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Short communication

Gas chromatographic/mass spectrometric profiling of luteolin and its metabolites in rat urine and bile

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1. Introduction

Ajuga decumbens Thunb is a medicinal herb which has been used in oriental medicine as a remedy for cough, phlegm, asthma and inflammation. A sugar-coated tablet prepared from the acidic extracts of this herb has been used in clinical practice. From the extract, luteolin (**I**), a flavone compound, was isolated and identified as the active component [1]. Besides its antitussive and antiasthmatic effects, luteolin has also been recognized as an effective antiperoxidative compound [2,3], an active hydroxyl radical scavenger [4], a potential antitumor [5] and antiviral [6] agent, and a potent Protein Kinase C inhibitor [7]. To our knowledge, there are no reports concerning the metabolism of **I**. Several investigators [8,9] have studied the metabolites of some flavanols in vivo; there is evidence to suggest that the related 3′,4′-*O*-dihydroxy flavanols are largely metabolized in the liver by 3′-*O*-methylation, which might be of importance in protecting the body against the carcinogenic action of naturally-occurring flavonoids of dietary origin. Other results [10,11] revealed the presence of the well-known catechin-*O*-methyltransferase in liver which transfers a methyl group directly to the 3-hydroxyl group of catechin. In view of the above evidence, it was of interest to establish whether the luteolin is metabolized via the action of an *O*-methyltransferase to give 3′-methylluteolin. In addition, through epoxidation of an aromatic double bond, catalyzed by hepatic monoxygenase enzymes [12], epoxidized or hydroxylated metabolites might arise, which have greater biological effects [4,13,14] than luteolin.

The objectives of the present study were to characterize the metabolic and excretory pathways of luteolin in rat. The sensitivity and resolving power of capillary GC coupled with the ability of MS to carry out identification make the GC/MSD technique particularly suitable for this purpose. Trimethylsilyl derivatives of the flavonoid aglycones were prepared for GC/MS analysis, because of the superior derivatization reaction with the tetrahydroxy flavonoids [15].

2. Experimental

2.1. Chemicals

Luteolin was kindly donated by Professor K.H. Shin of the Natural Products Research Institute, Seoul National University, South Korea. XAD-2 resin (0.15–0.2 mm, Serva, Germany) was pretreated with acetone,

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methanol and distilled water several times. The following materials were purchased: β-glucuronidase (E. Coli) from Boehringer Co., Germany; *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), TMS-imidazole, dithioerythritol (DTE) and estriol from Sigma, St. Louis, MO, USA; polyethyleneglycol (PEG, average MW 200) from Aldrich, Milwaukee, WI, USA. All other reagents were of analytical grade.

2.2. Sample preparation

Two adult male Sprague-Dawley rats (180–240 g) were injected i.p. with luteolin (40 mg kg^{-1}) in 1.0 ml of PEG–saline (1:1, v/v), and 24 h urine samples were collected in two separated metabolic cages (Nalgene Co., NY, USA). The rats were maintained under standardized conditions of light and temperature, and food and water were provided ad lib. Blank urines were collected 24 h before the injection of luteolin. The volume of each urine sample was measured and stored at −20 °C until analysis.

Following the collection of 2 h blank bile, luteolin (20 mg kg^{-1}) in 1.0 ml of PEG–saline (1:1, v/v) was injected i.p. into two bile-duct-cannulated rats; bile was then collected every 30 min for 7 h, and the sample tubes were filled with nitrogen and subsequently stored at 20 °C until analysis.

A stock solution (1.00 mg ml^{-1}) of luteolin was prepared by dissolving in methanol; a series of standard solutions of 2.5–50 μg ml^{-1} was then obtained. A standard solution (0.2 mg ml^{-1}) of estriol was prepared by dissolving in methanol.

