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Identification of chemicals and their metabolites from PHY906, a Chinese medicine formulation, in the plasma of a patient treated with irinotecan and PHY906 using liquid chromatography/tandem mass Spectrometry (LC/MS/MS)

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Abstract

Traditional Chinese Medicine (TCM) is increasingly being used in combination with Western medicine. In general, TCM is comprised of multiple components in sharp contrast to Western medicine, where a single active chemical is used. Presently, there are no well-established standards for most of the chemical compounds of TCM and their respective metabolites. Moreover, there are no formal analytical methods for the identification of these chemicals, especially in trace amounts. The ability to measure the pharmacokinetic behaviors of chemicals and their metabolites from these herbal formulations are critical in understanding of the action of TCM. This paper describes the use of LC/MS/MS along with enzyme treatments and n-octanol/water partition coefficient, to investigate the chemical components of PHY906 and their metabolites in the plasma of a patient with metastatic colorectal cancer (mCRC) treated with irinotecan and PHY906. The chemicals from an aqueous extract of PHY906 and the plasma from a patient was prepared and separated on a Agilent ZORBAX-SB C₁₈ column, and eluted with acetonitrile/0.05% (v/v) formic acid. From the PHY906 aqueous extract, a total of 57 compounds and 27 metabolites were identified and tentatively assigned structures based on their identified mass spectrometry, enzyme digestion and n-octanol/water partition coefficient. In contrast, analysis of patient plasma identified only 33 chemicals and new metabolites. These findings demonstrated that LC/MS/MS was an effective and reliable method for studying the parent chemicals of the Chinese herbal medicine PHY906 and their metabolites in a patient with metastatic colorectal cancer.

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Keywords

LC/MS/MS; Pharmacokinetics; Traditional Chinese Medicine; Metabolites

1. Introduction

PHY906 is a traditional Chinese botanical formulation that is composed of 4 herbs, which include *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, and *Ziziphus jujuba* Mill. This herbal medicine has been used for over 1800 years in the Orient to treat a wide range of gastrointestinal ailments, including nausea/vomiting, diarrhea, and abdominal cramps. Interestingly, these same symptoms are also commonly observed side effects in cancer patients receiving chemotherapy. We previously reported that PHY906 reduced the *in vivo* toxicities of Irinotecan (CPT-11) including body weight loss and mortality using BDF-1 mouse models. Despite having a protective effect against host toxicity, PHY906 enhanced the *in vivo* antitumor activity of CPT-11 as well as a number of other anticancer agents, such as 5-fluorouracil, capecitabine, *gemcitabine*, *sorafenib*, etoposide, and troxacitabine [1-2]. The botanical drug PHY906 was manufactured under good manufacturing practice (GMP) and the consistency of preparations from batch to batch was well-established. A phase I/II clinical trial in U.S. as a modulator of capecitabine for hepatocellular carcinoma and pancreatic carcinoma was conducted [3-6]. Presently, PHY906 is being studied in a phase I/II clinical trials as a modulator of CPT-11 monotherapy in patients with previously treated mCRC.

In order to understand the mechanism of action of PHY906, it is critical to know the chemicals and their metabolites present in plasma after oral administration of PHY906. Only some of the chemicals from PHY906 are orally absorbable and some of these are metabolized. Baicalein and baicalin were two of the major chemicals of PHY906. Various analytical methods have been described for the measurement of baicalein, baicalin, and other flavonoids in biological fluids [7-9]. Unfortunately there are no well-established standards for all of the chemicals contained within PHY906 and their metabolites. The absence of such standards represents a major challenge to investigate the metabolism and pharmacokinetic behavior of these chemicals and metabolites in patient plasma after oral administration of PHY906.

A major goal of this study was to develop novel methodologies to identify PHY906 chemicals and their metabolites in patient plasma after oral administration of PHY906. Our approach was to use high-performance liquid chromatography method coupled with Q-trap® mass spectrometer. Several analytical methods have been established to purify and identify the metabolites from TCM *in vivo* [10-12]. However, these methods have been primarily developed for pre-clinical studies where significantly lower doses of herbal medicine were used than what would be typically administered in the clinical setting. In the current study, mass spectrometry along with enzymatic treatments and n-octanol/water partition coefficient was used to identify PHY906 chemicals and metabolites in plasma. Given the high sensitivity and reliability of these methods, this approach should then be applied to investigate the chemicals composition of other herbal remedies along with their metabolites in patients who are undergoing active treatment.

2. Materials and Methods

2.1 Chemicals and Reagents

PHY906 is the spray-dried aqueous extract of a formula containing *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, and *Ziziphus jujuba* Mill. The

drug materials appeared as a fine brown powder, and were provided by PhytoCeutica Inc. (New Haven, CT). Sulfatase from *Helix pomatia* (S9626) was purchased from Sigma (St. Louis, MO). The sulfatase preparation was capable of deconjugation of sulfatase as well as β -glucuronidase activity on flavonoids. HPLC-Mass grade methanol, acetonitrile, water, and 0.05% formic acid were obtained from J.T.Baker (Phillipsburg, NJ).

