A C₈-Modified Graphene@mSiO₂ Composites Based Method for Quantification of Gallic Acid in Rat Plasma after Oral Administration of Changtai Granule and Its Application to Pharmacokinetics

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A rapid, effective extraction technique has been established for measuring the gallic acid in rat plasma by using sandwich-structured graphene/mesoporous silica composites with C₈-modified interior pore-walls as adsorbent. The unique characteristics of the graphene-silica composites excluded large molecules, like proteins, from the mesopore channels as a result of size exclusion effect, leading to a direct extraction of drug molecules from protein-rich biological samples such as plasma without any other pretreatment procedure. Followed by elution and centrifugation, the gallic acid-absorbed composites were rapidly isolated before LC-MS/MS. Serving as a reliable tool for analysis of Traditional Chinese Medicine: Changtai Granule, the newly developed method was fully validated and successfully applied in the pharmacokinetic study of gallic acid in rat plasma. Extraction recovery, matrix effect and stability were satisfactory in rat plasma. According to the results of pharmacokinetic studies, Changtai Granule exhibited greater adsorption, distribution and clearance properties of gallic acid in the treatment of ulcerative colitis. Hence, this study may offer a valuable alternative to simplify and speed up sample preparation, and be useful for clinical studies of related preparations.

Key words C₈-modified graphene@mSiO₂ composite; Changtai Granule; gallic acid; LC-MS/MS; pharmacokinetics

Changtai Granule, a traditional compound Chinese medicinal formula in the treatment of ulcerative colitis (UC, which is a refractory, chronic and non-specific disease, and one of the most severe gastrointestinal diseases), has generated interest in recent years. The prescription consists of four medicinal materials, including Phellodendro Chinense Willd., Sanguisorba officinalis L., and Polygonum hydropiper Linn. A series of comprehensive studies on Changtai Granule started from 2003, as well as the product development. 1–2 This Chinese medicinal formula has been proved to be very simple, safe and effective in the treatment of ulcerative colitis with less harmful side effects and has been approved by the U.S. Food and Drug Administration (FDA) to enter clinical trial for the treatment of ulcerative colitis. In our previous investigation, 1–2 Changtai Granule possesses a variety of pharmacological effects including analgesic–antipyretic, anti-inflammatory, antibacterial and anti-diarrhea actions, as well as the effect of adjusting gastrointestinal function. Evidence from animal models 1–2 and the history of use in folk medicine suggest a potential role for oral administration of Changtai Granule in prevention and amelioration of ulcerative colitis. Furthermore, administration of Changtai Granule can significantly decrease the frequency of diarrhea and promote survival in UC rats. Most of our previous studies 1–2 revealed that after analyzing the bioactive ingredients absorbed into the rat plasma, the most important ingredient was gallic acid (GA), which might originate from Sanguisorba officinalis L. and Euphorbia humifusa Willd. 3–5 The pharmacological studies have demonstrated that GA is the index bioactive constituents in Changtai Granule. The pharmacokinetic properties of the active components, however, in their pure forms are significantly different from that in herbal medicines. To date, several studies on pharmacokinetics of GA in its pure form have been reported. 4,6–8 However, to the best of our knowledge, no study on pharmacokinetic behaviors of GA after oral administration of Changtai Granule has ever been reported.

It is well known that Traditional Chinese Medicine (TCM) has thousands of years of history in China that play a crucial role in disease protection, control, and treatment. But there is a considerable lack in sample pretreatment techniques which severely delays development and application of TCM.9,10 The most common and traditional procedures for TCMs are solvent extraction, Soxhlet extraction, heat-reflux extraction, and ultrasound-assisted extraction, which are most widely used currently, but these methods involve large solvent consumption or long extraction time.11–16 New techniques like microwave-assisted extraction and supercritical fluid extraction are alternative method which can also be employed.17–21 However, both of these two pretreatment techniques need high-cost specific equipment. On the other hand, all the methods described above need an initial purification (deproteinization) step, to allow analysis of pharmacokinetic studies using biological samples. Therefore, for the sake of high selectivity, fast and efficient separation, we chose to examine the utility of newly emerging graphene-based composites.

