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Characterization of emodin metabolites in Raji cells by LC–APCI-MS/MS

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ABSTRACT

A rapid, simple, and sensitive liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS/MS) method was developed for the identification and quantification of emodin metabolites in Raji cells, using aloe-emodin as an internal standard. Analyses were performed on an LC system employing a Cosmosil $5C_{18}$ AR-II column and a stepwise gradient elution with methanol–20 mM ammonium formate at a flow rate of 1.0 mL/min operating in the negative ion mode. As a result, the starting material emodin and its five metabolites were detected by analyzing extracts of Raji cells that had been cultivated in the presence of emodin. The identification of the metabolites and elucidation of their structures were performed by comparing their retention times and spectral patterns with those of synthetic samples. In addition to the major metabolite 8-*O*-methylemodin, four other metabolites were assigned as ω-hydroxyemodin, 3-*O*-methyl-ω-hydroxyemodin, 3-*O*-methylemodin (physcion), and chrysophanol.

1. Introduction

The polyhydroxylated anthraquinone emodin (1,3,8-trihydroxy-6-methylanthraquinone) (**1**) is a widely occurring natural product found in several higher plant families, fungi, lichens, and animals. In higher plants emodin is present chiefly as the glycoside conjugate, which is an active component in various Chinese herbs, such as rhubarb, *Rheum palmatum* L., and *Polygonum multiflorum* Thunb. Pharmacological studies using crude and pure emodin have shown that emodin is a selective receptor tyrosine kinase inhibitor[1] and exhibits tumor cell-growth inhibition,[2] as well as antibiotic,[3] anti-viral,[4] and cytostatic[5] activity. Thus, emodin is expected to be a promising antitumor agent for patients who develop drug resistance to other traditional remedies.[6–9]

The biotransformation of emodin with rat liver microsomes has been studied, and six oxidative emodin metabolites (2-hydroxyemodin (**2**), 4-hydroxyemodin (**3**), 5-hydroxyemodin (**4**), 7-hydroxyemodin (**5**), ω-hydroxyemodin (**6**), and emodic acid (**7**)), generated by cytochrome P450 were observed.[10] The hepatic microsomes derived from various animal species transformed emodin into several anthraquinone metabolites.[11–15] Though emodin is a selective inhibitor of protein kinase, few biotransformation studies of emodin in human cells have been performed. We have previously shown that emodin inhibits Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells, which are derived from the human B-lymphoid cell line.[16] Oxidative emodin derivatives **2–7** possessed greater inhibitory effects compared with inhibition by emodin. Therefore, we are interested in characterization of the metabolites of emodin in Raji cells.

Because of their low concentrations in biological samples, quantification of the metabolites of emodin in cells requires a sensitive analytical method. High-performance liquid chromatography (HPLC)[17,18] and capillary electrophoresis (CE)[19] are used most commonly for the detection of anthraquinone metabolites. Liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) is a highly specific and sensitive method that has been widely used as a powerful analytical tool for the identification of drug metabolites in biological matrices.[15,20–23] MS technique has made possible the acquisition of structurally informative data from protonated molecules of analytes of interest.

In this paper, a simple, sensitive, and specific LC–APCI-MS/MS method was employed to investigate the in vitro metabolism of emodin (an overview of which is shown in Fig. 1). In Raji cells, five emodin metabolites, **6** and **8–11**, together with unchanged emodin were detected. The results were quite different from the reported results from the biotransformation of emodin incubated in rat liver microsomes.[10]

2. Materials and methods

2.1. Chemicals

Emodin (**1**) was purchased from Extrasynthese (Genay, France). Ammonium formate, acetonitrile, and methanol (MeOH) (HPLC

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grade) were purchased from Wako (Osaka, Japan). Aloe-emodin (**12**) was purchased from Sigma–Aldrich (St. Louis, MO). *S*-Adenosyl-L-methionine-d_3 was purchased from C/D/N Isotopes (Quebec, Canada). Chrysophanol (**11**) was isolated from the root

