Thin-Layer Chromatography and Multivariate Data Analysis of Willow Bark Extracts

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Abstract

In most cases the pharmacological activity of plant extracts is not assigned to single components and often not all active ingredients are known. Approaches other than those considering single compounds only to analyze plant material have proven helpful for a better characterization of extracts in their entirety. In this study extracts of willow bark are analyzed by high-performance thin-layer chromatography (HPTLC) and two different pharmacological tests [the 2,2'-azobis (2-amidinopropane) dihydrochloride reaction and the xanthine/xanthine oxidase reaction] with the help of multivariate data analysis. Described are two models using the results of the chromatographic study of 22 various extracts of willow bark and their pharmacological properties. The chromatographic data are obtained by a special TLC scanner that enables measurement of HPTLC tracks simultaneously in the range of λ = 200–400 nm. Additionally, the developed models are used to predict the activity of another three extracts of willow bark demonstrating the quality of the model.

Introduction

Willow bark is known as a remedy for arthritis and inflammatory diseases that shows few side effects in contrast to chemically defined anti-inflammatory agents. According to the European Pharmacopoeia (4th edition, 2002) the drug contains no less than 1.5% of total salicylic derivatives, expressed as salicin (1). Until now salicylates have been regarded as the most important component of willow bark extracts. The most well-known principal active ingredient of willow bark is salicin, which is metabolized to salicylic acid and is held partially responsible for the analgesic and antirheumatic effects. However, salicin is not sufficient to predict the therapeutic effectiveness of willow bark extracts (2,3). Willow bark extract, in the current therapy dose, leads to much lower serum salicylates levels than observed after analgesic doses of synthetic salicylates. The formation of salicylic acid alone is therefore unlikely to explain analgesic or antirheumatic effects of willow bark (4). Other chemical components seem to significantly influence the pharmacological properties of the extracts. To what degree these components are jointly responsible for the effectiveness is so far unknown.

In most cases the pharmacological activity is not assigned to single components. According to the prevailing opinion, the effectiveness of plant preparations depends upon the amounts of a complex mixture of components (5). The outcome of this is that the conventional standardization of plant extracts by use of marker substances (e.g., salicin) does not necessarily correlate with the effectiveness of the extract. Plant extracts are mixtures in a complex matrix that can be analyzed by multivariate data analysis. This is often performed in the food-technology and pharmaceutical industries (6–8). Extracts of St. John's wort have also been studied in this group by applying multivariate data analysis to high-performance thin-layer chromatography (HPTLC) evaluation (9). Because chromatographic methods (e.g., high-performance liquid chromatography or HPTLC) are commonly used and very well suited for separating and characterizing plant extracts, it proved to be advantageous to analyze plant extracts by HPTLC and to use multivariate data analysis considering additionally pharmacological properties.

In this study the chromatographic data of 22 various extracts of willow bark obtained by a thin-layer chromatography (TLC) scanner that enables measurement of HPTLC tracks simultaneously in the range of λ = 200–400 nm were chemometrically evaluated and calibrated using the partial least squares algorithm (PLS-1) and full cross validation ("leave-one-out" method). The spectroscopic pattern of 19 extracts were correlated with results of 2 chemiluminescence tests indicating their antioxidative properties: the 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) reaction that produces various species of radicals and the xanthine/xanthine oxidase (XOD) reaction that specifically generates superoxide anions. Quantitation of the activity was achieved by measuring the quenching time of the luminol enhanced luminescence triggered by AAPH and the inhibition of the XOD-dependent superoxide anion production (10,11).

Both tests were chosen because of the important factor of the generation of free radicals in the development and maintenance of inflammatory diseases, one of the main application fields of willow bark.

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Experimental

Plant material

Dried plant material was obtained from Steigerwald (Darmstadt, Germany). It was extracted with water or ethanol–water mixtures in various drug–extract ratios. The salicin content of the resulting 22 willow bark extracts varied in the range of 6.7% to 30.7% (w/w). The extracts (50 mg) were dissolved in methanol (5 mL). After 5 min of sonification the extracts were centrifuged at 3,000 rpm for 5 min at room temperature. The resulting extracts were characterized by HPTLC.

