



High-performance liquid chromatographic determination of licochalcone A and its metabolites in biological fluids

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Abstract

A high-performance liquid chromatographic method has been developed for the analysis of the novel antiparasitic agent, licochalcone A (Lica), and three of its glucuronic acid conjugates in plasma and urine. The high-performance liquid chromatography assay was performed using gradient elution and UV detection at 360 nm. The proposed technique is selective, reliable and sensitive. The limits of quantification for Lica are 0.2 µg/ml in plasma and 0.14 µg/ml in urine, 1.2 µg/ml for the 4'-glucuronide in plasma and 1.4 µg/ml in urine, and 2.0 µg/ml for the 4-glucuronide in plasma and 3.2 µg/ml in urine. The reproducibility of the analytical method according to the statistical coefficients is 7% or below. The accuracy of the method is good, that is, the relative error is below 10%. The stability of Lica and its glucuronides in urine and plasma samples has been assessed during storage in the autosampler and freezer. The applicability of the assay for determining Lica and its intact glucuronide conjugates in biological fluids was shown using a single dose study in rat.

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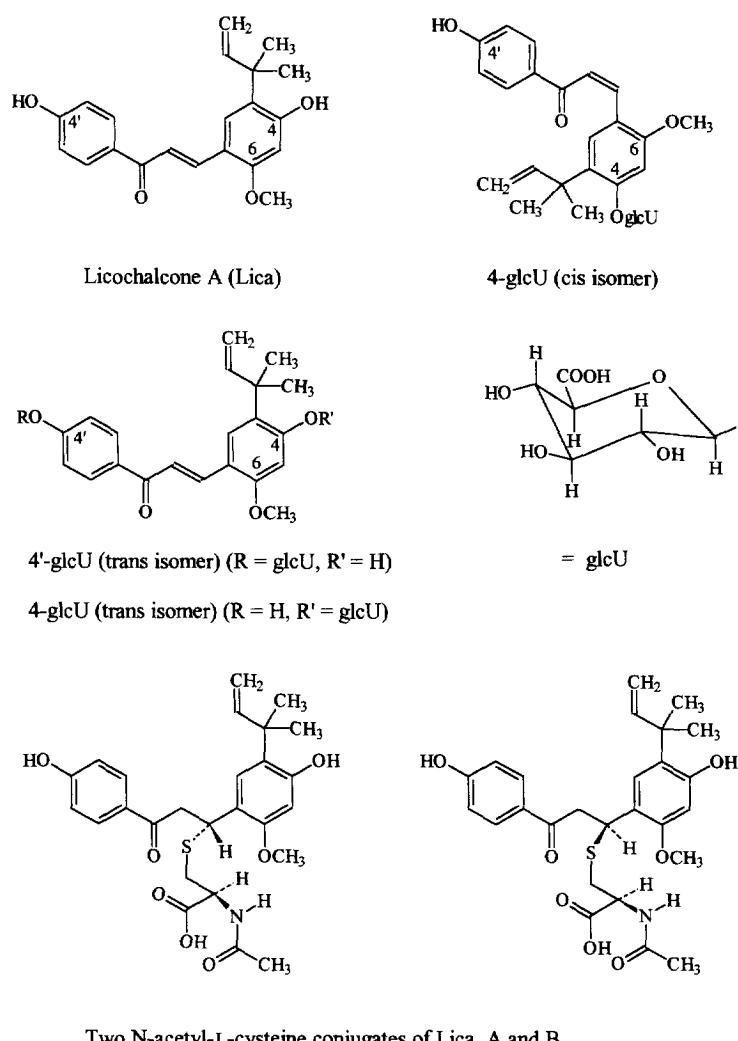
1. Introduction

Licochalcone A (Lica) (Fig. 1) is an oxygenated chalcone isolated from the roots of Xinjiang liquorice. Lica has been shown to be a potential drug candidate for the treatment of the parasitic diseases malaria and leishmaniasis [1-3] and its mechanism of action appears to be by altering the ultrastructure and function of the parasite mitochondria [4].

There are several reports on the separation of chalcones using normal and reversed-phase high-performance liquid chromatography (RP-HPLC),

where the effects of changing the column packing material and the mobile phase constituents have been investigated [5-9]. Casteele and coworkers [10] have investigated the separation of flavonoids, including several chalcones, on a Lichrosorb RP-18 column using isocratic as well as gradient elution. The analysis of commercial hesperidin methyl chalcone has been reported using a HPLC system consisting of a C₁₈ column and gradient elution with an eluent consisting of methanol and phosphoric acid [11]. A similar HPLC method has also been reported for the determination of chalcone constituents in liquorice root [12]. A HPLC method for the quantification of Lica in liquorice extract using a C₁₈ column and

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Two N-acetyl-L-cysteine conjugates of Lica, A and B

Fig. 1. Chemical structures of Lica, its phenolic glucuronides *trans*-4'-glcU and 4-glcU (*cis* and *trans*), and its two N-acetyl-L-cysteine conjugates.

isocratic elution with a mobile phase consisting of acetonitrile and acetic acid has also been described [13]. However, no assay has previously been presented for the quantification of Lica in biological fluids.

