Quantitation Assay for Absorption and First-Pass Metabolism of Emodin in Isolated Rat Small Intestine Using Liquid Chromatography-Tandem Mass Spectrometry

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Emodin has numerous biochemical and pharmacological activities, though information about its intestinal absorption and first-pass metabolism is scarce. The purpose of this study was to evaluate intestinal absorption and metabolism of luminally administered emodin in an isolated rat small intestine using the method of LC/MS/MS. About 22.55% of the administered emodin appeared at the vascular side, chiefly as free emodin (12.01%), but some emodin glucuronide (8.69%) and sulfate (1.84%) were also detected. Free glucuronide (5.23%) and sulfate (1.08%) moieties were found in the luminal perfusate. This model serves as a valuable tool for understanding intestinal handling of emodin, and our results confirm absorption and first-pass metabolism of emodin in the rat small intestine. Phase II metabolic enzymes such as glucuronyl transferase or sulfate transferase may also play an important role in the first-pass metabolism of emodin in the small intestine, which may ultimately reduce the bioavailability (and thus the efficacy) of orally administered emodin.

Key words emodin; absorption; small intestine; first-pass metabolism; LC/MS/MS

Polyphenols, a group of complex naturally occurring compounds, are widely distributed throughout the plant kingdom and thus are readily consumed by humans.[1,2] As members of the polyphenol family, dietary anthraquinones have received much attention as potential protectors against a variety of human diseases, in particular cardiovascular disease and cancer.[3,4] Emodin (1,3,8-trihydroxy-6-methylanthraquinone), the active components of many herbal laxatives such as aloe, senna, cascara sagrada, and rhubarb, belongs to the anthraquinone family.[5,6]

Intestinal absorption is a prerequisite for a possible causal relationship between emodin intake and its proposed chemopreventive action. However, our limited knowledge of how emodin is absorbed and metabolized has hampered our understanding of its effect in the body. In addition, some similar compounds have poor bioavailability due to weak absorption and significant degradation by enzymes.[7] Furthermore, to date, information on *in vivo* absorption and metabolism of emodin is scarce—there are no reports of evidence of extensive first-pass metabolism of emodin in the gut—and thus intestinal handling of emodin requires further investigation to appraise its beneficial action *in vivo*.

To address this issue, we evaluated the contribution of the small intestine to the absorption and first-pass metabolism of emodin using an isolated rat small intestine perfusion model. This model facilitates direct investigation of luminal disappearance and venous appearance of administered emodin and analyses the metabolites in both perfusate and mesenteric blood, thereby allowing the direct estimation of intestinal absorption and first-pass metabolism of this drug.[8,9]

MATERIALS AND METHODS

Reagents and Chemicals Emodin and chrysophanol was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) at the highest purity available (98%, as determined by HPLC). Emodin was dissolved in DMSO (0.1% v/v final concentration) because it is hydrophobic and poorly soluble in water. β-Glucuronidase (catalog no. G-0251) and sulfatase (catalog no. S-9754) were purchased from Sigma. All chemicals and reagents used were of analytical grade or HPLC grade.

Animals Male Sprague-Dawley rats (70—110 d old) weighing between 270 and 320 g were obtained from the experimental animal center of the Fourth Military Medical University, Xi'an, China. The rats were fasted overnight with free access to water before the day of the experiment.

Animal Surgery The rats were anesthetized with an intra-abdominal injection of a mixture containing 40 mg/kg phenobarbital sodium. The animals were then heparinized (90 U/kg^{-1}) *via* the vena caudalis. During surgery, the body temperature was maintained at 37 °C by a heating lamp.

The small intestine was prepared as described.[10—12] Figure 1 schematically illustrates the vascular-luminal perfused rat intestine preparation. Briefly, the small intestine was exposed by midline incision, and a 7- to 11-cm-long segment of the intestine from 5 cm below the ligament of Treitz was identified and separated. Silicone tubing (i.d. 3.0 mm, o.d. 5.0 mm) was placed inside both ends of the segment, and the tube at the proximal side was connected to a peristaltic pump for luminal perfusion. The segment was then flushed (0.5 ml/min) with warm saline to remove intestinal contents.

