Prediction of Cyclooxygenase Inhibitory Activity of Curcuma Rhizome from Chromatograms by Multivariate Analysis

Ken TANAKA,[a] Yoshiaki KUBA,[a] Atsutoshi INA,[a] Hiroshi WATANABE,[b] and Katsuko KOMATSU*,[a,c]

[a] *Division of Pharmacognosy, Department of Medicinal Resources, Institute of Natural Medicine, University of Toyama;* [c] *21st Century COE program, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan: and* [b] *International Research Center for Traditional Medicine, Toyama International Health Complex; 151 Tomosugi, Toyama 939–8224, Japan.* Received February 24, 2008; accepted May 1, 2008; published online May 8, 2008

The potential use of partial least square regression (PLS-R) models for the prediction of biological activities of a herbal drug based on its liquid chromatography (LC) profile was verified using various extracts of *Curcuma phaeocaulis* and their cyclooxygenase-2 (COX-2) inhibitory activities as the model experiment. The correlation of practically measured inhibitory activities and predicted values by PLS-R analysis was quite good (correlation coefficient=0.9935) and the possibility of transforming chromatographic information into a measure of biological activity was confirmed. In addition, furanodienone and curcumenol were identified as the major active anti-inflammatory constituents of *C. phaeocaulis*, through detailed analysis of the regression vector, followed by isolation of these compounds and their COX-2 inhibitory assays. The selectivity indices (SI), IC_{50} of COX-1/IC_{50} of COX-2, of both compounds were higher than that of indomethacin and it is considered that furanodienone and curcumenol are the most promising compounds as lead anti-inflammatory agents.

Key words partial least square regression; *Curcuma phaeocaulis*; cyclooxygenase-2 inhibitory activity; furanodienone; curcumenol

Traditional herbal drugs and their preparations have been practiced to maintain good health and cure diseases in many oriental countries for thousands of years. In the past decade they have attracted the attention of researchers in western countries because of their high pharmacological activities with low adverse effects. However, herbal drugs have not been officially recognized world-wide yet because qualitative and quantitative data on their safety and efficiency are not sufficient to meet the general criteria of medicines, as defined in "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines".[1)]

It is well known that the therapeutic effect of a herbal drug is based on the synergic effect of its mass constituents and this may be the main reason why quality control of herbal drugs is more difficult than that of western medicines.[2,3)] In traditional standardizations, a few marker or pharmacologically active constituents are generally employed to assess the quality and authenticity of a complex herbal drug. However, those compounds are not sufficient to fully represent the complex pharmacological activities of herbal drugs and preparations.[4)]

Recently, chromatographic fingerprint analysis methods have been introduced and accepted by the WHO and other governmental organizations as a strategy for the assessment of the quality of herbal drugs.[5—7)] A chromatographic fingerprint is a chromatogram representing all the detectable chemical components in an extract of herbal drugs. Chromatographic fingerprinting gives some confidence in measuring the chemical identities of herbal drugs. However, this quality control and quality assurance methods can not provide information on the biological activities of herbal drugs, because the marker compounds may not be the ones responsible for the bioactivities of herbal drugs.[4)] Therefore, it is still necessary to develop new methods for the quality control of herbal drugs from both chemical and pharmacological view points.

Multivariate data analysis allows the extraction of additional information from huge data matrices generated by chromatographic or spectrometric analyses. In recent years, multivariate calibrations such as principal component regression (PCR) and partial least squares regression (PLS-R) have been applied to the determination and quantitation of certain compounds in mixtures.[8)]

Useful pharmacological properties of *Curcuma* drugs including Turmeric, Zedoary and Chinese Ezhu, such as anti-inflammatory and immunological effects, have been reported. Tohda *et al.* have reported that oral administration of the methanol extract of *C. phaeocaulis* to adjuvant arthritic model mice significantly inhibited paw swelling and decreased the serum haptgloblin concentration. Furthermore, it showed significant cyclooxygenase (COX, EC; 1. 14. 99. 1) inhibitory activity in comparative studies of the anti-inflammatory activities of 6 *Curcuma* rhizomes.[9)]