2.3. Sample pretreatment

One milliliter of urine of 0.05 ml of bile was added to an XAD-2 resin column (5 mm i.d., 2–3 cm height), 10 μl of estriol solution was added to the bile samples, 2 ml of distilled water was used to wash the column, and 3 ml of methanol was employed as an eulent. Following evaporation of the eluate with a Rotavapor (Buchi, Switzerland), 1 ml of 0.2 mol l^{-1} phosphate buffer (pH 7.0) and 25 μl β-glucuronidase (200 U ml^{-1}) were added and the solution was incubated at 50 °C for 1.5 h. After cooling at room temperature, the incubated solution was extracted with 3 × 3 ml of ethyl ether, the aqueous layer was frozen at −30 °C in a freezing block (Lauda RC6, Germany); the ether layer was transferred into another tube and was evaporated to dryness. The residue was redissolved in 200 μl of methanol, and the solution was transferred into a test vial containing 0.1 mg DTE, evaporated to dryness under a gentle stream of nitrogen and dried over P_2O_5–KOH overnight. The vial was then filled with N_2, tightly capped, 50 μl of MSTFA–TMCS–TMS-imidazole (100:5:2, v/v/v) added, vortex mixed, and maintained at 80 °C for 30 min using a heating block. A 1 μl volume of the solution was injected into the GC/MSD instrument.

In order to analyze the unconjugated fractions, following the addition of phosphate buffer, the aqueous solution was extracted with 3 × 3 ml of ethyl ether, the ether layer dried, and the residue redissolved and analyzed using GC/MSD.

2.4. Gas chromatography/mass spectrometry

GC/MS analyses were carried out with an HP 5890B GC-HP 5970 MSD system equipped with a SE-30 fused-silica cross-linked capillary column (17 m × 0.2 mm × 0.3 μm) (Hewlett-Packard, CA, USA). The flow rate of helium carrier gas was 0.83 ml min^{-1}. The column head pressure was 12 psi. The oven temperature was programmed as follows: 100 °C (for 1 min) to 220 °C at 15 °C min^{-1}, then at 4 °C min^{-1} to 290 °C (for 2 min); the total running time was 28 min. The temperature of the injection port was 280 °C, and that of the transferline was 300 °C. The EI/MSD was operated with an electron energy and emission current of 70 eV and 200 μA, respectively.

3. Results and discussion

3.1. Chromatographic separation and structure elucidation of luteolin and its metabolites

All samples were subjected to GC/MSD analysis; the total ion current (TIC) chromatographic profiles obtained for the trimethylsilyl ethers of luteolin and its metabolites, all of which were absent from the blank samples, are shown in Figs. 1 and 2. Following administration of luteolin to rat, three possible metabolites and the parent compound in urine and bile were detected. Two differences were observed between urine and bile samples. The first was