A polyethylene cannula (i.d. 0.5 mm, o.d. 0.8 mm), connected to a peristaltic pump for the vascular perfusion, was inserted into the superior mesenteric artery. The single-pass perfusion of perfusate, Krebs–Ringer bicarbonate buffer (pH 7.4) including 5.5 mM D-glucose, 3%BSA, 2.5% dextran-70 and 0.8 mM L-glutamine, was begun immediately at 2.0 ml/min. The mesenteric vein for collecting blood from the specified segment of intestine was cannulated with a 6-

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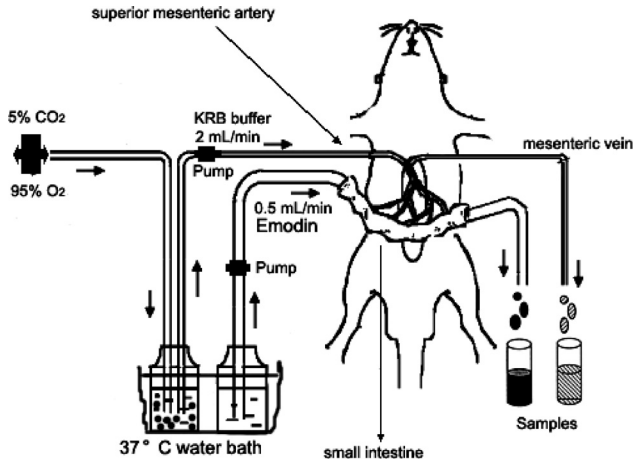

Fig. 1. Schematic Diagram for Vascular-Luminal Perfusion of Rat Small Intestine

cm-long polyethylene tubing (i.d. 0.86 mm, o.d. 1.27 mm). After cannulation of the superior mesenteric artery and the mesenteric vein, the small intestine was vascularly perfused with an artificial oxygen carrier. Blood was collected into tubes at appropriate time intervals.

To maintain a constant temperature of the perfusate, the inlet cannula was insulated and kept warm by a 37 °C water bath. The solutions on both sides were circulated by gas lift with 95% O_2 and 5% CO_2 throughout the transport studies. Samples obtained from the outlet of the mesenteric vein and luminal aliquots were collected into the preweighted microtubes every 10 min and stored at −70 °C until analysis.

Sampling and Sample Preparation Samples were analyzed for emodin and emodin conjugates by LC/MS/MS after sample preparation as described below. The vascular (venous) and luminal samples 0.2 ml were extracted with 1 ml ethyl acetate by vigorous vortex mixing for 1 min and centrifuged at 3000×***g*** at room temperature for 10 min. The supernatants were evaporated to dryness under N_2 gas, reconstituted in 100 μl of methanol, and analyzed by LC/MS/MS.

Analytical Procedures A Quattro Premier LC/MS/MS system (Waters Corp., Milford, MA, U.S.A.) operating under Masslynx 4.1 software was used. We developed a high-throughput and sensitive bioanalytical method using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) equipped with an electrospray ionization interface used to generate negative ions $[M-H]^-$ for the estimation of the concentration of emodin and its metabolites. The compounds were separated on a reversed-phase column (Symmetry C18 column, 50×2.1 mm, 5 μm) with an isocratic mobile phase consisting of 0.1% acetic acid and acetonitrile (10 : 90, v/v). The column temperature and flow rate were 25 °C and 0.2 ml/min, respectively.

Quantitation was performed by multiple reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion for emodin using the internal standard (IS) method[13,14] with peak area ratio. Collision-induced dissociation was achieved using argon as the collision gas. A standard solution of emodin 1 μg/ml and IS (chrysophanol, 1 μg/ml) was applied to optimize the detection condition in the presence of mobile phase. The mass transitions used for emodin and IS (chrysophanol) were *m/z* 269.4→225.4 (cone voltage, 55 eV; collision energy, 25 eV; dwell time, 400 ms) and 253.3→225.2 (cone voltage, 50 eV; collision energy, 28 eV; dwell time, 400 ms), respectively.