Previous studies with Lica have resulted in the synthesis, isolation and identification of five conjugates of Lica, three phenolic glucuronides (*cis*- and *trans*-4-glcU and *trans*-4'-glcU) and two N-acetyl-L-cysteine conjugates (see Fig. 1) [14].

The aim of the present study was to develop a HPLC method which would enable the separation of Lica and its conjugates and also allow the simultaneous quantification of Lica and its phenolic glucuronides in urine and plasma. This method was validated according to Good Laboratory Practice (GLP) guidelines [15] and was used successfully to determine Lica and its phenolic glucuronides in rat urine and plasma after intraperitoneal (i.p.) administration of a single dose.

2. Experimental

2.1. Chemicals

Lica was synthesized by a previously published method [16] according to Good Laboratory Manufacturing (GMP) rules by Clauson-Kaas, Chemical Research Laboratory, Farum, Denmark. The synthesized Lica possessed a melting point of 176 to 177°C. The identity of the synthetic Lica was established by recording the ¹H NMR (200 MHz) and ¹³C NMR (50 MHz) spectra. The purity of Lica was greater than 99.4% as determined by HPLC. The phenolic glucuronides of Lica (4-glcU (a mixture of the *cis*- and *trans*-isomers) and *trans*-4'-glcU) were synthesized using rabbit or pig microsomes as previously described [14]. The two N-acetyl-L-cysteine conjugates of Lica were also synthesized, isolated and identified as described previously [14]. β -Glucuronidase (EC 3.2.1.31) type HP-2 from *Helix pomatia* possessing approximately 100 000 units per ml of β -glucuronidase activity and 1000–5000 U of sulphatase activity was obtained from Sigma (St. Louis, MO, USA). Trisodium citrate dihydrate was purchased from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade, and all other chemicals were of analytical grade. Pooled blank plasma was obtained from the University Hospital, Copenhagen, Denmark.

2.2. Animals

SPF Wistar male rats of the strain Mol:Wist from Møllegaard Breeding and Research Center (Lille Skensved, Denmark) were fed on a complete rodent diet, Altromin 1314 (Chr. Pedersen, Ringsted, Denmark). At the commencement of the study the rats weighed 200±5 g. Lica was administered intraperitoneally as 1 ml of a 10 mg/ml solution in dimethyl sulphoxide. Rats were kept in individual Techniplast metabolism cages (150–300 g rats) (Techniplast Gazzada, Buguggiate, VA, Italy) for 24 h before dosing and for three days afterwards with free access to food and water. Urine was collected separately night and day. Venous blood samples (180 μ l), obtained from the orbital plexus of the rat, were collected into sampling tubes containing 20 μ l 0.13

M trisodium citrate dihydrate solution. Plasma samples were prepared by centrifugation at 2200 g for 15 min at 5°C. Plasma preparation was performed within 10 min of blood collection. All samples were frozen to –20°C immediately after preparation and stored until analysis (max. 1 week).

2.3. Standard solutions

Standard solutions of Lica, 4-glcU (a mixture of the *cis*- and *trans*-isomers) and *trans*-4'-glcU were prepared in human plasma or urine from a stock solution in acetonitrile for Lica and in acetonitrile–water (1:1, v/v) for the glucuronides. The final concentration of acetonitrile in the standard solutions did not exceed 3%. All stock solutions were stored at 5°C for several weeks without any detectable degradation and all standard solutions were prepared freshly each day. The standards were treated in exactly the same way as the urine and plasma samples.

2.4. Sample preparation

After thawing, plasma and urine samples were vortex mixed and 100- μ l aliquots were taken. Acetonitrile (100 μ l) was added to all samples and these were mixed by vortex and subsequently centrifuged at 9000 g for 10 min. A 100- μ l aliquot of the supernatant was transferred to a brown glass autosampler vial containing 200 μ l glass liner and a 20- μ l volume was injected onto the chromatographic column.

Cleavage of the glucuronides in urine and plasma samples was performed by the addition of 10 μ l β -glucuronidase (*Helix pomatia*) to a 100- μ l aliquot of the urine/plasma sample. The samples were incubated at 37°C for 24 h and the reaction was terminated by the addition of 100 μ l acetonitrile. The Lica glucuronides were shown to be completely hydrolyzed after 24 h incubation at 37°C. Furthermore the Lica glucuronides were shown to be stable when incubated at 37°C with β -glucuronidase and the inhibitor D-saccharic acid lactone for 24 h. The samples were treated as described above prior to analysis.

2.5. Chromatography

Liquid chromatography was performed with a Kontron (Tegimenta, Switzerland) HPLC system consisting of two HPLC pumps Model 420, an autosampler Model 460, a column oven Model 480 and a plotter Model 800. A Shimadzu (Kyoto, Japan) SPD-6A UV-detector operated at 254 nm and a Jasco 875 UV/VIS-detector (Tokyo, Japan) operated at 360 nm, were used. Data was collected using Kontron Data system 450 software.