2.2 Instrumentation

The chromatographic system used consisted of an Agilent 1200 HPLC series, including a binary pump (Model G1312B), a vacuum degasser (Model G1379B), an autosampler (Model G1367C), and a column oven (Model G1316B). The mass spectrometer was an Applied Biosystems Sciex 4000 Q-trap® mass spectrometer (Applied Biosystems Sciex; Foster, CA). Data acquisition was carried out by Analyst 1.4.2® software on a DELL computer.

2.3 LC–MS conditions

The chromatographic separation was achieved on a ZORBAX-SB-C18 column (100 mm $\times$ 2.1 mm i.d., 3.5 μ m, Agilent, Palo Alto, CA). Mobile phase A was water with 0.05 % formic acid and mobile phase B was acetonitrile. The liquid flow-rate was set at 0.3 mL/min, and the column temperature was maintained at 30°C. The mobile phase consisted of linear gradients of 0.05% (v/v) formic acid (A) and Acetonitrile (B): 0.01–2.5 min, 12–20% B (v/v); 2.5–12 min, 20–28% B; 12–18 min, 28–36% B; 18–20 min, 36–50% B; 20–25 min, 50–55% B; 25–27 min, 55–80% B; 27–30 min, 80–80% B; 30–32 min, 80–12%; 32–40 min, 12–12% B.

2.4 Analysis of patient plasma samples

Heparinized blood samples were obtained from a patient with mCRC before and 2 hours after taking 2400mg PHY906 orally and one and 1.5 hours after intravenous infusion of 180mg/m² CPT-11. The sample was centrifuged for 15min at 2000 $\times$ g. The resulting plasma was transferred to polypropylene tubes and stored at –80 °C until analysis. The patient was enrolled in a clinical protocol (HIC #0808004167) that had been approved by the Yale Cancer Center Scientific Review Committee and the Yale Human Investigation Committee, and the patient had given written, informed consent before enrollment on study.

2.5 PHY906 sample preparation

To 100 mg of PHY906 drug powder was added 1 mL of water. The mixture was vortexed vigorously for 1min, and then maintained at 80°C for 30 min. The sample was centrifuged at 12,000 rpm for 10 min and the supernatant was removed. This sample was immediately cooled to room temperature. The extract was kept in –20°C until analysis. In a 1.5 mL centrifuge tube, an aliquot 10 μ L of PHY906 extract was spiked with 90 μ L of solvent acetonitrile: water=12:88), and the tubes were vortex mixed for 1 minute. After centrifugation at 14,000 rpm for 10 min, the supernatant was filtered through a 0.2-mm membrane before use. An aliquot of 10 μ L of each sample was injected into the LC/MS/MS instrument for analysis.

2.6 Patient plasma sample preparation

The plasma was prepared for HPLC/MS/MS by removing protein with a precipitation method. To an aliquot of 100 μ L of human plasma in a 1.5 mL microfuge tube, 400 μ L of extraction solvent (acetonitrile: methanol=2:1) was added. The tubes were vortex mixed for 1 minute and then centrifuged at 14,000 rpm for 10 min in a Microfuge® 18 Centrifuge (Beckman Coulter, Inc.; Palo Alto CA) in the cold room (4 °C). The clear supernatant fluid was then transferred into another centrifuge tube and evaporated to dryness in a Speed Vac

apparatus (Speed Vac SC 110, Savant Instruments; Farmingdale, NY). The dry residue was dissolved in 100 μ L solution (acetonitrile: water=12:88) with vortex-mixing for 0.5 minutes. The prepared samples were kept in an autosampler at 4°C until injection, at which time 10 μ L of supernatant fluid was injected into the HPLC/MS /MS system.

2.7 Acquisition of enhanced product ion (EPI) spectra

MS/MS spectra for metabolites can be obtained using information dependent acquisition (IDA) of EPI. The IDA threshold was set to 500 counts per second (cps) for the multiple reaction monitoring (MRM) scans, and 500,000 cps for the Enhanced mass spectrum (EMS). The EPI scan rate was 1000 amu/s. The CE was set at 50eV with a CE spread of 15 eV. Dynamic exclusion, which defines the time for which a transition is excluded after acquiring an EPI scan, was set to 2 s after 3 appearances to allow detection of co-eluting substances. When dynamic exclusion was used, MS/MS spectra could be collected for only 5-6 co-eluting metabolite ions in the EPI mode. The turbo ion spray source was set at 50 V DP, and dynamic fill time (DFT) function was used to ensure that the linear ion trap was not overfilled. Other parameters were set as follows: curtain gas, 35; ion source: -4250 V; temperature, 550 °C; gas 1, 55 psi; gas 2, 50 psi; CAD gas, high.

2.8 EMS-EPI

The EMS survey scan was conducted at mass range from 120 to 1000 amu. The CE was set at 5 eV for EMS. The total cycle time was approximately 1.1 s. The PHY906 solution (10mg/ml) was analyzed to get product ion by using EMS-EPI mode.