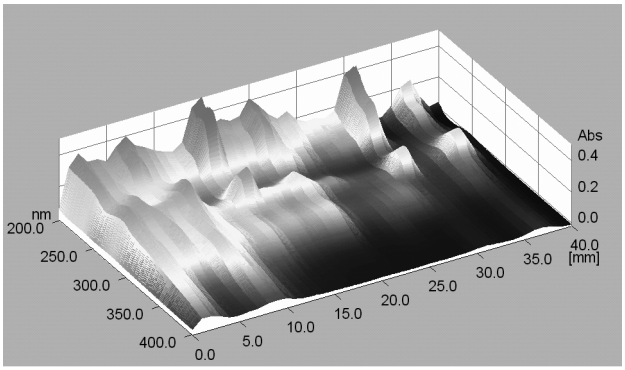

Figure 1. Typical three-dimensional chromatogram obtained from willow bark extract.

TLC method

Sample application was carried out by means of an automatic sample applicator Camag Linomat IV (Berlin, Germany). A 10-µL aliquot of each extract (50 mg/5 mL) was applied 3 times on 10- × 10-cm silica gel K60 F254 HPTLC plates, previously prewashed with acetone, as 3-mm bands. The HPTLC plates were developed in an automatic development chamber (Camag) with ethyl acetate–ethyl methyl ketone–formic acid–water (60:20:2:2) (12). The separation distance was 40 mm. The tracks were measured simultaneously in a range of λ = 200 to 400 nm.

Pharmacological tests

AAPH reaction

By decomposition of AAPH, different species of radicals are generated that can be observed by luminol enhanced chemiluminescence. The radical scavenging properties of the extracts are shown by the time of the suppression of the light signal.

The AAPH reaction was measured in a six-channel luminometer (Berthold MULTI-BIOLUMAT LB 9505 C) (Berthold, Technology, Bad Wildbad, Germany) under the following conditions: phosphate buffered saline (490 µL, pH 7.4), luminol (113µM), and AAPH (10mM). After reaching a constant rate of chemiluminescence, extracts in final concentrations from 0.63 to 20 µg/mL were added. The measuring time was 60 min. The lag-times to reach the initial rate of chemiluminescence were evaluated and then correlated with the concentrations of the extracts. The slope of the corresponding regression line was used for characterization of the radical scavenging properties of the extracts.

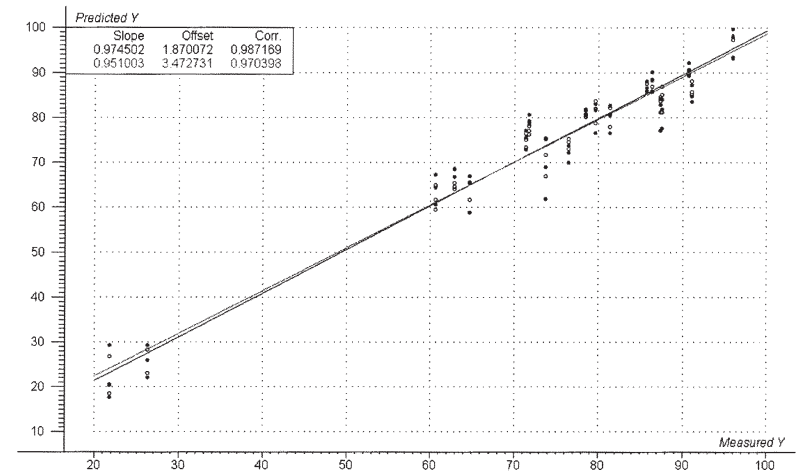

Figure 2. Regression plot of the PLS-1 calculation and validation (prediction) with XOD test as pharmacological test.

