Determination of Dihydroseselins in Fruits and Extracts of *Ammi visnaga* L.

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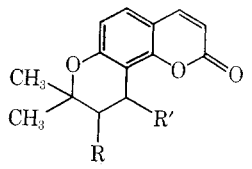
Abstract □ Three methods for the assay of dihydroseselins in the fruits and extracts of *Ammi visnaga* are proposed. The first is based on measuring the absorption maximum of the dihydroseselins at 323 mμ, with a sensitivity range of 2–20 mcg./ml. The second and the third are colorimetric in which visnadin was cleaved by cold ethanolic sodium hydroxide and then either coupled with the diazol solution or reacted with 2,6-dibromoquinone chlorimide. The intensity of the resulting colors was then measured spectrophotometrically at 500 and 630 mμ, respectively, with a sensitivity range of 1–12 mcg./ml. in both methods. The dihydroseselins were separated from *Ammi visnaga* by TLC, quantitatively eluted, and comparatively assayed by the above methods. The results were concordant.

Keyphrases □ Dihydroseselins, assay □ *Ammi visnaga*, fruits and extracts—assay of dihydroseselins □ UV spectrophotometry—analysis □ TLC—separation, identification

Since the isolation of visnadin (II), samidin (III), and dihydrosamidin (IV), which are derivatives of the parent coumarin dihydroseselin (I), from the fruits of *Ammi visnaga*, considerable attention has been directed toward their pharmacological and therapeutic actions. The newly isolated coumarin compounds proved to be more strongly vasodilatory than khellin and papaverine hydrochloride (1). Visnadin being 10 times as active as khellin, has been successfully employed as a vasodilator in the treatment of angina pectoris (2, 3).



I —dihydroseselin R = H ; R′ = H

II —visnadin R = O·CO·CH(CH$_3$)(C$_2$H$_5$) ; R′ = O·CO·CH$_3$

III —samidin R = O·CO·CH=C(CH$_3$)(CH$_3$) ; R′ = O·CO·CH$_3$

IV —dihydrosamidin R = O·CO·CH$_2$·CH(CH$_3$)(CH$_3$) ; R′ = O·CO·CH$_3$

Survey of the literature failed to show any reports concerning the quantitative determination of the dihydroseslins content in the fruits and extracts of *Ammi visnaga*. Hence, the present work was carried out in order to establish suitable and simple methods for this determination. The proposed methods made use of UV spectrophotometry, Erlich's diazo test (4), and Gibb's test (5). The dihydroseselins, being closely related chemically and biologically, were represented in this communication by visnadin which is the most biologically active and which appears to be the predominant dihydroseselin derivative present in *Ammi visnaga* (6).

EXPERIMENTAL

Reagents—Analytical grade reagents were used whenever necessary. Authentic samples of visnadin,[1] samidin, and dihydrosamidin[2] were supplied by external sources.

Standard Visnadin Solution—Ten milligrams of visnadin was dissolved in ethanol 95% and adjusted to 100 ml. with the same solvent.

Sodium Hydroxide Solution—One percent solution of sodium hydroxide in CO_2-free water was prepared, filtered, and kept in a plastic bottle.

Sulfanilic Acid Solution—Nine grams of sulfanilic acid was dissolved in 9 ml. of hydrochloric acid and 200 ml. of water with the aid of heat. The warm solution was filtered into a 1-l. volumetric flask, diluted with 700 ml. of water, cooled to room temperature, and adjusted to 1 l. with water.

Sodium Nitrite Solution—Five grams of sodium nitrite was dissolved in 100 ml. of water.

Diazol Solution—Five and one-half milliliters of sulfanilic acid solution was introduced into a 50-ml. volumetric flask and cooled in an ice bath. Ice-cooled sodium nitrite solution (0.5 ml.) was pipeted dropwise, while shaking, on the sulfanilic acid solution. The mixture was allowed to stand in an ice bath for 10 min., adjusted to volume with ice-cold water, well mixed, and kept in an ice chest. The solution is stable for 8 hr.

Chlorimide Solution—Fifty milligrams of 2,6-dibromoquinone chlorimide was dissolved in 50 ml. of chloroform in a 100-ml. volumetric flask, adjusted to volume with chloroform, and kept in a brown bottle in a refrigerator. The color of the solution must be pale yellow. In case the color darkens, the solution must be rejected and a fresh one should be prepared. The solution is stable for at least 1 month.

0.2 N Acetic Acid, (Approx.)—Eleven and one-half milliliters of glacial acetic acid was diluted to 1 l. with water.

Sodium Acetate Solution—Twenty percent aqueous solution of sodium acetate was prepared.