2.9 MRM-EPI

The MRM survey monitored PHY906 metabolites detected by EMS-EPI. Each transition was performed with 80 ms dwell time and a 5-ms pause time at a CE of 50 eV. We firstly used the MRM-EPI model to determine the PHY906 extraction solution in order to certify the accuracy of selecting MRM. All the compounds listed in the Table1 were found in PHY906 extraction solution using MRM-EPI. In addition, the PHY906 10 μ L (100mg/ml) was mixed with 90 μ L blank plasma. The spiked plasma samples were then treated according to the “patient plasma sample preparation” procedures described above.

2.10 Cloning and purification of *Escherichia coli* β -glucuronidase

The gene encoding the *Escherichia coli* β -glucuronidase was amplified by PCR. The PCR product was ligated to pET21b(+) vector to obtain the expression construct. The expression construct was introduced into *Escherichia coli* BL21(DE3) (Invitrogen) and a transformant was used to inoculate Luria-Bertani (LB) broth supplemented with ampicillin (100ug/ml) at 37 °C. When the culture reached an optical density of 0.5 at a wavelength of 600 nm, isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mM to induce expression. After 3 hours, bacterial cells were harvested, lysed, and centrifuged to collect the soluble recombinant β -glucuronidase. The enzyme was purified by applying the clarified lysate to a HisTrap HP column (GE Healthcare, Piscataway, NJ) and eluted with an imidazole gradient (20mM to 200M) in buffer (50mM Tris (pH 7.4), 500mM sodium chloride, 10% glycerol, 10mM imidazole, and 0.05 % triton 100). The enzyme removed the respective glucosyl and glucuronic acid residue from the flavonides molecule.

2.11 Enzymatic deconjugation of metabolites by β -glucuronidase

A total of 100 μ L of patient plasma was incubated with Tris HCL (100 μ L, 0.1M, pH 6.8). After 5 μ L of β -glucuronidase (2 mg/mL) was added, the sample was sealed in a microfuge tube and gently mixed [13]. Deglucuronidation of PHY906 in plasma by β -glucuronidase

was accomplished by incubating at 37°C for 1.0 hr. The sample was prepared according to the patient plasma sample preparation procedure described above.

2.12 Enzymatic deconjugation of metabolites by sulfatase

A total of 100 μ l of patient plasma was incubated with sodium acetate buffer (100 μ l, 0.2M, pH 5.0). After 1 μ l of sulfatase (2150 units/mL) was added, the sample was sealed in a microfuge tube and gently mixed. Deglucuronidation of PHY906 in plasma by sulfatase was accomplished by incubating the sample at 37°C for 2 hours. The sample was prepared according to the patient plasma sample preparation procedure described above.

3. Results and discussion

3.1. Mass spectrometry

In contrast to Western drugs, which are usually composed of a single chemical, PHY906 is a complex mixture consisting of multiple compounds. The concentrations of most active compounds in PHY906 and their metabolites at clinical relevant dosage are too low to be determined by the UV detector or by even a total ion-scan method of mass. We found no pronounced differences in the fingerprint of plasma from the patient before and after treatment with an oral dose of 2400mg of PHY906 using the UV detector or full-scan model of mass (Fig.1). Recently, a new triple quadrupole-linear ion trap mass spectrometer (Q-TRAP) was developed for analysis of small molecules. This hybrid mass spectrometer has MS/MS scan functions of both the traditional triple-quadrupole instruments and the linear ion trap instruments, including neutral loss (NL) scan, precursor ion (PI) scan, multiple reaction monitoring (MRM), etc [14-17]. MRM is a particularly powerful technique for the analysis of complex drug metabolic pathways where both the parent drug and multiple metabolites can be monitored simultaneously with high sensitivity and precision. However, it is a targeted, hypothesis-driven technique, rather than a discovery-based global expedition. To use MRM, one needs to know what chemical you are looking for. An NL, PI or MRM scan can also serve as a survey scan to trigger information-dependent acquisition (IDA) of enhanced product ion (EPI) spectra. These MS/MS survey-scanning capabilities have been utilized for rapid detection and characterization of oxidative metabolites, glucuronide conjugates, glutathione (GSH) adducts and N-acetyl-L-cysteine conjugates. The hybrid instrument has been widely employed in laboratories where a single LC/MS platform is used for both determination of drug plasma concentrations in pharmacokinetic studies and metabolite identification in drug metabolism studies. In this experiment, we used the Q-TRAP feature to determine and indentify the compound from PHY906 in the patient plasma.

3.2 EMS-EPI

In the present study, we used EMS-EPI to optimize MS parameters since the standards of most the compounds contained within the PHY906 formulation and its metabolites were not readily available. Previously, 64 compounds were detected in a PHY906 preparation using electrospray ionization time-of-flight mass spectrometer [18]. In order to increase the sample throughput, a critically important feature for clinic studies, the HPLC run time was shortened. With this modification, only 57 compounds were identified (Table 1).