In this study, we verify the potential use of PLS-R models for the prediction of COX-2 inhibitory activities of various extracts and their solvent partitioned fractions of Chinese Ezhu derived from the rhizome of *C. phaeocaulis* from the LC chromatographic profiles of them, aiming at the establishment of a new procedure for standardization of herbal drugs from both chemical and biological view points. In addition, a new approach for investigation of bioactive constituents in the herbal drugs using PLS-R models was examined.

Experimental

Materials and Analytical Sample Preparation Four crude drug samples were purchased from pharmaceutical companies or markets (Table 1). All samples were deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW).

A sample was pulverized and the powder screened through 850 μm sieves. Fifty grams of the fine powder was accurately weighed and extracted three times with 50 ml of methanol under reflux conditions for 30 min. The organic solvents were combined and evaporated *in vacuo* to give an extract. Five hundred milligrams of the extract was dissolved in 20 ml of methanol and extracted with hexane (25 ml) to separate the methanol extract into hexane and methanol soluble fractions. The amounts of the methanol extracts, hexane fractions and methanol fractions obtained from samples

* To whom correspondence should be addressed. e-mail: katsukok@inm.u-toyama.ac.jp

Table 1. Materials Used in Present Study

Botanical source	Place of production	Collected date	TMPW No.[a)]	COX-2 inhibitory activity (%)		
				Methanol extract	Hexane fraction	Methanol fraction
Curcuma phaeocaulis VALETON	Sichuan, Chong Zhou, China	2000. 8	20237	60.4	73.0	13.3
	Sichuan, Chong Zhou, China	2000. 8	20238	39.6[b)]	70.4	16.0
	Sichuan, China	2003. 3	22297	60.6	80.4	26.0
	Sichuan, China	2007. 5	25036	66.9	74.2	25.0

a) The specimen reference number of the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW). *b*) This value was removed as an outlier in PLS regression analysis.

Table 2. Weight of the Extracts and Fractions Obtained from *Curcuma phaeocaulis* Samples and Contents of Furanodienone and Curcumenol

	Weight of extracts and fractions[a)]	Contents (%)	
		Furanodienone	Curcumenol
Methanol extract			
20237	3.4 g	13.2	11.1
20238	3.1 g	12.5	11.0
22297	3.1 g	14.7	12.0
25036	3.8 g	13.2	13.3
Hexane fraction			
20237	178.5 mg	19.2	16.9
20238	151.1 mg	24.5	20.1
22297	210.8 mg	20.9	15.8
25036	146.6 mg	17.8	20.6
Methanol fraction			
20237	303.4 mg	2.8	5.7
20238	351.6 mg	0.6	1.6
22297	281.9 mg	2.6	5.8
25036	328.4 mg	1.8	5.4

a) Weight of methanol fraction obtained from 50 g of *C. phaeocaulis* sample and weight of hexane and methanol fraction partitioned 500 mg of methanol extract.

were shown in Table 2.

Standard Samples and Reagents Furanodienone (13 mg), curzerenone (12 mg), curcumenol (43 mg), 4,5-epoxygermacrone (1 mg), curcumenone (3 mg) and isofranodienone (3 mg) were isolated from 200 mg of the extract of *C. phaeocaulis* (TMPW No. 25036) by preparative HPLC. All isolated compounds were identified by comparison of $[\alpha]_D$, NMR and mass spectral data with those reported.[10—15)]

All chemicals were of analytical grade, and chromatographic solvents were of HPLC grade.

