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ORIGINAL RESEARCH PAPER

Analytical method development for exploring pharmacokinetic profile of ursolic acid in rat tissues by high-performance thin-layer chromatography

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Abstract
A simple and rapid high-performance thin-layer chromatographic (HPTLC) determination of ursolic acid (UA) in tissue is necessary for its utilization in therapeutic survey. In the present work, an attempt has been made to develop a method for the estimation of UA disposited in the liver, heart, spleen, lung, kidney, brain, stomach, intestine, ovary and uterine tissues at various requisite time intervals after oral administration. The biosamples were prepared based on protein precipitation by methanol with a recovery ranged from 91.38% to 98.47%. The mobile phase with the best resolution was achieved with toluene−ethyl acetate−methanol (4:1:1, *V/V*) and toluene−ethyl acetate−methanol−formic acid (4:1:0.7:1, *V/V*) solvent systems for normal and amine-coated plates, respectively. The mobile phase resolved UA efficiently from other constituents with R_F of 0.60 and 0.50 in normal and amine-coated TLC plates. The method exhibited satisfactory linearity ($r^2 > 0.99$) in the range of 9.6 to 38.4 µg/mL, i.e., 96 to 384 ng/spot. It was observed that UA mainly disposited in liver and uterine tissues indicating biliary extraction. This assay range is adequate for analyzing disposited UA after a single oral dose (40 mg/kg) administration for which the lower limit of quantification (LLOQ) values in different biological matrices are in the range of 2.63–7.88 nmol/mL. For routine quality control analysis, this method showed reliability by a rapid, reproducible, cheap and eco-friendly manner.

Keywords High-performance thin-layer chromatography (HPTLC) · Ursolic acid · Tissue disposition

1 Introduction

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) (UA), a triterpenoid compound, is present ubiquitously in herbal formulations and it is an integral part of the human diet and cosmetics preparations [1], possessing a number of pharmacological effects [2–10], like hepatoprotective, anti-inflammatory, hypoglycemic, anti-tumor, anti-HIV, antimicrobial, antifungal, anti-ulcer, gastroprotective, hypolipidemic and estrogenic activities. With the growing significance of a potential beneficial role of UA in human health, there is an increasing demand for analyzing it in vivo. Quantification of drug in the target organ is essentially required for its therapeutic survey and is based on the proposition that the concentrations in the target tissues are directly proportional to the intensity of the effect. It is not sufficient to ascertain the concentration of UA in different tissues from the data of its concentration in the blood. But due to the high lipophilicity of UA, it might be stored in different tissues for a long time. There is a report on bioavailability/quantification of tissue disposited UA by high-performance liquid chromatography (HPLC) after intravenous administration of UA-phospholipid nanoparticle [11], without any validation data. As UA has nutraceutical values and absorbed by intra-gastric route, an attempt has been made to develop a simple and rapid method to estimate UA disposited in the different target tissues after oral administration.

In the present work, modern thin-layer chromatography (TLC) is chosen as it is a simple, reproducible, rapid, economic and eco-friendly tool of analysis. Several samples can be analyzed upon one TLC plate, using a small amount of mobile phase and with a small analysis time. With the

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requisite time and solvent per sample analysis by conventional HPLC [12–19], analysis of more than ten samples can be done by high-performance thin-layer chromatography (HPTLC). Samples can be applied directly upon TLC plates without any pre-treatment, which is further advantageous over HPLC, liquid chromatography−mass spectrometry (LC−MS) [20, 21] and gas chromatography−mass spectrometry (GC−MS) [22]. As UA is not a volatile entity, silylation, i.e., derivatization is necessary to make it more volatile and thermo stable for its GC−MS analysis. But due to matrix effect and ion-suppression, reproducibility is hardly achieved. At the same time, analysis time is also longer. For metabonomics study, i.e., analysis of more than fifty components at a time, GC−MS or LC−MS will be the best alternatives. But for uni-component analysis, these must be replaced by a simple analytical method.

2 Experimental

2.1 Materials and reagents

UA (CAS No: 77-52-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the solvents used were of HPLC grade.

2.2 Animals and drug administration

Swiss female albino rats (200 ± 20 g) were housed in polyethylene cages in a room maintained at 22 °C for a 12 h light/dark period. 30 rats were randomly divided into five groups. After an overnight fasting with free access of water ad libitum, aqueous solution containing 10% (*V/V*) DMSO (dimethyl sulfoxide) of UA (10 mg/mL) was administered at 40 mg/kg orally to test groups, whereas the control group received the same without UA.

2.3 Sampling and sample processing

The target organs (liver, heart, spleen, lung, kidney, brain, stomach, intestine, ovary and uterus) were collected after 3, 6, 24 and 48 h of drug administration by sacrificing animal via cervical dislocation under ether anesthesia. Organs were weighed rapidly and placed into normal saline solution to remove the blood and extraneous fat, soaked by tissue paper and weighed again. Gastrointestinal tissues were thoroughly washed and luminal fluids were expressed from the uterus. Each tissue was sliced, homogenized with 5 mL methanol and centrifuged (9000 rpm for 15 min). The supernatant was aspirated and evaporated in freeze dryer, followed by addition of methanol in a ratio of 1 mL per g of tissue.

Simultaneously, an amount of 2 mL of blood sample was collected just before sacrificing animals followed by serum separation by centrifugation at 4500 rpm for 15 min. An aliquot of 0.5 mL serum was aspirated in a separate Eppendorf tube and the same volume of methanol was added. Here, methanol was used as an astringent and extracting solvent. The mixture was recentrifuged at 9000 rpm for 15 min, the supernatant was aspirated, evaporated and reconstituted with 100 µL of methanol.

Finally, an aliquot of 10 µL of the aspirated supernatant was taken and analyzed by HPTLC. The Departmental Animal Ethical Committee approved the entire protocol of the animal experiment (Reg. No.: 506/01/a/CPC SEA).