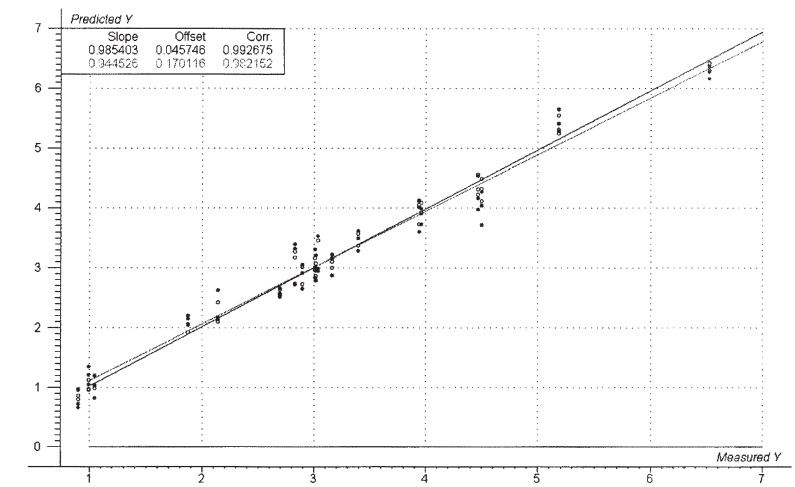

Figure 3. Regression plot of the PLS-1 calculation and validation (prediction) with AAPH test as pharmacological test.

XOD test

The xanthine oxidase catalyzed the oxidation of hypoxanthine to xanthine and of xanthine to uric acid, producing specifically superoxide anions. The generation of the radicals can be determined by luminol-enhanced chemiluminescence. The antioxidative properties of the extracts are shown by the decrease of the luminescence.

The XOD reaction was measured in a 1-channel luminometer (Berthold Biolumat LB 9500) by first pipetting phosphate buffer (570 µL, pH 7.8), luminol (94µM), xanthine (80µM), and 10 µL of the sample (solvent or willow bark extract in a final concentration of 2.08 µg/mL) and then starting the reaction by adding 20 µL of xanthine oxidase solution (final concentration, 14.5

mU/mL). The measuring time was 60 s, providing counts per minute. The pure solvent was used for calibration (= 100%). The inhibition of chemiluminescence was used for characterization of O_2^- scavenging properties of the extract.

Data treatment and analysis

Each track was measured simultaneously in a range of λ = 200 – 400 nm by a J&M (Aalen, Germany) TLC scanner. These data were imported into the multivariate analysis software, the Unscrambler version 7.6 (Camo AS, Trontheim, Norway), and analyzed by partial least squares regressions using the PLS-1 algorithm. Data pretreatment was performed by a multiplicative scatter correction (MSC), a method developed to correct the enormous light scattering variations in reflectance spectroscopy. Varying light scattering levels yield enormous interference effects with an additive and multiplicative component effect (13).

Results and Discussion

Raw evaluation of the chromatogram

A typical chromatogram of a willow bark extract is depicted in Figure 1. No obvious relation between the intensities of the spots and the effectiveness of the extracts concerning the radical scavenging properties can be detected by raw evaluation of the chromatogram. Due to the fact that the salicylates of the willow bark extracts are not sufficient to predict the therapeutic effectiveness, time- and cost-consuming pharmacological tests are necessary to estimate the effectiveness of the extracts.

PLS-1

The multivariate data analysis is highly convenient for the analysis of complex multivariate data sets such as plant extracts. The PLS algorithm is a decomposition technique and similar to PCA and PCR. With this technique, the dimensionality of the data was reduced by combining correlated variables (spots on the TLC track) to form a new, smaller set of independent variables called principal components (PCs). By these PCs the variance within the data set can be explained in relation to a target variable. In this study the target variable was obtained by two different pharmacological tests.