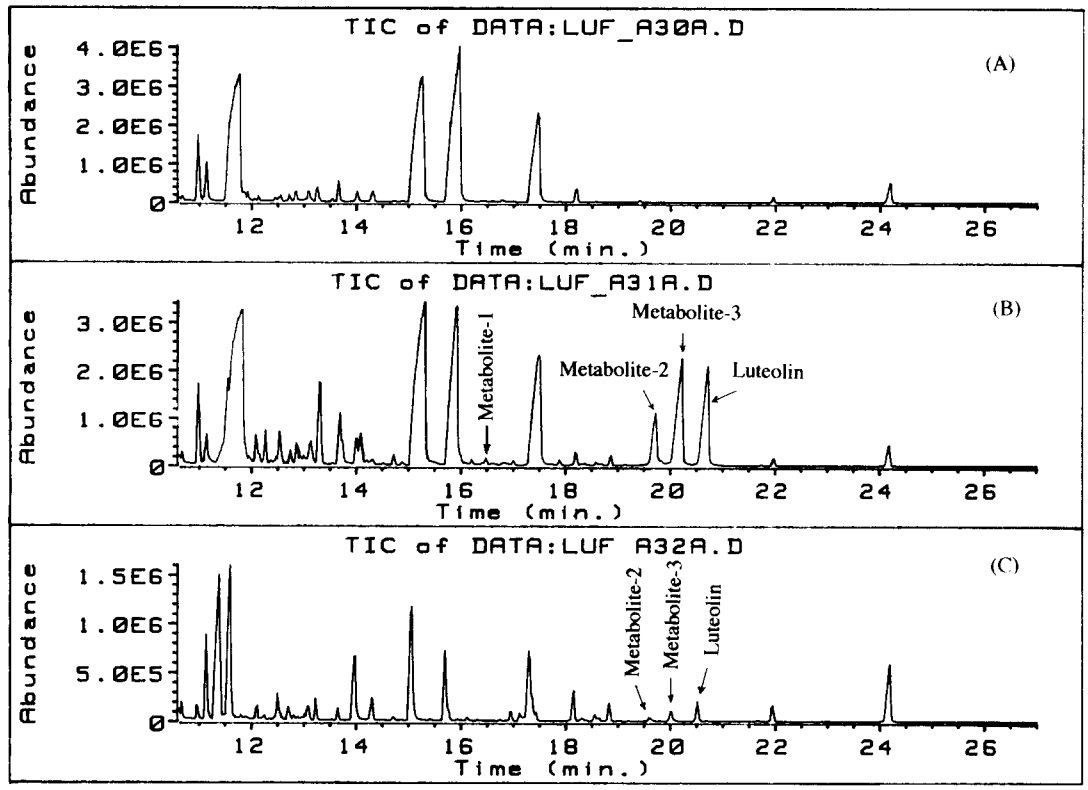

Fig. 1. Total ion current (TIC) chromatograms of trimethylsilyl derivatives of luteolin and its metabolites in rat urine. (A) blank urine, (B) conjugated forms in positive urine (24 h), (C) free forms in positive urine (24 h).

that metabolite-1 (Met-1) was present in very small amount in urine; it could not be detected in bile even in the selective ion monitoring (SIM) mode. The second difference was that, in urine, luteolin and its metabolites were present mainly in conjugated forms; about 5.2% of luteolin and 3.0% of metabolite-2 (Met-2) and metabolite-3 (Met-3) were excreted as aglycones, wherein in bile (0–2 h), free aglycones were not detected either in full scan or in the SIM mode. All of these compounds were neither detectable in urine after 24–48 h, nor in bile after 7 h. Mass spectral and chromatographic data for the TMS derivatives are given in Table 1.

In the TIC chromatograms, the TMS-derivatized luteolin gave a single peak, its mass spectrum gave the molecular ion of m/z 574, and the major associated fragment ions were m/z 559 (base peak), 471, 399, and 297, corresponding to $[M\text{-}CH_3]^+$, $[M\text{-}(CH_3 + OTMS)]^+$, $[M\text{-}(CH_3 + OTMS + TMS)^+$, and C-ring cleavage, respectively. In contrast to the mass spectrum of TMS-derivatized genistein [16], since the two OH groups on ring B are meta to each other, ions were detected at m/z 574 (M^+), 559 ($[M\text{-}CH_3]^+$), 487 ($[M\text{-}CH_3 - TMS]^+$), 415 ($[M\text{-}CH_3 - 2TMS]^+$), with no ions at m/z 471, 399, and 297 (Fig. 3).

The mass spectrum of the TMS derivative of Met-2 was quite similar to that of Met-3, both of them showing a molecular ion at m/z 516, and characteristic ions at m/z 501, 471, 399, 297, and 228, indicating that the two metabolites are methylated isomers of luteolin. Owing to the presence of m/z 471, 399, and 297, the same fragments as those of luteolin (Fig. 3), it seems that the methylation occurred on ring B to give 3′- or 4′-*O*-methylluteolin. However, because of the lack of sufficient fragmentation information on the TMS ethers and the absence of authentic standards, it was not possible to determine whether they were 3′-methylluteolin or the 4′-methylisomers. HPLC/MS, and ^1H NMR were therefore employed to enrich and identify Met-2 and Met-3, and the results will be reported elsewhere.

The TMS-derivatized Met-1 showed a molecular ion of m/z 590 and a base ion of m/z 575, and had a shorter retention time than TMS-luteolin; its structure has not been identified.

