A PHOTOELECTRIC COLORIMETRIC METHOD FOR THE ESTIMATION OF KHELLIN

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Khellin, $C_{14}H_{12}O_5$ (2-methyl-5 : 8-dimethoxy-6 : 7-furano-chromone[1,2]) m.pt. 154° to 155°C., the main active principle of the fruits of *Ammi Visnaga* Linn., is identified by the rose red colour which it gives with potassium or sodium hydroxide[3]. This test has been used as a spot reaction by Abdel-Rahman[4] for the determination of khellin in solutions. It makes use of the fact that one drop of khellin solution gives with solid sodium hydroxide a rose red colour, only, if the quantity of khellin is not below a certain limit (1 : 505,000), which is the identification limit, Accordingly, 0·066 μg. of khellin can be detected in one drop, assuming that 1 ml. of the solution equals 30 drops.

Applying this method quantitatively, a given test solution is progressively diluted with constant testing of drops taken from the various dilutions until the colour reaction fails. The concentration of khellin in the original solution can then be calculated from the dilution required to reach this limit.

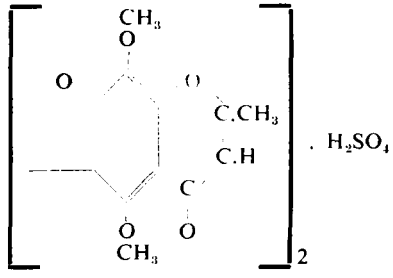
The repeated dilutions and testing of drops required by this procedure, are time consuming, and the results are only approximate, even if a special standardised dropper is used; this is because the " identification limit " is not the same for different observers; there is almost always a certain range of variation which depends on the individual characteristics of the observer. The range of dilution in which a test sometimes succeeds and sometimes fails is called by Feigl the " region of uncertain reaction[5]," and a quantitative estimation based on the examination for the failure of a test always extends into that region and therefore cannot be exact. Furthermore, even slight experimental errors become quite significant because the subsequent computation involves a multiplication. On applying this method, the results obtained by the authors varied to a great extent. In some determinations the results obtained were 40 to 60 per cent. lower than the amount of khellin taken.

The method of Anrep and co-workers[6] for assaying khellin, using a saturated solution of potassium hydroxide, is a modification of the above method. Its advantage lies in the fact that it makes use of controls, the colour given by the unknown being matched with that of a standard solution; but a saturated solution of potassium hydroxide is not a stable reagent as it easily absorbs carbon dioxide from the atmosphere leading to precipitation of potassium carbonate, the result being a turbid solution which interferes with correct comparison of colours. By applying this method on a photoelectric colorimeter, the authors found that the transmission values of the coloured solution are different for the same amounts of khellin, when examined under the same experimental conditions. This shows that this method is not reliable for the quantitative

estimation of khellin. In this communication, a photoelectric colorimetric method is described. This method has the advantage of being accurate, simple and suitable for the assay of pharmaceutical preparations.

The Photoelectric Colorimetric Method

Khellin gives with sulphuric acid a citron yellow colour which is stable in relatively dilute solutions. This colour is due to the formation of an oxonium salt (khellin sulphate) which has been isolated by the authors in a pure crystalline form as golden yellow needles having the structural formula.



The yellow colour of khellin sulphate is a property of the non-ionised salt. In presence of excess of water, khellin sulphate splits into khellin and sulphuric acid and the colour disappears as shown by the following equation:—

$$K + H_2SO_4 \rightleftarrows K\,S$$

where K and K S represent khellin and khellin sulphate respectively.

This is a reversible reaction which follows the law of mass action. This equation in terms of concentration becomes:

$$\frac{(K\,S)}{(K).\,(H_2SO_4)} = \text{Constant.}$$

brackets being used to represent concentrations.

If the concentration of sulphuric acid or of khellin is increased; the concentration of khellin sulphate increases proportionally; and as khellin sulphate is responsible for the citron yellow colour produced; the optical density of the solution will be proportional to the concentration of khellin and of sulphuric acid present in the solution.

On keeping the concentration of sulphuric acid constant, the concentration of khellin sulphate and consequently the optical density of the solution will be proportional to the concentration of khellin.

$$\frac{(K\,S)}{(K)} = \text{constant or } \frac{D}{(K)} = \text{constant.}$$

where D represents optical density.

This is true within certain limits of concentration, as shown in the experimental part.