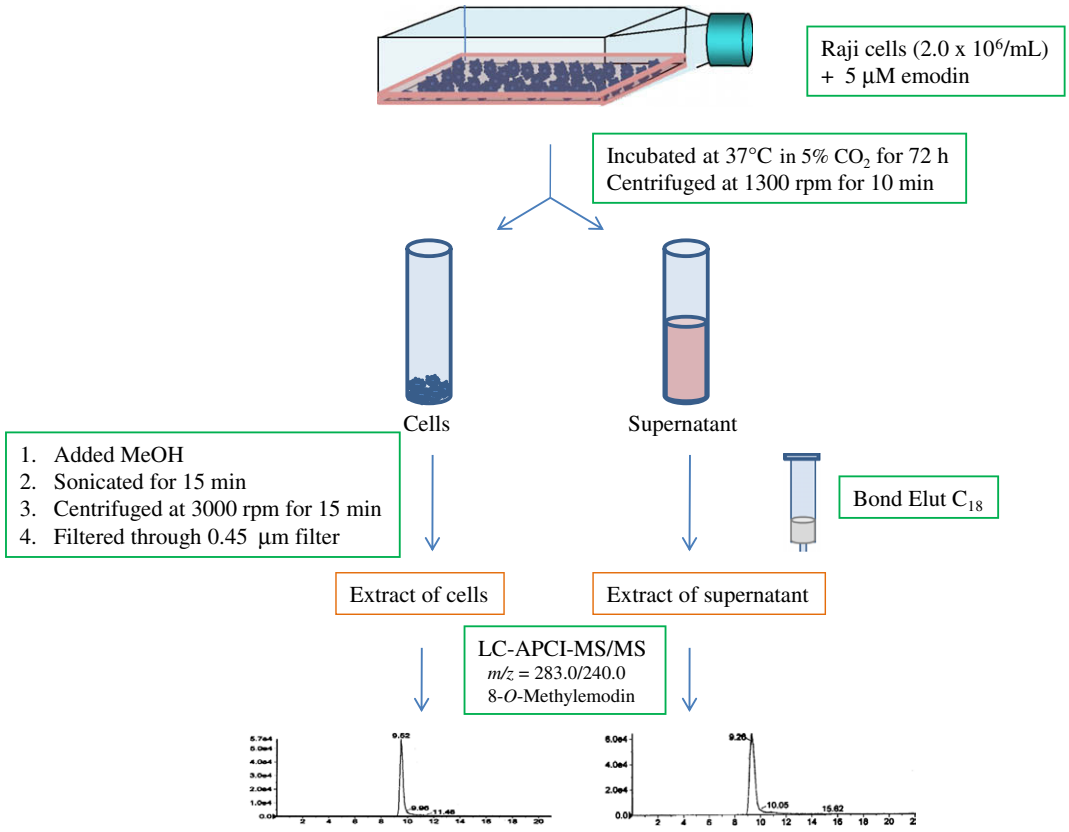
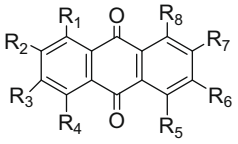
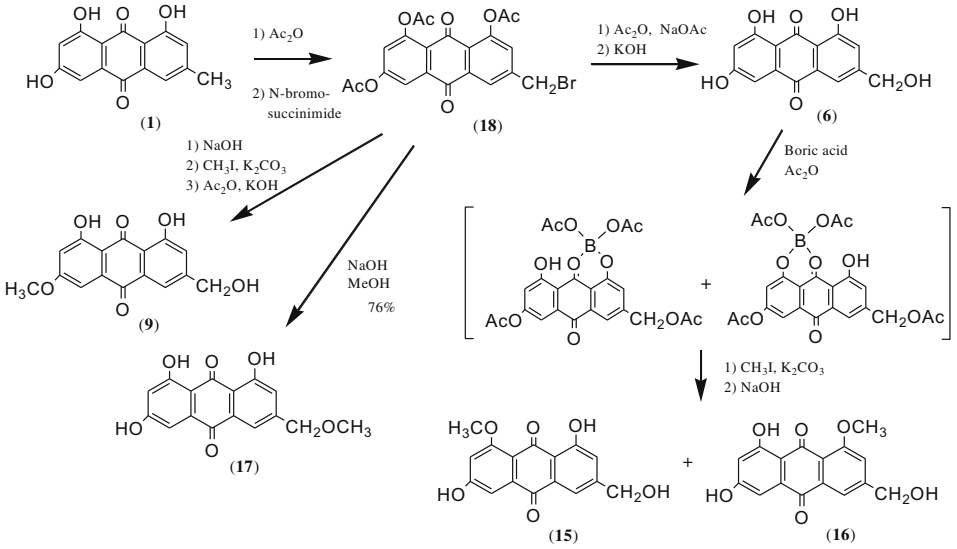


Figure 1. Schematic representation of the sample preparation and the determination of metabolites of emodin in Raji cells by LC–APCI-MS/MS.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	OH	H	OH	H	H	CH_3	H	OH
2	OH	OH	OH	H	H	CH_3	H	OH
3	OH	H	OH	OH	H	CH_3	H	OH
4	OH	H	OH	H	OH	CH_3	H	OH
5	OH	H	OH	H	H	CH_3	OH	OH
6	OH	H	OH	H	H	CH_2OH	H	OH
7	OH	H	OH	H	H	COOH	H	OH
8	OH	H	OH	H	H	CH_3	H	OCH_3
9	OH	H	OCH_3	H	H	CH_2OH	H	OH
10	OH	H	OCH_3	H	H	CH_3	H	OH
11	OH	H	H	H	H	CH_3	H	OH
12	OH	H	H	H	H	CH_2OH	H	OH
13	OH	H	OH	H	H	CHO	H	OH
14	OCH_3	H	OH	H	H	CH_3	H	OH
15	OCH_3	H	OH	H	H	CH_2OH	H	OH
16	OH	H	OH	H	H	CH_2OH	H	OCH_3
17	OH	H	OH	H	H	CH_2OCH_3	H	OH

Figure 2. Structures of emodin derivatives.



