39. **Komei Mizuno and Satoru Kuwada**: Application of Chromatography. XXIV.* Chromatographic Identification of Santonins and their Homologs.

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As is generally known, santonin has four asymmetric carbon atoms. Therefore, it may be thought to have sixteen isomers, but from the stereochemical reason only twelve isomers are possible to exist. In this laboratory, Abe and co-workers[1] have succeeded in synthesizing all the isomers, including the one which is in complete agreement with natural santonin in all respects.

The authors assumed that if the synthetic santonin, its isomers, and homologs could be identified by paper chromatography, it would greatly contribute to studies in this field.

So far as the authors are aware, there is found no report which deals with the identification of compounds of santonin series by paper chromatography. Since the compounds in this series, especially those which have a lactone ring, are sparingly soluble in water, they cannot be developed if water is used as the stationary phase. Therefore, various other solvents were examined referring the paper chromatography of steroidal compounds and it was found that dioxane-water (4:6) (all ratios described in this paper are by volume) and petroleum ether-dioxane (70:6) are the most suitable as stationary and mobile phases, respectively. However, what is most important is to know the ratio between the two phases which gives the optimal result, because, if the quantity of the stationary phase is too small to the applied sample, it is oversaturated with the sample and the latter is washed away by the mobile phase, causing "tailing" towards the origin, while if the quantity of the mobile phase is too small to the sample, it is oversaturated with the sample, and as a result "tailing" is formed in the opposite direction. As mentioned above, therefore, it is prerequisite to decide the most appropriate ratio between the two phases in a unit area of paper.

A sheet of Tōyō filter paper No. 131 of a definite size was weighed and then soaked in dioxane-water (4:6). The paper was then blotted between sheets of filter paper to remove the excess solvent and quickly weighed again. The weight obtained by subtracting the second weight from the first was taken as the quantity of the solvent saturating the paper and from this value a_s (μL) was calculated in a unit area of the paper. The wet paper was then dried at room temperature (22~28°) in an atmosphere of a definite relative humidity (60~80%) to observe how the weight decreases. When the time (t) required for evaporation of the solvent is taken as the abscissa and the variable quantities of the remaining solvent in unit area [a_w (μL)] as the ordinate, the evaporation curves shown in Figs. 1 and 2 were obtained.

Since dioxane resembles water in boiling point and melting point, the velocities of their evaporation also seem to resemble each other. Hence, the constitution of the remaining stationary phase after the above operation of drying would be almost the same with that before the operation.

As in the actual development, a sheet of filter paper saturated with stationary phase is used after air drying for a given time, the decreased quantity of the stationary phase (a_s-a_w) would be refilled with the mobile phase. Previously the authors pointed out that in paper partition chromatography the ratio between stationary and

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* Part XXIII: J. Pharm. Soc. Japan, **75**, 385 (1955).

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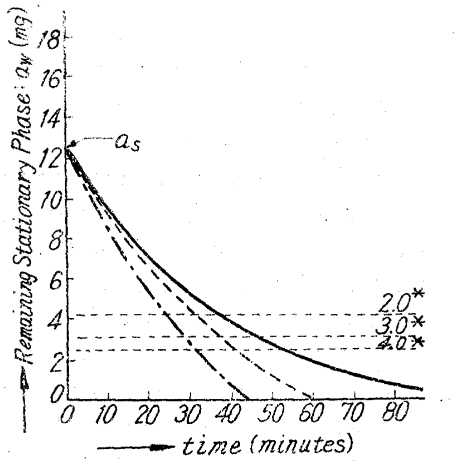
[1] Y. Abe, *et al.*: J. Am. Chem. Soc., **75**, 2567(1953); Proc. Japan Acad., **30**, 116, 119(1954); J. Am. Chem. Soc.,(1955) in press.

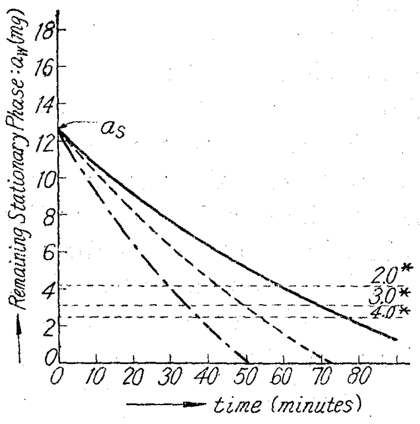
mobile phases, i.e. a_L/a_w, in a unit area of paper is almost constant[2] and this has a great significance. Also in the present study $(a_s-a_w)/a_w$ is considered to have the same meaning.