Calculations Fluxes [nmol×min^{-1}×(g dry intestine)$^{-1}$, mean±S.D.] were calculated from concentration differences (ΔC, arteriovenous, and proximodistal), the corresponding flow rates, and the dry weight (DW) of the entire small intestine used in the experiment according to the following equation[15]:

$$\text{flux} = \frac{\Delta C\,[\text{nmol}\cdot\text{ml}^{-1}] \times \text{flow}\,[\text{ml}\cdot\text{min}^{-1}]}{\text{DW}\,[\text{g}]}$$

To calculate the flux, we used the actual luminal and vascular flow rates, which were somewhat different from the theoretical flow rates of 0.5 (luminal) and 2.0 ml/min (vascular). Viability parameters were compared using Student's *t*-test for unpaired observations. *p* values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Mass Spectra In addition to emodin, two metabolites could be detected in the luminal and vascular samples. Negative ion mass spectra of emodin and its two biotransformation products showed stable molecular ions at *m/z*=269.4 (Fig. 2A), *m/z*=349.7 (Fig. 2C), and *m/z*=446 amu (Fig. 2E) with subsequent loss of 176 amu (glucuronic acid moiety) and 80 amu (sulfuric acid moiety), in agreement with the molecular weight of emodin, emodin-glucuronide, and emodin-sulfate. Precursor/product ion pairs for emodin and its two metabolites were *m/z* 269.1/225.1 (Fig. 2B), *m/z* 349.7/269.3 (Fig. 2D), and *m/z* 446/269.6 (Fig. 2F), respectively. Treatment of samples with β-glucuronidase prior to MRM analysis led to a concomitant increase in the concentration of the parent emodin, indicating that the metabolites contained glucuronide. After incubation of the samples with sulfatase, the parent emodin also increased, indicating the presence of a conjugate containing sulfuric acid.

Quantitation of Emodin The method used a chromatographic run time of 2.5 min and a linear calibration curve over the concentration range of 0.1—200 ng/ml for emodin. There were no interfering peaks at the elution times for either analyte (emodin, 1.17 min) or IS (chrysophanol, 1.57 min) (Fig. 3). The lowest limit of quantification came to 0.05 ng/ml at a signal-to-noise ratio of 10. Such a low limit of detection might be attributed to the efficient cleanup process of the biological specimen by ethyl acetate, which seemed to improve the ionization and detection of emodin in the sample. The equation of the regression line was $y=0.278x+0.148$ ($r^2=0.9996$). The coefficients of variation of the precision of the intra- and inter-day validation were less than 2.3 and 4.1% at 1 ng/ml of emodin, respectively.

The Absorption and Metabolism of Emodin Conjugates of emodin such as glucuronides and sulfates were analyzed as emodin after enzymatic cleavage. For cleavage of glucuronides, 0.2 ml β-glucuronidase solution was added to 0.2 ml sample solution. Cleavage of sulfate conjugates was performed with 0.1 ml sulfatase solution and 0.2 ml sample solution. Mixtures were incubated for 120 min at 37 °C.[16]