Apparatus—A spectrophotometer (Beckman DU) was used for UV and colorimetric determinations.

Spectrophotometric Method—Visnadin, samidin, and dihydrosamidin were found to have absorption maxima at 323–324 mμ and absorption minima at 264 mμ in ethanol 95% (1). A standard curve was constructed by measuring the UV absorbance of visnadin solutions of variable concentrations (2–20 mcg./ml. of ethanol 95%), in 1-cm. silica cells at 323 mμ. The average a of visnadin at 323 mμ was found to be 365.

Diazol Colorimetric Method—The orange-red color resulting from coupling the cleaved visnadin with diazol solution, was investigated. It was found that the maximum absorption of the color produced occurred at 500 mμ. The suitability of the color reaction for quantitative assay of visnadin was investigated along two main lines, namely, the quantitative hydrolysis of visnadin into the corresponding phenolic compound, and the formation of a measurable stable color of the azo-dye resulting from the coupling reaction.

Quantitative Hydrolysis of Visnadin—The complete cleavage of the lactone ring of visnadin to the corresponding phenolic derivative (*O*-hydroxycinnamic acid derivative), was detected by reaching a maximum and stable absorbance value of the formed color. Different factors which were expected to affect the hydrolysis of visnadin were studied. These were: the effect of concentration of visnadin, medium of hydrolysis, volume of solvent, concentration of alkali, temperature, and time. The optimum conditions of a

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quantitative hydrolysis of visnadin, taking into consideration the above factors were as follows: visnadin (50–500 mcg.), dissolved in 5 ml. of ethanol 95% was completely hydrolyzed by the action of 1 ml. of 1% sodium hydroxide solution when kept at room temperature (20–30°) for 60 min.

Color Formation—The factors that may affect the stability of color of the azo-dye were studied. These factors were: the effect of concentration of ethanol and of diazol solution, temperature, time of different steps of the color reaction, pH, and aging of the diazol solution. The optimum experimental conditions to obtain precise and reproducible results were as follows: the hydrolyzed visnadin solution was diluted with 15 ml. of 50% ethanol and the solution was cooled in an ice bath for 3 min. One milliliter of freshly prepared ice-cold diazol solution was added, the mixture was shaken, and allowed to stand at room temperature for an additional 10 min., diluted with water to 25 ml., mixed, and the absorbance of the color was measured against a blank at 500 mμ. The pH of the final solution was 12.3 and the color remained stable for more than 24 hr. unaffected by light.

Standard Curve—A standard curve was plotted using variable concentrations of visnadin. Taking into consideration the above precautions, the following method is recommended: introduce variable concentrations of visnadin (25–300 mcg.) into 25-ml. volumetric flasks. Adjust the volume in each flask to 5 ml. by the addition of 95% ethanol and mix well. To each flask add 1 ml. of 1% sodium hydroxide solution and shake frequently during 60 min. Then add 15 ml. of 50% ethanol, mix well, and allow to stand in an ice bath for 3 min. Remove the flasks from the ice bath, add to each flask 1 ml. of ice-cold, freshly prepared diazol solution, mix well, allow to stand for 10 min., adjust the volume to 25 ml. with water, and mix well. Measure the absorbance of the color at 500 mμ against a blank sample.

The amount of visnadin in solutions of unknown concentration can be deduced from the standard curve or more simply by adaptation of a K factor as follows:

$$K = \frac{\text{mcg. visnadin}}{A} = 368^{3}$$

mcg. visnadin/100 ml. solution = $A \times 36800/V$, where A = absorbance value at 500 mμ and V = volume of visnadin solution.

Application of this K value in analysis of solutions of unknown concentration of visnadin gave reproducible results.

Chlorimide Colorimetric Method—The method is based on the color reaction between 2,6-dibromoquinone chlorimide and the phenolic derivative produced from hydrolysis of visnadin with sodium hydroxide.

Color Formation—The chloroformic solution of chlorimide reagent was more stable than that made with methanol, ethanol, or isopropanol. For a concentration range of 25–300 mcg. of visnadin, 1 ml. of 0.05% chlorimide solution was found to be adequate. The color was best developed at pH 8.5 which was obtained by using acetic acid-sodium acetate buffer system.

Under these conditions, the blue color was fully formed after about 2–3 min., remained stable for 2 hr., exhibited absorption maximum at 630 mμ, and obeyed Beer's law in a visnadin concentration range of 25–300 mcg./25 ml.