After many experiments under various conditions it was found that if the $(a_s-a_w)/a_w$ value is found to be more than 4.0, the quantity of the stationary phase is too small and if it is less than 2.0, the quantity of the mobile phase is too small, and in either case the separation is incomplete, causing "tailing". In Figs. 1 and 2 the points of the $(a_s-a_w)/a_w$ value corresponding to 2, 3, and 4 are shown by the horizontal dotted lines. Therefore, if the room temperature and relative humidity are known, the time of air drying necessary to put the $(a_s-a_w)/a_w$ value between 2 and 4 is easily presumed, but the exact value must be determined by experiment.



——— Relative humidity 78~80%
.... 〃 68%
– – – – 〃 60%
* $(a_s-a_w)/a_w$

Fig. 1. Evaporation Curves of Dioxane-Water (4 : 6) from the Surface of Tōyō Filter Paper No. 131, at 28°



——— Relative humidity 78~80%
.... 〃 70%
– – – – 〃 60%
* $(a_s-a_w)/a_w$

Fig. 2. Evaporation Curves of Dioxane-Water (4 : 6) from the Surface of Tōyō Filter Paper No. 131, at 22°

With neutral santonin compounds 1~2 μL. each of their 1% solution in dioxane-water (10:3) was applied on the starting line at intervals of 2 cm. in the manner mentioned above and developed with petroleum ether-dioxane (70:6) by one-dimensional ascending method for 3~4 hours.

When the chromatogram thus obtained was observed under ultraviolet rays (2537 Å), the spots were detected as dark ones. In this case, if the chromatogram is put together with a sheet of printing paper and irradiated with the above-mentioned light from a distance of 10~20 cm. for 150~200 sec. (the time varies with the nature of the paper), the developed printing paper shows the spots as white ones with various shades and spreads depending upon the amount of the absorbed light on the black background. In this way, as small as a few γ of a sample can be detected and the data of the chromatography is conveniently recorded and stored. The color reaction with a solution of sodium alkoxide in 50% ethanol, which has been used for the detection of santonin, can also be utilized.

The results obtained by this method with various stereoisomers of santonin and carboxylic acid ester intermediates are shown in Table I. However, as is obvious from this Table, racemic santonins cannot be distinguished from their optically active isomers by this method because their Rf values are the same under these conditions. This is

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2) S. Kuwada, K. Mizuno, H. Nawa: Vitamins (Japan), **3**, 137(1950).

true of various racemic ester intermediates.

When acid intermediates of santonin were subjected to paper chromatography under the foregoing conditions, most of them caused "tailing". In such a case, an alcoholic solvent such as 70% hydrous methanol, 70% hydrous ethanol, 84% hydrous butanol, or butanol-saturated 3% ammonia water, was suitable as the mobile phase. The resulting spots could be detected by the formation of colored hydrazones with hydrochloric acidic 2,4-dinitrophenylhydrazine solution, besides the ultraviolet ray absorption method, but the former was inferior to the latter in sensibility.

Of these acids, racemic 11-carboxy-3-oxoeusantona-1,4-dienic acid was detected as one spot on the chromatogram. If this compound could be resolved into its optically active components on paper, such a paper chromatography would be utilized more effectively. For this purpose the following process was attempted.

A sheet of Tōyō filter paper No. 131 was soaked in a saturated solution of brucine in warm water, the excess solution was removed with dry filter paper, and the wet paper was dried at 22° in an atmosphere of less than 80% relative humidity. In this case, differing from the case where dioxane-water was used as the stationary phase, all the moisture except that inherent in the paper must be removed, and therefore, it is necessary to dry the paper for 4~10 hours. About 20 γ of *dl*-11-carboxy-3-oxoeusantona-1,4-dienic acid was applied as an aqueous solution or as 1~3% solution in aqueous brucine solution on the paper impregnated with brucine and developed by the one-dimensional, ascending method with the same mobile phase as in the case of acid compounds. When the resulting chromatogram was sprayed with hydrochloric acidic 2,4-dinitrophenylhydrazine solution, two colored spots were observed and the spot with smaller Rf value was in accord with that obtained by developing *d*-11-carboxy-3-oxoeusantona-1,4-dienic acid with 70% ethanol (Table III). In the paper chromatography of this kind the main factor for the separation seems to be partition and displacement, but it is regrettable that only one instance is available. However, the authors are confident that this method would offer reference data quickly and simply in confirming the result of resolution by fractional crystallization or in judging the purity of optically active compounds.

