Journal of Traditional Chinese Medical Sciences (2015) 2, 264–269

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Fourier transform infrared spectroscopy analysis of the active components in serum of rats treated with Zuogui Pill

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Received 15 December 2014; accepted 20 August 2015
Available online 15 March 2016

KEYWORDS
Zuogui Pill;
Fourier transform infrared spectroscopy;
Serum;
Fitting

Abstract *Objective:* To investigate the active components Zuogui Pill, a typical recipe for nourishing kidney essence in the traditional Chinese medicine.
Methods: Adult male Sprague Dawley rats were treated with the traditional Chinese herbal medicine Zuogui Pill and the active components found in the serum of the animals were analyzed by Fourier transform infrared (FTIR) spectroscopy. FTIR spectra of serum samples of treated and untreated rats were analyzed and the A_{2960}/A_{2931} and A_{1540}/A_{1080} ratios were calculated.
Results: A_{2960}/A_{2931} ratios of the serum samples collected following the administration of Zuogui Pill were significantly higher than those of the normal serum samples. FTIR data were then fitted using a Gaussian equation for wave numbers in the range of 1140–1000 cm^{-1}. A_{RNA}/A_{DNA} ratios in the serum of rats treated with Zuogui Pill were higher than those found in normal rat serum.
Conclusion: FTIR spectroscopy could be used as an analytical tool to detect the active components in serum of animals treated with Zuogui Pill.

Introduction

Zuogui Pill is a traditional Chinese herbal medicine that has been used for centuries to nourish kidney essence.[1] Results of several studies have shown that Zuogui Pill possesses interesting pharmacologic activities. For example, Zuogui Pill has been reported to improve the effectiveness of osteoporosis treatments, enhance secretion of sex hormones, promote recover from ovary injury, inhibit apoptosis in nerve cells by improving hyperplasia, differentiation and recovery in nerve cells, improve immunity and restore equilibrium to the immune system.[2–8]

Research has shown that he active components of Zuogui Pill in rat serum have been found to affect secretion of bone Gla-protein (BGP) in osteoblasts. Further studies have

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Peer review under responsibility of Beijing University of Chinese Medicine.

http://dx.doi.org/10.1016/j.jtcms.2016.01.006

indicated that Zuogui Pill also inhibits hyperplasia and the differentiation of osteoblasts, most likely through estrogenic effects involving modification of the ERK/Smad signal pathways. In addition, Zuogui Pill was found to increase expression of alkaline phosphatase in the bone marrow of mesenchymal stem cells, leading to a significant increase in conversion of bone marrow stromal cells to hepatic cells and improvement in differentiation of marrow cells to hepatic cells.[9–13]

Serum samples collected from rats treated with Zuogui Pill contain a complicated mixture of compounds with activities against a variety of different targets. Fourier transform infrared (FTIR) spectroscopy is a sophisticated analytical technique for detecting the vibrational characteristics of different functional groups in organic molecules. FT-IR has been widely used to study the biomolecular characteristics and biochemical properties of tissues and cells because of its sensitivity to changes in structural and environmental factors. FT-IR spectroscopy can therefore be used to provide important information concerning changes in the serum of an animal following administration of a drug compound, such as variations in the serum protein, lipid, carbohydrate, and nucleic acid structures.[14]

However, it can be difficult to determine the differences in the FTIR spectra of different serum samples because of the complexity of the different components. FTIR techniques have also been used in combination with pattern recognition methods relating to stoichiometry in taxonomy-based studies. Pattern recognition can be studied in two ways, supervised and unsupervised taxonomies. In this study, we used the unsupervised pattern recognition method known as principal component analysis (PCA) to analyze the raw data. This process provided information pertaining to the relationship between the primitive distribution and the different groups. The supervised pattern of orthogonal partial least-squares discriminant analysis (OPLS-DA) model was also applied during this analytical process to remove any irrelevant data in the variable matrix (X) and the dependent variable matrix. The ability of the discriminant analysis was accordingly improved, with the results highlighting the maximum discrepancy between the two groups.