Lately, graphene has drawn much attention due to its extraordinary electrical, thermal and mechanical properties arising from its unique structure of two-dimensional monolayer of carbon atoms arranged in hexagonal rings in a honeycomb
network. Considerable effort has been devoted to the development of graphene-based composites by integrating graphene nanosheets with other nanomaterials into various multifunctional materials or nanodevices for applications in diverse fields. To improve the properties of graphene, particularly related to its hydrophobicity and difficulty in interfacial interaction with targeted matrix, chemical modification has been found to be important.

For the past few decades, mesoporous silica has been demonstrated to be an ideal adsorbent for extraction and enrichment, thanks to its large surface area, regular pore volume, tunable pore size and modifiable silanol group. The ordered mesopores with small diameters and narrow size distributions can exclude large proteins outside and selectively extract the analytes. We hypothesized that the combination of graphene nanosheets and mesoporous silica would generate a hybrid nanomaterial that could improve the interfacial properties of graphene and integrate the advantages of both components. Recently, our group has done a good deal of work to synthesize magnetic mesoporous silica microspheres for the extraction of glucocorticoids from milk, which shows the possibility of drug extraction from plasma samples by such composites.

Therefore, the aim of this work was to demonstrate the feasibility of using this material for extraction and analysis of drugs in protein-rich biological samples. The C₈-modified graphene@mSiO₂ composite has been applied in the extraction and enrichment of gallic acid in rat plasma after oral administration of the traditional compound Chinese medicinal formula: Changtai Granule, and in pharmacokinetic studies.

MATERIALS AND METHODS

**Chemicals and Reagents** Changtai Granule were provided by Tenth People’s Hospital of Tongji University (Shanghai, China). Reference standards of gallic acid (GA) and salicylic acid (SA, used as internal standard (IS)) were purchased from Sigma-Aldrich Chemical Company (Shanghai, China). Purity of all the analytical standards were ≥98.0%. HPLC-grade methanol was obtained from TEDIA (OH, U.S.A.). Ammonium hydroxide, concentrated nitric acid, sodium hydroxide, phosphoric acid, disodium hydrogen phosphate, acetic acid, ammonium acetate, monopotassium phosphate, hydrochloric acid, ethanol and acetone were all purchased from Sinopharm Chemical Reagent (Shanghai, China). Graphene, tetraethyl orthosilicate (TEOS) and cetyltrimethyl ammonium bromide (CTAB) were bought from Shanghai Chemical Corp. (Shanghai, China). n-Octyltriethoxysilane (abbreviated as C₈TES, purity >95%) was purchased from Alfa Aesar (Tianjin, China). Deionized water used for HPLC-MS/MS was purified with a Milli-Q plus system (Millipore, Bedford, MA, U.S.A.). All other chemicals and reagents were of the highest grade commercially available.

**Experimental Animals** Pathogen-free adult male Sprague–Dawley (SD) rats weighing 300±30 g were purchased from Changsha Tianqin Bio-technology Co., Ltd. (Changsha, China, Certificate no. SCXK 2009-0012). All rats were acclimated for at least a week in environmentally controlled quarters (24±1°C and 12/12 h light/dark cycle) with free access to standard chow and water. The rats were fasted overnight but supplied with water *ad libitum* before the experiments. All experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animal (National Institutes of Health Publication 85-23, revised edition 1985).

**Preparation of C₈-Modified Graphene@mSiO₂ Composites** The synthesis of C₈-functionalized graphene-mesoporous silica composites involves three steps (Fig. 1A). Firstly, the pristine graphene flakes were dispersed into 70 mL concentrated nitric acid at 60°C for 6 h under mechanical stirring. Afterwards, the acidic reaction solution was collected by centrifugation, rinsed with deionized water, and dried in vacuum at 60°C followed by adjusting the pH value to neutral by 0.1 M NaOH solution.