A Knauer (Berlin, Germany) 120×4.6 mm I.D. column packed with Inertsil ODS-2, 5 µm particles (GL Sciences, Tokyo, Japan) was used. The mobile phases consisted of acetonitrile–2% acetic acid (8:92 v/v) and (81:19 v/v) for mobile phase A and B respectively, and were degassed ultrasonically prior to use. The following gradient elution program was used: a linear gradient from 100 to 70% A was applied for 5 min, then a linear gradient from 70 to 65% A was used for 15 min, a linear gradient from 65 to 0% A was used for 5 min and finally isocratic elution with 100% A was used for 10 min. The column was thermostated at 45°C and a flow-rate of 1.5 ml/min was used. The retention times for *cis*-4-glcU, *trans*-4-glcU, *trans*-4'-glcU and Lica, were 10.6, 11.1, 18.3 and 23.4 min, respectively.

Knauer columns 120×4.6 mm I.D. packed with Spherisorb ODS-2, 5 µm particles (Phase Separations, Cheshire, UK); Nucleosil C₁₈ (Machery-Nagel, Düren, Germany); Kromasil C₁₈ (Ika Nobel, Surte, Sweden); Polygosil C₁₈ (Machery-Nagel); Spherisorb C₈ (Phase Separations) and Kromasil C₈ (Ika Nobel) were used in order to investigate the column selectivity's towards Lica and its metabolites.

2.6. Assay validation

Calibration data were generated by spiking blank plasma and urine samples with Lica, *trans*-4'-glcU and 4-glcU (a mixture of the *cis*- and *trans*-isomers) respectively, in the concentration range 0.25–20 µg/ml in plasma and 0.25–10 µg/ml in urine for Lica, 1–60 µg/ml in plasma and 5–60 µg/ml in urine for *trans*-4'-glcU, and 4–16 µg/ml in plasma and 4–60 µg/ml in urine for 4-glcU (as the total amount of *cis*- and *trans*-isomers). The peak areas were plotted

against analyte concentration, and standard calibration curves were generated by unweighted least-squares linear regression analysis of the data. The linearity of the method was investigated by linear regression analysis.

Intra-day precision and accuracy were assessed using six samples of Lica, *trans*-4'-glcU or 4-glcU (a mixture of the *cis*- and *trans*-isomers) at three different concentration levels. Inter-day precision and accuracy were determined using six samples of Lica, *trans*-4'-glcU or 4-glcU (a mixture of the *cis*- and *trans*-isomers) at three concentrations levels analyzed on three different days.

Precision and accuracy were characterized by the relative standard deviation (R.S.D.) and by the mean recovery (%) respectively.

The limits of detection were estimated as three times the standard deviation (σ) of the peak to peak noise (N_{p-p}), where $\sigma = N_{p-p}/5$. The limits of quantification were estimated as ten times σ of the peak to peak noise (N_{p-p}). The limits of detection and limits of quantification were estimated using six replicate samples of Lica and the glucuronides respectively in urine and plasma samples. Verification of the determined limits of quantification was performed using spiked blank urine and plasma.

2.7. Stability studies

The stability of Lica and its phenolic glucuronides *cis*- and *trans*-4'-glcU and *trans*-4'-glcU in the standard solutions prepared in plasma and urine and placed in the autosampler at ambient temperature was assessed for 24 h. The stability of Lica and its glucuronide conjugates was tested after storage at –20°C for 1, 2 and 3 months using spiked blank urine and plasma samples.

3. Results and discussion

The only chromatographic system described in the literature for the analysis of Lica [13] uses a C₁₈ column and acetonitrile–2% acetic acid (45:55) as the eluent. This method has been used for the quantitative analysis of Lica in liquorice. The separation of Lica and its conjugates in urine samples was therefore investigated using the described HPLC

method. It was not possible to obtain separation of the conjugates of Lica in this system. HPLC methods using a Spherisorb ODS-2 column, isocratic elution and mobile phases consisting of various mixtures of acetonitrile–acetic acid, methanol–acetic acid and tetrahydrofuran–acetic acid were studied. It was observed that eluents containing acetonitrile as the organic modifier resulted in improved separation and peak shape of the conjugates compared to mobile phases based on methanol or tetrahydrofuran. These isocratic systems did however not allow simultaneous separation of all five conjugates and peaks from blank urine samples were also shown to interfere. Thus a method based on gradient elution was developed.