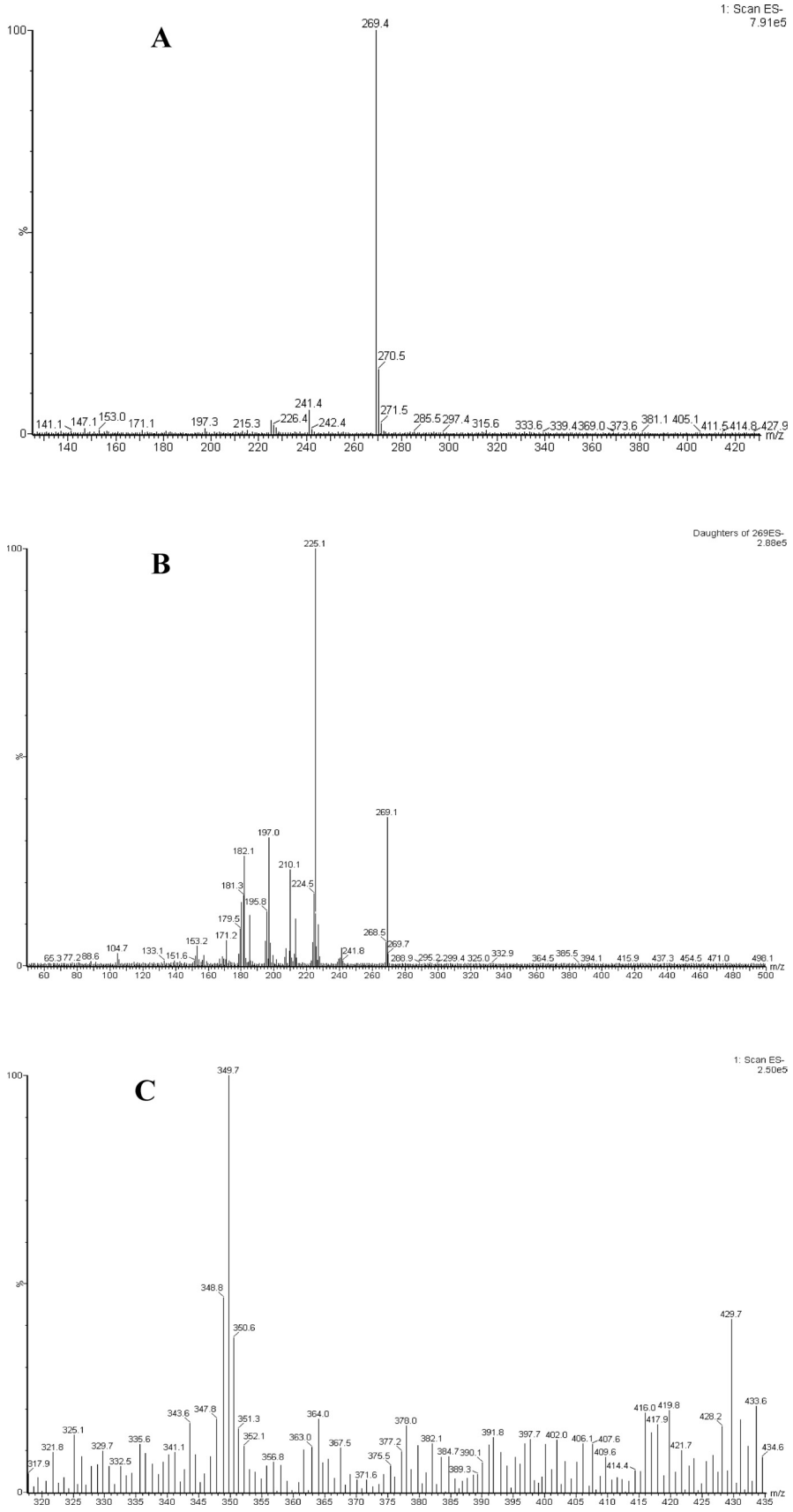
A
1: Scan ES-
7.91e5
269.4
270.5
241.4
271.5
B
Daughters of 269ES-
2.88e5
225.1
269.1
197.0
182.1
210.1
224.5
181.3
195.8
179.5
171.2
153.2
268.5
269.7
C
1: Scan ES-
2.50e5
349.7
348.8
350.6
429.7
433.6
%
m/z