3.3 compounds screening by MRM-EPI

Sample preparation is a critical step for accurate and reliable detection by the LC/MS/MS method. When the water extract was spiked into a sample of human plasma, 50 compounds were observed (Table 1). The fact that seven compounds were missing may be due to the process of sample preparation, where co-precipitation with protein may have occurred. However, when the patient plasma was extracted 2 hours after oral administration, only 9

compounds from PHY906 could be found. It is conceivable that the 41 compounds that were not detectable might not have been absorbed by the human gut mucosa or they might have been metabolized into different compounds or and/or excreted rapidly. An extra 18 transitions were added to MRM-EPI that were predicted based on biotransformation pathways and metabolism data previously reported. Software chembiodraw ultra environment (Cambridgesoft®; Cambridge, MA) that calculated the n-octanol/water partition coefficient was used to help in the tentative assignment of the structure of the various PHY906 metabolites. The n-octanol/water partition coefficient has been useful as a means to predict partitioning between water and sediments, soil adsorption, biological uptake, lipophilic storage, and toxicity [19-22]. In this study, the n-octanol/water partition coefficient was used to predict the relative retention time of structural isomers. In general, the large n-octanol/water partition coefficient should have longer retention time on the reverse phase-HPLC. We have used the structural elucidation of baicalin and its respective metabolites as a model for PHY906 metabolism as bacailin is a major compound in this herbal formulation.

3.3.1 Metabolites (MRM 621-445)—When compared with control patient plasma, two new metabolites (MRM 621-445) were eluted from the PHY906-treated patient plasma at the retention times of 6.49 and 8.16 min (Fig. 2A), respectively. Both products showed a deprotonated molecule ion at 621, which was two glucuronic acid moieties or 2×176 Da more than baicalein. The MS/MS spectrum showed their main product ions at m/z 445(M-H-GluA) and 269(M-H-GluA-GluA) (Fig. 2B). According to this information both products were likely double glucuronidated metabolites of baicalein. The difference between these metabolites lies in the position of glucuronidation in baicalein. In considering the metabolism of baicalin, the potential metabolites included baicalein-5-O-glucuronide-7-O-glucuronide or baicalein-6-O-glucuronide-7-O-glucuronide. Both metabolites were found to be susceptible to β -glucuronidase treatment yielding the spectra seen in Fig. 2A. The n-octanol/water partition coefficient of M1 (6.49 min) and M2 (8.16 min) were -1.60 and -2.39, respectively. According to the retention time of the reference and their n-octanol/water partition coefficients, M1 was tentatively assigned as baicalein 5-O-glucuronide-7-O-glucuronide and M2 was assigned as baicalein 6-O-glucuronide-7 O-glucuronide [23].

3.3.2 Metabolites (MRM 445-269)—Three metabolites (MRM 445-269) were identified in patient plasma at the 2-hour time point at the retention times of 10.93, 13.51 and 14.22 min, respectively (Fig. 3A). Each product showed a deprotonated molecule ion at 445Da, which is one sugar or 176 Da more than baicalein, suggesting that they were isomers of β -glucuronidated metabolites of baicalein. As seen in Fig. 3A, each metabolite was digested by β -glucuronidase. The MS/MS spectrum showed their main product ions at m/z 269 (Fig. 3B). Baicalein has three hydroxyl groups, so there should be three isomers of β -glucuronidated baicalein metabolites. The n-octanol/water partition coefficients of baicalein-5-O-glucuronide, baicalein-6-O-glucuronide, and baicalein-7-O-glucuronide (baicalin) were -0.061, 0.52 and 0.77, respectively. The retention time of standard of baicalin was 11.1 min consistent with the fact that a large n-octanol/water partition coefficient would have less retention on the reverse phase chromatography. Thus, M3 (10.93), M4 (13.51) and M5 (14.22) were temporarily assigned as baicalin, baicalein-6-O-glucuronide, and baicalein-5-O-glucuronide, respectively.

3.3.3 Metabolites (MRM 283-268)—Metabolites (MRM 283-268) were eluted from the PHY906 patient plasma at the retention times of 18.62, 23.56 and 24.2 min, respectively (Fig. 4A). Each of these metabolites showed a deprotonated molecule ion at 283Da, which is 15 Da or the mass of a methyl group more than baicalein, suggesting that they were isomers of the methylated metabolites of baicalein. These metabolites were stable to treatment with

β -glucuronidase (Fig. 4A). The MS/MS spectrum revealed their main product ion at m/z 268 (Fig. 4B). According to the established standards of wogonin and oroxylin A (6-methylated baicalein), M7 and M8 were characterized as wogonin and oroxylin A, respectively. The *n*-octanol/water partition coefficients of wogonin and oroxylin A were 3.08 and 3.33 respectively. The *n*-octanol/water partition coefficients of 5-methylated baicalein and 7-methylated were 2.5 and 3.3 respectively. According to the retention time of reference standard and their *n*-octanol/water partition coefficients, M6 was tentatively characterized as 5-methylated baicalein.

