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Densitometric Determination of Salicin in Willow Stem Bark

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Summary

Most of the salicin present in willow stem bark occurs as labile glycosides (e.g. salicin, salicortin, 2-*O*′-acetylsalicortin, 3-*O*′-acetylsalicortin, tremulacin, and fragilin). On alkaline hydrolysis, these acylated derivatives decompose to salicin; in this way, the total amount of salicin can be determined. A HPTLC method has been developed for the quantitative determination of salicin in stem bark before and after alkaline hydrolysis. The mobile phase used for the separation is a modification of one described in the literature [1]. A double or a triple migration is necessary to separate salicin from other components of the extract.

The results have been compared with those obtained after bidimensional chromatography using two phases described for the quantitative evaluation of salicin [2,3]. To check that separation was complete, the spectrum of salicin obtained from willow extracts was superimposed on that of a salicin standard. Quantification was performed at λ = 270 nm, the wavelength of maximum absorption in the UV spectrum of salicin.

1 Introduction

The phenolic glycoside salicin is of widespread occurrence in the genus Salix and has been used in medicine in the treatment of acute rheumatism and influenza [4,5]; a German monograph [6] on willow bark recommends a daily dosage of 60-120 mg salicin. The quality of analgesic herbal remedies containing vegetable drug preparations of dried willow can be determined by the total content of salicin after hydrolysis: most of the salicin is present as labile glycosides such as salicortin, 3-*O*′- and 2-*O*′-acetylsalicortin, and tremulacin (**Figure 1**) [7]. These acylated derivatives, precursors of much of the salicin found in willow stem bark, decompose to salicin [7].

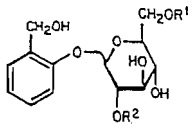
Because the mobile phase described in a densitometric method for the quantitative evaluation of salicin [2] gives incomplete separation from another component of some willow extracts, we have tried to find an appropriate phase for the separation, and to determine the identity of the component which coeluted with salicin.

2 Experimental

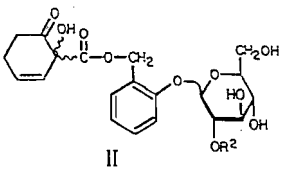
2.1 Materials

2.1.1 Chemicals and Solvents

Salicin of high purity was purchased from Aldrich (Milwaukee, WI, USA). The IR and UV spectra and the melting point were similar to those described in the literature [7,8]. All solvents were of analytical grade and were used without further purification: dichloromethane, ethyl acetate, methyl ethyl ketone, and formic acid were obtained from Merck (Darmstadt, FRG); methanol was purchased from Labscan (Dublin, Ireland). Sodium hydroxide (Pleuger, Wijnegem, Bel-



I



II

Figure 1

Phenolic glycosides

		R^1	R^2
I	**Salicin**	H	H
	Fragilin	CH_3CO	H
II	**Salicortin**	–	H
	2′-*O*-acetylsalicortin	–	CH_3CO
	Tremulacin	–	C_6H_5CO

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gium) was used for the alkaline hydrolysis, and hydrochloric acid (Merck) was necessary for neutralization after hydrolysis.

2.1.2 Plant Materials

Commercial batches of willow stem bark (sold under the general name of *Salix alba* without accurate botanical identification) were obtained from Denolin (Braine-l'Alleud, Belgium), Pharmaflore (Lessines, Belgium), and Tilman (Bomal, Belgium). Other batches were collected by members of the botanical department of our university. They were authenticated in the author's department according to the description in Teedrogen [9] and the German Pharmacopoeia X [10]. Batches not in agreement with these specifications were excluded from the standardization.

2.2 Preparation of Solutions

A standard solution was prepared by dissolving salicin (10 mg) in methanol – water (1:1; 5 ml).

A sample solution was prepared by extracting willow stem bark (500 mg) with methanol – water (1:1; 3 × 25 ml); each extraction was performed for 20 min. The solution was filtered, evaporated, and dissolved in methanol – water (1:1; 5 ml). The solution was purified on Adsorbex C_{18} extraction columns (400 mg; Merck) [11] which had been prewashed with methanol (2 ml). The solution was evaporated and the residue dissolved in methanol – water (1:1; 4.0 ml). Half of this solution (2.0 ml) was used for hydrolysis by adding sodium hydroxide (0.1 N; 2.0 ml), heating at 60 °C for 60 min, and adding hydrochloric acid (1 N; 0.20 ml). The solution was transferred to a 10 ml volumetric flask and filled to the mark with methanol.

2.3 Chromatography

TLC was performed on 20 × 10 cm silica gel 60 HPTLC plates (Merck).