Experimental

Preparation of Khellin Sulphate. 10 g. of khellin is dissolved in 50 ml. of glacial acetic acid in the cold; then 25 ml. of concentrated sulphuric

acid is gradually added and the mixture is kept in the ice-chest for 24 hours. 150 ml. of absolute alcohol is then added slowly to the mixture, stirred and again kept in the ice-chest for 1 week. The golden yellow crystals of khellin sulphate which separate, are filtered by suction, washed twice with ethyl acetate, and dried at room temperature. Yield, 9·5 g. (95 per cent.); m.pt. 175° to 185°C.

The crystals when treated with water split into pure khellin, m.pt. 154° to 155°C., and sulphuric acid. Acid released, 15·635 per cent. w/w of H_2SO_4. Khellin sulphate $C_{14}H_{12}O_5)_2,H_2SO_4$ requires 15·85 per cent. w/w.

Relation Between the Concentration of Khellin, the Concentration of Sulphuric Acid and the Optical Density of the Solution

Solutions required:—

(1) 10N sulphuric acid (A.R.).

(2) Standard stock solution of khellin (0·5 per cent. w/v). 0·5 g. of pure crystalline khellin, m.pt. 154° to 155°C. dissolved in 100 ml. of alcohol (60 per cent. v/v).

(3) Standard dilute solution of khellin (0·025 per cent. w/v); freshly prepared by diluting 5 ml. of the standard stock solution to 100 ml. with distilled water.

The relation between the concentrations of khellin and sulphuric acid and the optical density was determined at room temperature (25°C.).

The concentration of sulphuric acid in the solution is controlled by diluting known volumes of 10N sulphuric acid with distilled water to a constant volume, and the optical density of the solution is calculated from the equation:

$$D = 2 \log_{10} T$$

where D represents the optical density and T the percentage transmission of the solution. T is read directly on the scale of the colorimeter. Blue filter 420 is found to be the most suitable, as it gives minimum transmission values and a better spread of readings.

The following general procedure has been adopted. Into a volumetric flask of 50 ml. capacity, containing x ml. of a standard solution of khellin, n ml. of 10N sulphuric acid is added. The volume is then completed to 50 ml. with distilled water, well mixed, and left to stand for about 5 minutes; about 10 ml. of the solution is transferred to a colorimeter tube; and its percentage transmission is read in a Lumetron Photoelectric colorimeter Model 400-A using blue filter 420 against water as the blank set at 100 per cent. transmission, x = 1 ml. of the standard stock solution of khellin (0·5 per cent. w/v), representing a concentration of 10 mg. per cent. w/v of khellin, or 1, 2, 4, 8 and 10 ml. of the standard dilute solution of khellin (0·025 per cent. w/v) representing concentrations of 0·5, 1, 2, 4 and 5 mg. per cent. w/v of khellin respectively; n = 10, 15, 20, 25, 30 and 40 ml. of 10 N sulphuric acid, representing concentrations of 98·08, 14·712, 19·616, 24·52, 29·424 and 39·232 g. per cent. w/v of sulphuric acid respectively.

From the results obtained it may be concluded:—

(a) For the same concentration of khellin; the optical density of the solution increases with an increase in the concentration of sulphuric acid.

(b) For the same concentration of sulphuric acid the optical density of the solution increases with an increase in the concentration of khellin.

(c) For concentrations up to 4 mg. per cent. w/v of khellin the optical density is proportional to the concentration of khellin provided that the concentration of the acid is more than 29·424 per cent. w/v of sulphuric acid.

(d) Transmission readings obtained with a concentration of 39·232 per cent. w/v of sulphuric acid are spread enough to allow a determination of khellin to be done within concentrations ranging from 0·5 to 10 mg. per cent. w/v of khellin.

(e) The graph shows that within the above concentrations (0·5 to 10 mg. per cent. w/v of khellin) there is a slight deviation from Beer's Law. Therefore, a calibration table will replace more conveniently the graph and give more accurate results, when the calibration table and the estimations are made at the same room temperature and under the same conditions.

Calibration Table

As a concentration of 39·232 per cent. w/v of sulphuric acid may be practically obtained by mixing 10 ml. of 10N sulphuric acid with 2·5 ml. of aqueous khellin solution the following procedure has been adopted for the preparation of the calibration table.