The authors ars grateful to Dr. Yasuo Abe and his co-workers for their kindness in offering the materials for the present works.

Experimental

Paper Chromatography of Neutral Santonin and Its Stereoisomers—1) Stationary phase and mobile phases: A mixture (5 : 6 : 7) of dioxane: water: petroleum ether (b.p. 40~80°) forms at 22~28° two layers in a ratio of 8:10. The lower layer consisting of dioxane: water (4:6) is used as the stationary phase. The upper layer consisting of petroleum: ether dioxane (68 : 12) is mixed with water in a ratio of 8 : 2 and, of the resulting two layers, the upper layer consisting of petroleum ether : dioxane (70 : 6) is used as the mobile phase.

2) Range of $(a_S-a_w)/a_w$ suitable for separation: A sheet of Tōyō filter paper No. 131 with a definite weight is soaked in the above-mentioned stationary phase and after removing the excess solvent the paper is weighed again. The weight of the stationary phase (s.g. 1.0) saturating the papes is converted into volume value, and this value, which is always constant (12.5 μL), is taken as a_S in a unit area. The wet paper is dried at various room temperatures and relative humidities, the weight (a_w) of the solvent remaining in a unit area is measured by weighing the paper at given times, and from the weights of a_s and a_w, the value of $(a_s-a_w)/a_w$ is calculated.

In the present study all the experiments were carried out under the conditions that the value of $(a_s-a_w)/a_w$ remains within a range of 2~4, because it has been found that paper chromatography of this kind is successful only when conducted within that range and because the value of $(a_s-a_w)/a_w$ may be considered similar to the paper constant a_L/a_w.

3) Detection of bands: Detection of the resulting bands by ultraviolet light (2537 Å) and by printing was already referred to in the general description.

4) Rf values of neutral compounds of santonin series are listed in Table I.

Paper Chromatography of Acid Compounds of Santonin Series(See also Tables IIa and IIb)——Acid compounds obtained as intermediates in the course of the synthesis of santonin series compounds gave the chromatograms shown in Tables IIa and IIb, which show the success of the experiments.

TABLE I. Rf Values of Neutral Compounds of Santonin Series

Room temp.	30°		28°		22°		18°		
Relat. humidity (%)	75	68	78	65	65	60	75	65	60
Compound[3] $(a_S-a_W)/a_W$	4.0	3.0	3.6	2.3	2.4	2.1	4.0	3.5	3.0
dl-6α-Hydroxy-3-oxoisoeusantona-1,4-dienic acid lactone			0.43	0.33					
dl-6α-Hydroxy-3-oxo-11-*epi*-isoeusantona-1,4-dienic acid lactone			0.53	0.44					
l-6α-Hydroxy-3-oxo-11-*epi*-isoeusantona-1,4-dienic acid lactone			0.53	0.44					
dl-Methyl 3-oxo-11-*epi*-isoeusanton-4-enate			0.61						
dl-α-Santonin	0.63	0.61	0.63					0.40	
l-α-Santonin	0.63	0.61	0.63	0.55		0.28 "tailing"		0.40	
dl-β-Santonin	0.57	0.54							
dl-6β-Hydroxy-3-oxo-11-*epi*-eusantona-1,4-dienic acid lactone			0.67	0.60		0.37	0.50	0.46	
dl-6β-Hydroxy-3-oxoeusantona-1,4-dienic acid lactone			0.71	0.66		0.50	0.56	0.52	
dl-6-Hydroxy-3-oxoeusantona-4,6-dienic acid lactone			0.76						
d-6α-Hydroxy-3-oxoeusanton-4-enic acid lactone		0.79							
dl-6α-Hydroxy-3-oxoeusanton-4-enic acid lactone		0.79			0.73	0.70	0.75	0.70	
dl-6β-Hydroxy-3-oxoeusanton-4-enic acid lactone					0.76	0.73	0.78	0.73	
dl-6β-Hydroxy-3-oxo-11-*epi*-eusanton-4-enic acid lactone					0.70	0.76	0.77	0.67	
dl-11-Ethoxycarbonyl-6α-hydroxy-3-oxo-11-*epi*-eusanton-4-enic acid lactone (m.p. 124°)		0.87					0.84		0.79
Ethyl 11-ethoxycarbonyl-3-oxoeusanton-4-enate		0.97							0.94
Ethyl 11-ethoxycarbonyl-11-nor-3-oxoeusanton-4-enate		0.94							
Ethyl 3-benzoxy-11-ethoxycarbonyleusantona-3,5-dienate									0.97

