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Development of GC-MS based cytochrome P450 assay for the investigation of multi-herb interaction

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ABSTRACT

As drug interactions with cytochrome P450 enzymes become increasingly important in the field of drug discovery, a high-throughput screening method for analysing the effects of a drug is needed. We have developed a simple and rapid simultaneous analytical method using a cocktail approach for measuring the activities of seven cytochrome P450 enzymes (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4). Human liver microsomes were used as a source for the seven cytochrome P450 enzymes, and a gas chromatography-mass spectrometry (GC-MS) was used for analysing their activities. Kinetic studies and inhibition assays of CYP enzymes were performed using known substrates and inhibitors for validating and comparing the reaction rates and time-dependent activities between methods using each substrate versus a method using a cocktail solution. The optimized cocktail method was successfully applied to evaluate the effects of the decoction of Socheongryong-tang (SCRT) on cytochrome P450 enzymes. Our cocktail method provides a simultaneous high-throughput activity assay using GC-MS for the first time. This method is applicable for analysing the drug interactions of various plant-derived mixtures.

1. Introduction

Cytochrome P450 (CYPs, P450) enzymes play a critical role in major phase I metabolism. CYPs metabolize 75% of drugs and are involved in the biotransformation of xenobiotics (carcinogens, toxins, drugs, plants and endogenous molecules, such as steroids and eicosanoids) [1,2]. Five major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) metabolize approximately 90% of the drugs on the market, including synthetic and plant-derived compounds, these five CYPs are critical to the oxidative metabolism of humans [3].

Herbal medicines have gained wide popularity in complementary and alternative medicine (CAM) worldwide. Over 70% of the world population has used CAM for medicinal purposes [4]. Herbal medicine is often co-administered with synthetic drugs or other herbs, potentially causing herb-drug interactions (HDIs) that could lead to adverse effects, such as alternating CYP metabolisms, such as induction or inhibition. Several studies have been published on the HDIs between natural compounds (ginkgo, St. John's Wort, garlic and ginseng), a single active compound from plant extracts [5–7], herbal mixtures [8,9] and synthetic drugs [2,10–12]. Similar to the HDIs, the herb-herb interactions (HHIs) in complex plant mixtures, e.g., a decoction, may also have pharmacological and toxicological effects. To date, these interactions have not been well studied.

To evaluate the interaction of CYPs with the components in

Abbreviations used in this paper: CAM, Complementary and alternative medicine; CV, Coefficient of variation; CYP, Cytochrome P450; GC-MS, DTE; dithioerythritol, Gas chromatography-mass spectrometry; HDI, Herb-drug interaction; HHI, Herb-herb interaction; HLM, Human liver microsome; HPLC, High-performance lipid chromatography; LC-MS/MS, Lipid chromatography tandem mass spectrometry; LLOQ, Low limit of quantitation; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide; NADPH, β-nicotinamide adenine dinucleotide 2'-phosphate-reduced tetrasodium salt hydrate; QTOF, Quadrupole time-of-flight; SCRT, Socheongryong-tang; SIM, Selected ion monitoring; QC, Quality control; UPLC-MS/MS, Ultra-performance lipid chromatography tandem mass spectrometry; 3′-OH-Cotinine, 3′-Hyfroxycotinine; 4′-OH-Diclofenac, 4′-Hydroxydiclofenac; 4′-OH-Mephenytoin, 4′-Hydroxymephenytoin; 6′-OH-Chlorzoxazone, 6′-Hydroxychlorzoxazone; 6β-OH-Testosterone, 6β-Hydroxytestosterone; 7′-OH-Coumarin, 7′-Hydroxycoumarin.

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herbal medicine, it is essential to develop a rapid high-throughput *in vitro* CYP inhibition assay method that is not labour-intensive. The Food and Drug Administration (FDA) has recommended many strategies for developing high-throughput screening for the *in vitro* inhibition of CYPs. Among these strategies, the cocktail approach using human liver microsomes (HLMs), pooled CYP enzymes, has been developed recently, this approach does not use only a single recombinant CYP enzyme [13–19]. The cocktail method is faster, more efficient and more cost-effective than the single substrate approach [20]. In addition, it better reflects the *in vivo* data [10].

Conventional methods for measuring CYP enzyme activities involve the quantitation of specific CYP enzyme metabolites using a fluorescence technique with high-performance liquid chromatography (HPLC) and liquid chromatography tandem mass-spectrometry (LC-MS/MS). LC-MS/MS is currently used because the fluorescent probe is not specific to each CYP isoform. Recent CYP enzyme studies have improved upon previous methods by employing high resolution instrument systems, including ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) and UPLC-quadrupole time-of-flight (QTOF) [20,21]. A method incorporating simultaneous high-throughput screening and the cocktail approach using LC-MS/MS has been validated by several researchers [13–19]. In addition to the above techniques, the screening of CYP enzyme metabolites using gas chromatography mass-spectrometry (GC-MS) has been reported [22–24]. However, these methods have only been developed with a few CYPs, including CYP2D6 and CYP3A4 [22]. To date, a simultaneous method for measuring the activity of various CYP isoforms in HLMs using GC-MS has not been reported.