In this work, we used FTIR in combination with stoichiometric analysis to evaluate serum samples of rats treated with Zuogui Pill to develop a deeper understanding of its effects on a specific organism and to establish a scientific method for evaluating the effects of Zuogui Pill in serum.

Methods and materials

Preparation of Zuogui Pill

Zuogui Pill was prepared using the following raw herbs: 24 g cooked rehmannia root (*Rehmannia glutinosa* (Gaertn.) DC.), 12 g Chinese yam rhizome (*Dioscorea oppositifolia* L.), 12 g cornus fruit (*Cornus officinalis* Sieb. et Zucc.), 12 g wolfberry fruit (*Lycium chinense* Mill.), 9 g cyathula root (*Cyathula officinalis* K.C.Kuan), and 12 g Chinese dodder seed (*Cuscuta chinensis* Lam.). All aforementioned herbs were soaked in 810 mL of tap water at 60 °C for 1 h. The herbs were then decocted (boiled) for 1.5 h and filtered. This process of soaking, decoction, and filtration was repeated two more times. The three filtrates were combined to form an extract. Then a liquid mixture made of 12 g of deerhorn glue and 12 g of tortoise plastron glue, which were first liquefied in a hot water bath, was poured into the herbal extract. The resultant mixture was concentrated to obtain 1 g of crude drug, thus forming the Zuigui Pill remedy.

Experimental animals

Twenty specific-pathogen-free adult male Sprague Dawley rats weighing 200 g each were purchased from the Academy of Military Medical Sciences (Beijing). The rats were randomly divided into two groups of 10 rats each. One group of rats was fed a normal diet, and will be referred to hereafter as the normal group. The other group of rats was also fed a normal diet, but with the addition of Zuogui Pill at a dosage of 20 g/kg per day. This group will be referred to hereafter as the drug group. All rats had free access to food and tap water and were housed in conventional cages at a temperature range of 18—22 °C under a 12-h light—dark cycle.

Serum samples

After 7 days, all rats in both groups were sacrificed with a high dose of diethyl ether within 30 min of its last feed. Blood of each animal was collected directly from its heart and incubated at −4 °C for 30 min, followed by centrifugation at 4000 $\times g$ for 10 min. The serum was collected and immediately dried under vacuum at room temperature for X min prior to being analyzed by FTIR spectroscopy.

FTIR spectroscopy of serum

FTIR spectra of the different serum samples were recorded on the Frontier model FTIR spectrometer equipped with a deuterated-triglycine sulfate (DTGS) detector (PerkinElmer, City, Country Waltham, MA, USA). Each spectrum was recorded with a resolution of 4 cm^{-1} with 32 co-added scans over the range of 4000—400 cm^{-1}. Each sample was recorded in triplicate.

Statistical analysis

FTIR data were collected using Spectrum software package (version 5.0; PerkinElmer). Data were then processed with Omnic software (version 6.0; Thermo Electron, Madison, WI, USA), including baseline corrections in the range of 4000—500 cm^{-1}, automatic smoothing, and the min—max normalization of the amide I band.

Bands observed in the range of 1140—1000 cm^{-1} were mainly attributed to nucleic acids. IR curves in this region were subsequently fitted using a Gaussian formula to allow for these peaks to be investigated in greater detail. Secondary derivative IR spectra were calculated and three fitting curves resulted by applying Origin software (version 8.0; OriginLab, Northampton, MA, USA). This same software

was used to carry out statistical analysis to obtain the SD (mean) values.

An independent t-test was used to compare the differences in the serum samples of the normal and drug groups. Differences were considered to be significant for $P < .05$.

IR data of each sample in the range of 500–1500 cm^{-1} were imported into SIMCA-P software (version 11.5; MKS Umetrics, Umea, Sweden) for multivariate analyses. Unsupervised principal component analysis (PCA) was performed to observe any intrinsic and obvious outliers. Supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) was employed to visually discriminate between the serum samples of the normal and drug groups.