Standard Curve—Taking into consideration the above conditions, an absorbance/concentration curve was plotted using the following method: visnadin in varying concentrations ranging between 25 and 300 mcg. was hydrolyzed in exactly the same way as described under the diazol method. After hydrolysis, add successively 15 ml. of 50% ethanol, 1.25 ml. of 0.2 N acetic acid, 1 ml. of 20% sodium acetate solution, and 1 ml. of 0.05% chlorimide solution, shaking the liquid after each addition. Allow to stand for 10 min., make up the volume to 25 ml. with water, and mix well.[4] Measure the absorbance of the color at 630 mμ in a 1-cm. silica cell against a blank. Deduce the results from the standard curve or from the average K value of 455.

Quantitative TLC Recovery of Dihydroseselins—After many chromatographic trials on a mixture of authentic constituents of *Ammi visnaga* fruit extracts, a successful separation of the dihydroseselins from other constituents was achieved on Silica Gel G plates, employing chloroform–ethanol (98.5:1.5) as the developer.

Table I—Recovery of Pure Visnadin from Silica Gel G Plates

Visnadin Applied, mcg.	Visnadin Recovered: UV Method Rec., mcg.	%	Diazol Method Rec., mcg.	%	Chlorimide Method Rec., mcg.	%
50.0	49.6	99.2	48.3	96.6	48.3	96.6
100.0	98.0	98.0	101.8	101.8	100.3	100.3
150.0	142.5	95.0	153.0	102.0	148.4	98.9
200.0	200.0	100.0	207.5	103.7	200.0	100.0
250.0	243.5	97.4	244.9	97.9	248.4	99.4
300.0	308.4	102.8	306.5	102.1	298.2	99.4

The spot (R_f 0.66) of dihydroseselins fluoresced violet under UV (350 mμ) and was located above that of khellin (R_f 0.43 and yellowish brown fluorescence) (see Fig. 1). As a result of this separation quantitative TLC became possible.

Silica Gel G plates[5] (20 × 20-cm. and 0.25-mm. thick) were spotted with volumes of absolute alcoholic solution corresponding to amounts of visnadin ranging from 50 to 300 mcg., with an agla micrometer syringe. The plates were developed with chloroform–ethanol (98.5:1.5) until the solvent front had ascended 15 cm. The developed plates were allowed to dry spontaneously at room temperature, and the dried plates were examined under UV light. The violet fluorescing areas of visnadin were separately scraped with a thin spatula or removed quantitatively by means of a zone extractor, and eluted with 12 ml. of chloroform into a 25-ml. volumetric flask. At the same time blank areas having the same dimensions and at the level of visnadin were treated in the same way. The chloroform in each flask was evaporated to dryness on a water bath. The residues were then comparatively assayed by the three methods outlined above and the results are compiled in Table I.

Determination of Dihydroseselins in the Fruits and Extracts of Ammi visnaga—The above TLC method was applied for the quantitative determination of the dihydroseselins content of the fruits and extracts of *Ammi visnaga*.

Two and one-half grams of powdered *Ammi visnaga* fruits was transferred to the surface of 5 g. of acid alumina in the thimble

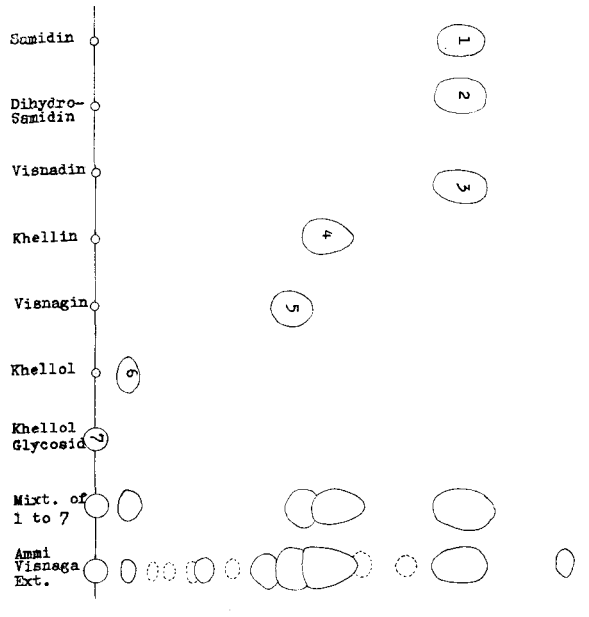

Figure 1—*TLC of* Ammi visnaga *fruit extract and authentic reference substances. Layer: Silica Gel G. Solvent: chloroform–ethanol (98.5:1.5).*

[3] The mean of six different concentrations, each of six readings.