Separation on C_8 and C_{18} RP columns using gradient elution was investigated in order to obtain separation of the N-acetyl-L-cysteine conjugate II and *trans*-4'-glcU peaks. The use of C_{18} columns resulted in an improved resolution of these two conjugate peaks compared to the C_8 columns (see Table 1). The column packing material was also shown to influence the peak shape, e.g., Polygosil C_{18} resulted in broad peaks compared to Inertsil ODS-2 (see Fig. 2). The complete separation of all five conjugates in urine samples was obtained using an Inertsil ODS-2 column and gradient elution with the mobile phases acetonitrile–2% acetic acid (8:92 v/v) A and (81:19 v/v) B. The gradient was as follows: a linear gradient from 100–70% A was

applied for 5 min, followed by a linear gradient from 70–65% A for 15 min, 65% to 0% A was used over 5 min and finally isocratic elution with 100% A for 10 min. The overall analysis time including re-equilibration was 35 min.

3.1. Applications

A single dose of 10 mg Lica was administered to six rats by i.p. injection. Plasma samples were collected 0, 5, 10, 15, 20, 25, 30, 45, 60 and 120 min after the administration of Lica. Urine samples were collected from six rats which received a single dose of Lica, as described above. The urine was collected separately as day and night urine for three days after administration. Representative chromatograms of the urine and plasma samples are shown in Fig. 3 and compared to blank rat plasma or urine, respectively. The chromatograms show that there were no interfering peaks in blank plasma or urine at the retention time of the respective analytes and a good separation of the analytes was obtained.

3.2. Linearity

The calibration curves obtained in spiked urine and plasma showed good linearity in the concentration ranges mentioned above (Table 2). It was observed that the linearity range for Lica in urine was 0.25–10 $\mu\text{g}/\text{ml}$ as concentrations above 10

Table 1

The separation of N-acetyl-L-cysteine conjugate II and *trans*-4'-glcU of Lica in spiked urine, on C_{18} and C_8 columns

Column packing material	Capacity factor (k')	Selectivity (α)	Resolution (R_s)
Spherisorb ODS-2	$k'_1=18.5$ $k'_2=19.6$	1.06	1.0
Nucleosil C_{18}	$k'_1=12.5$ $k'_2=13.9$	1.11	1.0
Kromasil C_{18}	$k'_1=19.2$ $k'_2=21.3$	1.11	0.8
Polygosil C_{18}	$k'_1=15.7$ $k'_2=16.7$	1.06	0.8
Inertsil ODS-2	$k'_1=19.5$ $k'_2=22.8$	1.17	3.5
Spherisorb C_8	$k'_1=12.3$ $k'_2=12.6$	1.02	0.3
Kromasil C_8	$k'_1=18.7$ $k'_2=19.5$	1.04	0.4

k'_1 is the capacity factor for the N-acetyl-L-cysteine conjugate II peak and k'_2 is the capacity factor for the *trans*-4'-glcU peak.