Scheme 1. Synthesis of methyl ethers of ω-hydroxyemodin.

bark of *Cassia siamea*.[24] 2-Hydroxyemodin (**2**), 4-hydroxyemodin (**3**), 5-hydroxyemodin (**4**), and 7-hydroxyemodin (**5**) were prepared according to the method of Banks.[25] Synthesis of ω-hydroxyemodin (**6**) was accomplished according to Hirose's method,[26] and emodic acid (**7**) was prepared according to Wolfbeis's method.[27] Emodin aldehyde (**13**) was obtained by oxidation of **6** with MnO_2. 1-*O*-Methylemodin (**14**), 3-*O*-methylemodin (**10**), and 8-*O*-methylemodin (**8**) were obtained by methylation of **1** with iodomethane. 1-*O*-Methyl-ω-hydroxyemodin (**15**)[28] and 8-*O*-methyl-ω-hydroxyemodin (**16**)[29] were obtained by methylation of **6**. 3-*O*-Methyl-ω-hydroxyemodin (**9**) was prepared from 1,3,8-triacetoxy-6-bromomethylanthraquinone (**18**).[30] The structures of all emodin derivatives are provided in Figure 2, and the chemical transformations are shown in Scheme 1.

Water (H_2O) was purified using a Millipore water purification system (Bedford, MA) with a resistance >18 MΩ/cm. Solid phase extraction (SPE) cartridges (Bond Elut C_{18}, 500 mg, 6 mL) were purchased from Varian (Lake Forest, CA).

2.2. Synthesis of 1,3,8-trihydroxy-6-methoxymethylanthraquinone (17)

1,3,8-Triacetoxy-6-bromomethylanthraquinone (**18**) (50 mg) was refluxed with NaOH (50 mg) in MeOH (20 mL) for 1 h. After evaporation of the solvent, water was added, and the mixture was stirred for 30 min. The aqueous solution was extracted with chloroform, and the organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness. The residue was purified with PTLC to afford 1,3,8-trihydroxy-6-methoxymethylanthraquinone (**17**) as yellow crystals (76%). Mp 195–197 °C (decomp.). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.46 (3H, s, CH_3), 4.53 (2H, s, CH_2), 6.60 (1H, d, J = 2.0 Hz, H-2), 7.23 (1H, d, J = 2.0 Hz, H-4), 7.26 (1H, s, H-7), 7.72 (1H, s, H-5). ^{13}C NMR ($CDCl_3$) δ: 58.7 (OCH_3), 73.5 (CH_2O), 108.8 (C2), 109.2 (C1a), 110.2 (C4), 115.2 (C9a), 118.4 (C5), 122.4 (C7), 133.7 (C10a), 135.3 (C4a), 147.8 (C6), 162.4 (C8), 165.4 (C1), 166.8 (C3), 182.7 (C10), 190.3 (C9). HRMS *m/z*: 300.0641 (Calcd for $C_{16}H_{12}O_6$, 300.0633).

2.3. Cell culture and sample extraction

Raji cells (EBV genome-carrying lymphoblastoid cells) derived from Burkitt's lymphoma were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (GOLD, ICN Biomedicals, Aurora, Ohio), 100 units/mL penicillin G, 100 μg/mL streptomycin, and 10 mM HEPES buffer. Raji cells were cultured at 37 °C in a humidified 5% CO_2 incubator. The cells were inoculated and maintained, and the medium was changed every 3–4 days. Raji cells (2.0×10^6 cells mL^{-1}) were incubated in 25 mL of the serum-free medium containing 5 μM emodin at 37 °C in a CO_2 incubator. Because the viability of Raji cells was 70% when exposed to 8.5 μM emodin, the emodin concentration could not exceed this threshold for the metabolic assay. Incubation with a medium containing 100 nM emodin was insufficient, making the identification of the metabolites impossible. Further, emodin inhibits EBV-EA activation with an IC_{50} of 2.6 μM.[31] After incubation of the Raji cells with emodin for 72 h, the cell suspensions were centrifuged at 1300 rpm for 10 min, and the supernatant and cells were separated. The cell pellet and supernatant were processed separately to provide the cell extract and supernatant extract for analysis.