3.3.4 Metabolite (MRM 607-431)—Metabolite (MRM 607-431) was eluted from the PHY906 treated patient plasma at the retention time of 8.36 min (Fig. 5A). The MS/MS spectrum shows its main product ions at m/z 431 and 269 (Fig. 5B). The loss of 162 Da and 176 Da suggested that this metabolite contains glucosyl and glucuronic acid residues. Indeed, this metabolite was easily digested with β -glucuronidase enzyme (Fig. 5A). Based on the metabolites of baicalin in rats, M9 was characterized as baicalein-6-O-glucose-7-O-glucuronide instead of the double β -glucuronidated metabolite liquiritigenin that has the same molecular weight as M8.

3.3.5 Metabolites (MRM 349-269)—Metabolites (MRM 349-269) were eluted at the retention times of 15.37, 17.39, and 18.12 min (Fig. 6A). The MS/MS spectrum shows the main product ions to be at m/z 269 (Fig. 6B). The loss of 80 Da suggested that this was a sulfated residue. According to the metabolites (MRM 445-269), the M10, M11, and M12 were characterized as baicalein-7-sulfate, baicalein-6-sulfate and baicalein-5-sulfate. These metabolites were stable when treated with β -glucuronidase but treatment with sulfatase resulted in the formation of baicalein. (Fig. 6A)

3.3.6 Metabolites (MRM 525-445)—Metabolites (MRM 525-445) from the PHY906 treated plasma were eluted at the retention times of 10.05 and 10.78 min. (Fig. 7A) The MS/MS spectrum shows the main product ions at m/z 445 and 269 (Fig. 7B). The loss of 80 Da again suggests it has sulfated residue. According to the metabolites (MRM 445-269), the M13 and M14 were characterized as M4-sulfate and M5-sulfate, as these metabolites were digested by β -glucuronidase and sulfatase treatment (Fig. 7A).

3.4 Confirmation of metabolites using β -glucuronidase and sulfatase

In this study, β -glucuronidase and sulfatase were used for confirmation of metabolites proposed by mass spectrometry. After β -glucuronidase treatment, the peak of baicalein appeared after glucuronidase treatment from the Fig. 8. These results are consistent with the assignation of metabolites. The peak of M4 has an approximately 50% decrease even when the time of incubation was extended to 4 hours. This finding suggests that β -glucuronidase has less activity on the 6 O-glucuronide. The sulfated and glucuronidated metabolites disappeared after the sulfatase treatment. This observation suggests that sulfatase has glucuronidase activity. The combination of both LC-MS/MS methods and enzymes permitted a conclusive identification of baicalein even in low concentrations. These results are more reliable as compared with methods that only use a single chromatographic trace and retention time. Based on the metabolite profile of baicalin in plasma of the patient treated with PHY906, we have proposed the metabolic pathways of baicalein and baicalin as seen in Fig. 9. The structural assignations of other metabolic components were similar with baicalin and the results are presented in Table 2.

4. Conclusion

In summary, we have developed a novel approach using LC/MS/MS to identify the individual chemical components and metabolites of a Chinese herbal medicine, PHY906. Using this methodology, we were able to identify a total of 57 compounds in the parent formulation of PHY906 and 27 new metabolites in plasma following oral administration. In this experiment, the n-octanol/water partition coefficients were tentatively used to assign the region-isomers of PHY906 metabolites. This type of analysis represents the first step to identifying the active compounds and/or metabolites of PHY906 that mediate the PHY906 biological activity *in vivo* and in patients. In the future the authentic standards will have to be prepared for conclusive identification. Thus, quantitative analysis of the major compounds and metabolites in plasma of patients taking PHY906 orally is feasible when the authentic standards are already available. In the case of liquiritin, baicalin, and wogonin, we were able to detect plasma concentrations over a 3-log range going down to as low as 1 ng/mL. Moreover, such an approach may also be extended to investigate the composition and metabolism of other herbal medicines. Chemical databases are already available for a broad range of herbs that are being used clinically. Thus, the integration of LC/MS/MS analysis with chemical databases should greatly facilitate studies that investigate the clinical pharmacology and metabolism of herbal medicines. This method can also be used to investigate the chemical component and pharmacokinetic behaviors of other TCM even when the standards for these herbs are not available.