Fig. 2

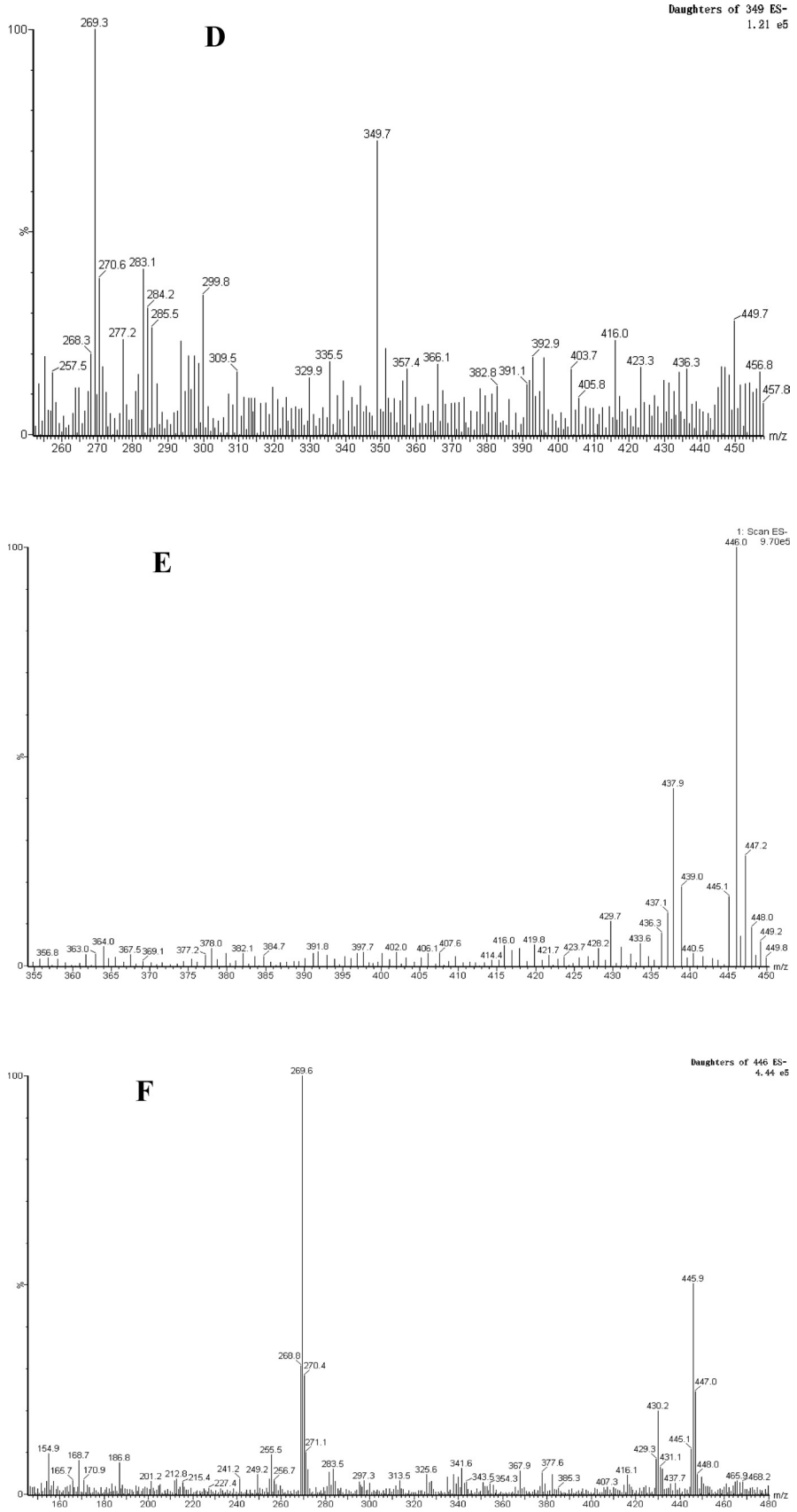

Fig. 2. Precursor/Product Ion Mass Spectra of Emodin and Its Metabolites in the Vascular and Luminal Compartment Taken 1 h after Luminal Loading

(A) Emodin, *m*/*z* 269.4; (B) daughter of emodin, *m*/*z* 269.1→225.1; (C) sulfated emodin, *m*/*z* 349.7; (D) daughter of sulfated emodin, *m*/*z* 349.7→269.3; (E) glucuronidated emodin, *m*/*z* 446; (F) daughter of glucuronidated emodin, *m*/*z* 446→269.6.

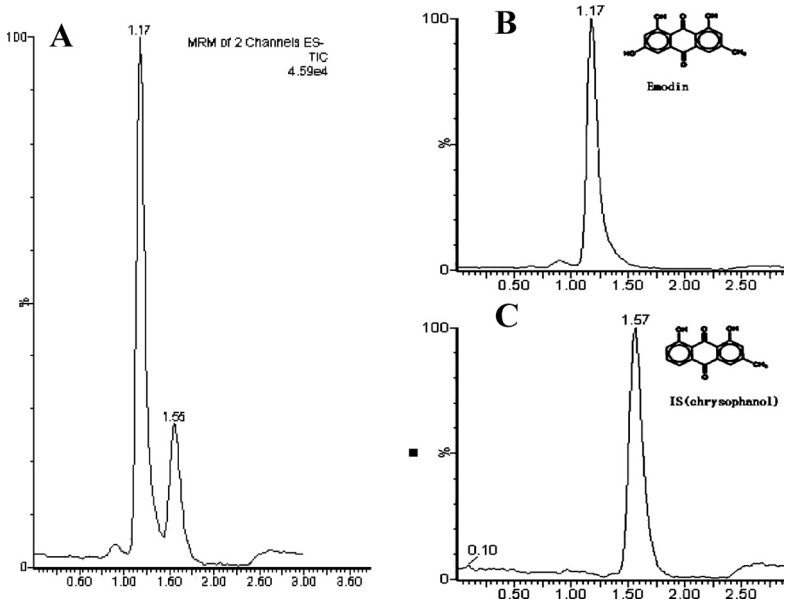

Fig. 3. MRM Chromatograms of Emodin and IS (Chrysophanol) in the Vascular and Luminal Sample