2.4 Preparation of standard solution and quality control of sample

Standard solution (0.96 mg/mL) of UA was prepared in methanol. Aliquots of stock solution equivalent containing 9.6, 19.2, 28.8 and 38.4 µg of UA were taken in Eppendorf tubes. Methanol was evaporated and 1 mL of the control homogenate, i.e., UA free tissue homogenate obtained from the control group, was added to each. The concentration was maintained in the range of 9.6–38.4 µg/mL for preparation of standard calibration curve.

2.5 Validation

To study the accuracy and suitability of the method, recovery studies were performed. Standard concentrations (9.6 and 38.4 µg/mL) within the linearity range were selected and added during the extraction procedure to the control homogenate. Quantification was done by comparing with standard calibration curve in each plate.

The lower limit of quantification (LLOQ) was calculated in different biological matrices. It is defined as the lowest concentration at which the accuracy level is less than or equal to ± 20% [23].

To investigate the stability of UA in the various tissue matrixes in vitro, spiked biosamples containing 9.6 and 38.4 µg/mL of UA were taken. Analysis was done after three freeze (− 4 °C) and thaw (room temperature) cycles and they were stored at room temperature for 6 h and 24 h, respectively.

2.6 HPTLC conditions

A CAMAG (Muttenz, Switzerland) HPTLC system with a Linomat 5 sample applicator, a CAMAG twin-trough plate development chamber, CAMAG TLC Scanner 3 and winCATS integration software (Version: 1.4.1.8154) were used in the present work. Aluminum-backed TLC plates of 0.2 mm layer of normal and amine-coated silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) were pre-washed with methanol−hexane mixture (1:1, *V/V*). Laboratory

temperature during analysis was maintained at 21 °C ± 1 °C and the relative humidity at 40%.

An aliquot of 10 µL each of the blank and standard, and 5, 10 and 15 µL of each sample solutions were applied 8 mm above the edge of the plate with a band width of 4 mm. The chromatogram was developed up to 68 mm in chamber saturation with suitable solvent system of twin-trough chamber. The solvent system developed for TLC separation was toluene−ethyl acetate−methanol (4:1:1, *V/V*) with a chamber saturation time of 1 h. For amine-coated plate, the solvent system comprising toluene−ethyl acetate−methanol−formic acid (4:1:0.7:1, *V/V*) was selected. Solvent evaporation followed by dipping of the plate was done in a chamber of freshly prepared solution of 5% (*V/V*) ethanolic sulphuric acid. After soaking the reagent upon silica gel layer, the plates were heated (120 °C) in an oven for 3 min, followed by dipping in a solution of 5% (*V/V*) liquid paraffin in hexane for intensifying and stabilizing the developed color. After that, the plates were scanned in remission/fluorescence mode at 366 nm by a Hg lamp, with 20 mm/s scan speed, with slit dimensions of 3.00 × 0.45 mm.

3 Results and discussion

The mobile phase toluene−ethyl acetate−methanol (4:1:1, *V/V*) resolved UA efficiently from other constituents with R_F of 0.60 in normal silica gel plate. In the case of amine-coated plate, a sharp peak was observed at 0.50 R_F, with a solvent system of toluene−ethyl acetate−methanol−formic acid (4:1:0.7:1, *V/V*). Figures 1 and 2 are depicting the representative chromatograms showing good resolution of UA in biological matrix and developed TLC plate (chromatograph), respectively. Figure 3 is the representative chromatogram depicting good resolution of UA from other constituents present in biological matrix.

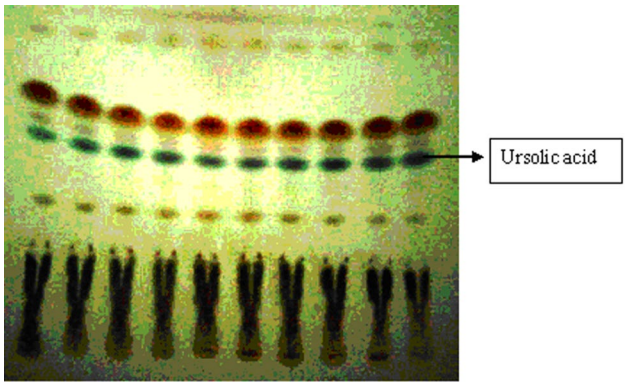

Fig. 2 Representative image of the developed TLC plate (chromatograph)

The calibration plots were linear in the range of 9.6–38.4 µg/mL, i.e., 96–384 ng/spot of spiked UA with a correlation coefficient > 0.99 (Table 1). The analysis was merely based on the color development by thermal activation and the color intensity was varied in plate to plate. For that reason, spiked standards were applied for comparison. A representative calibration curve is presented in Fig. 4.

From the analysis, the UA content/g of liver and uterine tissues after 6 h of its oral administration was 67.87 µg and 67.05 µg ($n = 6$), respectively, i.e., approximately five times higher than the content in per mL of serum. The concentrations of UA in other tissues were relatively low as depicted