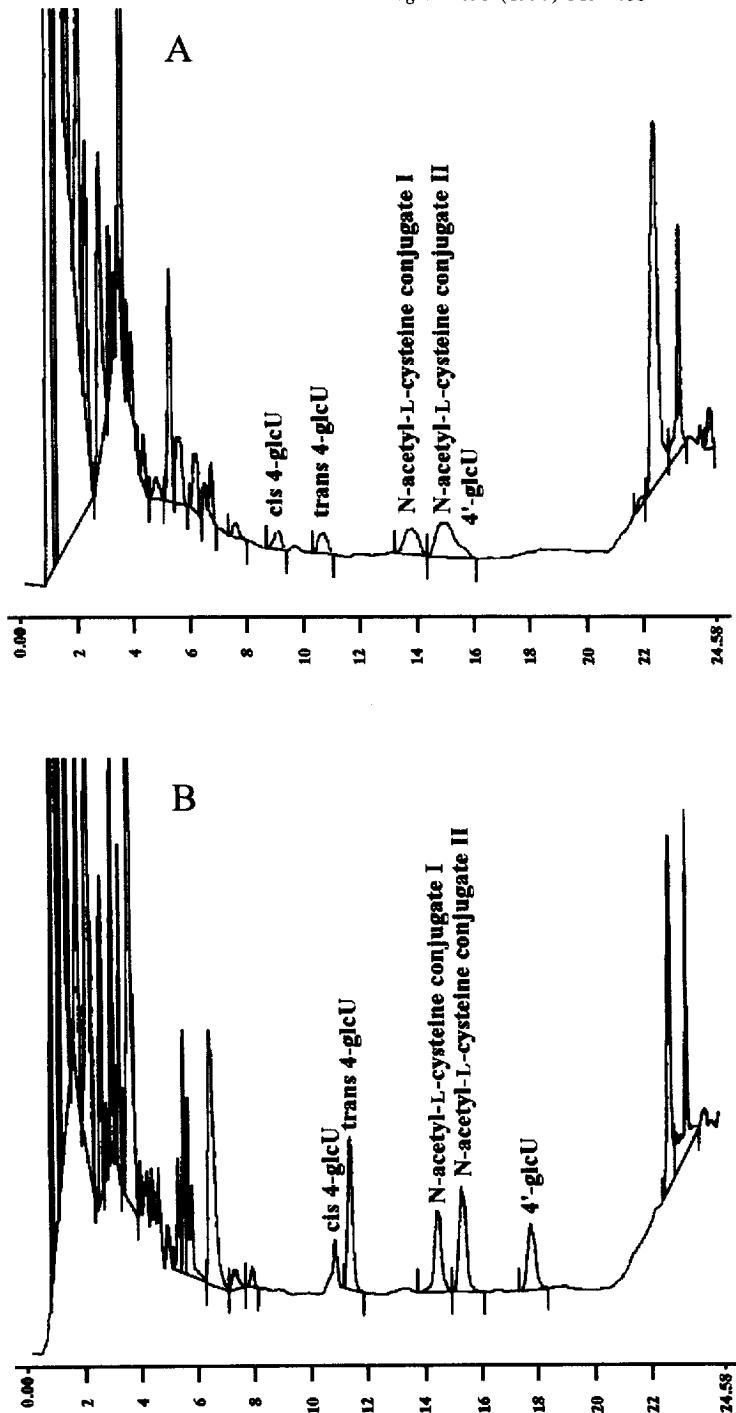


Fig. 2. Chromatograms in spiked urine samples, showing the separation of five Lica conjugates on (A) Polygosil C₁₈ and (B) Inertsil ODS-2 columns. Chromatographic conditions: column, 5 μ m, 120 \times 4.6 mm I.D.; mobile phases consisting of acetonitrile–2% acetic acid (8:92) (v/v %) for mobile phase A and (81:19) (v/v %) for mobile phase B; flow-rate of 1.5 ml/min; column temperature, 45°C; UV detection at 254 nm and an injection volume of 20 μ l. The gradient profile was as follows: a linear gradient of 100–70% A for 5 min, followed by 70–65% A for 15 min, 65% to 0% A for 5 min and finally isocratic elution with 100% A for 10 min.