From the standard stock solution of khellin (0·5 per cent. w/v) are prepared standard dilutions so that 2·5 ml. of each dilution contain an amount of khellin ranging from 0·05 to 1 mg. and increasing in the order of 0·05 mg.; 2·5 ml. of each dilution are accurately measured in a dry colorimeter tube; 10 ml. of 10N sulphuric acid are added, well mixed; left to stand for about five minutes, then the percentage transmission of the solution is read, in a Lumetron Photoelectric colorimeter using blue filter 420 against water as the blank set at 100 per cent. transmission.

The results obtained at room temperature (25°C.) are shown in Table I.

TABLE I

Calibration table

Concentration of Khellin in mg. per cent.	Amount of Khellin in mg.	Percentage Transmission	Concentration of Khellin in mg. per cent.	Amount of Khellin in mg.	Percentage Transmission
0·4	0·05	93·0	4·4	0·55	49·0
0·8	0·10	87·0	4·8	0·60	47·0
1·2	0·15	80·5	5·2	0·65	44·0
1·6	0·20	75·5	5·6	0·70	41·0
2·0	0·25	71·0	6·0	0·75	39·0
2·4	0·30	65·0	6·4	0·80	37·5
2·8	0·35	61·0	6·8	0·85	35·0
3·2	0·40	57·0	7·2	0·90	33·0
3·6	0·45	54·0	7·6	0·95	31·5
4·0	0·50	51·0	8·0	1·00	30·0

ESTIMATION OF KHELLIN

METHOD OF ASSAY

(1) For the estimation of khellin in a test solution the following method is recommended. Make a dilution of the test solution so that 2·5 ml. contain an amount of khellin ranging between 0·05 and 1 mg. Accurately measure 2·5 ml. of this dilution into a dry colorimeter tube, add 10 ml. of 10N sulphuric acid; mix well; leave to stand for about 5 minutes; measure the percentage transmission of the solution and read the amount of khellin corresponding to the percentage transmission from the calibration table. The amount of khellin in the original test solution can then be obtained by calculation.

(2) For the estimation of khellin in oily preparations the following method is recommended. Dilute a known volume of the oily preparation with light petroleum so that 10 ml. of this dilution contains an amount of khellin ranging between 1 and 8 mg. Accurately measure 10 ml. of this dilution in a dry separating funnel; add 100 ml. of 39·232 per cent. w/v of sulphuric acid (obtained by diluting 80 ml. of 10N sulphuric acid to 100 ml. with distilled water); shake the mixture for about 15 minutes, then leave to stand for 15 minutes to separate. Filter about 15 ml. of the aqueous layer into a dry colorimeter tube; measure the percentage transmission of the solution and read the concentration of khellin corresponding to the percentage transmission from the calibration table. The figure obtained is the amount in mg. of khellin contained in 10 ml. of the diluted oil solution.

DISCUSSION

It has been found experimentally that, on mixing at room temperature 2·5 ml. of an aqueous solution of khellin with 10 ml. of 10N sulphuric acid the optical density of the solution becomes stable after leaving the mixture to stand for about 5 minutes at room temperature; and it remains stable for more than 24 hours.

On mixing the solution of sulphuric acid with the solution of khellin a rise of about 2°C., in the temperature and a contraction in the volume of about 0·5 ml. per cent. of the solution takes place. The rise in temperature and the contraction in the volume of the solution within these limits do not affect, to any appreciable extent, the optical density of the solution, i.e., a difference of about 2°C. in the temperature and a variation in the volume up to 0·5 ml. per cent. of the solution does not affect the optical density of the solution to any appreciable extent. On applying this method to accurately weighed amounts of pure khellin the results obtained did not differ by more than ±2 per cent.

Visnagin, another constituent of the fruits of *Ammi Visnaga* Linn., may be present, as an impurity, in pharmaceutical preparations of khellin to an extent of about 5 to 10 per cent. of the weight of khellin present. As visnagin gives with sulphuric acid a yellow colour which, when compared with that given by khellin, is found to be about 50 per cent. less in intensity. In this case, the results obtained will not differ by more than ± 5 per cent.

Moreover, alcohol was found to interfere to a certain extent with the proper development of the colour. Therefore, alcoholic solutions of

khellin should be diluted with water before applying this method, so that the alcohol content of the coloured solution does not exceed 1 per cent. v/v.

SUMMARY

(1) A photoelectric colorimetric method for the assay of khellin is described.

(2) This method is recommended for the assay of pure khellin in pharmaceutical preparations.

(3) The assay is carried out within the limits of 0·1 to 1 mg. of khellin.

Work is proceeding for the application of this method on galenical and other pharmaceutical preparations. The results will be compared with those obtained by the other colorimetric methods.

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