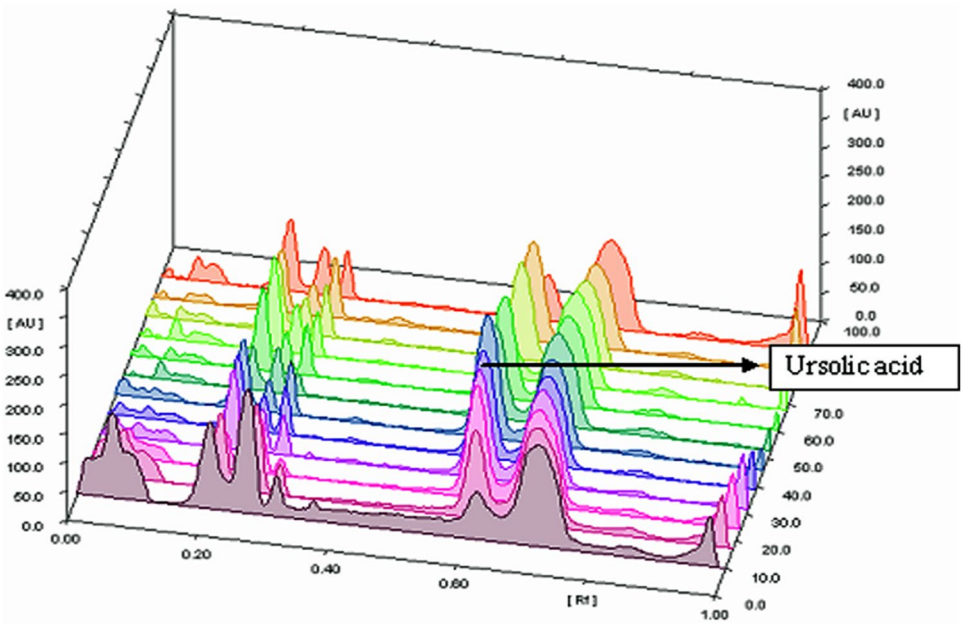

Fig. 1 Representative chromatogram showing good resolution of ursolic acid (UA) in liver tissue homogenate

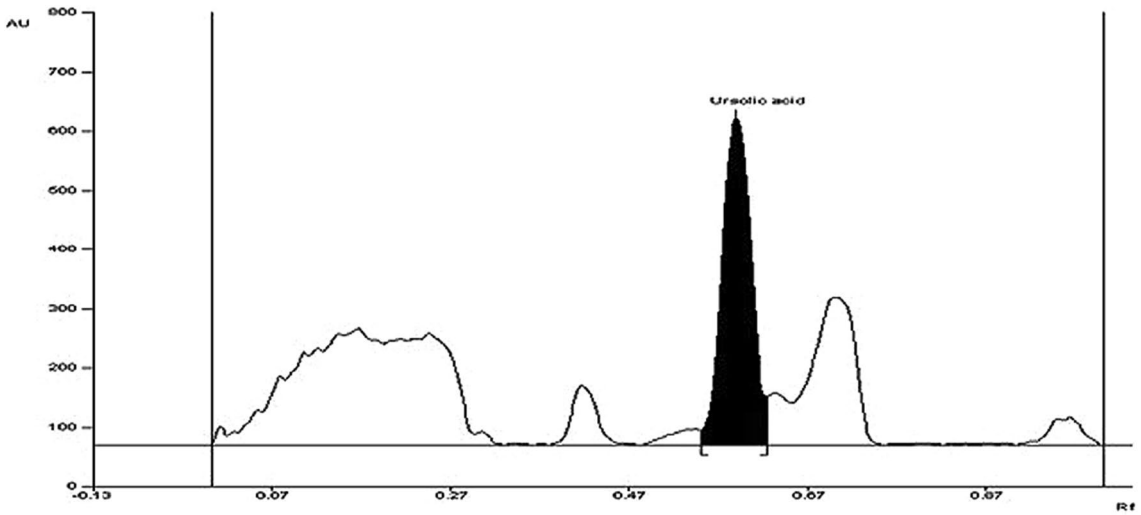

Fig. 3 Representative chromatogram depicting good resolution of ursolic acid (UA) from other constituents present in biological matrix (uterine tissue homogenate)

Table 1 Standard curves, correlation coefficients of ursolic acid (linear ranges 9.6–38.4 µg/mL, i.e., 96–384 ng/spot) in biological samples

Tissue homogenate	Standard curve ($Y=$)	Correlation coefficient
Serum	$34.667X+556\times10^3$	0.998
Liver	$28.541X+1.17\times10^3$	0.999
Liver (amine)	$9.137X+1.49\times10^3$	0.999
Uterus	$20.440X+5.07\times10^3$	0.998
Ovary	$20.68X+2.41\times10^3$	0.994
Stomach	$46.877X+3.03\times10^2$	0.999
Intestine	$20.092X+1.88\times10^3$	0.998
Heart	$23.261X+8.03\times10^2$	0.999
Lung	$40.942X+4.66\times10^3$	0.998
Spleen	$41.85X+3.26\times10^3$	0.999
Brain	$21.79X+2.42\times10^3$	0.995
Kidney	$21.764X+1.74\times10^3$	0.999

in Table 2. The LLOQ in different biological matrices was in the range of 1.2 to 3.6 µg/mL (Table 3), i.e., 2.63–7.88 nmol/mL.

A subtle change in sample processing was performed due to the lesser disposition of UA in a large number of organs. In such cases, final reconstitution was done with methanol in a ratio of 200 µL/g of the respective tissues, except liver, uterus, stomach and intestine.

In the case of body distribution, it was observed that after 3 h of oral administration, UA reached to almost every tissue. After 6 h, the UA concentrations in the liver and uterus increased. In the stomach and intestine, the levels were comparable with the serum concentration, whereas in other tissues they decreased gradually. After 24 h, the concentration in the liver was slightly elevated, but in the uterus, ovary, stomach, intestine, spleen and kidney the level decreased. In other organs, there was no occurrence of UA after 24 h. After 48 h, UA was not traced in any tissue excluding blood and intestine. There, a concentration near to its LLOQ value was detected. The female rat model was used in the present work for investigating the estrogenic and hepatoprotective potential of UA as phytomedicine, and it was found that UA had greater affinity with liver and uterus (after 3, 6 and 24 h, the contents per g of these tissues were approximately 4, 5 and 10 times higher than the content per mL of serum) along with a low affinity to kidney. The observations further indicated its biliary excretion, supported by the work of *Zhou* et al. [11]. Figures 5, 6 and 7 portray the representative chromatograms of UA distributed in a total of eleven tissues and biological organs inside animal subjects obtained by our HPTLC method.

The recovery of UA was tested at 9.6 and 38.4 µg/mL levels by comparing the peak areas from the extracted target tissues homogenate samples with those found by direct injection of standard solution at the same concentration. The mean recovery value obtained from liver and uterine tissues homogenate were 94.72% to 97.70% and 91.47% to 96.97%, respectively ($n=3$), indicating the reliability and reproducibility of the method (Table 4).

Further, the method accuracy was checked at 9.6 and 38.4 µg/mL upon each plate along with the LLOQ level. SD and RE (standard deviation and relative error) were within ± 20% (Table 3).