Next 50 mg acidized graphene was dispersed into 50 mL deionized water with 500 mg CTAB, followed by ultrasonication treatment for 30 min to form a homogeneous dispersion. An additional 400 mL deionized water and 50 mL of 0.01 M NaOH solution was added, followed by an additional 10 min ultrasonication and 30 min heating under mechanical stirring at 60°C. 2.5 mL TEOS–ethanol (v/v, 1:4) solution was added drop by drop subsequently, followed by heating at 60°C for another 30 min with stirring. With the injection of 150 µL TEOS–C₈TES (v/v, 2:1) mixture into the dispersion, the reaction was allowed to continue at 60°C for 12 h while stirring.

The products were finally collected by centrifugation, washed with deionized water, and refluxed in acetone several times for thorough removal of CTAB templates. Thereafter, the C₈-modified graphene@mSiO₂ composite was dried at 50°C for 24 h in vacuum for future use. Technical support of the whole synthesis process was provided by Department of Chemistry, Fudan University.

**Plasma Sample Preparation Using C₈-Modified Graphene@mSiO₂ Composite** An aliquot of 90 µL plasma sample was transferred into a 1.5 mL Eppendorf tube (EP tube), and 10 µL IS solution (200 ng/mL) together with 50 µL of 0.14 M H₃PO₄–Na₂HPO₄ buffer (pH 2.0) were individually added. The mixture was vortexed for 60 s and 80 µg C₈-modified graphene@mSiO₂ composite was added. The analyte and IS were extracted from plasma by vortex for 20 min; by ultrasonication for 5 min. Then the sample was centrifuged at 5000 rpm for 5 min. The precipitate was mixed with 100 µL of 70% methanol by vortex for another 20 min and ultrasonication for 5 min. After being centrifuged at 10000 rpm for 5 min, the supernatant was transferred into a clean 1.5 mL EP tube and evaporated to dryness under a nitrogen stream at 40°C. The dried extract was dissolved in 20 µL deionized water and centrifuged. A 10 µL aliquot was injected into LC-MS/MS for analysis (Fig. 1B).

**Analytical Method and Statistical Analysis** The LC-MS/MS system was composed of an Agilent 1200 HPLC system equipped with an AB SCIEX 4500 triple-quadrupole (QqQ) mass spectrometer (AB SCIEX™, Massachusetts, U.S.A.).

The chromatographic separation was achieved on a Shiseido® Capcell Pak C₁₈ column (4.6×150 mm, 5 µm) (Shiseido®, Tokyo, Japan) with a Dikma® C₁₈ guard cartridge (4.6×10 mm, 5 µm) (Dikma®, Beijing, China) at the temperature of 30°C. The mobile phase consisted of methanol (A) and water (B) (both containing 1.0% NH₃) with a gradient of 20–80 (v/v), together with a flow rate of 0.35 mL/min and an injection volume of 10 µL.
Mass spectrometric analyses were performed with an electrospray ionization source (ESI) source interface set in negative ionization mode. Both of the analyte and IS were monitored under multiple reactions monitoring (MRM) mode with transitions of \( m/z \) 169.2→125.1 for GA, and \( m/z \) 136.9→93.2 for IS. The optimal MS parameters of the mass spectrometer were as follows: ion spray voltage 4500 V; N\(_2\) sheath gas 25 psi, and turbo heater temperature 550°C. High purity nitrogen served as the collision gas.

All of the peak integration and mass spectrometry data processing was performed with AB SCIEX Analyst Software (Version 1.6) (AB SCIEX™, Massachusetts, U.S.A.). Statistical analysis was performed using Microsoft Office 2013 (Microsoft Corp., Redmond, WA, U.S.A.) and SPSS 18.0 software (SPSS, Inc., Chicago, U.S.A.). Data were expressed as mean±standard deviation (S.D.) and a \( p \) value <0.05 was considered to be statistically significant.

**Preparation of Calibration Standard and Quality Control (QC) Samples** Stock solutions of GA and IS at the concentration of 1000 \( \mu \)g/mL were prepared by dissolving the accurately weighed reference standards in methanol, respectively. A series of standard working solutions of GA was obtained by future diluting the stock solutions into 5.0–100.0 ng/mL. The IS solution was prepared by diluting with deionized water to a final concentration of 200 ng/mL. All the solutions were stored at 4°C before analysis.