[4] Increasing the aqueous phase caused turbidity, and raising the concentration of alcohol lowered the stability and sensitivity of the color.

[5] In case of the spectrophotometric assay, the Silica Gel G should be washed three times with 3 parts of boiling methanol, dried at 110° before spreading the layer in the usual manner. Negligible blank values were obtained by this treatment.

Table II—Analysis of Commercial Samples of *Ammi visnaga* Fruits

Sample No.	% of Total Dihydroseselins Calculated as Visnadin UV Method	Diazol Method	Chlorimide Method
1	0.475	0.477	0.481
2	0.471	0.475	0.479
3	0.472	0.477	0.471
4	0.490	0.486	0.483
5	0.481	0.484	0.477
6	0.509	0.517	0.513

of a small continuous extraction apparatus and extracted with chloroform until exhaustion (about 4 hr.). This was detected by the disappearance of the dihydroseselins spot in the chromatogram of the extract. The chloroform extract was concentrated to 2 ml. and adjusted to 5 ml. with chloroform in a volumetric flask. Aliquots of the chloroform solution, expected to contain from 100–200 mcg. of the dihydroseselins (50 μl.) were applied in the form of a band (0.5 × 4 cm.) on a Silica Gel G plate. After development, the band area corresponding to dihydroseselins was removed by the zone extractor, and dihydroseselins were extracted as described in the preceeding section. The extracted dihydroseselins were then estimated by the three methods.

In case of extract of *Ammi visnaga*, 50 μl. was applied directly on a Silica Gel G plate and the dihydroseselins content was estimated as described above.

The blank values corresponding to the diazol and chlorimide methods were found to be too small so that they could be neglected. The results of the assays were then deduced from standard curves of reference sample of visnadin recovered from Silica Gel G plates and they are listed in Tables II and III.

RESULTS AND DISCUSSION

The dihydroseselins, visnadin, samidin, and dihydrosamidin were successfully separated by TLC in the form of a single spot from other constituents of *Ammi visnaga* fruits. It was also found that samidin and dihydrosamidin reacted in the same way and gave colors having the same absorption maximum and color intensity as visnadin in the two proposed colorimetric assay methods.

The diazol and chlorimide reagents were also utilized for the detection of dihydroseselins as well as other coumarins of *Ammi visnaga* on TLC plates. This was done by spraying the dry chromatogram with a 10% methanolic solution of potassium hydroxide, setting aside the plates for 30 min. or for about 5 min. under UV, then spraying with diazol or chlorimide solutions; the spots corresponding to coumarins were colored orange-red or blue, respectively.

Table I shows that visnadin can be quantitatively recovered from

Table III—Analysis of Commercial Samples of Liquid Extract of *Ammi visnaga*

Sample No.	% of Total Dihydroseselins Calculated as Visnadin UV Method	Diazol Method	Chlorimide Method
1	0.227	0.221	0.228
2	0.230	0.231	0.229
3	0.210	0.208	0.213
4	0.209	0.203	0.210
5	0.229	0.237	0.232
6	0.306	0.300	0.297

Silica Gel G plates and that the results obtained by the three proposed methods are comparable. In the light of the results shown in Tables II and III, the three proposed methods—*viz.*, the UV absorption, the diazol and the chlorimide method can be used in the determination of the dihydroseselins in the fruits of *Ammi visnaga* and its extracts. The data also reveal that the dihydroseselins, calculated as visnadin, occur in *Ammi visnaga* fruits in considerable amounts ranging from 0.471–0.517%. They are present in approximately half the above values in the liquid extracts of *Ammi visnaga* prepared according to the Egyptian Pharmacopoeia 1963. Their dihydroseselins content ranged from 0.203–0.306%.

REFERENCES

(1) E. Smith, N. Hosansky, W. G. Bywater, and E. E. van Tamelen, *J. Am. Chem. Soc.*, **79**, 3534(1957).

(2) J. M. de Bettencourt, A. C. Ralha, F. P. Gomes, and H. P. Monteiro, *Presse Med.*, **64**, 1468(1956).

(3) M. Mouquin and C. Macrez, *ibid.*, **68**, 257(1960).

(4) P. Erlich, *Z. Klin. Med.*, **5**, 285(1882); through F. Feigcl, "Spot Tests in Organic Analysis," 7th ed., Elsevier, New York, N. Y., 1966, p. 143.

(5) H. D. Gibbs, *J. Biol. Chem.*, **72**, 649(1927).

(6) E. Smith, L. A. Pucci, and W. G. Bywater, *Science*, **115**, 520 (1952).

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