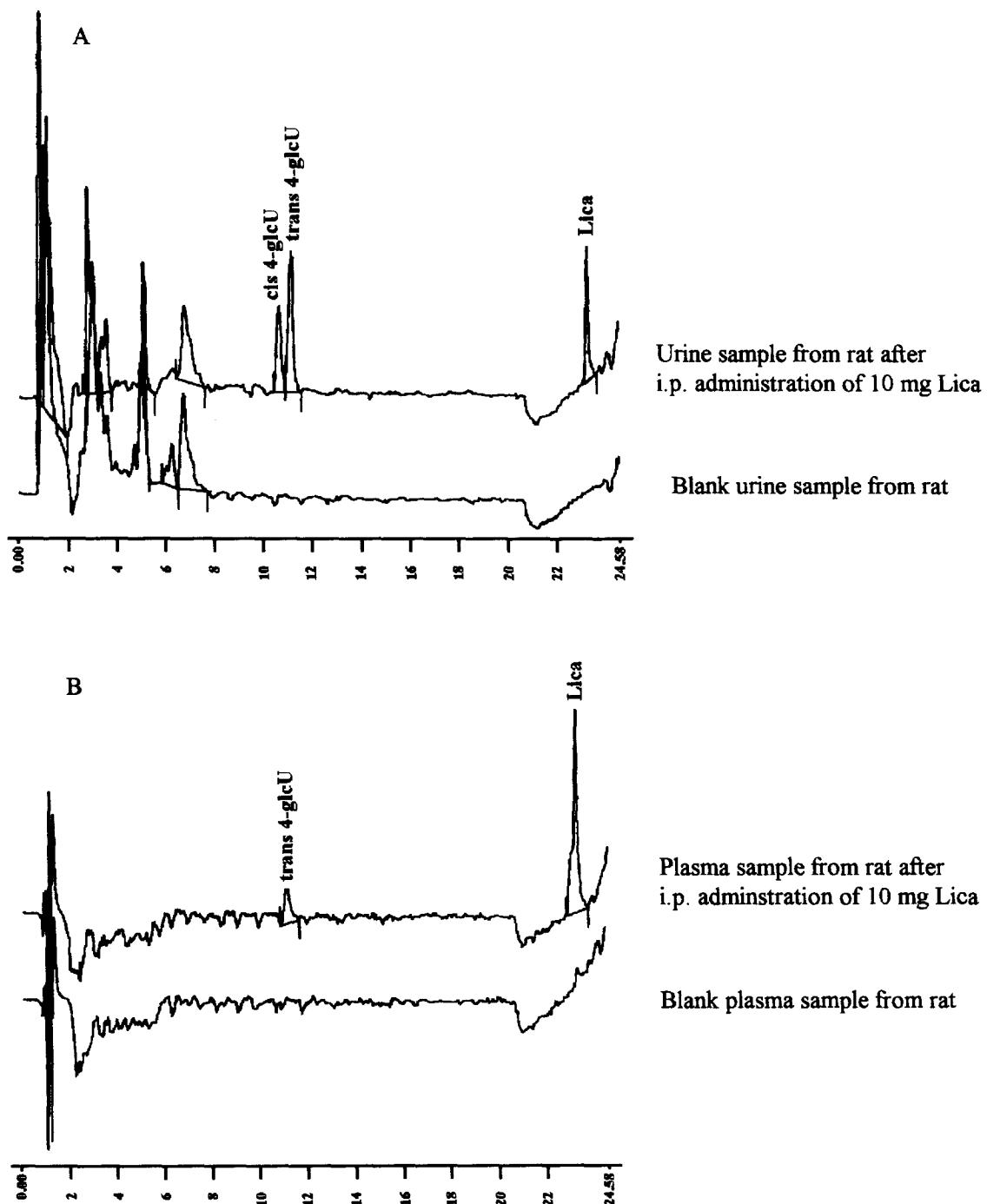


Fig. 3. Chromatograms of a urine sample from rat after i.p. administration of 10 mg Lica and a blank urine sample (A), a plasma sample after i.p. administration of 10 mg Lica and a blank plasma sample (B). Chromatographic conditions: an Inertsil ODS-2, 5 μ m, 120 \times 4.6 mm I.D. column; other conditions as described in Fig. 2.

Table 2

The linearity of the calibration curves for Lica, 4-glcU and 4'-glcU in urine and plasma (n=6)

	Correlation coefficient ±S.D. [R.S.D. (%)]	Intercept±S.D. [R.S.D. (%)]	Slope±S.D. [R.S.D. (%)]
Lica in plasma (0.25–20 µg/ml)	0.9994±1.2×10 ⁻⁴ (0.012%)	0.0444±0.029 (65.6%)	0.8231±0.0055 (0.7%)
Lica in urine (0.25–10 µg/ml)	0.9981±5.5×10 ⁻⁴ (0.055%)	-0.0460±0.0080 (17.5%)	0.5958±0.0051 (0.9%)
4'-GlcU in plasma (1–60 µg/ml)	0.9993±6.0×10 ⁻⁴ (0.060%)	0.0325±0.036 (110.0%)	0.0962±0.0040 (4.1%)
4'-GlcU in urine (5–60 µg/ml)	0.9998±0.5×10 ⁻⁴ (0.005%)	-0.0667±0.027 (40.9%)	0.1255±0.0016 (1.3%)
4-GlcU in plasma (4–16 µg/ml)	0.9978±26×10 ⁻⁴ (0.260%)	-0.0284±0.018 (63.1%)	0.1064±0.0020 (1.9%)
4-GlcU in urine (4–60 µg/ml)	0.9998±1.3×10 ⁻⁴ (0.013%)	-0.0472±0.015 (31.0%)	0.1017±0.0006 (0.6%)

µg/ml Lica resulted in non-linear responses. Problems with a limited linear range were also observed for 4-glcU in plasma as concentrations above 16 µg/ml resulted in non-linear responses. The linearity ranges mentioned above were shown to be more than adequate for the analysis of Lica and 4-glcU in urine and plasma samples from rats after i.p. administration of 10 mg Lica.

Linear regression analysis of the data resulted in correlation coefficients equal to or better than 0.998 and showed that the intercept was not statistically different from zero for each calibration curve (see Table 2).

3.3. Precision and accuracy

The concentrations in the spiked samples were calculated using the equation of the linear regression curves (found concentration) and expressed as a percentage of the theoretical concentrations (mean recovery %). The results of the intra-day and inter-day variations are shown in Tables 3 and 4. R.S.D.s were below 8% for all concentrations for intra-day as well as inter-day precision.

3.4. Limit of detection and quantification

The limit of detection and limit of quantification for Lica and the glucuronides were determined as described above using UV detection at 254 as well as

360 nm. The limits of detection at 360 nm were 0.1 µg/ml in plasma and 0.04 µg/ml in urine for Lica, 0.4 µg/ml in plasma and urine for 4'-glcU, and 0.6 µg/ml in plasma and 1.0 µg/ml in urine for 4-glcU. The limits of quantification for Lica were 0.2 µg/ml in plasma and 0.14 µg/ml in urine, and 1.2 µg/ml for 4'-glcU in plasma and 1.4 µg/ml in urine, and 2.0 µg/ml 4-glcU in plasma and 3.2 µg/ml in urine. It was observed that detection at 360 nm resulted in lower detection limits and therefore increased the sensitivity of the assay. A detection wavelength of 360 nm was therefore suitable for the quantification of Lica and the glucuronide conjugates *trans*-4'-glcU and *trans*-4-glcU.

The specificity of the method for Lica and its three phenolic glucuronides is also increased by using a detection wavelength of 360 nm as opposed to 254 nm as many possible interfering peaks from the biological matrix are not seen at this wavelength. It is however necessary also to perform detection at 254 nm in order to detect all five conjugates of Lica as the two N-acetyl-L-cysteine conjugates show absorption at 254 nm but not at 360 nm. The *cis*-4-glcU also shows greater UV absorption at 254 nm compared to 360 nm.