Table 5 depicts the results of the stability study that indicated the UA content was almost unaltered (RE within ± 15%) after three successive freeze and thaw cycles. The analytes were also shown to be stable in rat

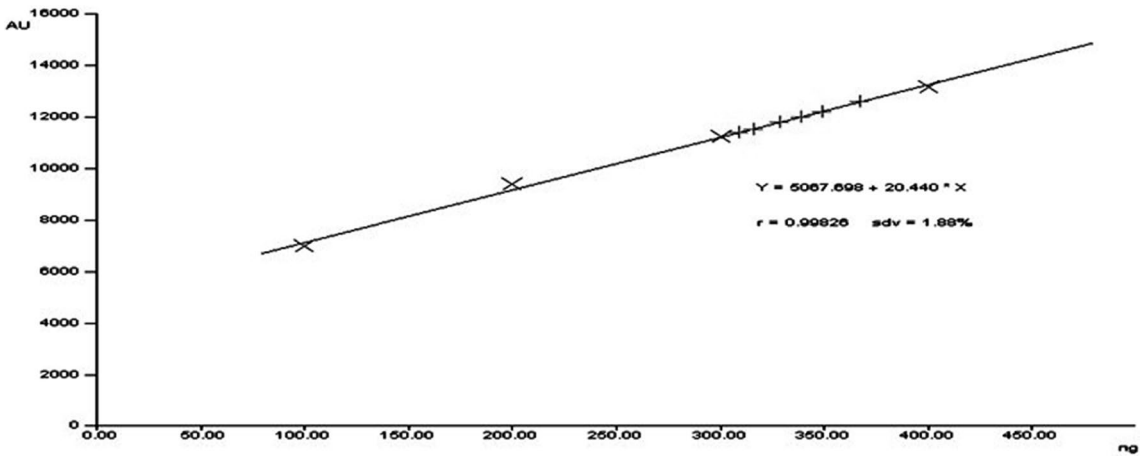

Fig. 4 Representative calibration curve of ursolic acid (UA) in biological matrix

Table 2 Distribution of ursolic acid (UA) in tissues after oral administration of 40 mg/kg in rats ($n=6$)

Tissue	Content of UA (μg/g of tissue ± SD) at the time (h) interval			
	3	6	24	48
Liver	27.24 ± 6.32	67.87 ± 7.42	69.77 ± 5.30	ND
Liver (amine)	28.34 ± 4.23	66.79 ± 3.33	71.84 ± 4.81	ND
Uterus	36.25 ± 4.41	67.05 ± 4.95	29.12 ± 3.78	ND
Ovary	3.28 ± 0.80	3.69 ± 0.58	4.18 ± 0.50	ND
Stomach	7.10 ± 0.77	12.28 ± 2.04	4.34 ± 0.71	ND
Intestine	7.92 ± 0.75	11.52 ± 1.59	6.20 ± 0.84	2.10 ± 0.70
Heart	4.68 ± 0.78	2.70 ± 0.67	ND	ND
Lung	2.61 ± 0.97	2.90 ± 0.76	ND	ND
Spleen	4.51 ± 1.47	3.86 ± 0.66	2.54 ± 0.59	ND
Brain	3.50 ± 0.74	ND	ND	ND
Kidney	5.86 ± 1.40	3.76 ± 1.65	2.24 ± 0.83	ND
Serum (μg/mL)	7.41 ± 3.37	13.47 ± 6.13	7.55 ± 3.44	2.95 ± 0.90

SD standard deviation, *ND* not detected

tissue homogenates, stored at room temperature for 6 h and 24 h, respectively (RE within ± 15%).

As UA is devoid of ultraviolet (UV)-absorbing chromophores, for visual observation it is necessary to derivatize the plate. If there is a requirement of thermal activation for color development, then for uniformity and reproducibility, at least four standards must be applied upon each plate and quantification should be done by the standard curve, prepared from the peak area of the standards applied to that respective plate. By using a 20 × 10 cm plate with four standards, more than 20 samples can be analyzed at a time which makes it again advantageous over other methods.

However, two important disadvantages, which may be associated with this method include the following:

1. The TLC plate after dipping in the derivatizing reagent is bended during the thermal activation at 120 °C and for that uniformity in heating could not be achievable which was observed during this study.
2. Biological samples can be directly applied in plate without any pre-treatment, which is advantageous. But due to short path-length for separation, i.e., only 60 mm on TLC plate, as compared to 60 m in GC, matrix effect might occur, which may be responsible for improper separation from numerous diverse biological constituents/impurities, resulting in an error in analysis.

To overcome these two problems, i.e., non-uniform thermal activation and matrix effect, conventional methodology has been modified. The sample solution was applied in three different proportions, i.e., 5, 10 and 15 μL instead of applying replica of the same amount of sample. From each, the quantity of UA was calculated in per mL of tissue homogenate, i.e., per g of the respective tissue.

For detection of UA, there is a requirement of thermal activation during the derivatization step with a reagent containing sulphuric acid. Sulphuric acid is not evaporated and for that noise level might be increased, resulting in higher LOQ. To overcome this, upon each plate four spiked standards along with one blank were applied, i.e., standard curve prepared upon each plate including accuracy checking. Application of three different concentrations of sample facilitates to overcome such problems.