The retention times for emodin and chrysophanol were 1.17 min and 1.57 min, respectively. (A) Chromatogram of the biological sample containing emodin and chrysophanol; (B) emodin chromatograms and structure; (C) IS (chrysophanol) chromatograms and structure.

In control perfusion experiments with emodin-free basic perfusion media, no emodin or its conjugates were detected. The vascular appearance rate of emodin increased during perfusion (significantly for the first 50 min) (Fig. 4A). Emodin conjugates were quantified based on emodin concentration after enzymatic incubation of luminal and vascular effluents. Glucuronide and sulfate were identified and quantified by enzymatic hydrolysis with glucuronidase and sulfatase using LC/MS/MS. Approximately 22.55% of emodin that was administered appeared at the vascular side, chiefly as free emodin (12.01%), but also as emodin glucuronide (8.69%) and emodin sulfate (1.84%). The main compound in the luminal effluent was emodin, accompanied by lesser amounts of emodin glucuronide and emodin sulfate. Emodin glucuronide (5.23%) and emodin sulfate (1.04%) were found at the luminal side. The mean total recovery of emodin and metabolites was 96.49±2.2% (Table 1).

DISCUSSION

In this study, we validated the use of the LC/MS/MS method to qualify emodin and its metabolites. The method proved to be sensitive, specific, precise and suitable for the quantitation of absorption and first-pass metabolism of emodin in isolated rat small intestine. The liquid–liquid extraction method gave good and consistent recoveries for emodin and IS from biological specimens with no detected interference from other metabolites. The method saved a lot of time that normally would be required to pretreat the biological specimen for analysis by LC/MS/MS. Viability was maintained during the entire perfusion as indicated by no significant differences between emodin and control perfusions for perfusion pressure, lactate–pyruvate ratio, oxygen uptake and acid–base homeostasis.

Systemic blood sampling cannot completely elucidate the contribution of intestinal first-pass metabolism during the absorption process of a drug, and thus we used an isolated preparation of a vascularly and luminally perfused rat small intestine to assess the intestinal absorption and first-pass metabolism of emodin. Intact mucosal morphology without loss of villous tip cells after a 120-min perfusion was previously demonstrated in histologic specimens of isolated intestinal preparations perfused in the same way as in the present study. As in earlier studies, the viability and functional integrity of the intestinal preparation were carefully and continuously controlled and the viability was confirmed.[17,18]

Our present results clearly quantify the absorption and first-pass metabolism of emodin in the rat small intestine. The steady increase in the vascular appearance of free emodin and its metabolites reflects the transport and metabolic activity of the small intestinal tissue. Glucuronide and sulfate conjugates were quantified as emodin equivalents after enzymatic cleavage. It is generally accepted that glucuronic acid and sulfate conjugates are water-soluble detoxification products intended for excretion.[19,20] The conjugates formed in the small intestinal tissue were preferentially absorbed to the serosal side, with a part of the glucuronide and sulfate secreted into the luminal perfusate. However, the absorption of emodin did not result in a rapid and high-level appearance of emodin in the mesenteric blood. On the contrary, over 46.72% of the administered emodin that appeared at the vascular side was rapidly converted to emodin-glucuronide and emodin-sulfate in the mesenteric blood when emodin was perfused into the rat intestine.

About 22.55% of the administered emodin appeared at the vascular side, chiefly as free emodin (12.01%), but some emodin glucuronide (8.69%) and sulfate (1.84%) was also detected. Free glucuronide (5.23%) and sulfate (1.08%) moieties were found in the luminal perfusate. The transfer of the free and glucuronidated or sulfated emodin from the gut tissue to the vascular side showed a steady, significant increase until about 50 min post-administration when an apparent steady state was established. Thereafter, the luminal disappearance and vascular appearance rates were well balanced.