Results

Peak height ratios of serum FTIR spectra

No obvious differences were observed between the spectra of serum samples (range of 400–4000 cm^{-1}) of normal and drug group rats (Fig. 1). The main peaks observed in the one-dimensional FTIR spectra of the serum samples collected from both groups of rats were around 3300–3400, 2960, 2931, 1655, 1540, 1450, 1398, 1311, 1240, 1159 and 1080 cm^{-1}.

Bands around 3300–3400 cm^{-1} were attributed to the stretching vibrations of the OH and NH bonds of proteins and amino acids present in the serum samples. Bands at 2960 and 2931 cm^{-1} were attributed to stretching vibrations of CH_3 (asymmetric) and CH_2 groups, respectively. The band at 2873 cm^{-1} was attributed to the stretching vibrations of the CH_2 (symmetric). The band at 1655 cm^{-1} was attributed to the C═O stretching vibration of an amide I bond, while the band at 1540 cm^{-1} was attributed to the $\delta_{NH} + \delta_{CN}$ bending vibrations of an amide II bond. The band at 1450 cm^{-1} was attributed to the scissoring C–H bending vibrations of the CH_2 groups of the lipids found in the serum. Furthermore, the band at 1398 cm^{-1} was attributed to the symmetric and asymmetric stretching vibrations of the CH_3 groups of the lipids found in the serum samples. The band at 1311 cm^{-1} was attributed to the stretching vibration of $\gamma_{NH} + \nu_{CN} + \gamma_{CO} + \nu_{CO}$ for the serum protein amide III bond. The band at 1240 cm^{-1} was attributed to the P═O asymmetric stretching vibrations of the $-PO_2^-$ groups of the nucleic acids in the serum samples, which indicated the presence of DNA and RNA. The band at 1079 cm^{-1} was attributed to the P═O symmetric stretching vibration of the $-PO_2^-$ groups. Finally, the band at 1159 cm^{-1} was attributed to the C–O stretching vibrations of the amino acid residues bearing hydroxyl groups in the serum proteins, including leucine, threonine and tyrosine.[15]

Table 1 Summary of absorption ratio data for characteristic peaks.[a]

Ratios	Drug serum	Normal serum	Independent t-test (*P*-value)
A2960/A2931	1.002 ± 0.004	0.996 ± 0.004	.049[b]
A1540/A1080	0.334 ± 0.025	0.323 ± 0.002	.290

[a] All data expressed as mean (SD).
[b] $P < .05$, comparison with normal group serum.

The absorption ratio, which was defined as the ratio of the absorbance values (%) of two different peaks, was used in the following discussion. This ratio can be used to provide important structural information of biologic significance. To compare the differences in the FTIR spectra of serum samples collected from both groups, we analyzed changes in the composition of the different samples at the molecular level based on the methods by Sheng et al.[16] The absorption ratios of A_{2960}/A_{2931} and A_{1540}/A_{1080} were calculated for the serum samples collected from the drug and normal groups (Table 1).

The absorption ratio of A_{2960}/A_{2931} can be used to represent the ratio of the asymmetric stretching vibrations between the CH_3 and CH_2 groups. The mean A_{2960}/A_{2931} value for the drug group serum samples was 1.002, while the value for the normal group serum samples was 0.996. The significance of this difference between the A_{2960}/A_{2931} values of the two groups was determined to be 0.049 ($P < .05$), indicating that the ratio was significantly higher in the treated group than it was in the normal group.

The absorption ratio of A_{1540}/A_{1080} can be used to provide an indication of the differences in the protein and nucleic acid contents of the serum samples. The A_{1540}/A_{1080}