The major disadvantage of HPTLC is its off-line pattern, i.e., it is an open system. It was tried to convert this into an advantage by simultaneous application of blank and standards with samples upon each plate. This step facilitated

Table 3 Method accuracy for ursolic acid estimation in rat serum and tissues ($n=3$)

Sample matrix	Spiked concentration		Concentration measured			Accuracy	
	(µg/mL)	(ng/spot)	S1	S2	S3	(Mean ± SD)	(Mean ± SD, %)
Serum	1.2	12	12.67	11.22	10.88	11.59 ± 0.95	96.58 ± 7.92
	9.6	96	88.54	85.42	91.24	88.4 ± 2.91	92.08 ± 3.03
	38.4	384	364.26	370.86	374.81	369.98 ± 5.33	96.35 ± 1.39
Liver	1.2	12	10.84	11.61	12.04	11.50 ± 0.61	95.81 ± 5.07
	9.6	96	94.45	88.72	92.26	91.81 ± 2.89	95.64 ± 3.01
	38.4	384	394.22	386.84	402.24	394.43 ± 7.70	102.72 ± 2.01
Liver (amine)	1.2	12	13.11	12.26	11.89	12.42 ± 0.63	103.50 ± 5.21
	9.6	96	94.11	98.82	94.26	95.73 ± 2.68	99.72 ± 2.79
	38.4	384	388.22	390.14	376.87	385.08 ± 7.17	100.28 ± 1.87
Uterus	1.2	12	10.77	11.34	12.80	11.64 ± 1.05	96.97 ± 8.73
	9.6	96	84.68	92.14	88.65	88.49 ± 3.73	92.18 ± 3.89
	38.4	384	374.22	390.78	376.85	380.62 ± 8.90	99.12 ± 2.32
Ovary	2.4	24	22.06	21.70	21.75	21.84 ± 0.20	90.99 ± 0.81
	9.6	96	76.44	94.27	91.33	87.35 ± 9.56	90.99 ± 9.96
	38.4	384	366	362.29	382.43	370.24 ± 10.72	96.42 ± 2.79
Stomach	1.2	12	10.53	11.65	12.16	11.45 ± 0.83	95.39 ± 6.95
	9.6	96	84.76	96.82	100.25	93.94 ± 8.14	97.86 ± 8.47
	38.4	384	352.24	368.42	390.17	370.28 ± 19.03	96.43 ± 4.96
Intestine	1.2	12	11.72	10.28	12.15	11.38 ± 0.98	94.86 ± 8.16
	9.6	96	86.47	88.26	100.71	91.81 ± 7.76	95.64 ± 8.08
	38.4	384	374.82	362.48	375.54	370.95 ± 7.34	96.60 ± 1.91
Heart	2.4	24	22.62	22.24	21.19	22.02 ± 0.74	91.74 ± 3.09
	9.6	96	92.48	86.77	100.25	93.17 ± 6.77	97.05 ± 7.05
	38.4	384	378.9	364.28	391.72	378.3 ± 13.73	98.52 ± 3.58
Lung	2.4	24	21.86	21.58	25.22	22.89 ± 2.03	95.36 ± 8.44
	9.6	96	108.26	107.34	94.27	103.29 ± 7.83	107.59 ± 8.15
	38.4	384	368.25	379.9	392.72	380.29 ± 12.24	99.03 ± 3.19
Spleen	2.4	24	22.61	25.54	21.80	23.32 ± 1.97	97.15 ± 8.20
	9.6	96	88.74	95	93.17	92.30 ± 3.22	96.15 ± 3.35
	38.4	384	394.42	371.05	378.26	381.24 ± 11.97	99.28 ± 3.12
Brain	3.6	36	32.28	34.65	35.82	34.25 ± 1.80	95.14 ± 5.01
	9.6	96	86	90.05	96.91	90.99 ± 5.51	94.78 ± 5.74
	38.4	384	372.65	391.17	378.28	380.7 ± 9.49	99.14 ± 2.47
Kidney	1.2	12	12.84	12.25	11.19	12.33 ± 0.47	102.78 ± 3.92
	9.6	96	104.46	94.44	93.02	97.31 ± 6.24	101.36 ± 6.50
	38.4	384	377.19	382.64	369.28	376.37 ± 6.72	98.01 ± 1.75

SD standard deviation

to overcome the problem arises with non-uniform thermal heating and other various problems encountered with any off-line application.

In closed systems, like LC/GC, if a problem arises in the column it will not be detectable during operation. The time for each sample run is much longer, generally between 40 and 60 min; for that validation parameters are generally checked before and after sample quantifications. But HPTLC can analyze six samples with different concentrations along with standards in different concentrations in the same plate for sample quantification and accuracy check in a lucrative manner. Analysis of six samples along with standards with different concentrations can be done in the same plate. Sample quantification with accuracy checking was performed simultaneously in a lucrative manner. Thus the process became much more a valid, miniaturized analytical platform.

In the present work, an attempt has also been made to establish one simplified extraction method for the analysis of UA disposited in different tissue matrices. There is only one reported method for the analysis of UA [24] in biological fluid, i.e., in serum constituents. Chloroform, as described by *Shetty* et al. [24] is futile in this situation,