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References

- [1]. Liu SH, Jiang ZL, Su T, Gao WY, Leung CH, Lee Y, Cheng Y. Proc. Am. Assoc. Cancer Res. 2004; 45:557.
- [2]. Saif MW, Liu S, Elfiky A, Jiang ZL, Cheng YC. J. Clin. Oncol. 2007; 25:15116.
- [3]. Saif MW, Lansigan F, Ruta S, Lamb L, Mezes M, Elligers K, Grant N, Jiang ZL, Liu SH, Cheng YC. Phytomedicine. 2010; 17:61.
- [4]. Saif MW. J. Pancreas. 2008; 9:430.
- [5]. Saif, MW.; Li, J.; Chu, E.; lamb, L.; Kaley, K.; Elligers, K.; Jiang, ZL.; Bussom, S.; Liu, SH.; Cheng, YC. Gastrointestinal Cancers Symposium; 2008. p. 246
- [6]. Yen Y, So S, Rose M, Saif MW, Chu E, Liu SH, Foo A, Jiang ZL, Su T, Cheng YC. Anticancer Res. 2009; 29:4083. [PubMed: 19846955]
- [7]. Kim YH, Jeong DW, Paek IB, Ji HY, Kim YC, Sohn DH, Lee SH. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2006; 844:261.
- [8]. Lai MY, Hsiu SL, Tsai SY, Hou YC, P.D. J. Pharm. Pharmacol. 2003; 55
- [9]. Taira Z, Yabe K, Hamaguchi Y, Hirayama K, Kishimoto M, Ishida S, Ueda Y. Food Chem. Toxicol. 2004; 42:803. [PubMed: 15046826]
- [10]. Li HB, Chen I F. J. Chromatogr. A. 2005; 1071:107. [PubMed: 15941045]
- [11]. Bao KD, Li P, Qi LW, Li HJ, Yi L, Wang W, Wang YQ. Chem. Pharm. Bull. (Tokyo). 2009; 57:144. [PubMed: 19182403]
- [12]. Kesting JR, Huang J, Sørensen D. J. Pharm. Biomed. Anal. 2010; 51:705. [PubMed: 19850434]
- [13]. Inoue K, Kawaguchi M, Funakoshi Y, Nakazawa H. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2003; 789:17.
- [14]. Yao M, Ma L, Duchoslav E, Zhu M. Rapid Commun. Mass Spectrom. 2009; 23:1683. [PubMed: 19418486]

- [15]. Ma S, Zhu M. Chem. Biol. Interact. 2009; 179:25. [PubMed: 18848531]
- [16]. Wen B, Fitch WL. J. Mass Spectrom. 2009; 44:90. [PubMed: 18720456]
- [17]. Yao M, Ma L, Humphreys WG, Zhu M. J. Mass Spectrom. 2008; 43:1364. [PubMed: 18416441]
- [18]. Ye M, Liu SH, Jiang Z, Lee Y, Tilton R, Cheng YC. Rapid Commun. Mass Spectrom. 2007; 21:593.
- [19]. Klopman G, Zhu H. Mini Rev.Med.Chem. 2005; 5:127. [PubMed: 15720283]
- [20]. Huang J, Yu G, Zhang ZL, Wang YL, Zhu WH, Wu GS. J. Environ. Sci. (China). 2004; 16
- [21]. Zheng G, Huang WH, Lu XH. Anal. Bioanal. Chem. 2003; 376:680. [PubMed: 12761606]
- [22]. Kitagawa S, Li H, Sato S. Chem. Pharm. Bull. (Tokyo). 1997; 45:1354. [PubMed: 9301035]
- [23]. Feng NP, Di B, Liu WY. Chem. Pharm. Bull. (Tokyo). 2005; 53:978. [PubMed: 16079531]

Fig. 1. Representative TIC and UV chromatograms of PHY906 and metabolites in human plasma samples. Blue represents the plasma sample before administration of PHY906; Red represents the plasma sample 2 hours after administration of PHY906.

Fig. 2.

(A) Extraction ion current chromatograms of 621>445 in the patient plasma (1) before administration ;(2)2.0 hr after administration of PHY906; (3) sample (2) after β -glucuronidase treatment (B) LC-MS² spectra of 621>445 in PHY906-treated patient plasma (1) M1; (2) M2.

Fig. 3.

(A) Extraction ion current chromatograms of 445>269 in patient plasma (1) before administration; (2) 2.0 hr after administration of PHY90 ; (3) sample (2) after β -glucuronidase treatment. (B) LC-MS² spectra of 445>269 in PHY90-treated patient plasma (1) M3; (2) M4; (3) M5.

Fig. 4.

(A) Extraction ion current chromatograms of 283>268 in the patient plasma (1) before administration; (2) 2.0 hr after administration of PHY906; (3) sample (2) after β -glucuronidase treatment. (B) LC-MS² spectra of 283>268 in PHY906-treated patient plasma (1) M6; (2) M7; (3) M8.

Fig. 5.
 (A) Extraction ion current chromatograms of 607>431 in patient plasma (1) before administration; (2) 2.0 hr after administration of PHY906; (3) sample (2) after β -glucuronidase treatment. (B) LC-MS² spectra of M9 in PHY906-treated patient plasma.

Fig. 6.

(A) Extraction ion current chromatograms of 349>269 in patient plasma (1) before administration; (2) 2.0 hr after administration of PHY906; (3) sample (2) after β -glucuronidase treatment; (4) sample (2) after sulfatase treatment. (B) LC-MS² spectra of 349>269 in PHY906-treated patient plasma (1) M10; (2) M11; (3) M12.

Fig. 7.

(A) Extraction ion current chromatograms of 525>445 in patient plasma (1) before administration; (2) 2.0 hr after administration of PHY906; (3) sample (2) after β -glucuronidase treatment. (B). LC-MS² spectra of 525>445 in the PHY906 treated patient plasma (1) M13; (2) M14.

Fig. 8. Extraction ion current chromatograms of baicalein in PHY906-treated plasma before and after β -glucuronidase treatment.