To generate the extract of cells, an internal standard (IS; 10 μM aloe-emodin) was added to the cell pellet followed by the addition of MeOH to provide a final volume of 0.5 mL, and the mixture was processed ultrasonically for 15 min. After centrifugation at 3000 rpm for 15 min, the supernatant was filtered through a 0.45-μm filter and transferred into a vial to provide the extract of cells. To generate the extract of the supernatant, the supernatant containing 2 μM internal standard was loaded onto the SPE cartridge that had been activated and conditioned with 1.5 mL MeOH followed by 1.5 mL water. The supernatant was drawn through the column by using a low vacuum, then a full vacuum was applied briefly, and the retained components were eluted from the cartridge with 1.5 mL MeOH into a vial to provide the extract of the supernatant. The extracts (50 μL injection volume) were analyzed by LC–APCI-MS/MS. Controls for the extracts were obtained according to the above method using samples without emodin in cells cultures and without Raji cells in a serum-free medium containing emodin.

2.4. Instrumentation

The LC–APCI-MS/MS system used for the assays was a Shimadzu HPLC system (Shimadu, Kyoto, Japan) consisting of a binary pump (LC-10AD liquid chromatograph), an automatic solvent degasser (DGU-14A degasser), and an autosampler (SIL-10AD auto injector) coupled to an API3000 LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with an APCI interface. All MS data were measured in the negative ion mode. Typically, source condi-

tions were set as follows: nebulizer gas (N_2), 14; curtain gas, 10; ionspray voltage, 5.0 kV; vaporizer temperature, 400 °C. Analyst software version 1.4.2 (Applied Biosystems) was used for the quantitation procedure.

2.5. LC–APCI-MS/MS conditions

A Cosmosil 5C_{18} AR-II column (5 μm, 4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan) was used for the LC–APCI-MS/MS. LC separation was carried out using a mobile phase consisting of 20 mM ammonium formate (solvent A) and MeOH (solvent B). The following stepwise gradient was employed at a flow rate of 1.0 mL/min: initial A:B = 40:60, 30 min 20:80, 35 min 5:95. Sample volumes of 50 μL were injected.

2.6. *S*-Adenosyl-L-methionine-dependent methylation

Raji cells (2.0×10^6 cells mL^{-1}) were incubated in 25 mL of the serum-free medium containing 5 μM emodin and 300 μM *S*-adenosylmethionine-d_3 (SAM-d_3) at 37 °C in a CO_2 incubator. After 72 h, the cell suspensions were centrifuged at 1300 rpm for 10 min, and the supernatant and cells were separated. The extracts were obtained according to a method similar to the one described in Section 2.3. The control was acquired using the extract that was obtained according to the above method without Raji cells in the serum-free medium containing emodin and SAM-d_3.

2.7. Quantification

Quantitative analysis was carried out using the APCI-MS/MS (selected reaction monitoring: SRM) mode for the precursor/product ion of 8-*O*-methylemodin (283.0/240.0), ω-hydroxyemodin (285.0/210.8), 3-*O*-methyl-ω-hydroxyemodin (299.0/256.0) and aloe-emodin (269.9/183.0). To generate the cell extracts for making calibration curves, an internal standard (IS; 10 μM aloe-emodin) and standard 8-*O*-methylemodin, ω-hydroxyemodin, or 3-*O*-methyl-ω-hydroxyemodin (5, 50, 200, and 1000 nM, respectively) were added to the cell pellet followed by the addition of MeOH to provide a final volume of 0.5 mL. The mixtures were treated as the similar method in Section 2.3 to obtain the cell extracts. The supernatant extracts were generated according to the similar method in Section 2.3 from the supernatant (25 mL) containing 2 μM IS and standard 8-*O*-methylemodin, ω-hydroxyemodin, or 3-*O*-methyl-ω-hydroxyemodin (1, 5, 50, and 500 nM, respectively). The ratio of the standard to IS peak area was used for calibration by linear regression analysis.

3. Results and discussion

The LC–MS analyses described in the report represent the first assay conditions allowing the chromatographic separation and MS identification of the main metabolites of emodin transformed in the human B-lymphoid cell line.

3.1. Method development

An LC–APCI-MS/MS method was developed using a reverse-phase LC C18 column (Cosmosil 5C_{18} AR-II) and a stepwise gradient elution system composed of 20 mM ammonium formate as solvent A and MeOH as solvent B (initial A:B = 40:60, 30 min 20:80, 35 min 5:95) at a flow rate of 1.0 mL/min. Several mobile phases were evaluated for the ability to detect six oxidization metabolites of emodin obtained from treatment of emodin with hepatic microsomes. Evaluation of formic acid, acetic acid, ammonium formate, and ammonium acetate as potential components of the elution system indicated that ammonium formate was preferable. Table 1 provides an overview of MS parameters, including SRM transitions and energies of the metabolites, and a listing of product ions from the MS/MS spectra. These product ions were generated from low-energy collision-induced dissociation of the protonated molecules.