For the validation and pharmacokinetic study of the assay, quality control (QC) samples at different concentration levels were also prepared in the same manner by spiking 100 \( \mu \)L aliquots of blank plasma with 10 \( \mu \)L working solutions of corresponding concentrations. The concentrations of QC samples (low, middle and high) were 1.0 ng/mL (2–3 times of lower limit of quantity (LLOQ), 20 ng/mL (between QC1 and QC3)), and 80 ng/mL (80% upper limit of quantity (ULOQ)), respectively.

The standards and QC samples were extracted on each analysis day with the same processes for plasma samples preparation as described in “Plasma Sample Preparation Using C8-Modified Graphene@mSiO\(_2\) Composite.”

**Pharmacokinetics Study** Based on the previous studies of Changtai Granule\(^{11}\) and taking the exact concentration of GA in Changtai Granule into consideration, 6 male SD rats were orally administrated with 10 g/kg Changtai Granule. Blood samples (about 300 \( \mu \)L) were collected in heparinized EP tubes via the suborbital vein from each rat at pre-dose (0 min) and at 10, 20, 30, 45, 60, 120, 240, 360, and 480 min post-dose. The plasma was immediately centrifuged at 3000 rpm for 5 min, transferred into clean EP tubes, and stored at −80°C until analysis. The pharmacokinetic parameters were calculated by the non-compartmental analysis of plasma concentration versus time data using the DAS 2.1 software package (Chinese Pharmacological Society).
RESULTS AND DISCUSSION

Optimization of Chromatographic and Mass Conditions

A few columns were screened before Shiseido® Capcell Pak C18 column (4.6×150 mm, 5 µm) (Shiseido®) was finally chosen, together with a Dikma® C18 guard cartridge (4.6×10 mm, 5 µm) (Dikma®, Beijing, China) for column protection. Various mobile phase conditions were tried to obtain high detection sensitivity, suitable retention times, good peak symmetry and appropriate ionization of the analyte. Methanol, acetonitrile, and different proportions of formic acid, ammonium hydroxide in water were tested as potential mobile phases. It was found that methanol gave better resolution, lower background noise and lower cost than acetonitrile. The addition of 1% NH3 to the mobile phase can improve the response of GA. As a result, satisfactory resolution values, sharp and symmetrical peaks were gained by using methanol–water (both containing 1% NH3) system.

MS analytical parameters were carefully optimized for the determination of GA in rat plasma. Both positive and negative ionization modes were tuned. From Figs. 2A, B, the ionization of GA and IS gave relatively more intense signals in negative ion mode than in positive ion mode in the full-scan analytical signal. To get the richest relative abundance of precursor and product ions, the parameters for fragment energy and collision energy were further optimized. The MS/MS product ion spectra of GA and IS with the chemical structures combined are shown in Fig. 2C.

Investigation of Sample Pretreatment

Validation of C8-Modified Graphene@mSiO2 Composites

By using the facile one-pot-sol-gel approach, the C8-modified graphene@mSiO2 composites were synthesized through the simultaneous condensation of TEOS and C8TES in the presence of graphene as the seeds and CTAB as the template. According to our previous work26–29, the mesopore channels in the shell are perpendicular to the surface of the as-prepared composites, and the pore size is about 3.0 nm with narrow pore size distribution. Theoretically, these mesopores on the surface of the composites can prevent irreversible adsorption of the proteins from the sample matrix due to size exclusion effect.

Several experiments including maximum loading volume, repeatability and stability, and sensitivity of enrichment, had been validated for the performance testing of the as-made C8-modified graphene@mSiO2 composites before putting into use.

To acquire the maximum extraction efficiency of the target analyte, the amounts of the plasma sample were optimized first ranged from 10 to 200 µL when the same amount of C8-modified graphene@mSiO2 composites (40 µg) was used for extraction. As Fig. 3A displayed, when 50 µL plasma spiked with 100 ng/mL GA was loaded, the composites were saturated, which implied the maximum loading volume was 50 µL for 40 µg C8-modified graphene@mSiO2 composites.

Following the procedure in Fig. 1B, the extraction was repeated 10 times to verify repeatability and stability. Relative
standard deviations (RSDs) for these 10 extractions were all below 4.2%, suggesting desired repeatability and stability was achieved.