Two chromatographic methods were used for quantitative determination of salicin in willow bark. The first entailed bidimensional chromatography with different mobile phases. 2, 3, and 4 μl aliquots of standard solution were applied repeatedly, 10 mm apart on the left of the plate, 15 mm from its lower edge. Standard solution (3 μl) was spotted at the same position as (*i.e.* superimposed on) an aliquot of the sample solution before hydrolysis at a point 15 mm from the lower and right edges of the plate (**Figure 2**). The solution after hydrolysis (6 μl) was applied without superimposition of salicin.

After drying the applied spots, separation was performed in a normal unsaturated chamber (20 × 10 cm twin-trough chamber; Camag, Muttenz, Switzerland) at room temperature (23 °C) using dichloromethane – methanol – water (37 + 12 + 1, *v/v*) as mobile phase for development in the first dimension and ethyl acetate – methanol – water (100 + 13.5 + 10, *v/v*) for the second. The development distance was 7 cm in both dimensions (development time 45 min).

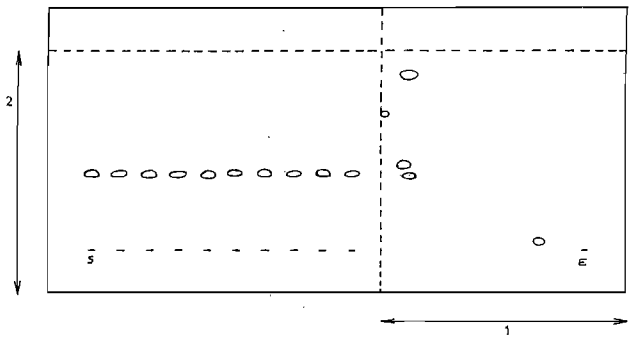

Figure 2

Disposition of spots on the plate before and after bidimensional chromatography: S, salicin; E, extract.

After each development, the plate was dried for 60 min in a stream of cold air.

The second chromatographic method entailed spotting 2, 3, and 4 μl aliquots of standard, 3 μl of the salicin solution superimposed on 1 μl of solution before hydrolysis, and 6 μl of the solution after hydrolysis spotted without superimposition of salicin; double (occasionally triple) development in an unsaturated chamber was then performed at room temperature with ethyl acetate – methyl ethyl ketone – formic acid – water (60 + 20 + 2 + 2, *v/v*), each time to a distance of 7 cm. After each development, the plate was dried for 60 min in a stream of cold air.

For both methods samples were applied to the plates by means of a Desaga (Heidelberg, FRG) AS 30 TLC Applicator. The width of bands was 2 mm; the distance between the bands, 10 mm; the step volume, 1 μl; the rate of application, 25 s μl^{-1}; and the break between application, 15 s. For bidimensional chromatography, the standard solution was spotted on the left of the plate only.

HPLC was performed with a Pharmacia LKB (Bromma, Sweden) liquid chromatograph.

2.4 TLC Quantification

The chromatogram was scanned by means of a CD 60 densitometer (Desaga) supplied with user-friendly software operating *via* a personal computer. For bidimensional chromatography scanning was performed in zig-zag mode (meander, 0.5 mm) using remission – absorbance, with an excitation wavelength of 270 nm (deuterium lamp); the slit width and slit height were both 0.4 mm; the resolution, 0.100 mm; the number of measurements per position, 32; and the signal factor, 20. For unidimensional chromatography scanning was performed in linear mode; using remission – absorbance, with an excitation wavelength of 270 nm (deuterium lamp); the slit width was 0.4 mm, the slit height 4 mm; the scanner was operated in spot optimization mode with the resolution 0.100 mm; the number of measurements per position, 32; and the signal factor, 20.

Peak area measurement was employed for both types of chromatography.

3 Results and Discussion

3.1 Choice of Chromatographic System

Different chromatographic systems previously described for the separation of salicin from other components of willow extracts were tested using silica gel HPTLC plates and five different mobile phases: dichloromethane – methanol – water (37 + 12 + 1, *v/v*) [2], ethyl acetate – methanol – water (100 + 13.5 + 10, *v/v*) [3], toluene – chloroform (10 + 10, *v/v*) [12], dichloromethane – acetic acid – methanol – water (50 + 30 + 20 + 5, *v/v*) [5], and ethyl acetate – methyl ethyl ketone – formic acid – water (60 + 12 + 4 + 4, *v/v*) [1]. None enabled the separation of salicin from the mixture. Measurement of the spectra of a standard solution of salicin and the extract from willow after development with these mobile phases showed that the two were different. For example, spectra obtained after development with the mobile phase reported by *Vanhaelen et al.* [2] contained bands the intensities of which were interchanged, as shown in **Figure 3**, owing to the background and, in some cases, the presence of picein **Figure 4**.