Table I. Statistical Data from the PLS-1 Regression Plots of Both Developed Models

PLS-1 with both pharmacological tests	XOD		AAPH	
	Calibration	Validation	Calibration	Validation
Slope	0.975	0.951	0.985	0.945
Offset	1.870	3.473	0.046	0.170
Correlation coefficient (r^2)	0.987	0.970	0.993	0.982
Root mean square error	3.109	4.708	0.170	0.266
S.E.	3.137	4.748	0.172	0.269
Bias	1.0 x 10-6	–0.121	1.3 x 10-7	–0.004
PCs	6	6	6	6

A crucial aspect for a significant and robust developed model is the determination of the number of PLS factors. The optimum number in this model was six, which was determined by full cross validation. In this method one sample is left out for the model calculation of each principal component and the target property of this sample is then predicted. The procedure is repeated until each sample has been left out once. Consequently, two fully characterized regression plots are obtained: one for the calibration step and one for the validation. For both developed models the resulting regression plots are depicted in Figures 2 and 3. The accuracy of the calibration model is described by the squared correlation coefficient. Both models showed a good correlation for the chromatographic data and both pharmacological data sets with a correlation coefficient of 0.970 (XOD, validation) and 0.982 (AAPH, validation). Another important criterion to confirm the quality of a model is the root mean square error of calibration and the root mean square error of prediction (RMSEP). They represent the average difference between predicted and measured response values at calibration and validation step. With RMSEP of 4.708 (XOD, validation) and 0.266 (AAPH, validation), both models promised good predictive abilities. Table I summarizes the statistical parameters of the calibration equations.

Prediction of an external test set

To further evaluate the quality of the calculated models, the predictive ability should be demonstrated by an external test set. Therefore results of both pharmacological tests of another three extracts of willow bark were studied. On the basis of the chromatographic data, the results of both pharmacological tests of

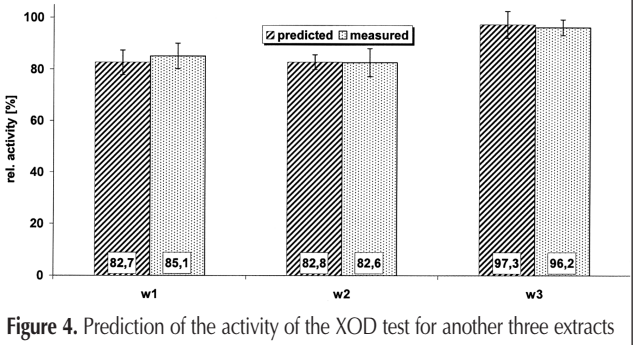

Figure 4. Prediction of the activity of the XOD test for another three extracts of willow bark calculated with the help of the developed PLS-1 model.

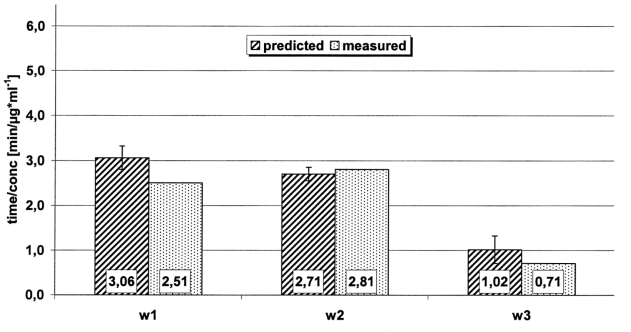

Figure 5. Prediction of the outcome of the AAPH-test for another three extracts of willow bark calculated with the help of the developed PLS-1 model.

these extracts were predicted by the PLS-1 model. The results of the prediction were confirmed by the pharmacological tests and are shown in Figures 4 and 5.

Conclusion

The extracts are successfully characterized in an unusual way, taking into account not only one characteristic constituent but also the complete chromatographic data as well as results of two pharmacological tests.

In the future we intend to develop a model with a kind of test battery by simultaneously correlating the chromatographic data of the extracts with results of more than two pharmacological tests. The possibilities and limitations of this method as an analytical tool for phytochemical analysis need to be evaluated.

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