Analytical Instruments LC analyses were performed using a Shimadzu LC-10AS LC system equipped with a Shimadzu SPD-M10A photodiode array detector. A Waters Symmetry C_{18} column (4.6 mm i.d.×150 mm, 5 μm) was used. The column temperature was set at 40 °C and eluted compounds were detected by monitoring the UV absorbance at 254 nm. The mobile phase was a binary eluent of (A) 5 mM ammonium formate–formic acid (0.1%) solution, (B) CH_3CN under following gradient conditions: 0—30 min linear gradient from 10 to 100% B, 30—40 min isocratic at 100% B. Flow rate was 1.0 ml/min.

Multivariate Data Analysis Before statistical analysis of the data by means of multivariate methods, LC chromatographic data were converted to ASCII format by the Shimadzu LC solution software. Peak alignment was carried out using chromatographic data processing software (TS Lab., Tokyo, Japan).

The PLS model can be presented as follows:

$$\mathbf{X}=\mathbf{T}\cdot\mathbf{P}^{\mathrm{T}}+\mathbf{E}$$

$$\mathbf{y}=\mathbf{T}\cdot\mathbf{q}+\mathbf{f}$$

where $\mathbf{X}(n,p)$ represents the chromatographic data matrix, vector $\mathbf{y}(n,1)$ is inhibition values of the extracts, $\mathbf{T}(n,n)$ is the score matrix, $\mathbf{P}^{\mathrm{T}}(n,p)$ denotes the transposed loading matrix, $\mathbf{q}(n,1)$ is a loading vector, $\mathbf{E}(n,p)$ and $\mathbf{f}(n,1)$ are the residuals.

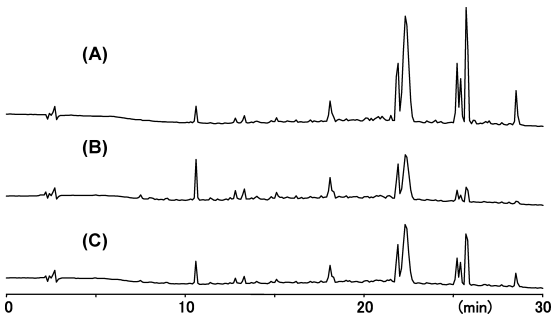

Fig. 1. Chromatograms of the Extract of *Curcuma phaeocaulis* (TMPW No. 20237) and Solvent Partitioned Fractions

(A) Hexane fraction of the methanol extract, (B) methanol fraction, (C) methanol extract.

In order to predict y_i for a new chromatogram $\mathbf{x}_i(1,p)$, the following equation is used:

$$\hat{y}_i=\bar{y}+\mathbf{x}_i\cdot\mathbf{b}$$

where $\hat{y}_i$ is the predicted inhibition value for the ith new sample, $\bar{y}$ denotes the mean of the inhibition values for the calibration samples, and $\mathbf{b}(p,1)$ is the vector of PLS regression coefficients computed as:

$$\mathbf{b}=\mathbf{P}\cdot\mathbf{q}$$

All statistical analyses were carried out by Piroruet software (GL Science Inc., Tokyo).

COX Inhibition Assay COX inhibitory activity was measured using a Colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, MI, U.S.A.). The assay kit was used to measure the peroxidase activity of COX to monitor the oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). All samples and positive control (indomethacin) were added as methanol solutions to assay solutions. All procedures were performed as indicated in the assay kit instructions. Oxidized TMPD was measured using an Immuno Reader at 590 nm (NJ-2100, Inter Med, Tokyo). Absorbance of assay buffer (160 μl of 100 mM Tris–HCl (pH 8.0), 10 μl of Heme and 10 μl of methanol) and assay buffer with COX enzyme were measured as background and 100% initial activity.