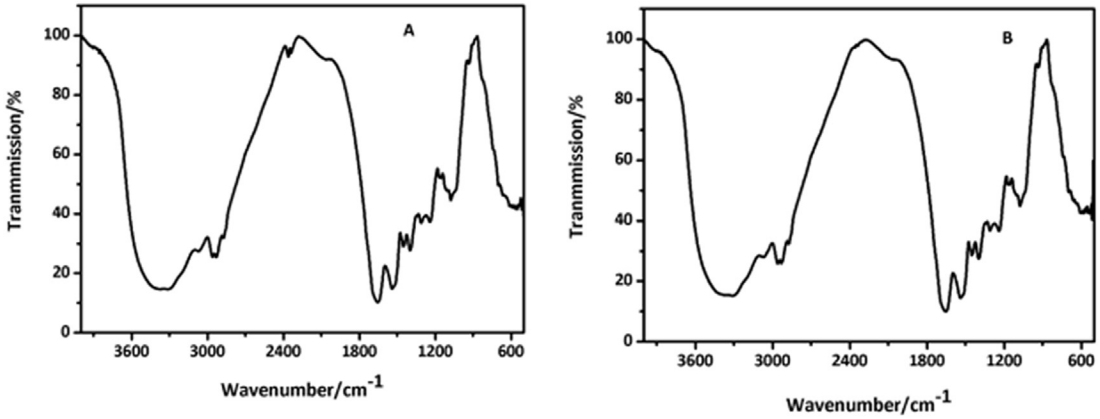

Fig. 1 FTIR spectra of the serum samples from rats in the (A) drug and (B) normal group.

ratio of the drug group serum samples was in the range of 0.316—0.377, whereas the ratio for the normal group was in the range of 0.321—0.325. Statistical analysis revealed that the difference between these two absorption ratios was not significant. However, the protein content of the serum samples from rats treated with Zuogui Pill was higher than that of rats in the normal group.

FTIR spectra of nucleic acid

The vibrations corresponding to the nucleic acids present in the serum samples were observed in the range of 1000—1140 cm^{-1}. These vibrations are especially important, because they can be used to conduct a detailed analysis of the chemical composition of a specific serum sample. FTIR data for serum samples collected from rats in the drug and normal groups were analyzed using several methods, including the second derivative method, baseline correction and Gaussian fitting, as well as statistical methods (Figs. 2 and 3).

The characteristic peaks at 1030, 1076 and 1119 cm^{-1}, which were obtained by the second derivative method and Gaussian fitting, were attributed to the vibrations of the DNA, nucleic acid and RNA molecules present in the serum, respectively. There were significant differences in the IDNA/IRNA ratios between the drug group serum samples and those of the normal group ($P < .05$). The IDNA/IRNA ratio of the serum samples collected from the rats in the drug group was lower than that of the normal group serum, suggesting that there had been a significant decrease in DNA content of the serum in the rats treated with Zuogui Pill or that there had been a significant increase in RNA content.

Nucleic acid is the basic genetic material of humans and important for the biosynthesis of proteins. Nucleic acids can form macromolecular structures composed of C, H, O, N and P, which represent the primary substances of life (i.e. RNA and DNA). One sequence of nucleic acid molecules differs from another primarily in terms of its chemical composition and the sequence of its constituent nucleotides. Based on their chemical composition, nucleic acids can be RNA or DNA. DNA is primarily involved in the storage, copying and transferring genetic information, whilst RNA plays an active role in protein synthesis. The relative content of protein was high in serum samples of the rats treated with Zuogui Pill, indicating that the level of RNA would also be higher in this group.

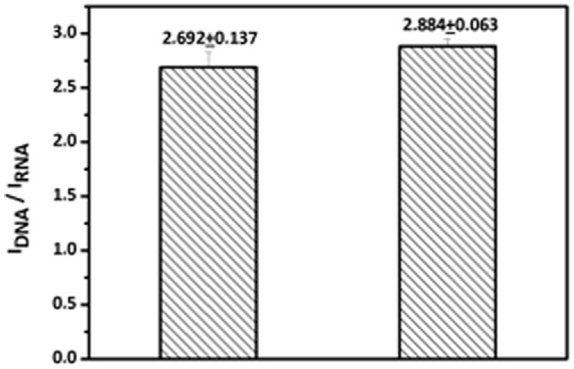

Fig. 3 Statistical analysis of the IDNA/IRNA ratios of serum samples collected from animals in the drug and normal groups.