Fig. 9.
Proposed metabolites of baicalein and baicalin in plasma following oral administration of PHY906.

Table 1

Compounds in PHY906 identified in plasma after oral administration of PHY906.

No	TR	Identification	Transition	PHY906	PHY906 spiked in plasma	patient plasma	source
1	1.2	gallic acid	168.9>125	+	–	–	P
2	1.9	paeoniflorin sulfonate	542.8>421.3	+	+	–	P
3	3.2	liquiritigenin 7-O-glucoside-4'-O-Apiosyl-O-Glucoside	711.2>549.2	+	+	–	G
4	3.4	viscidulin I 2'-O-Glucoside	463.2>301.1	+	+	–	S
5	4.9	albiforin	479-121	+	+	–	P
6	5.6	paeoniflorin	479>121	+	+	–	P
7	5.6	apigenin 6,8-DI-C-Glucoside	593.3>353.2	+	+	–	S
8	5.7	3,5,7,2',6'-pentahydroxyflavanone	303.1>177.1	+	–	–	S
9	5.8	apigenin 6-C-Glucoside-8-C-Arabinoside	563.2>353.2	+	+	–	S
10	6.3	chrysin 6,8-DI-C-Glucoside	577.2>337.2	+	+	–	S
11 *	6.8	isomer of Chrysin 6-C-Arabinoside-8-C-Glucoside	547.2>337.2	+	–	–	S
12	7.0	rutin	609>301	+	+	–	Z
13	7.0	chrysin 6-C-Arabinoside-8-C-Glucoside	547.1>337.3	+	+	–	S
14	7.1	liquiritigenin 4'-O-Apiosyl-O-Glucoside	549.3>255.2	+	+	+	G
15	7.1	liquiritin	417.2>255.2	+	+	–	G
16	7.5	Chrysin 6-C-Glucoside-8-C-Arabinoside	547.2>337.2	+	+	+	S
17	7.6	Acteoside	623.4>461.2	+	–	–	S
18	7.8	Scutellarin	461>285	+	+	–	S
19	7.8	isomer of Chrysin 6-C-Arabinoside-8-C-Glucoside	547.1>337.3	+	–	–	S
20	8.0	isomer of Chrysin 6-C-Arabinoside-8-C-Glucoside	547.1>337.2	+	+	+	S
21	8.2	isomer of Acteoside	623.3>461.3	+	+	–	S
22	9.1	5-hydroxyliquiritin	433.2>271.1	+	+	–	G
23	9.7	isomer of Hydroxyl Oroxylin A 7-O-glucuronide or hydroxyl wogonoside	475.2>299.1	+	+	–	S
24 *	10.3	isomer of liquiritigenin 4'-O-Apiosyl-O-Glucoside	549.2>255.1	+	–	–	G
25	10.9	baicalin	445.2>269.0	+	+	+	S
26	11.0	isoliquiritin	417.1>255.2	+	+	–	G
27	12.0	5,6,7-trihydroxyl flavanone 7-o-Glucuronide	447.2>271.1	+	+	–	S
28	12.3	7-hydroxy-2'-methoxyflavanone 7-o-glucuronide	445>269	+	+	–	S
29	12.5	isomer of baicalin	445>269	+	+	+	S
30	13.6	isomer of Hydroxylwogonin glucuronide	475.0>299.2	+	+	–	S
31 *	13.6	isomer of wogonoside	459.1>283.1	+	+	–	S
32	14.7	Wogonoside	459.1>283.2	+	+	+	S
33	14.9	licorice saponin J2	823.5>351.0	+	+	–	G
34	16.5	22β-Acetoxylicorice saponin G2	895.6>895.6	+	+	–	G
35	17.0	licorice saponin H2 or K2	821.0>351.2	+	+	–	G
36	17.8	22β-formyl licorice saponin G2	849.4>351.2	+	+	–	G
37	17.8	licorice saponin A3	983.5>821.6	+	+	–	G

No	TR	Identification	Transition	PHY906	PHY906 spiked in plasma	patient plasma	source
38	18.4	4'-Hydroxylwogonin	299.1>284.0	+	+	–	S
39	18.8	Baicalein	269.1>269.1	+	+	–	S
40	18.9	23-hydroxyl licorice saponin E2	835.5>835.5	+	+	–	G
41	18.9	22 β -acetoxyglycyrrhizic	879.5>351.2	+	+	–	G
42	21.0	licorice saponin G2	837.6>351.2	+	+	–	G
43	21.1	licorice saponin E2	819.7>819.7	+	+	–	G
44	21.4	isomer of licorice saponin E2	819.7>819.7	+	+	–	G
45	22.0	22 β -Acetoxylicorice saponin J2	881.7>881.7	+	+	–	G
46	22.2	isomer of licorice saponin G2	837.6>351.2	+	+	–	G
47	22.5	glycyrrhizic acid	821.2>351.4	+	+	+	G
48	22.8	22 β -Acetoxylicorice saponin B2	865.6>865.6	+	+	–	G
49	23.1	isomer of licorice saponin B2	807.6>351.3	+	+	–	G
50	23.2	isomer of Glycyrrhizic acid	821.5>351.3	+	+	–	G
51	23.5	isomer of licorice saponin H2 or K2	821.5>351.3	+	–	–	G
52	23.6	wogonin	283.0>268.0	+	+	+	S
53	23.7	isomer of licorice saponin J2	823.6>351.2	+	+	–	G
54	23.8	chrysin	253.1>209.2	+	+	–	S
55	24.0	licorice saponin C2	805.4>351.1	+	+	–	G
56	24.1	Oroxylin	283.2>268.1	+	+	+	S
57	24.2	licorice saponin B2	807.5>351.2	+	+	–	G