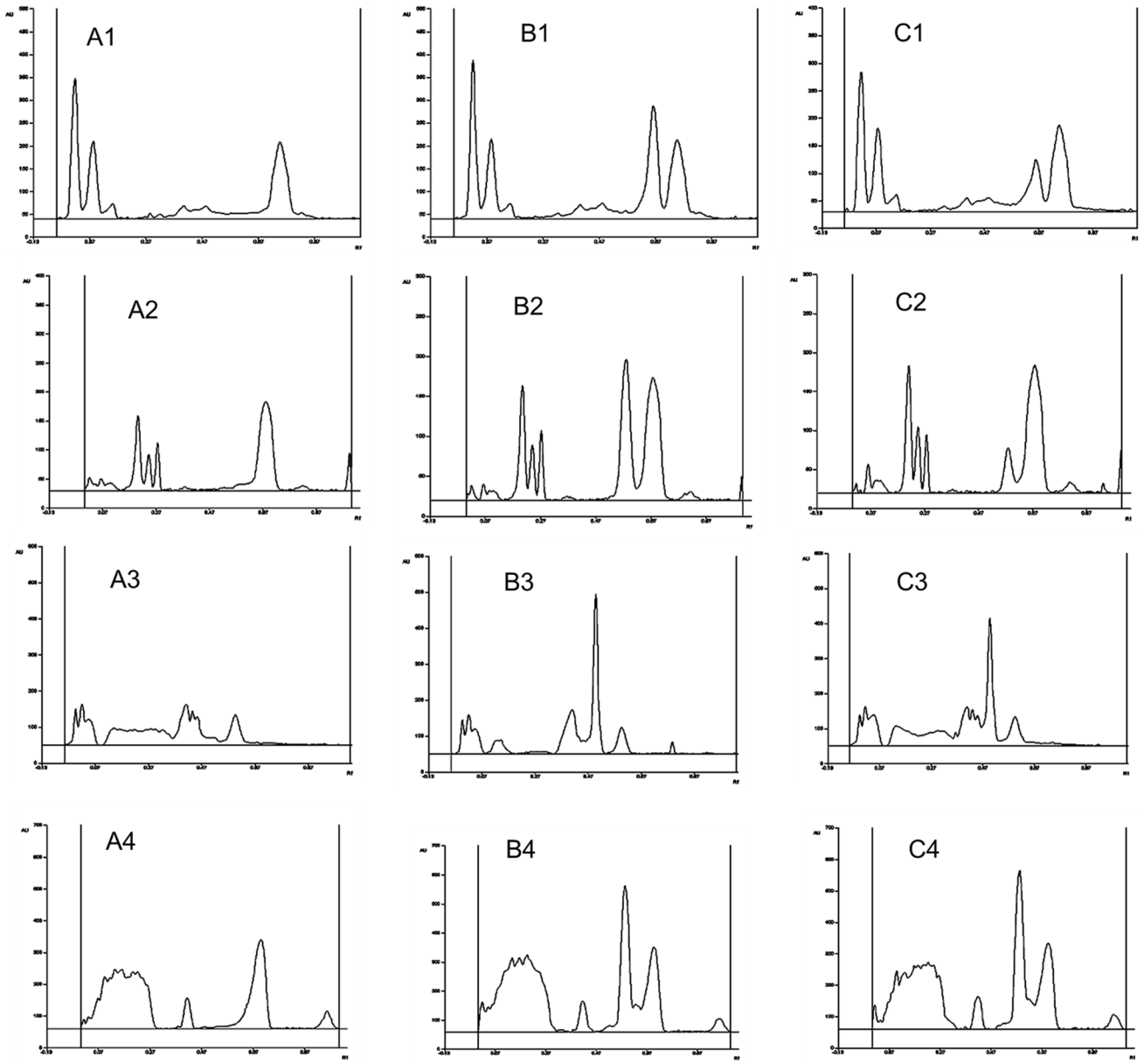

Fig. 5 Representative chromatographic (HPTLC) profile of tissue ursolic acid. (A) Blank, (B) spiked standard, (C) sample. (1) Serum, (2) liver, (3) liver (amine-coated plate), (4) uterus. The peak corresponding to R_F of 0.60 and 0.50 in normal and amine-coated TLC plate is the peak of ursolic acid

as it does not have any astringent property. This method may be reliable for extracting UA from serum constituents, but could not separate UA disposited in tissue matrix, i.e., in solid state. In such case, saponification prior to extraction will be the essential step and/or the volume of the extracting solvent must be higher. At the same time, as the commercially available chloroform contains a trace of phosgene that may influence stability during the analysis of UA in matrix tissues. *Zhou* et al. [11] used ethyl acetate as extracting solvent. But, it was found to extract only 70%–88% of UA from different biological matrices, compared to methanol with a recovery of 91%–98% (Table 4). Different protein precipitants like isopropanol, acetonitrile and methanol were added during the recovery of UA from liver tissue matrix. Methanol, in a minimum volume, gave maximum and reproducible recovery without any prior saponification step, making this extraction procedure miniaturized and much more simpler than other reported methods.

There are some reported methods for the analysis of UA in phyto- and biological fluids by HPTLC [24–26]. But the solvent systems mentioned in those works could not