The N-acetyl-L-cysteine conjugates of Lica have so far not been detected in vivo studies of Lica. These conjugates have therefore not been included in the validation procedure.

The limits of quantification for the glucuronides

Table 3

The intra-day variability for Lica, 4-glcU and 4'-glcU in urine and plasma (n=6)

	Added ($\mu\text{g}/\text{ml}$)	Found ($\mu\text{g}/\text{ml}$) (mean \pm S.D.)	Mean recovery (%)	R.S.D. (%)
Lica in plasma	1.0	0.97 \pm 0.04	97.0	3.7
	10.0	9.89 \pm 0.18	98.9	1.8
	20.0	19.97 \pm 0.18	99.9	1.1
Lica in urine	1.0	1.06 \pm 0.03	106.0	2.8
	5.0	5.08 \pm 0.12	101.6	2.3
	10.0	9.96 \pm 0.46	99.6	4.6
4'-GlcU in plasma	10.0	9.84 \pm 0.38	98.4	3.9
	20.0	20.24 \pm 1.3	101.2	6.5
	60.0	59.94 \pm 1.7	99.9	2.8
4'-GlcU in urine	10.0	10.22 \pm 0.39	102.2	3.8
	20.0	19.33 \pm 0.39	96.7	2.0
	60.0	60.04 \pm 1.4	100.1	2.4
4-GlcU in plasma	4.0	4.26 \pm 0.17	106.5	4.0
	8.0	7.62 \pm 0.34	95.3	4.4
	16.0	16.27 \pm 0.60	101.7	3.7
4-GlcU in urine	10.0	9.90 \pm 0.44	99.0	4.4
	20.0	19.63 \pm 0.38	98.2	1.9
	60.0	59.22 \pm 0.99	98.7	1.7

Table 4

The inter-day variability for Lica, 4-glcU and 4'-glcU in urine and plasma (n=18)

	Added ($\mu\text{g}/\text{ml}$)	Found ($\mu\text{g}/\text{ml}$) (mean \pm S.D.)	Mean recovery (%)	R.S.D. (%)
Lica in plasma	1.0	0.97 \pm 0.03	97.0	3.3
	10.0	9.76 \pm 0.22	97.6	2.2
	20.0	19.53 \pm 0.65	97.7	3.3
Lica in urine	1.0	1.09 \pm 0.03	109.3	3.2
	5.0	4.83 \pm 0.21	96.6	4.3
	10.0	10.31 \pm 0.45	103.1	4.3
4'-GlcU in plasma	10.0	10.21 \pm 0.34	99.5	3.8
	20.0	20.45 \pm 1.3	102.3	6.2
	60.0	59.90 \pm 2.0	99.9	3.4
4'-GlcU in urine	10.0	10.21 \pm 0.34	102.1	3.3
	20.0	20.01 \pm 1.1	100.1	5.4
	60.0	62.32 \pm 2.2	103.9	3.5
4-GlcU in plasma	4.0	4.37 \pm 0.32	109.3	7.3
	8.0	7.79 \pm 0.56	97.4	7.2
	16.0	16.37 \pm 0.82	102.3	5.0
4-GlcU in urine	10.00	10.12 \pm 0.51	101.2	5.0
	20.0	20.44 \pm 1.0	102.2	5.1
	60.0	59.60 \pm 0.94	99.3	1.6

were shown to be 10–20 times higher than for Lica in terms of $\mu\text{g}/\text{ml}$. However, since the urinary concentrations of the glucuronides were found to be at least twenty times higher than that of the parent drug, these limits of quantification are sufficient for the quantification of 4-glcU in rat urine. The plasma concentration levels of 4-glcU were slightly higher than the limit of quantification, indicating that the assay can also be used to determine 4-glcU in plasma. However, an increase in sensitivity of the method towards 4-glcU in plasma would increase the applicability of the method for monitoring the glucuronide in plasma. The limit of quantification for Lica was shown to be sufficient for the analysis of Lica in plasma and urine. The R.S.D. was found to be 20% or less at the limit of quantification.

3.5. Stability of samples during storage

The stability of Lica and the glucuronides in the spiked samples was assessed in the autosampler by comparing the chromatograms and the peak areas after 0 and 24 h, using a UV detection wavelength of 254 nm. No signs of degradation were observed for Lica and *trans*-4'-glcU as the decrease in peak areas for the respective compounds was less than 3%. The only signs of degradation seen in the chromatograms for 4-glcU was the *cis/trans*-isomerization of the compound. The 4-glcU was present as the *cis*- and *trans*-isomers in both urine and plasma samples. The ratio of the *cis/trans*-isomers present in the samples was shown to be stable during the intra-day assay, however after storage in the autosampler for 24 h the ratio of the *cis/trans*-isomers present was shown to change from 70/30% to 85/15%. This could therefore explain the decrease in the total peak area of the *cis*- and *trans*-4-glcU peaks by up to 10% after 24 h. These results also indicate that the samples should be analyzed within the same day in order to minimize the isomerization.