Afterwards, a simulation sample of GA with a low concentration (100 pg/mL) was measured for evaluating the sensitivity of the enrichment. After enrichment by C₈-modified graphene@mSiO₂ composites, the S/N ratio of GA was significantly improved (S/N=5), leading to a sensitive detection of GA with an interference-low background.

Study on C₈-Modified Graphene@mSiO₂ Composites Extraction Conditions

Considering the weak acidity of GA, an appropriate type of buffer solution is fundamental for the extraction. A series of buffer solutions of various pH was observed, including 1.0 M HAc–NH₄Ac buffer (pH 4.5), 0.67 M HAc–NH₄Ac buffer (pH 3.6), 0.73 M KH₂PO₄–HCl buffer (pH 2.5), and 0.14 M H₃PO₄–Na₂HPO₄ buffer (pH 2.0) (Fig. 3B). The 0.14 M H₃PO₄–Na₂HPO₄ buffer (pH 2.0) was finally selected for its satisfactory extraction efficiency of GA, whose pKₐ value was 4.5.

For the sake of enhancing extraction efficiency, the selection of elution solvent is important in the procedure. In this study, five solvents including acetonitrile, methanol, ethanol, water and acetone were investigated for improving the extraction efficiency of GA. As depicted in Fig. 3C, the extraction efficiency reached the maximum when using methanol as the elution solvent. This could be explained as the similar polarity of methanol and GA, thus leading to easier elution compared with other four solvents.

After methanol was chosen as the elution solvent, further study on methanol–water ratio was carried out. Different ratios of methanol and water were investigated and 70% methanol was found to be most suitable for elution of GA from the C₈-modified graphene@mSiO₂ composite, as be seen in Fig. 3D.

The time of vortex and ultrasonication also important during the extraction process. Parallel experiments were conducted to define the optimal time for the two-time vortex and ultrasonication. The proper vortex time was found to be 20 min for both; while ultrasonication times were 5 and 10 min, respectively.

Assay Validation
Selectivity and Specificity

Under the developed chromatographic and mass spectrometry conditions, there were no interfering peaks at retention times for GA and IS. The typical chromatograms of the spiked plasma sample with GA and IS are represented in Fig. 2.

Linearity and Limit of Detection (LOD)/LLOQ

To evaluate linearity, calibration solutions of GA (5.0–100.0 ng/mL) were prepared and assayed on three consecutive days. The peak area ratios of GA to IS in rat plasma varied linearly over the concentration ranges. The best linear fit the least-square residual for the calibration curve was achieved with a 1/x² weighting factor. The regression equation for GA was Y=0.0027X+0.0034 (r=0.9995, n=5), where Y refers to the peak area ratio (GA/IS) and X is the concentration of GA. The LOD and LLOQ for GA was 0.08 and 0.30 ng/mL. This was sufficient for pharmacokinetic studies of the analyte following oral administration of Changtai Granule to rats.

Accuracy and Precision

As shown in Table 1, the results for intra- and inter-day precision and accuracy for GA indicated that the intra- and inter-day RSDs were all less than 2.4 and 6.2%, respectively, while the Relative Efficiencies (REs) ranged from −7.1 to 4.9%, respectively. All the assay values were within the ac-
ceptable criteria, demonstrating an accurate, reliable, and reproducible method was established for the determination of GA in rat plasma.

Recovery and Matrix Effect

Table 1 summarizes the extraction recovery and matrix effect of GA. Based on the data displayed, the extraction recovery of the investigated component in plasma at three concentration levels ranged from 84.5 to 98.2%. The assessment of matrix effect of the assay was performed systematically and all the ratios of the analyte were found to be within the acceptable range (94.4–99.8%), which indicated that there was no significant ion suppression or enhancement from plasma for this assay.

Stability

The stability of the assay was conducted strictly under different circumstances (room temperature, post-preparation, and freeze-thaw). The results presented in Table 1 indicated that GA was stable under routine laboratory conditions.