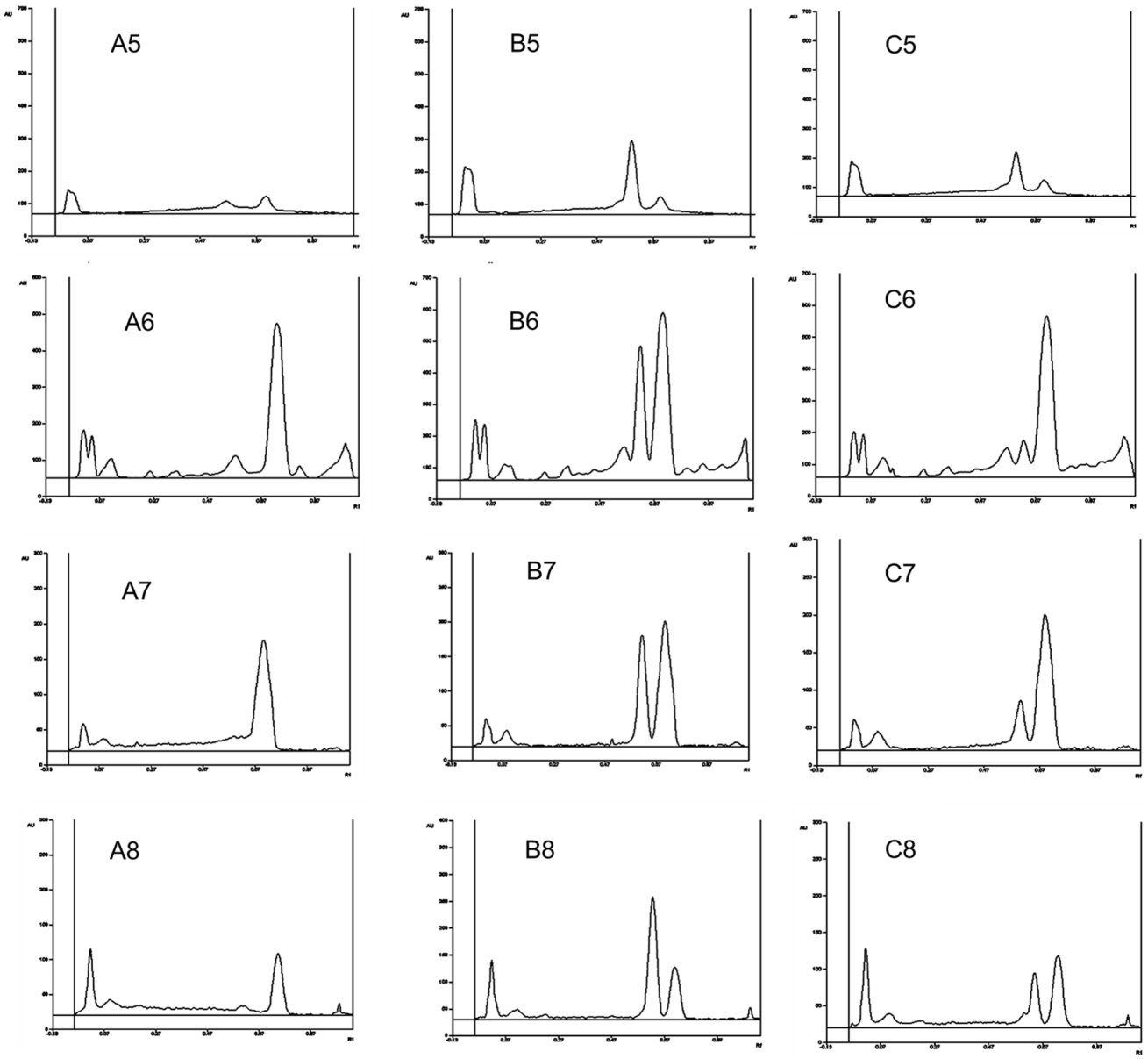

Fig. 6 Representative chromatographic profile (HPTLC) of tissue ursolic acid. (A) Blank, (B) spiked standard, (C) sample. (5) Ovary, (6) stomach, (7) intestine, (8) heart. The peak corresponding to R_F of 0.60 and 0.50 in normal and amine-coated TLC plate is the peak of ursolic acid

resolve UA from the liver and uterine tissue homogenates observed at the time of this study.

Further, *Kosior* [26] used pre-derivatization step using iodine–chloroform and spraying the post-derivatizing reagent which makes the process more cumbersome. Dipping of the developed plate in derivatizing reagent makes the process much simpler.

The most important, by applying 5% (*V/V*) liquid paraffin in hexane, the developed color is stabilized for 1 h. Scanning in fluorescence mode facilitates approximately a ten times higher response than the previous report [26].

Kosior [26] used 10% (*V/V*) ethanolic sulphuric acid reagent, which was found to create a higher noise level. The use of freshly prepared 5% (*V/V*) reagent eliminates such problem.

The robustness of the method was studied by determining the effects of small variations of mobile phase composition. No significant change of R_F and resolution to UA was observed, indicating the reliability and robustness of the method.

Comparable results were obtained from normal and amine-coated TLC plates (Table 2). The amine-coated

