼70%). Liquid–liquid extraction using hexane alone and with different combinations of hexane (90–10, %v/v):ethyl acetate (10–90, %v/v) and hexane (98–95%):isopropyl alcohol (2–5, %v/v) resulted in either low recovery, high matrix suppression or both. Extraction using diethyl ether also resulted in low recoveries (30–55%) and high matrix suppression was observed. LC modifications by incorporating ammonium acetate buffer (10 mM, pH 6.0) with different compositions of mobile phase did not help much in matrix suppression problem. Therefore, solid phase extraction (SPE) using OASIS® HLB cartridges with their standard protocol was tried. The recoveries of picroside-I, kutkoside and IS were between 85 and 95% but there was significant suppression of ionization response of both picroside-I and kutkoside due to the plasma components. Different percentages of methanol (7.5, 10, 25, 40) washings did not help to eliminate matrix suppression. It was observed that washing with 1 ml of methanol ammonium acetate buffer (pH 4, 10 mM) solution [25:75, %v/v] eliminated matrix suppression completely. However, recoveries of analytes were compromised with recoveries ≥60% and ≥75% for picroside-I and kutkoside, respectively (Table 2). This was acceptable, as the LLOQ of analytes was 1.56 ng/ml, which is sensitive enough to detect analytes concentration in low plasma volumes (0.1 ml).

3.4. Assay validation

3.4.1. Linearity and calibration standards

The peak area ratios of picroside-I and kutkoside to IS in plasma varied linearly with the concentration over the range 1.56–400 ng/ml. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting factor for both the analytes. Moreover, the regression coefficient of both the analytes were greater than 0.998.

3.4.2. Specificity

High specificity was achieved using tandem mass spectrometry by monitoring the dissociation of the precursor ions to their respective product ions. LC–MS–MS analysis of eight lots of blank plasma samples indicated no endogenous peaks interference with analytes and IS. Representative chromatograms of extracted blank rabbit plasma fortified with picroside-I, kutkoside and IS overlaid with extracted blank rabbit plasma are shown in Fig. 3.

Table 2
Mean recoveries of picroside-I and kutkoside in spiked rabbit plasma

Concentration (ng/ml)	Absolute recovery (mean ± S.D., $n = 5$) (%)	
	Picroside-I	Kutkoside
3.13	67.78 ± 11.64	80.54 ± 8.31
12.5	60.32 ± 8.63	83.14 ± 9.22
25	63.70 ± 10.90	81.73 ± 7.04
200	58.53 ± 5.37	86.50 ± 7.74

Table 3
Accuracy (%Bias) and precision (%R.S.D.) of picroside-I and kutkoside in rabbit plasma

Analyte	Concentration (ng/ml)	%Bias		%R.S.D.	
		Intra-batch	Inter-batch	Intra-batch	Inter-batch
Picroside-I	3.13	−2.59	−0.95	4.81	4.46
	12.5	−5.86	−5.18	6.46	14.05
	25	−9.69	−10.58	5.71	10.64
	200	−1.70	−3.00	5.48	13.27
Kutkoside	3.13	4.65	4.27	3.38	12.06
	12.5	6.03	5.77	3.04	9.01
	25	10.93	11.66	3.67	4.97
	200	7.51	7.49	2.87	8.48

3.4.3. LOD and LLOQ

The method was sensitive with a LLOQ of 1.56 ng/ml for picroside-I and Kutkoside. The LOD for both analytes was 0.5 ng/ml. There was an increase in sensitivity by a factor of 32 for picroside-I, with a decrease in sample processing volumes by fivefold leading to higher sensitivity and throughput from the earlier reported HPLC-UV method [19]. The factor 32 indicates the decrease in LOQ of picroside-I from 50 ng/ml in the earlier reported method [19] to 1.56 ng/ml in the present method.

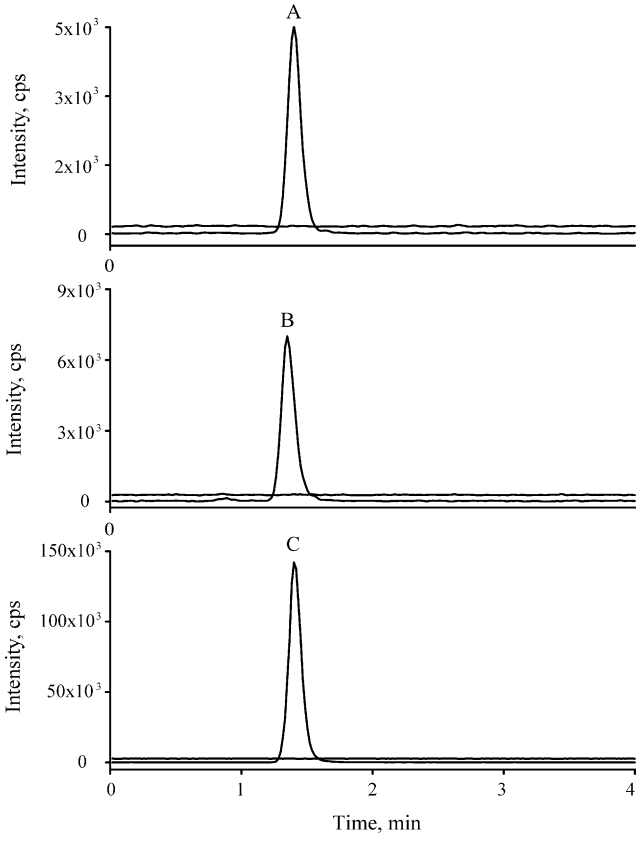

Fig. 3. Representative chromatograms of (a) picroside-I (1.56 ng/ml) (b) kutkoside (1.56 ng/ml) (c) IS in fortified blank rabbit plasma overlaid with extracted blank rabbit plasma.