3.2. Identification of metabolites

The extracted ion chromatograms for the negative LC–APCI-MS analysis of products obtained from the incubation of emodin in Raji cells are shown in Figure 3. Compared with the control, three major metabolites were detected in addition to unchanged emodin. Each metabolite was identified on the basis of chromatographic behavior and characteristic mass spectrometric fragmentation features observed in the APCI-MS/MS spectra of these compounds. The

Table 1
LC–APCI-MS/MS parameters and retention times (T_R)

	Compound	Product ion (*m*/*z*)	Precursor ion (*m*/*z*)/product ion (*m*/*z*)	DP[a] (V)	FP[b] (V)	CE[c] (V)	CXP[d] (V)	T_R (min)
1	Emodin	241.1, 225.0 182.0	268.9/225.0:- CO_2	−81	−290	−36	−13	21.5
2	2-OHEM	210.9, 201.2 183.0	284.9/210.9:- (2CO + H_2O)	−51	−170	−48	−11	12.5
3	4-OHEM	257.0, 201.2 187.1, 158.8	284.9/257.0:- CO	−51	−180	−38	−19	13.5
4	5-OHEM	256.9, 240.8 216.8, 213.0	284.9/256.9:- CO	−91	−310	−38	−15	30.0
5	7-OHEM	257.0, 240.9 213.0	284.9/240.9:- (2CO + H_2O)	−51	−170	−38	−13	10.9
6	ω-OHEM	268.0, 256.8 241.0, 210.8	285.0/210.8:- (2CO + H_2O)	−51	−170	−46	−9	6.6
7	Emodic acid	267.0, 254.9 227.1	298.9/254.9:- CO_2	−51	−170	−46	−21	3.8
8	8-*O*-MeEM	268.0, 240.0	283.0/240.0:- (CH_3 + CO)	−61	−190	−34	−19	9.5
9	3-*O*-Me-ω-OHEM	256.0, 227.0	299.0/256.0:- (CH_3 + CO)	−56	−190	−38	−15	15.0
10	3-*O*-MeEM	239.9, 183.1	282.9/239.9:- (CH_3 + CO)	−61	−190	−36	−15	35.5
11	Chrysophanol	225.0, 181.9	253.0/225.0:- CO	−51	−170	−38	13	30.4
12	Aloe-emodin	240.0, 183.0	269.0/183.0:- (2CHO + CO)	−51	−210	−42	−11	9.9
13	Emodin aldehyde	255.0, 239.0 182.9, 199.0	283.0/255.0:- CO	−56	−200	−38	−17	8.5
14	1-*O*-MeEM	240.0, 183.1	282.9/240.0:- (CH_3 + CO)	−61	−190	−34	−19	11.4
15	1-*O*-Me-ω-OHEM	256.0, 239.0	299.0/256.0:- (CH_3 + CO)	−71	−250	−38	−15	3.5
16	8-*O*-Me-ω-OHEM	256.0, 239.0	299.0/256.0:- (CH_3 + CO)					3.6
17	l,3,8-TriOH-6-methoxymethylAQ	268.3, 239.9	299.1/268.3:- OCH_3	−56	−270	−40	−20	16.7

AQ: anthraquinone; EM: emodin; Me: methyl; OH: hydroxy.
[a] Declustering potential.
[b] Focusing potential.
[c] Collision energy.
[d] Collision exit potential.