Results and Discussion

Three chromatograms of the methanol extract, its fraction partitioned with hexane and the remaining methanol fraction of *C. phaeocaulis* (TMPW No. 20237) are shown in Fig. 1. Their corresponding COX-2 inhibitory activities are listed in Table 1. In general, qualitative evaluation of herbal drugs is performed by the quantitative analysis of a few active constituents. As shown in Table 1, the COX-2 inhibitory activity of the hexane fraction of *C. phaeocaulis* (TMPW No. 20237) (COX-2 inhibitory activity: 73.0%) showed approximately 5.5 times higher inhibitory activities than the remaining

methanol fraction (COX-2 inhibitory activity: 13.3%). However, comparisons of the chromatographic fingerprints shown in Fig. 1 do not provide the quantitative information for evaluating the quality of herbal drugs. To compensate for this weakness of chromatographic fingerprints, additional quantitative information about the bioactivities of the drugs are required. It is considered that the components in the herbal drugs act in a synergistic manner with respect to their biological activities. This makes it difficult to create a model for predicting biological activity using concentration information of the individual components in herbal drugs by ordinary multivariate calibration.

Partial least squares (PLS) regression technique is a method for constructing predictive models and is especially useful in quite common cases where the number of descriptors (independent variables) is comparable to or greater than the number of compounds (data points) and/or there exist other factors leading to correlations between variables. To create the PLS model, methanol extracts of four samples of *C. phaeocaulis* were fractionated into hexane soluble fractions and remaining methanol fractions.

Removing of Leverage Objects and Outliers If a sample's profile differs greatly from the average training set profile, it will have a great influence on the model, drawing the model closer to its location in factor space. A sample's influence is quantified by its "leverage". In addition, if a sample's y value is extreme, it has a greater influence on the model than a sample close to an average y value (y). The extreme sample "pulls" the model toward it. Therefore, leverage objects and outliers have to be removed prior to constructing the calibration model. After peak alignment of the HPLC chromatographic profiles, examination of the presence of high leverage samples and the outlying observations in the data of chromatograms, X, and COX-2 inhibition values, y, were initially carried out. The outlier of the y data (outside of 95% line of Studentized Residuals) and X variables having high-leverage values were removed from the original data set to recreate the new analytical data set. In this study, the COX-2 inhibitory activity value of the methanol extract of the sample TMPW No. 20238 (Table 1, COX-2 inhibitory activity=39.6%) was removed as an outlier.

PLS Model In general, the optimal number of latent factors of the PLS model can be determined by the lowest root mean squared error of cross-validation (RMSECV) in the leave-one-out cross validation procedure. However, in many practical analyses, an apparent minimal RMSECV value can not be achieved. In the present study, we have used the change of the regression vector's shape depending on the number of latent factors. When the number of factors is small and each additional factor accounts for significant variation, the vector's shape changes dramatically with the number of factors. The point at which the changes are much less striking and appear random signaling is determined as the optimal number of latent factors of the PLS model. From observation of the regression vector's shape, four PLS factors were used in this study. The results of the correlation of measured COX-2 inhibitory activities and predicted inhibitory values, and the regression vector of the model are shown in Figs. 2 and 3, respectively. As shown in Fig. 2, the correlation of practically measured inhibitory activities and predicted values is quite good (correlation coefficient=0.9935) and the possibility of transforming chromatographic information into a biological activity is confirmed.

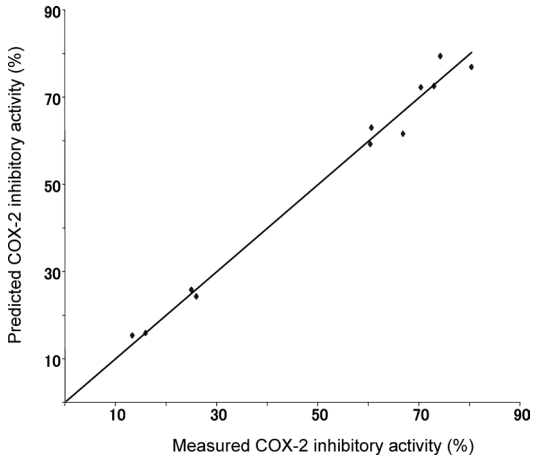

Fig. 2. The Results of the Correlation of Measured COX-2 Inhibitory Activities and Predicted Inhibitory Values