TABLE IIa. Rf Values of Acidic Compounds of Santonin Series

Compound*	Rf values	Color of 2,4-dinitrophenylhydrazones
dl-3-Oxoeusanton-4-enic acid	0.65	orange yellow
dl-3-Oxo-11-*epi*-eusanton-4-enic acid	0.62	orange red
dl-11-Carboxy-6α-hydroxy-3-oxoeusanton-4-enic acid lactone**	0.42	orange yellow
d-11-Carboxy-6α-hydroxy-3-oxoeusanton-4-enic acid lactone**	0.41	〃
dl-11-Carboxy-6α-hydroxy-3-oxoeusantona-1,4-dienic acid**		
m.p. 200°(decomp.)	0.48	orange red
〃 213°(〃)	0.36	〃
dl-11-Carboxy-3-oxoeusantona-1,4-dienic acid	0.17	〃

* These acidic compounds were chromatographed with butanol saturated with 3% aqueous ammonia, on strips of Tōyō filter paper No. 131 at 22~25°, by one-dimensional ascending method.

** The configuration at C_{11} is not yet definitely established.

TABLE IIb. Rf Values of *dl*-11-Carboxy-3-oxoeusatona-1,4-dienic Acid

Solvent	Rf Values
70% MeOH	0.87
70% EtOH	0.82
84% BuOH	0.71
BuOH satd. with 3% aq. NH_4OH	0.17

The compound was chromatographed with alcoholic solvents on strips of Tōyō filter paper No. 131 at 22°, by one-dimensional ascending method.

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3) Y. Abe, *et al.*: Chemistry & Industry, 91 (1955).

Resolution of Racemic Compound of Santonin Series by Paper Chromatography——Strips of Tōyō filter paper No. 131 are used.

As mentioned before, the compound is developed at 22° on the paper impregnated with brucine by the one-dimensional, ascending method with various alcoholic solvents, and detected by spraying 0.5% solution of 2,4-dinitrophenydrazine in 1 N hydrochloric acid.

Table III. Resolution of Racemic Compound by Paper Chromatogrophy

Applied samples	Solvent	Rf Value		
dl-11-Carboxy-3-oxoeusantona-1,4-dienic acid	70% MeOH		0.68	0.84
〃	70% EtOH	(0.62)	0.75	0.85
〃	84% BuOH		0.53	0.70
〃	BuOH satd. with 3% aq. NH_4OH	(0.15_5)	0.25	0.63
〃 + aqueous solution of brucine	70% EtOH		0.77	0.87
d-11-Carboxy-3-oxoeusantona-1,4-dienic acid + warm aq. soln. of brucine	〃		0.77	

Summary

Synthetic santonin, its stereoisomers and carboxylic acid ester intermediates, which are all neutral compounds, were developed using dioxane-water (4:6) and petroleum ether-dioxane (70:6) as the stationary and mobile phases, respectively, and the resulting bands were detected by ultraviolet light (254 mμ) as light absorption spots or by printing the spots on a sensitized paper. In this case, the volume ratio between stationary phase and mobile phase on paper must remain within a definite range. The change of the ratio at various room temperatures and relative humidities were investigated in detail, using Tōyō filter paper No. 131.

The acid compounds obtained in the course of the synthesis of santonin and its isomers were well separated when developed with alcoholic solvent of various percentages or with butanol saturated with 3% ammonia water.

Of the acid compounds, *dl*-11-carboxy-3-oxoeusantona-1,4-dienic acid gave two bands of its optically active components when developed on a paper impregnated with brucine. Paper chromatography of the brucine salt of this compound also gave the same result. Of the above two bands, the one with smaller Rf value was in complete agreement with that obtained by developing the brucine salt of *d*-11-carboxy-3-oxoeusantona-1,4-dienic acid.

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