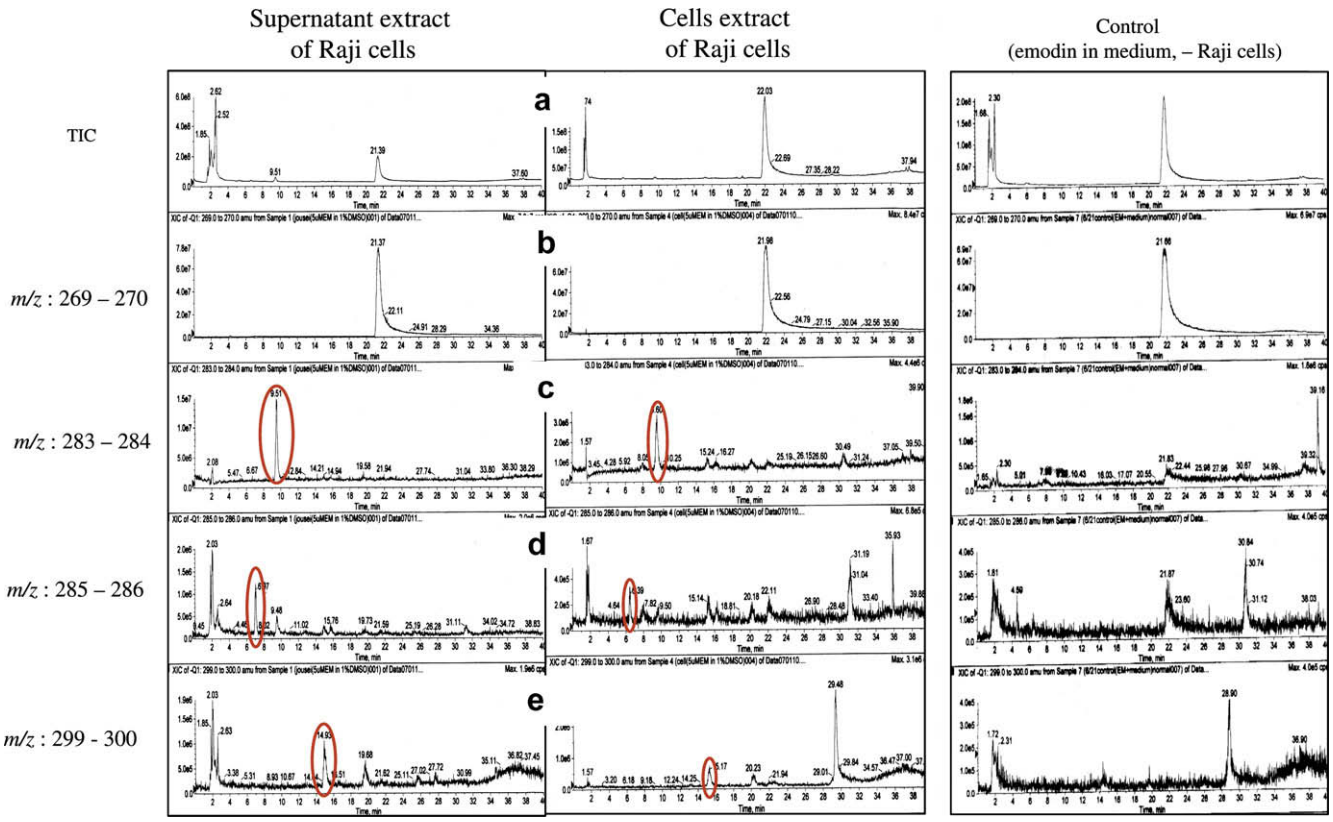


Figure 3. LC–APCI-MS chromatograms of emodin metabolites.

retention times and characteristic fragments used to identify the metabolites are summarized in Table 1.

The compound eluting at 6.6 min (Fig. 3d) gave a deprotonated molecule at *m/z* 285, 16 Da more than that of emodin (*m/z* 285–269), indicating that this compound was a monohydroxylated metabolite of emodin. The appearances of product ions at *m/z* 268 ([M−H−OH]), *m/z* 257 ([M−H−CO]), *m/z* 241 ([M−H−CO–O]), and *m/z* 211 ([241-HCHO]) suggested that this compound should be a metabolite generated by oxidation of the exocyclic methyl group of emodin. Thus, the metabolite was identified as ω-hydroxyemodin (**6**) from the information obtained from the MS/MS spectrum and from comparison with a synthesized authentic sample.

The compound eluting at 9.5 min (Fig. 3c) showed a deprotonated molecule at *m/z* 283, 14 Da more than that of emodin, indicating that this compound was a monomethylated metabolite of emodin. Authentic samples of three monomethylemodins (**8**, **10**, **14**) were prepared by chemical methylation of emodin to assist with identification of these metabolites. According to the retention times and MS/MS spectra of these authentic samples, the compound eluting at 9.5 min was identified as 8-*O*-methylemodin (**8**). The compound eluting at 35.5 min (not visible in Fig. 3c because of a very small peak) was identified as 3-*O*-methylemodin (**10**).

Another metabolite eluting at 15.0 min (Fig. 3e), exhibited a deprotonated molecule at *m/z* 299, which corresponds to *m/z* of emodic acid or to a mass shift of 14 Da (*m/z* 299–285) more than that of ω-hydroxyemodin. Identification of the metabolite as emodic acid was ruled out because the retention time of emodic acid was 3.8 min, not the observed retention time of 15.0 min. The MS/MS spectrum of this metabolite displayed ions at *m/z* 299, 256, and 227. The ion at *m/z* 256 was formed by the loss of CH_3 and CO from *m/z* 299, and an additional loss of CHO from the fragment with *m/z* 256 formed the fragment ion at *m/z* 227. Because anthraquinones having β-hydroxy groups are more acidic, it was thought that 1-*O*-methyl-ω-hydroxyemodin and 8-*O*-methyl-ω-hydroxyemodin would have earlier retention times in reverse-phase chromatography. In order to elucidate the structure of the metabolite at 15.0 min, samples of *O*-methylated ω-hydroxyemodins (**9**, **15, 16**) were prepared from emodin for comparison (Scheme 1). According to the information obtained from the MS/MS spectrum and the retention times of authentic samples, the compound at 15.0 min was identified as 3-*O*-methyl-ω-hydroxyemodin (**9**). In order to determine the direct precursor of 3-*O*-methyl-ω-hydroxyemodin, metabolites of ω-hydroxyemodin (**6**) and 3-*O*-methylemodin (**10**) in Raji cells were studied using LC–APCI-MS/MS method. These studies indicated that 3-*O*-methyl-ω-hydroxyemodin was generated as a metabolite from both **6** and **10**.