FTIR fingerprint region of serum

The fingerprint regions of the FTIR spectra of the drug and normal group serum samples were further analyzed. FTIR data collected in the range of 500—1500 cm^{-1} were imported to SiMCA-P software (version 11.5; MKS Umetrics) and subjected to multivariate statistical analysis. This was followed by PCA to study changes in the spectral models of the serum metabolic profile of the two groups and transform the resultant data into a graphical representation (Fig. 4).

Distributions of both serum samples were in the 95% confidence interval based on the results of the PCA. However, the two groups could not be distinctly separated because of the unsupervised nature of the model used for PCA recognition and analysis systems. PCA only provides primary data pertaining to the naturally occurring distribution and relationship of the observed samples.

Therefore, to eliminate random errors and within-group errors that occurred during PCA, we applied a supervised

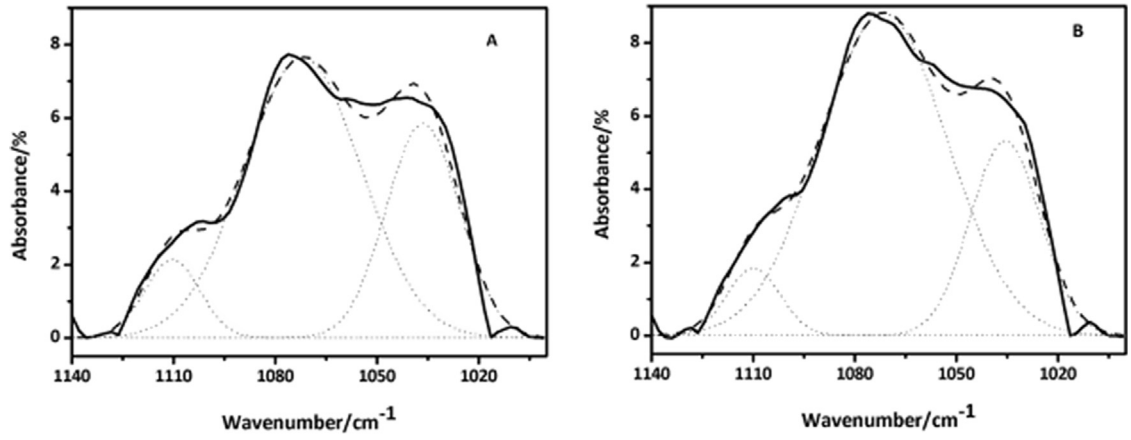

Fig. 2 Gaussian fitting of FTIR data collected in the range of 1000—1140 cm^{-1} for serum samples of the (A) drug and (B) normal groups. Note: Solid line: original spectrum; dashed line: fitted spectrum; dotted line: fitted bands.