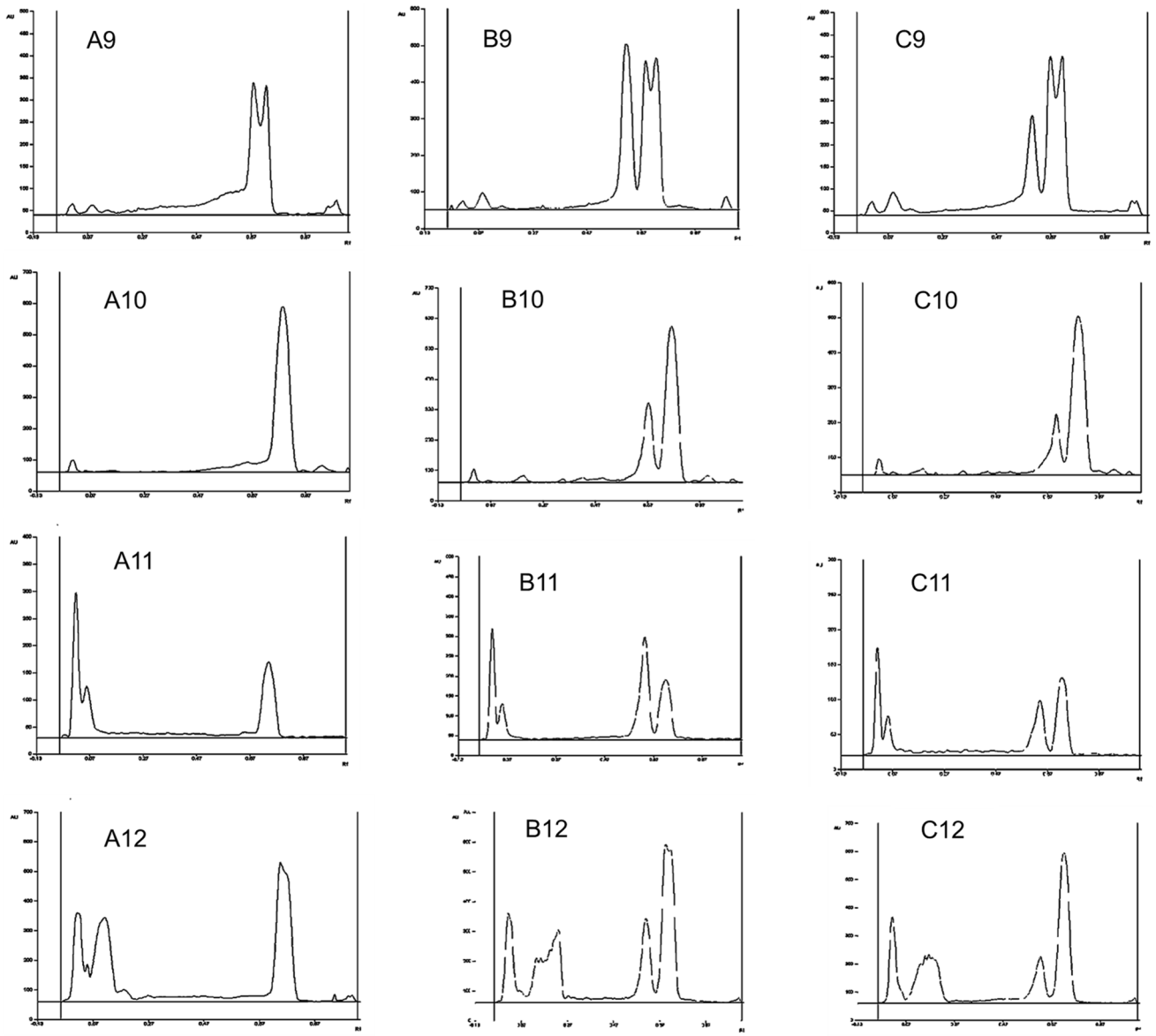

Fig. 7 Representative chromatographic profile (HPTLC) of tissue ursolic acid. (A) Blank, (B) spiked standard, (C) sample. (9) Lung, (10) spleen, (11) brain, (12) kidney. The peak corresponding to R_F of 0.60 and 0.50 in normal and amine-coated TLC plate is the peak of ursolic acid

plate gave sharper peak compared to the normal TLC plate. Amine-coated plates are mainly used for the detection of requisite compound without any derivatization. UA is devoid of any chromophoric group; hence derivatization is mandatory. During UA quantification, we tried with amine-coated plates to avoid this derivatization part, but we could not achieve the desired output. Even after using amine-coated plates, after development of chromatogram, derivatization is required for the quantification of UA. With normal silica gel plate, we got the desired and validated output. During our research work, we used amine-coated plates for checking whether we could avoid derivatization for the quantification of UA or not, just as data we represented the research output over here, which could be a reference point of future researchers.

With HPTLC analysis, there is significant reduction in estimation time and solvent consumption, which is further advantageous over other analytical methods devoid of complicating pre-treatment and storage problems.

4 Conclusion

A rapid, sensitive and simple method for the quantitative analysis in the range of 9.6–38.4 µg/mL of UA in rat tissues is described. This method will be very useful during

Table 4 Recovery of ursolic acid from biological samples ($n=3$)

Sample matrix	Spiked concentration		Concentration measured			Recovery	
	(μg/mL)	(ng/spot)	S1	S2	S3	(Mean ± SD)	(Mean ± SD, %)
Serum	9.6	96	84.47	92.24	95.28	90.66 ± 5.57	94.44 ± 5.81
	38.4	384	366.52	378.85	374.29	373.22 ± 6.23	97.19 ± 1.62
Liver	9.6	96	94.78	85.27	92.73	90.93 ± 5.00	94.72 ± 5.21
	38.4	384	358.29	374	379.04	370.44 ± 10.82	96.47 ± 2.82
Liver (amine)	9.6	96	97.24	94.08	89.91	93.74 ± 3.68	97.65 ± 3.83
	38.4	384	366	382.42	377.09	375.17 ± 8.38	97.70 ± 2.18
Uterus	9.6	96	80.14	92.22	91.07	87.81 ± 6.67	91.47 ± 6.95
	38.4	384	364.8	377	375.28	372.36 ± 6.60	96.97 ± 1.72
Ovary	9.6	96	98.02	92.22	93.36	94.53 ± 3.07	98.47 ± 3.20
	38.4	384	354.28	379	386.04	373.11 ± 16.68	97.16 ± 4.34
Stomach	9.6	96	85.52	88.08	99.46	91.02 ± 7.42	94.81 ± 7.73
	38.4	384	371.06	364.1	380.24	371.8 ± 8.10	96.82 ± 2.11
Intestine	9.6	96	86.21	82.46	94.49	87.72 ± 6.16	91.38 ± 6.41
	38.4	384	366	372.29	374.48	370.92 ± 4.40	96.59 ± 1.15
Heart	9.6	96	84.06	93.25	89.77	89.03 ± 4.64	92.74 ± 4.83
	38.4	384	368.24	355.59	376.2	366.68 ± 10.39	95.49 ± 2.71
Lung	9.6	96	90.17	94.75	89.92	91.61 ± 2.72	95.43 ± 2.83
	38.4	384	371.88	365.54	382.02	373.15 ± 8.31	97.17 ± 2.16
Spleen	9.6	96	99.74	88.28	95.55	94.52 ± 5.80	98.46 ± 6.04
	38.4	384	355.28	379.28	368.18	367.58 ± 12.01	95.72 ± 3.13
Brain	9.6	96	82.96	94.19	96.72	91.29 ± 7.32	95.09 ± 7.63
	38.4	384	364.4	375.52	362.18	367.37 ± 7.15	95.67 ± 1.86
Kidney	9.6	96	92.68	90.74	98.29	93.90 ± 3.92	97.82 ± 4.08
	38.4	384	355.79	382.58	374.26	370.88 ± 13.71	96.58 ± 3.57

SD standard deviation

Table 5 Stability of biological samples of ursolic acid ($n=3$)

Statistical variables	Theoretical concentration (μg/mL)					
	Serum		Liver		Uterus	
	9.6	38.4	9.6	38.4	9.6	38.4
Three freeze and thaw cycles						
Mean (μg/mL)	9.26	34.62	9.03	34.15	8.94	35.17
SD (%)	5.38	7.00	7.00	9.04	9.55	8.91
RE (%)	−3.54	−9.84	−5.94	−11.08	−6.91	−8.40
After storage at room temperature for 6 h						
Mean (μg/mL)	9.00	35.70	9.48	33.90	9.08	34.29
SD (%)	7.30	10.74	1.87	10.00	4.41	10.66
RE (%)	−6.22	−7.04	−1.22	−11.71	−5.42	−10.71
After storage at room temperature for 24 h						
Mean (μg/mL)	9.90	36.33	9.24	34.80	9.90	33.91
SD (%)	6.41	4.22	3.97	11.99	7.49	3.90
RE (%)	3.09	−5.40	−3.78	−9.38	3.125	−11.68

RE relative error, *SD* standard deviation

the therapeutic intervention of different herbal formulations containing UA. By this, HPTLC can also be applicable as a valuated tool in bioavailability and tissue disposition studies.

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Declarations

Conflict of interest There is no conflict of interest in the manuscript.

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