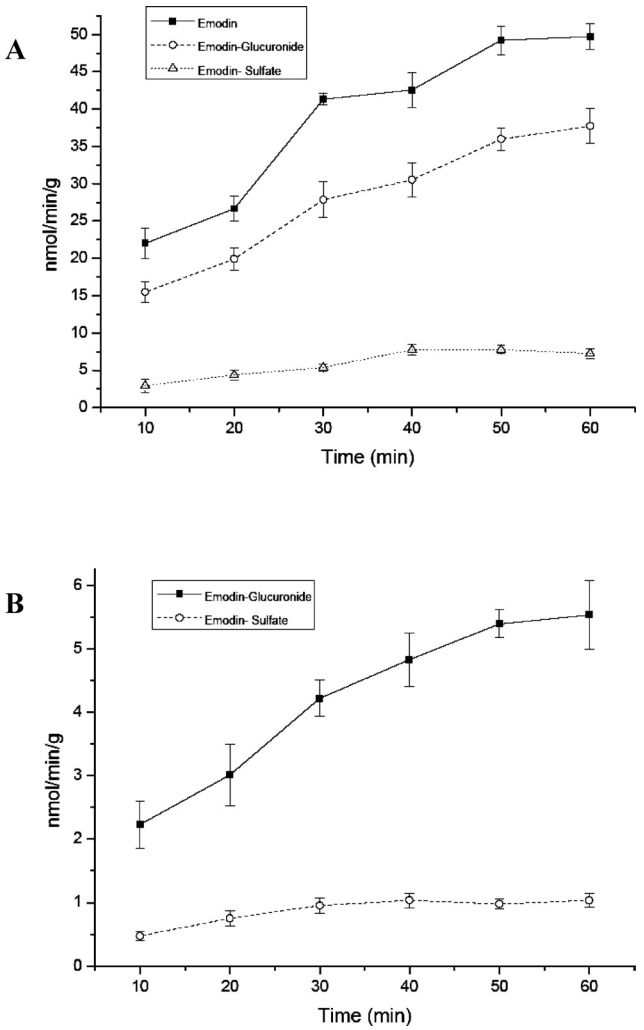

Fig. 4. Time-Dependent Appearance of Emodin and Its Metabolite, Respectively

(A) Emodin and emodin conjugates in the vascular compartments after perfusion experiments of emodin with isolated rat small intestine. (■) Emodin, (○) emodin glucuronide, (△) emodin sulfate. (B) Emodin conjugates in the luminal compartments after perfusion experiments. (■) Emodin glucuronide, (○) emodin sulfate. Each data point represents the mean and S.D. of three determinations.

Table 1. Distribution of Emodin and Emodin Conjugates in the Luminal, and Vascular Compartments after Perfusion Experiments of Emodin with Isolated Rat Small Intestine

	Luminal effluent		Vascular side	
	nmol	%[a)]	nmol	%[a)]
Emodin	1013.56±43.2	67.57[a)]	180.57±8.22	12.01[a)]
Glucuronide	78.65±7.29	5.23[a)]	130.63±8.86	8.69[a)]
Sulfate	16.31±1.97	1.08[a)]	27.71±3.03	1.84[a)]
Total	1108.52±52.46	73.75[a)]	338.91±20.11	22.55[a)]

Emodin (1503±52.1 nmol) was applied in three perfusion experiments of 60 min. Recoveries are given as means±S.D. Mean recovery over three experiments was 1447.43±47.2 nmol (96.49±2.2%); *a*) based on the dosage of 1503±52.1 nmol.

During the entire perfusion period, emodin glucuronide was preferentially released into the vascular perfusate—the vascular appearance rate was about six times that of the luminal appearance rate. By contrast, there was no significant difference between the vascular and luminal distribution of emodin sulfate during the perfusion experiments.

These results strongly support previous evidence showing that the anthraquinone emodin is extensively metabolized and conjugated during transfer from the gut lumen to the serosal surface. Although the major metabolites detected were glucuronides, sulfate was also detected in significant amounts. It is noteworthy, however, that glucuronyl transferase or sulfate transferase may also play an important role in the first-pass metabolism of emodin in the small intestine, which may ultimately reduce the bioavailability (and thus the efficacy) of orally administered emodin.

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