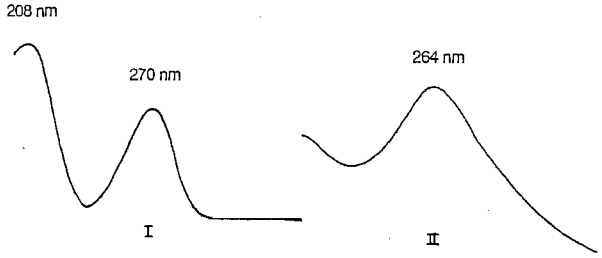

Figure 3

Comparison of the spectra, obtained after separation with the mobile phase described in reference 3, of (I) standard salicin and (II) salicin in the extract.

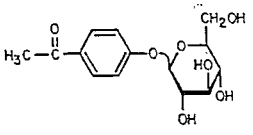

Figure 4

The formula of picein.

The spectra were identical after bidimensional chromatography (**Figure 5**) but because only one spot can be applied to plates used for bidimensional chromatography the cost of the method is high and the conditions for a complete validation are absent. Such a system can, however, be used to establish the performance of the chromatographic system proposed. After modification of the system described in reference 1, double development (or triple development in instances when the picein level was higher than usual) ensured good separation of salicin from other components of the extract and a reduced background. Comparison of spectra showed that they could be superimposed and the results obtained by this method were, moreover, similar to those obtained after bidimensional chromatography.

3.2 Validation Data

When ethyl acetate – methyl ethyl ketone – formic acid – water was used as the mobile phase for HPTLC, the calibration plot of peak area (λ = 270 nm) against amount of salicin showed good linearity for quantities between 1 and 8 μg (n = 10); the regression equation was $y = 64.643 + 58.342 \cdot x$.

The precision of the method was assessed by calculating the *RSD* of the results obtained for an extract containing 0.51 % salicin before hydrolysis and 1.71 % after hydrolysis. The

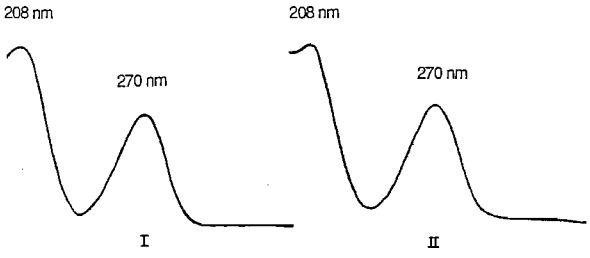

Figure 5

Comparison of the spectra, after bidimensional chromatography, of (I) standard salicin and (II) salicin in the extract.

within-day reproducibilities were 1.09 % (n = 6) and 2.01 % (n = 6), respectively, and the respective between-day reproducibilities were 1.17 % (n = 12) and 2.46 % (n = 12). The results obtained for one batch were compared with those obtained using the HPLC method described by *Meier* [7,11,13]; this confirmed the accuracy of the method.

The minimum detectable quantity at λ = 270 nm was 0.6 μg. After visualization with vanillin [6], 0.2 μg of salicin could be detected.

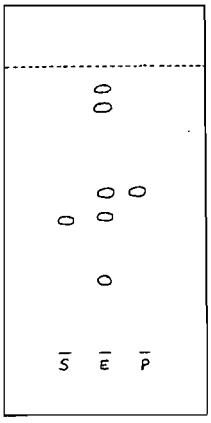

Figure 6

Chromatogram of the extract (E) obtained after development with ethyl acetate – methyl ethyl ketone – formic acid – water (60 + 20 + 2 + 2, *v/v*), the eluent ensuring the separation of salicin (S) from picein (P).

3.3 Amount of Salicin in the Samples of Willow Studied

The salicin content in batches of willow stem bark before and after hydrolysis ranged, respectively, from 0.1 % to 1.24 % and 1.6 % to 2.8 %. It is of note that these results are indicative of high variability in the amount of salicin (normally the salicin content of *Salix alba* L. is *ca* 0.5 %). Some commercial samples contain, in addition, picein – normally absent from *Salix alba* L. stem bark – and have a salicin content exceeding that described in the literature: *Salix alba* L. is a species reported to contain little salicin.

4 Conclusion

An HPTLC procedure has been developed which should, in the future, find successful application for quantification of salicin in willow stem bark and extracts.

The amounts of salicin found in six batches of willow before and after hydrolysis show high variability. Although this variability has been partially attributed to seasonal variation [17], the composition of some of these batches is different from that described for *Salix alba* L. stem bark. A German monograph on willow bark recommends a daily dose of 60-120 mg total salicin (amount found after alkaline hydrolysis) and the German Pharmacopoeia X [10] requires a minimum total salicin content of 1 % yet does not differentiate between species. The standardization of commercial batches should be based on these specifications.

Acknowledgments

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