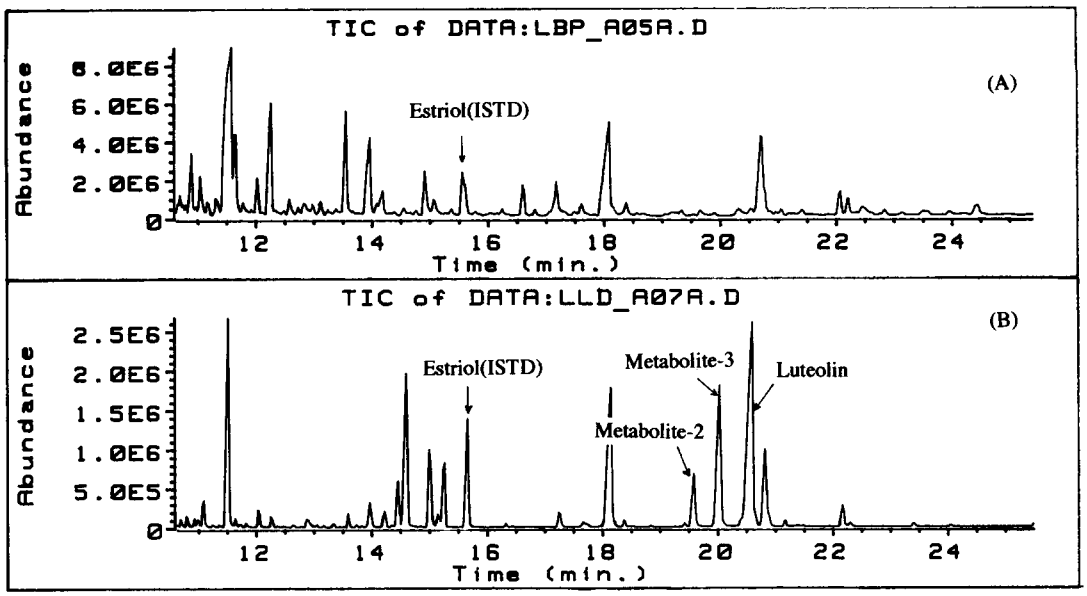

Fig. 2. TIC chromatograms of trimethylsilyl derivatives of luteolin (I) and its metabolites in rat bile. (A) blank bile, (B) positive bile (2.5–3 h).

Table 1
GC retention times and fragment ions in the mass spectra of trimethylsilyl derivatives of luteolin and its metabolites

Compound	*RT*(min)	*RRT*	Fragment ions m/z (%)
Metabolite-1	16.49	0.794	590(5) 575(100) 562(11) 477(5) 369(4) 147(3)
Metabolite-2	19.77	0.952	516(1) 501(100) 471(17) 399(8) 297(1) 228(4)
Metabolite-3	20.26	0.975	516(1) 501(100) 471(16) 399(6) 297(1) 228(2)
Luteolin	20.77	1.000	574(1) 559(100) 471(13) 399(6) 297(1)

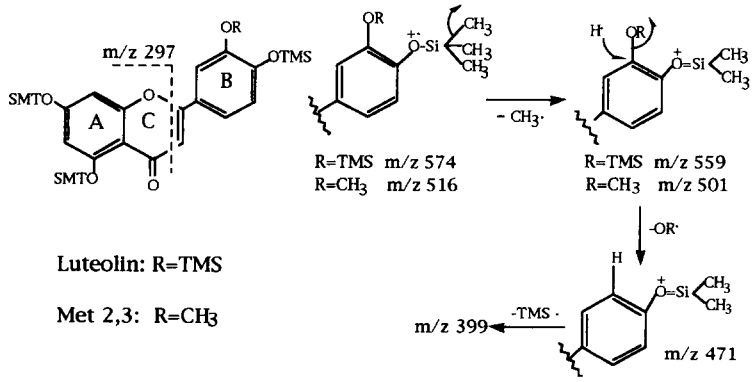

Fig. 3. Characteristic fragmentation pathways of trimethylsilyl luteolin and its metabolites.

3.2. Excretion of luteolin via urine and bile

Semi-quantitative analysis of luteolin and its Met-2 and Met-3 excreted from urine and bile was achieved by SIM analysis of the base peak (*m/z* 504) in the mass spectrum of TMS-estriol (internal standard) relative to ions *m/z* 559, 501, and 501 of TMS-derivatized luteolin, Met-2 and Met-3, respectively. The excretory data of these compounds are shown in Table 2, the results suggesting that nearly 15% of luteolin administered was excreted via urine (5.6% as the parent compound, 9.1% as methylated metabolites), and more than 28% of the parental dose was recovered from bile (14.7% as the unchanged drug, 13.7% as metabolites). Data for bile levels of luteolin and its metabolites indicated quick absorption (t_{max} = 1 h) and rapid transformation (t_{max} = 1 h), followed by a slow elimination phase (Fig. 4).