In addition to the metabolites described above, the compound eluting at 30.4 min showed a deprotonated molecule at *m/z* 253, 16 Da less than that of emodin. The product ion at *m/z* 225 formed by loss of CO and an ion at *m/z* 182 ([M−H−2CO–CH_3]) were observed in the MS/MS spectrum of this metabolite. According to the information from the MS analysis and a comparison with authentic samples, this compound was identified as chrysophanol (**11**).

The SRM chromatograms of three metabolites (**6**, **8**, **9**) are shown in Figure 4. The same metabolites were observed when the extract of cells was analyzed.

The results from our study did not agree with our original prediction that only ω-hydroxyemodin (**6**) and 3-*O*-methyl-ω-hydroxyemodin (**9**) would be obtained as oxidative metabolites of emodin.[10] The hepatic microsomes derived from various animal species transformed emodin into several oxidized metabolites catalyzed by cytochrome P450. The reason why only a few oxidative metabolites were observed in our study may be attributed to the

fact that the Raji cells have low cytochrome P450-related metabolizing activity.[32]

Although *O*-methyl emodins were isolated from many fungi, lichens, and higher plants, most reports described the isolation of 1-*O*-methylemodin (**14**) or 3-*O*-methylemodin (**10**). Only a few articles have reported that 8-*O*-methylemodin (**8**) was isolated as a metabolite from fungi.[33–36] From a biosynthetic point of view, emodin is the common key intermediate in the biosynthesis of many natural products, such as monomeric and dimeric anthraquinoids,[37] xanthones,[38] and benzophenones.[39] Methylation of the 1-hydroxy group of emodin is considered to be a key step in the conversion of anthraquinone to benzophenone in fungi. Methyltransferases are an important class of enzymes that catalyze the transfer of a methyl group from several donors including SAM, colin, betain, 5-methyltetrahydrofolic acid, and dimethylrutin, to a wide variety of substrates. SAM, the primary methyl donor present in all living organisms, is generated by methionine adenosyl transferase. SAM is involved in the methylation of DNA, proteins, and lipids, and in polyamine synthesis.[40,41] Emodin *O*-methyltransferase (EOMT) from *Penicillium frequentans* was described by Getenbeck and Malmstrom,[42] and Chen at al. reported EOMT derived from *Aspergillus terreus*.[43] EOMT catalyzes methylation of the 1-hydroxy group of emodin to form 1-*O*-methylemodin. Therefore, it was concluded that O-methylation of emodin to 8-*O*-methylemodin in Raji cells was catalyzed by another *O*-methyl transferase that uses SAM as the methyl donor. An isotopically labeled SAM (SAM-d_3) was used as the methyl donor to obtain a small amount of [CD_3]-8-*O*-methylemodin. It was concluded that *O*-methyl transferase in Raji cells is different from the previously reported EOMT.

3.3. Validation results for emodin metabolites

The linearity, precision, and sensitivity of the method were analyzed by applying methanolic solutions of ω-hydroxyemodin (**6**), 8-*O*-methylemodin (**8**), 3-*O*-methyl-ω-hydroxyemodin (**9**), and IS to the LC–APCI-MS/MS system. The linearity of the calibration curves was calculated from the representation of the ratio of the peak area of emodin metabolite to the peak area of IS versus emodin metabolite concentration. The data were collected for four different concentrations of emodin metabolites from the supernatant (1, 5, 50, 500 nM) and cells (5, 50, 200, 1000 nM) with IS. Table 2 shows the slope, intercept, and correlation coefficient values obtained from linear least-squares regression analysis of metabolites (**6**, **8**, **9**). As shown in Table 3, the relative standard deviation (RSD) values for intraday and interday retention times of metabolites and IS were 0.48–1.05% and 2.45–4.82%, respectively. The intraday RSD values of the area ratio were less than 6.59%. The detection limits were 71.5, 14.3, and 150.0 pg/mL for **6**, **8**, and **9**, respectively, and these values were three times the signal/noise (*S/N*) ratio. These results proved that the method for the measurement of emodin metabolites was reliable and reproducible.