Comparison with Other Routine Bioassay Pretreatment Methods

To illustrate the advantages of C₈-modified graphene@mSiO₂ composites as a novel extraction tool, the comparison of our developed method with other reported sample preparation procedures was performed. As presented in Table 2, the recoveries of the proposed method ranged from 84.5 to 98.5% with RSD between 0.6 and 2.6% with LLOQ of 0.30 ng/mL. These values, which reflect good sensitivity and precision, are substantially superior to those achieved via traditional extraction methods like protein precipitation and liquid–liquid extraction (LLE) previously reported,⁴,²⁹ which range from 78% to 83%, 80 to 89% with RSD between 4 and 7%, 7 and 10%, respectively. On the other hand, our present method gave LLOQ value for GA at 0.30 ng/mL, which was far more sensitive compared with the LLOQ value of 30 and 20 ng/mL given by the routine methods previously referenced.⁴,²⁹)

According to the literature, the recent presented method of GA extraction ³⁰ utilize acetonitrile to precipitate protein. We reproduced this experiment with some modification due to the different samples we used. As presented in Table 2, the recovery of this method ranged from 80.2 to 88.5%, with RSD of 4.3–8.2%, which was much lower than the results we get from our as-made material. In addition, the LLOQ of our method is 0.30 ng/mL, compared with the 5.0 ng/mL of the reported method, and showed high sensitivity and selectivity. These satisfactory results maybe probably due to the effective adsorption of hydrophobic compounds based on the interaction between C₈-modified pore-walls and GA. The distinctive structure of C₈-modified graphene@mSiO₂ composites provides extended plates with hydrophilic surface, which has the excellent dispersibility in solution. And the prepared nanosheets give a large number of C₈-modified mesopores with high surface in the interior pore-walls, which ensure the effective adsorption of hydrophobic compounds.

Application to the Pharmacokinetic Study

The developed C₈-modified graphene@mSiO₂ composites based LC-MS/MS method was successfully applied to pharmacokinetic studies on GA in rat plasma after oral administration of Changtai Granule with dose of 10 g/kg. The plasma concentrations of GA were determined at different time points, and the mean concentration–time curve (n=6) is illustrated in
treatment of ulcerative colitis. This apparent improvement in therapeutic effect might be the result of as yet unknown mechanisms arising from the mixture of the four TCMs: Phellodendro Chinense Schneid., Sanguisorba officinalis L., Euphorbia humifusa Willd., and Polygonum hydropiper Linn.

Proper Mechanisms of C8-Modified Graphene@mSiO2 Composites In this paper, we utilized a surfactant-mediated co-condensation sol–gel process to prepare a three layer structured graphene/mesoporous silica composites, which possesses extended plate-like morphology, good water dispensability, highly open pore structure, uniform pore size (2.8 nm), high surface area (632 m²/g), and unique C8-modified interior pore wall.26–28 By applying to real sample preparation, the C8-modified graphene@mSiO2 can be introduced directly into the original sample fluid. Afterwards, drug molecules like GA can enter the mesopore channels and be efficiently absorbed through hydrophobic interaction by interior C8-groups on the internal walls, while large molecules like proteins are excluded from the mesopore channels as a result of size exclusion effect. Thus target drug molecules could be directly extracted from protein-rich biological samples without any other pretreatment procedure.

CONCLUSION

Herein, we developed a graphene/mesoporous silica composite with C8-modified pore-walls. The as-made material was synthesized by coating acidized graphene with functional mesoporous silica, resulting in material endowed with regularly aligned pores, high surface area, hydrophilic exterior surface, and numerous hydrophobic interior pore-walls. This efficient and rapid extraction technique was successfully applied to the determination of GA in rat plasma after oral administration of Changtai Granule, coupled with LC-MS/MS detection. This method was proven to be simple, fast and reliable because of the straightforward sample pretreatment procedure and its relative short analysis time. The results obtained demonstrate that after being combined with different ingredients extracted from Changtai Granule, GA showed different pharmacokinetic behavior, compared with that of pure GA. This study contributes to understanding of Changtai Granule therapy and the described methods should provide a useful tool for pharmacokinetic investigation of Changtai Granule-related preparations.

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Conflict of Interest The authors declare no conflict of interest.

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