The stability of Lica and the glucuronides in the spiked samples was also investigated after storage at -20°C for 1, 2 and 3 months as described above. The peak areas corresponding to Lica and its *trans*-4'-glcU were shown to decrease by less than 5% during three months storage. The 4-glcU assessed as the total peak areas for the *cis*- and *trans*-isomers at 254 nm was shown to decrease by less than 15%. The only signs of degradation seen for the 4-glcU

was the *cis/trans*-isomerization of the compound. This could explain the observed decrease in peak area due to the different response factors of the two isomers. The ratio of the *cis*-isomer present in the samples was shown to increase and a corresponding decrease in the *trans*-isomer present was observed after one months storage. The ratio of the *cis/trans*-isomers present in the samples was however shown to stabilize as no further increase was observed after two and three months storage.

3.6. Stability during sample preparation

The results from the analysis of urine samples showed that the 4-glcU is present as two isomeric glucuronides (*cis* and *trans*), as seen in Fig. 3.

The chalcones may undergo *cis/trans*-isomerization. This isomerization of the *trans*-form is influenced by sunlight (photoisomerization) [17,18], and the *cis*- to *trans*-isomerization of chalcones is catalyzed by acid and base [19–23].

It is necessary to protect all samples from sunlight in order to limit the photoisomerization of the 4-glcU during sample preparation. It was observed that exposure to direct sunlight led to the presence of the 4-glcU mainly as the *cis*-isomer, whereas the *trans*-isomer is predominant when solutions were stored in darkness.

The presence of the *cis*-isomers of Lica and its 4'-glcU have however not been detected in the urine or plasma samples. The presence of a free hydroxy group at the 4-position (see Fig. 1) of the chalcones has been reported to inhibit photoisomerization [24]. This could therefore explain why the 4-glcU shows *cis/trans*-isomerization opposed to the 4'-glcU and Lica, which both have a free hydroxy group at the 4-position.

3.7. In vivo study in rat

The proposed HPLC method for the determination of Lica and its glucuronide conjugates in plasma and urine was found to be sufficiently sensitive to monitor urine and plasma levels of Lica and 4-glcU in rat after i.p. administration of a 10 mg dose.

The results from the analysis of the urine samples showed that between 1–3% of the administered dose was detected as the *cis*- and *trans*-isomers of 4-glcU in urine. Only trace amounts of the parent compound

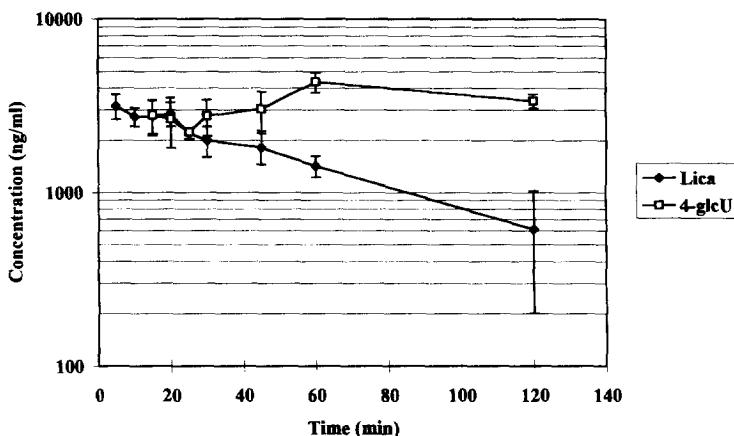


Fig. 4. Semi-logarithmic plot of mean concentrations (\pm S.D.) of Lica and *trans*-4-glcU in plasma after a single intraperitoneal dose of 10 mg Lica to rat ($n=6$).

were present (i.e., <0.1% of dose) and the *trans*-4'-glcU was not observed in the urine samples. Furthermore, only a small percent (approximately 2–3%) of the total dose was excreted in urine. These results indicate that the 4-glcU is the main urinary metabolite of Lica in rat.

It was also observed that only Lica and *trans*-4-glcU were detected in plasma and it was possible to monitor the plasma concentration profile of Lica and *trans*-4-glcU after i.p. administration of a single dose of Lica as shown in Fig. 4.

The treatment of the urine and plasma samples with β -glucuronides resulted in the hydrolysis of the *cis*- and *trans*-4-glcU and a corresponding increase in Lica peak area was observed.

A recent in vivo study of Lica in bile cannulated rats verified that Lica and its metabolites are excreted mainly in the bile. It was possible to detect large quantities of Lica and its three phenolic glucuronides in the bile after i.p. administration of a single dose of Lica [25]. These results could explain why only low concentration levels of Lica and its glucuronide conjugates are detected in urine and plasma samples.

4. Conclusion

The present HPLC method allows the simultaneous determination of Lica and its three phenolic conjugates in urine and plasma. The technique is simple, as sample preparation involves protein prepa-

ration only, and has good repeatability, recovery and accuracy. In addition, this is the first assay established for the determination of Lica and its main metabolites in biological fluids.

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