Prediction of the activity from a chromatogram is carried out by the calculation of the product of two vectors, the regression vector and chromatogram (Fig. 3). The chromatogram involves the quantitative information of the chemical components and the regression vector involves the information on the degree of the contribution strength of the compounds to the activity. Thus, it can be considered that the positive peaks indicate the positive effects of the compounds in biological activities. Generally, the earlier PLS factors in the model are most likely to be the ones related to the constituents of interest, while later PLS factors have less information that is useful for predicting concentration. However, if too few PLS factors are used to construct the model, the prediction accuracy for unknown samples will suffer since not enough terms are being used to model all the chromatographic variations that compose the constituents of interest. Therefore, it is very difficult to eliminate the effects from the noise or outlying peaks in the chromatogram completely, depending on the number of PLS factors used. Consequently, detailed evaluation of the regression vector at the every PLS factor numbers and the relationship between regression vector and corresponding peak intensities in the chromatogram is required. As shown in Figs. 3, 6 major peaks (peak 1, 21.5 min; peak 2, 21.9 min; peak 3, 22.3 min; peak 4, 25.2 min; peak 5, 25.4 min; peak 6, 25.7 min) were observed. In the regression vector shown in Fig. 3, there are 4 positive peaks at 20.1 min of the retention time and the points of peaks 1, 3 and 6. The peak at 20.1 min and peak 1 appear at the point of third PLS factor, and they correspond to the very weak peaks in the chromatograms. In addition, methanol extract and hexane fraction of sample 25036, having strong inhibition activity, provides the peaks at 20.1 min and peak 1 in the chromatogram, exceptionally. Thus, it is considered that the 2 peaks arise from outlaying observations in the chromatogram of sample 25036 and do not indicate the positive effects of the compounds in biological activities. On the other hand, the strong positive peaks at 22.1 and 25.7 min of retention time (corresponding to peaks 3 and 6) in the regression vector were observed from the point of first PLS factor, and it is predicted that peaks 3 and 6 contribute strongly to the COX-

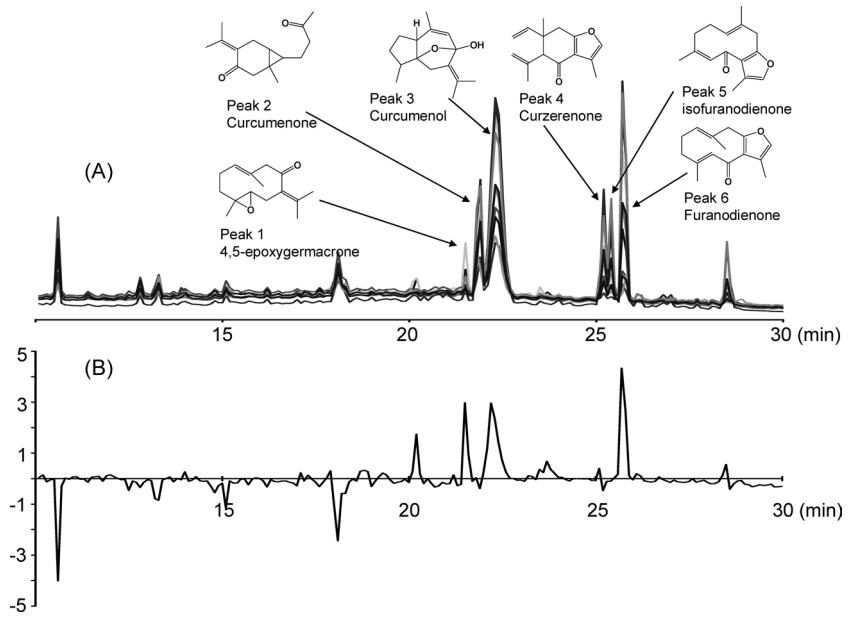

Fig. 3. Superimposed Chromatograms of the Extracts and Solvent Partitioned Fractions (A) and the Regression Vector of the PLS Model (B)

2 inhibitory activity of *C. phaeocaulis*.