Table 2
Excretory recovery of luteolin from rat urine and bile

Sample	Luteolin administered (mg)	Luteolin extreted (mg)			Excretory recovery (%)		
		Luteolin	Met-2	Met-3	Luteolin	Met-2	Met-3
Urine (0–24 h)	10.56	0.57	0.30	0.63	5.40	2.84	5.96
	10.32	0.61	0.31	0.65	5.91	3.00	6.30
Bile (0–7 h)	4.40	0.70	0.17	0.45	15.91	3.86	10.23
	4.65	0.63	0.21	0.41	13.55	4.52	8.82

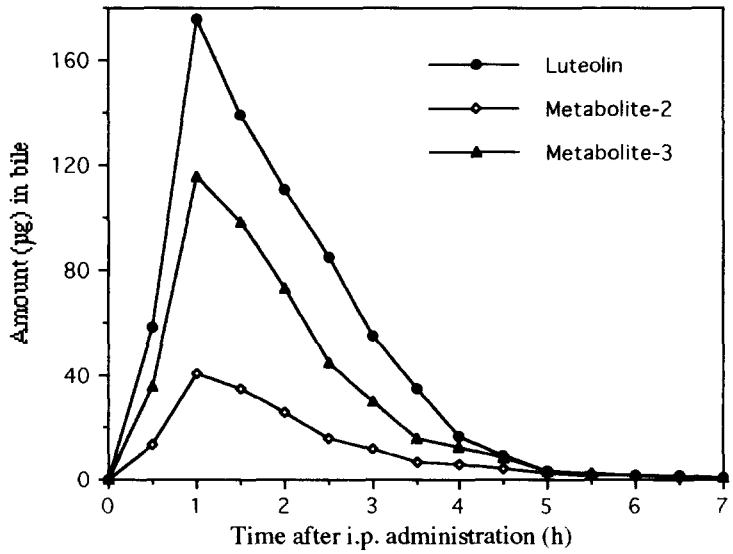

Fig. 4. Biliary excretion of luteolin and its metabolites.

From the results obtained, it seems important to note that the amounts of conjugated methyl ethers found in urine and bile exceeded those of conjugated luteolin. This would suggest that although the degree of absorption of luteolin from the gastro-intestinal tract is small, a considerable proportion of the absorbed flavone undergoes methylation in the tissues, blocking an essential structural feature required for inhibitory activity [7,13,17] as mentioned in the Introduction. Some investigators have pointed out that, owing to the degradation in the intestine by the microflora following biliary excretion, the methylated metabolites of some flavonols were not observed in urine [8]. The results obtained from luteolin by our method were not in good agreement with their implications; *O*-methylated metabolites were readily detected in rat urine, although their concentrations in urine were lower than those in bile.

Table 3
Recovery of the sample pretreatment method

Luteolin added ($+x$)[a] (μg ml)	Luteolin recovered ($+y$)[a] (μg ml)	Recovery (%)
2.50	1.95	79.7
	2.18	87.4
5.00	3.97	79.4
	4.57	91.4
10.00	7.40	74.0
	7.92	79.2
25.00	18.50	74.0
	20.25	81.0
50.00	38.18	76.4
	33.33	66.7
Mean		78.9
SD		5.68
RSD (%)		7.20

[a] Linear correlation gave the relationship $y = 0.482 + 0.719x$ ($r = 0.998$)

3.3. Recovery and detection limits

In our present method, the mean recovery from the sample pretreatment procedure was 78.9%, and the recovery was linear in the range

of 2.5–50 μg ml^{-1} in urine (Table 3). The reproducibility expressed as a relative standard deviation (RSD) was 7.2%. The lowest detectable concentration of luteolin was 500 ng ml^{-1} in full scan mode (10 ng was injected into GC/MSD) and 50 ng ml^{-1} in the SIM mode (1 ng was injected into GC/MSD), respectively.

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