3.4.4. Recovery

The average recoveries for picroside-I and kutkoside ranged from 59 to 68% and 75 to 86%, respectively. Mean recoveries of picroside-I and kutkoside at four QC levels are shown in Table 2.

3.4.5. Accuracy and precision

The overall percentage bias and R.S.D. at the four concentrations is presented in Table 3. The results show that the bioanalytical method is accurate and precise with deviation ≤20% at LLOQ and ≤15% at all other concentration levels [26].

3.4.6. Stability studies

3.4.6.1. Freeze–thaw stability. The deviation observed after one, two and three f–t cycles was within the acceptable limits of ≤ 20% at low and ≤ 15% at high concentration levels for picroside-I and Kutkoside (Table 4) [26]. It should be noted that these variations represent both stability parameters and the inherent inter- and intra-batch variations.

3.4.6.2. Dry residue stability. The dry residue samples stored at −60 °C after extraction were found to be stable for 15 days with percent deviation ≤10% at all concentration levels.

3.4.6.3. Auto injector stability. There was no significant difference between the responses of standards at time 0 and after 12 h kept at auto injector at 4 °C in terms of %CV (≤5%) for both picroside-I and kutkoside, indicating sufficient stability in auto injector for completing large set of analysis.

Table 4
Freeze–thaw (f–t) stability data for picroside-I and kutkoside.

Nominal concentration (ng/ml)	f–t 1	f–t 2	f–t 3
Picroside-I (percent deviation from reference)			
3.13	2.31	−1.54	−5.27
200	−8.61	−9.15	−14.21
Kutkoside (percent deviation from reference)			
3.13	2.75	−19.21	−2.75
200	9.75	−14.30	−12.5

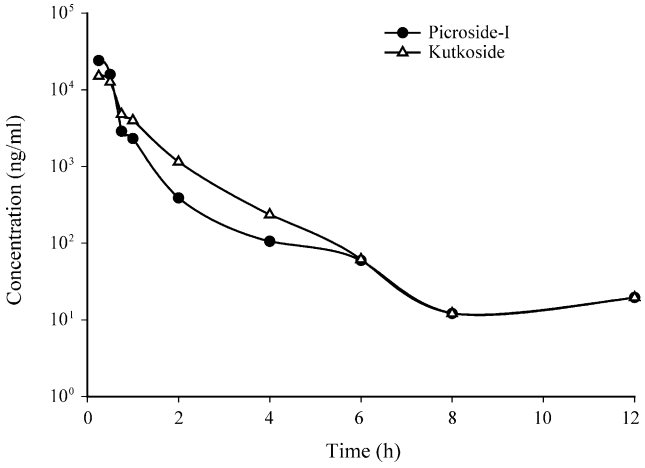

Fig. 4. Concentration-time profile ($n = 1$) of picroside-I and kutkoside after 30 mg/kg i.v. bolus administration of picroliv in rabbit.

3.5. Application to pharmacokinetic study

The method was applied to determine levels of picroside-I and kutkoside post i.v. bolus dosing in rabbit ($n = 1$). Since the hepatoprotective action of picroliv has been evaluated in rabbits in our institute (unpublished data), rabbit was chosen as the suitable animal model. Moreover, we will get the PK parameters based on single animal study, which will minimize the variation. Intravenous administration was chosen as it provides important information about the PK parameters like V_d (volume of distribution), Cl (Clearence) and half life of the drug candidates. Plasma concentration-time profile of picroside-I and kutkoside after 30 mg/kg i.v. bolus administration of picroliv is shown in Fig. 4. However, the PK parameters are not included in the present work as the study was carried out only in single animal just to show the application of the present method. Furthermore, complete pharmacokinetics studies in rabbits are in progress to obtain realistic PK parameters.

4. Conclusion

An LC–MS–MS bioanalytical method for simultaneous determination of picroside-I and kutkoside was developed and validated in rabbit plasma. The method was rapid, sensitive, selective and linearity was observed over a concentration range of 1.56–400 ng/ml for both analytes. The SPE extraction method gave good and consistent recoveries for analytes and IS from rabbit plasma, with no detected interference and matrix suppression. The results of validation indicate that method can be considered suitable for carrying out preclinical pharmacokinetic studies of picroliv in rabbits. Following partial validation, the present method can be adopted for the plasma of other experimental models also. The present assay method was extrapolated to rat plasma by performing partial validation.

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