Identification of Active Compounds and Their COX-2 and COX-1 Inhibitory Activities The compounds corresponding peaks 1 to 6 were isolated by preparative HPLC, and identified as 4,5-epoxygermacrone (peak 1), curcumenone (peak 2), curcumenol (peak 3), curzerenone (peak 4), isofuranodienone (peak 5) and furanodienone (peak 6) by comparison of our spectroscopic data and $[\alpha]_D$ with those reported previously.[10—15] The compound giving the peak at 20.1 min in the chromatogram could not be isolated due to its small content.

The COX-2 and COX-1 inhibitory activities of these compounds were measured. As shown in Table 3, furanodienone and curcumenol showed COX-2 and COX-1 inhibitory activity, and their IC_{50} values were 4.6 and 34.5 μM for COX-2, 52.6 and 159.9 μM for COX-1, respectively. 4,5-Epoxy germacrone, curcumenone and curzerenone did not show significant effects toward both COXs. These results were coincident with the prediction from the detailed analysis of regression vector. Although these inhibitory activities of furanodienone and curcumenol were weaker than those of indomethacin as a positive control, the selectivity indices (SI), IC_{50} of COX-1/IC_{50} of COX-2, of those compounds were higher than that of indomethacin (11.7 in furanodienone, 4.6 in curcumenol and 0.05 in indomethacin).

COX is known to be a biosynthetic enzyme responsible for the conversion of liberated phospholipid of cytomembrane to prostaglandins (PG). COX has two active sites, one that oxidizes arachidonic acid to PGG_2 and another where there is subsequent peroxidation of PGG_2 to PGH_2. It is well known that COX-2 is an inducible enzyme of cancer and inflammation, whereas COX-1 is a housekeeping enzyme expressed in the intracellular endoplasmic reticulum in the cells of digestive organs.[16,17] Therefore, a selective inhibitor of COX-2 is required as an anti-inflammatory agent. However, it has been reported recently that a highly selective COX-2 inhibitor, such as rofecoxib, has an associated serious cardiovascular risk.[18] Though the inhibitory activities and selectivity of furanodienone and curcumenol are not superior to "coxib" drugs, it is considered that the compounds have potential as anti-inflammatory agents obtained from nature.

Table 3. COX Inhibitory Activities of Isolated Compounds

Compound	IC_{50} (μM)		Selectivity index[a]
	COX-2	COX-1	
Franodienone	4.6	52.6	11.7
Curcumenol	34.5	159.9	4.6
Curzerenone	No effect	No effect	—
Isofranodienone	No effect	194.6	—
4,5-Epoxygermacrone	No effect	No effect	—
Curcumenone	No effect	No effect	—
Indomethacin (positive control)	1.1	0.06	0.05

a) Selectivity index was calculated by IC_{50} (COX-1)/IC_{50} (COX-2).

As shown in Table 2, the variation of the contents of furanodienone and curcumenol in the methanol extract and solvent partitioned fractions of *C. phaeocaulis* samples could not clarify the difference of activities in the respective extracts or fractions. The regression vector (Fig. 3) indicated the presence of several peaks having positive and negative contributions to the activity. Though the practical synergic effects of the compounds isolated in this study were not clarified, the variation of the activities of extract and fractions could be explained by present PLS model efficiently.

In conclusion, we verified the potential use of PLS-R models for the prediction of biological activities from LC chromatographic profiles using various extracts of *C. phaeocaulis* and their COX-2 inhibitory activities as the model experiment. Furanodienone and curcumenol were identified as the major constituents for the anti-inflammatory activity of *C. phaeocaulis*. It was confirmed that PLS-R models could be utilized for prediction of biological activities of herbal drugs from the information obtained from chemical fingerprint analysis. In addition, it was indicated that detailed analysis of

the regression vector could be utilized for effective investigation of active constituents in herbal drugs.

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