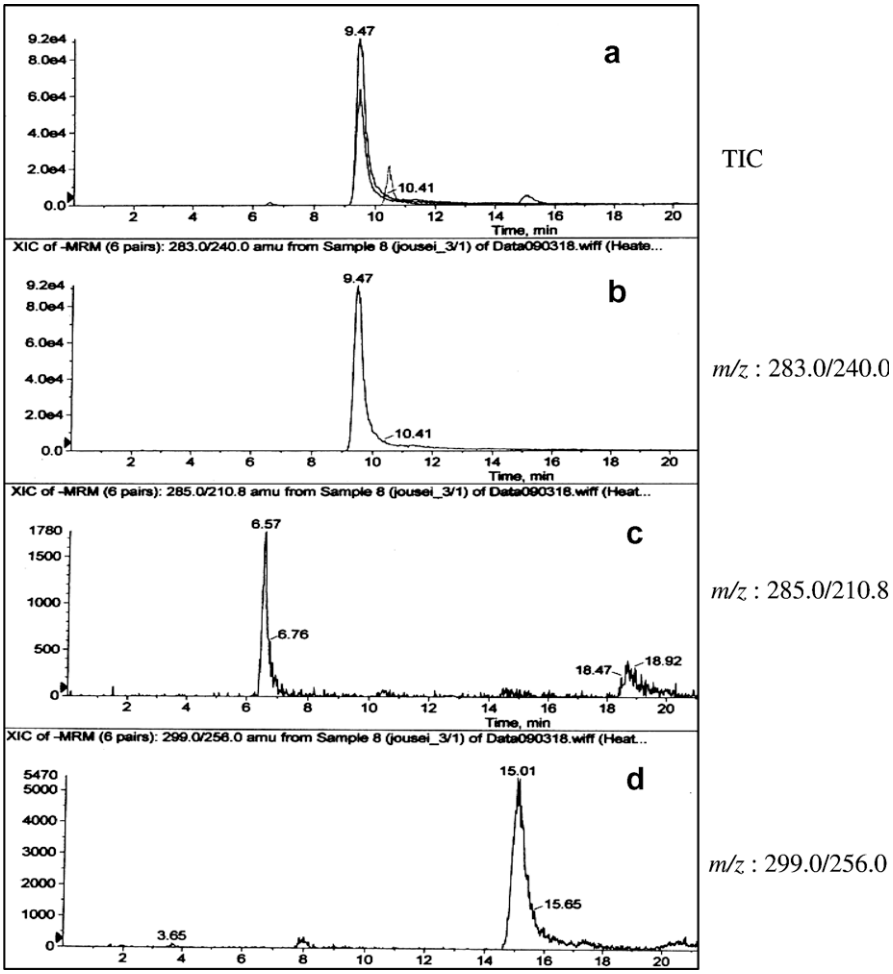


Figure 4. SRM chromatograms of supernatant extract solution in MeOH: (a) total ion chromatogram, (b) 8-*O*-methylemodin, (c) ω-hydroxyemodin, (d) 3-*O*-methyl-ω-hydroxyemodin.

Table 2
Regression analysis equations for emodin metabolites

Compound	Regression equation supernatant extract[b]	Correlation coefficient	Regression equation cells extract[c]	Correlation coefficient
6[a]	$y = 0.0021x - 0.004$	$r^2 = 0.9999$	$y = 0.0003x + 0.0069$	$r^2 = 0.9976$
8[a]	$y = 0.0466x + 0.1156$	$r^2 = 0.9998$	$y = 0.0103x + 0.1191$	$r^2 = 0.9991$
9[a]	$y = 0.0018x + 0.0063$	$r^2 = 0.9995$	$y = 0.0004x - 0.0066$	$r^2 = 0.9992$

y: peak area ratio of metabolite to IS, x: concentration in nM.
[a] Quantitative analysis was carried out using the precursor/product ion: m/z 285/211, 283/240, 299/256.
[b] 1, 5, and 50, 500 nM.
[c] 5, 50, 200, and 1000 nM.

Table 3
Relative standard deviation (RSD) and limit of detection (LOD) of emodin related compounds

Compound	RSD of Tr/ intraday (n = 10, %)	RSD of Tr/ interday (n = 5, %)	RSD of area ratio (supernatant, n = 3)	LOD (pg/ mL) (S/N = 3)
1	1.01	2.53*	2.00	13.5
6	1.05	4.82	5.45	71.5
8	0.82	2.58	6.59	14.3
9	0.55	2.45	4.26	150.0
IS	0.48	3.36	—	—

* n = 3.

The detection limit of emodin for HPLC, capillary electrophoresis (CE), and the present LC–APCI-MS/MS method were 40 ng/mL,[44] 100 ng/mL,[45] and 13.5 pg/mL, respectively. Thus, the sensitivity of LC–MS/MS analysis was superior to that of HPLC and CE. Compared with spectrophotometric detection methods of HPLC and CE, the LC–MS/MS method is more specific to the detection of prespecified structures because specific ions can be selected. Therefore, the LC–MS/MS method described herein is more useful for the detection of emodin derivatives than standard approaches.

4. Conclusion

A rapid, simple, and sensitive LC–APCI-MS/MS method for the determination of emodin metabolites in Raji cells, derived from a human B-lymphoid cell line, has been developed. The starting material emodin and its five metabolites were detected in the Raji cell culture. In addition to the major metabolite 8-*O*-methyl emodin, ω-hydroxyemodin, 3-*O*-methyl-ω-hydroxyemodin, 3-*O*-methylemodin (physcion), and chrysophanol were detected. This is the first report describing the elucidation of metabolites of emodin in a human cell line. Investigation of the novel *O*-methyl transferase species found in Raji cells will be continued, and the results will be reported in due course.

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