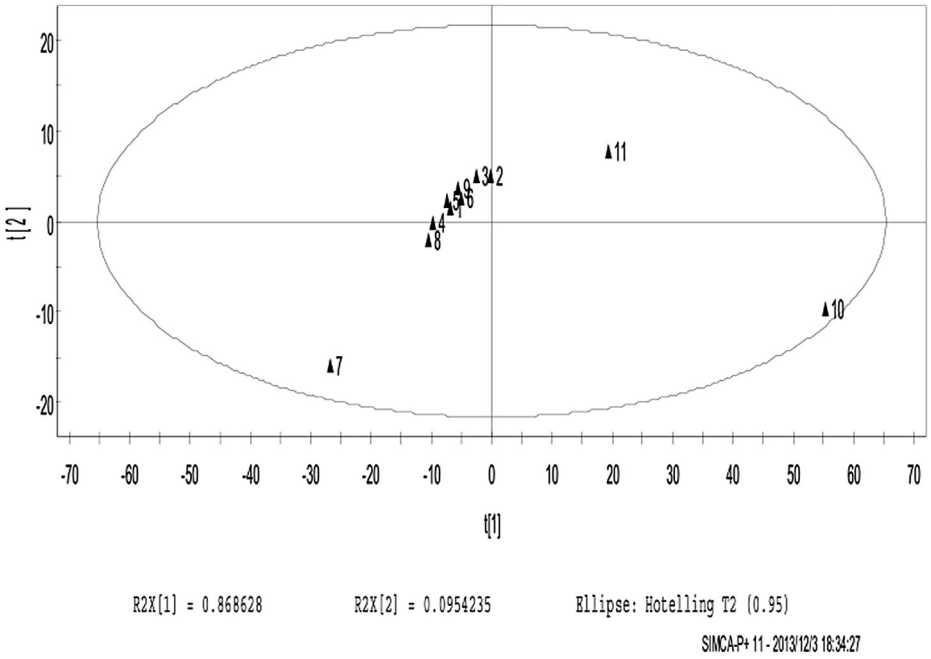

Fig. 4 Plot scores from principal component analysis (PCA) of the serum samples collected from the drug and normal groups.

model of OPLS-DA. The FTIR data in the fingerprint region of the serum samples of the normal and drug groups were separated to the greatest degree (Fig. 5). This result indicated that the compositions of the different serum samples were different. Furthermore, the serum samples of the drug group exhibited a much higher confidence than those of the normal group.

Discussion

Differences in the FTIR spectra of the serum samples collected from rats in the drug (Zuogui Pill) and normal groups can be related to the components of the herbs present in Zuogui Pill. Preliminary results of our analysis showed that the main chemical constituents of the serum samples of rats treated with Zuogui Pill were iridoids from the rehmannia root and cornus fruit.

Results of the fitting analysis for the IR data collected in the range of 1000—1140 cm^{-1} for the nucleic acids indicated that RNA content of the serum samples from the animals treated with Zuogui Pill was higher than that of serum samples from the normal group. This increase in RNA content appears to be attributable to the high protein content resulting from administration of Zuogui Pill.

OPLS-DA results revealed that there were considerable differences in the metabolic profiles of the serum samples of rats in the Zuogui Pill and normal groups. These data could therefore be used to differentiate between the two groups and indicated that there were significant

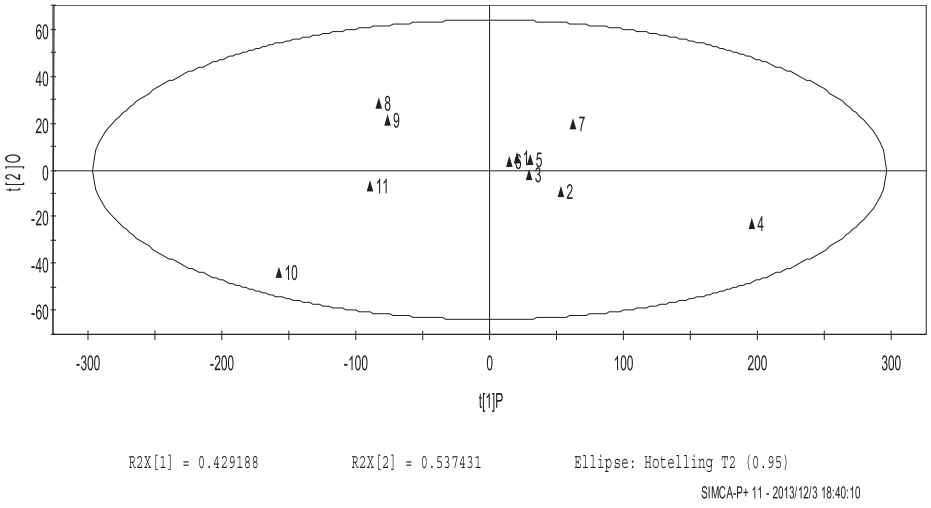

Fig. 5 Plot scores of orthogonal partial least-squares discriminant analysis (OPLS-DA) analysis of serum samples from the drug and normal groups.

differences between the physiologic functions and metabolic profiles of the rats treated with Zuogui Pill compared with the normal rats.

Acknowledgments

This work was financially supported by the National International Cooperation Science and Technology Support Program of the Ministry of Science and Technology of the People's Republic of China (2012DFA31330).

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