Herein, we disclose a high-throughput screening method for simultaneously measuring the activities of seven CYP enzymes using GC-MS. Substrates were carefully chosen that are specific to each CYP, the metabolites were detectable by GC-MS. The extraction method was optimized to detect all metabolites in the enzyme assay system. Kinetic and inhibition studies were performed using the substrates and their respective inhibitors using a HLMs incubation assay. With the optimized platform, the method was used to evaluate the multicomponent interactions in Socheongryong-tang (SCRT, Xiao-Qing-Long-Tang in China and Sho-seiryu-to in Japan), a traditional herbal medicine in a decoction form (consisting of a mixture of eight plants used to treat asthma, allergic diseases and bronchitis for more than hundreds of years in Asia) [25]. GC-MS analysis is suitable for examining the effects of traditional multi-herb decoctions on CYPs because it removes signal interferences through the process of extracting the product of the enzyme assay. In addition, GC-MS is convenient for routine analysis.

2. Materials and methods

2.1. Chemicals

Phenacetin, coumarin, diclofenac, (S)-mephenytoin, cotinine, chlorzoxazone, 4'-hydroxydiclofenac (4-OH-diclofenac), 7'-hydroxycoumarin (7-OH-coumarin), dextrorphan, acetaminophen, quinidine, ketoconazole, α-naphthoflavone, benzylnirvanol, 8-methoxypsoralen, sulfaphenazole, diethyldithiocarbamate, carbamazepine, potassium phosphate monobasic and dibasic, ammonium bicarbonate (NH_4HCO_3), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), dithioerythritol (DTE), magnesium chloride ($MgCl_2$) and β-nicotinamide adenine dinucleotide 2'-phosphate-reduced tetrasodium salt hydrate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pooled HLMs (150 donor pool) and 6β-hydroxytestosterone (6β−OH−testosterone) were obtained from BD Biosciences (Woburn, MA, USA). Dextromethorphan, 3'-hydroxycotinine (3-OH-cotinine), 6'-hydroxychlorzoxazone (6-OH-chlorzoxazone) and 4'-hydroxymephenytoin (4-OH-mephenytoin) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HPLC-grade methanol and acetonitrile were obtained from SK Chemicals (Ulsan, Republic of Korea). Ultrapure water (18.2 MΩ·cm) was obtained using a Milli-Q apparatus from Millipore (Milford, MA, USA). All chemicals were of analytical grade (over ≥98% in purity). All stock solutions were prepared at a 1000 μg/mL concentration in methanol and working solutions were diluted in methanol, excluding NADPH. HLMs were stored at −80 °C. Other chemicals were stored at −20 °C prior to use.

2.2. Procedure for the enzyme reaction

Depending on the reaction rate of each CYP isoform, the reaction was divided into three groups. In reaction group A, 1.25 μL of human liver microsomes (20 mg/mL protein) and 8.75 μL of a potassium phosphate buffer (0.1 M pH 7.4) were mixed together [26,27]. In group B, 2.5 μL of human liver microsomes (20 mg/mL protein) and 7.5 μL of a potassium phosphate buffer (0.1 M pH 7.4) were mixed together. For group C, 10 μL of human liver microsomes (20 mg/mL protein) and 10 μL of a potassium phosphate buffer (0.1 M, pH 7.4) were mixed together. Into the mixtures, 5 μL of $MgCl_2$ (100 mM) and 1 μL of substrates were added to each group to make up the final concentration as followings; Group A: 0.02 mM diclofenac, 0.02 mM mephenytoin, and 0.1 mM testosterone; Group B: 0.1 mM phenacetin and 0.005 mM coumarin; Group C: 0.1 mM cotinine, 0.02 mM dextromethorphan, and 0.02 mM chlorzoxazone as their final concentration. The resultant mixtures were diluted to 100 μL with ultrapure water. The total methanol content was less than 2% (v/v) in the reaction mixture [28]. After pre-incubation for 5 min at 37 °C, the reaction was initiated by adding 20 μL of 5 mM NADPH to the incubation mixture. The reaction mixture was placed in a shaking water bath at 37 °C for 15 min (Group C; 50 min). The reaction was terminated by adding 197 μL of cold NH_4HCO_3 solution (0.1 M, pH 9) to the reaction mixture, which was then immediately placed in ice. Then, the samples were vortexed for 1 min and centrifuged for 2 min at 3500 g, 4 °C after adding 3 μL of internal standard (IS: carbamazepine, final concentration: 0.5 μg/mL). More detailed information is shown in Table 1.

2.3. Extraction and derivatization of CYP products in the cocktail solution

One millilitre of ethyl acetate was added to extract the products, then the mixture was vortexed for 1 min and centrifuged for 2 min at 3500 g, 4 °C. To separate the supernatant from the inorganic phase, the reaction mixture was incubated for 30 s in a solvent-circulation freezer at −30 °C, and the organic phase was transferred to a new test-tube. After thawing the aqueous fraction, 1 mL of dichloromethane was added. The resultant solution was vortexed for 1 min and centrifuged for 2 min at 3500 g, 4 °C. The supernatant (aqueous phase) was removed from the organic phase using a vacuum pump and the remaining organic phase was mixed with the previously extracted organic phase. The extraction was repeated twice. The extracted organic phases for the three cocktail groups were combined into one tube prior to derivatization. The resultant mixture was concentrated by evaporating the solvent under a gentle stream of nitrogen gas at 37 °C and placed under vacuum in a desiccator for at a minimum of 30 min. For derivatization, 50 μL of the MSTFA:NH_4I:DTE (500:4:2 (v/w/w)) mixture was added to the residue [29] and reacted for 20 min at 60 °C. Finally, 1 μL of the derivatized solution was injected into the GC-MS.