* newly found in this LC/MS/MS condition; isomer of Chrysin 6-C-Arabinoside-8-C-Glucoside (RT=32.9min), viscidulin π 2'-O-glucoside (RT=38.0min), scutellarein (RT=54.2min), viscidulin π (RT=59.2min), isomer of baicalin (RT=72.7min), isomer of Hydroxylwogonin glucuronide (RT=72.7min), isomer of baicalin (RT=77.6min), Oroxylin A 7-O-glucuronide (RT=79.1min), 5,7-dihydroxy-8,2'-dimethoxyflavone 7-O-glucuronide (RT=80.6min) and 22 β -Acetoxylicorice saponin J2 (RT=111.9min) were not found in this condition.

Table 2

Metabolites of PHY906 detected and structurally characterized by MRM-EPI using Qtrap LC/MS

	TR (min)	Identification	Transition	major fragment	source	After glucuronidase treatment	After sulfatase treatment
M1	6.49	Baicalein-5-O-glucuronide-7-O-glucuronide	621>445	445,269	S	↓	↓
M2	8.16	Baicalein-6-O-glucuronide-7-O-glucuronide	621>445	445,269	S	↓	↓
M3	10.93	Baicalin ^{**}	445>269	269,241,167	S	↓	↓
M4	13.51	Baicalein-6-O-glucuronide [#]	445>269	269,241	S	↓	↓
M5	14.22	Baicalein-5-O-glucuronide [#]	445>269	269,241,167	S	↓	↓
M6	23.56	Wogonin ^{**}	283>268	283,268	S	↑	↑
M7	24.20	Oroxylin A ^{**}	283>268	283,268	S	↑	↑
M8	18.62	5-methylation baicalein.	283>268	283,268,240	S	→	↓
M9	8.36	Baicalein-6-O-glucose-7-O-glucuronide	607>431	431,269	S	↓	↓
M10	15.37	Baicalein-7-sulfation [#]	349>269	269,241	S	→	↓
M11	17.39	Baicalein-6-sulfation [#]	349>269	269	S	→	↓
M12	18.12	Baicalein-5-sulfation [#]	349>269	269,241	S	→	↓
M13	10.05	Baicalin or isomer with a sulfation [#]	525>445	445,269	S	↓	↓
M14	10.78	Baicalin or isomer with a sulfation [#]	525>445	445,269	S	↓	↓
M15	18.03	Wogonin or oroxylin A with a sulfation	363>283	283,268	S	↑	↓
M16	13.84	Wogonin 5-O-glucuronide or 7-O-glucuronide	459>283	283,268	S	↓	↓
M17	14.91	Oroxylin A 6-O-glucuronide or 7-O-glucuronide	459>283	283,268	S	↓	↓
M18	9.5	Wogonoside with a sulfation	539>459	459,283,268	S	↓	↓
M19	10.33	Oroxylin A with a glucoside and a sulfation	539>459	459,283,268	S	↓	↓
M20	6.42	Wogonin 5-O-glucuronide-7-O-glucuronide	635>459	459,283,268	S	↓	↓
M21	8.71	Oroxylin A-6-O-glucuronide-7-O-glucuronide	635>459	459,283,268	S	↓	↓
M22	13.79	Chrysin 5-O-glucuronide or 7-O-glucuronide.	429>253	253,209	S	↓	↓
M23	17.91	Chrysin-5-sulfation or 7-sulfation	333>253	253,209	S	↑	↓
M24	7.22	Chrysin 5-O-glucuronide - 7-O-glucuronide	605>429	429,253	S	↓	↓
M25	9.48	Hispidulin 5-O-glucuronide-7-O-glucuronide	651>475	475,299,284	S	↓	↓
M26	14.93	Liquiritigenin -5-sulfation [#]	335>255	ND	G	→	↓
M27	17.91	Liquiritigenin -7-sulfation [#]	335>255	ND	G	→	↓
M28	NF	Chrysin-6-O-β-glucose-7-O-glucuronide	591>429	ND	S	ND	ND
M29	NF	Wogonin-6-O-β-glucose-7-O-glucuronide	621>459	ND	S	ND	ND
M30	NF	Hispidulin-6-O-β-glucose-7-O-glucuronide	637>475	ND	S	ND	ND

^{**} identified by the standard[#] identified by n-octanol/water partition coefficients

NF: not found

ND: no data

↓: decrease

→: no change

↑: increase