USE OF ACID–DYE TECHNIQUE IN THE ANALYSIS OF NATURAL PRODUCTS. III. SPECTROPHOTOMETRIC MICRODETERMINATION OF KHELLIN AND BERGAPTEN

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Abstract.—A simple spectrophotometric determination of each of khellin and bergapten, based on the complexation reaction with bromothymol blue, was developed. The yellow complex, in either case, was extracted with chloroform over the pH 3–7 range. The solution of each complex in chloroform showed an absorption maximum at 420 nm and the optimum pH in both cases was pH 4.4. Beer's law is obeyed over the concentration range of 2–16 μg/ml for khellin and 2–14 μg/ml in the case of bergapten. The method was successfully applied to the estimation of total furocoumarins (calculated as bergapten) in *Ammi majus* fruits and extracts and to the estimation of total furanochromones (calculated as khellin) in fruits and extracts of *Ammi visnaga*. Estimation of khellin in pharmaceutical dosage forms (tablets and injections), both alone and in combination with either papaverine or atropine, was also accomplished by this acid-dye complexation technique.

Individual furanochromones or furocoumarins may be determined by this technique after their separation by preparative tlc.

Khellin and bergapten are reported by Farnsworth *et al.* (1) and by Farnsworth (2) to react with Dragendorff's reagent in a manner typical of alkaloids. It was determined that any non-nitrogenous organic compound having a conjugated ($\Delta^{\alpha-\beta}$) carbonyl (ketone or aldehyde) or lactone function would react positively with a number of alkaloidal reagents (1–2). This prompted the present investigation to determine whether such alkaloid-like behaviour of khellin and bergapten could be extended to acid-dye complexation technique.

It has been established that alkaloids are capable of forming chloroform-soluble alkaloidal complexes with acid-dyes, which can be used in their spectrophotometric determination (3–8). It was of interest to study the capability of of khellin and bergapten to form similar chloroform-soluble complexes with acid-dyes and the applicability of this method for their determination in the crude drug, in galenicals, and in pharmaceutical preparations.

Most of the existing methods for the estimation of khellin are essentially spectrophotometric (9), ir-spectrophotometric (10) or colorimetric (11–14) methods. Karawya *et al.* (15) oxidized khellin with nitric acid to the corresponding quinone and measured the violet color obtained from such quinone with NaOH.

Reported methods for the estimation of furocoumarins include gravimetric (16), polarographic (17), spectrophotometric (18–20), high-pressure liquid chromatographic (21–22), spectrofluorimetric (23–25), or colorimetric methods involving either coupling with diazonium salts (26) or with hydroxylamine hydrochloride (27).

This report describes a new sensitive spectrophotometric method for the estimation of khellin and bergapten based on the complexation reaction of each with bromothymol blue, followed by extraction of the complex in chloroform and measurement of the absorbance of the chloroform solution at 420 nm. The method described is simple, straightforward, and reproducible. It can be carried

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out with readily available equipment and compares well with other reported methods in terms of speed and sensitivity.

MATERIAL AND METHODS[2]

REAGENTS AND CHEMICALS.—All solutions were prepared from reagent grade chemicals.[3] Freshly prepared 2×10^{-4} *M* bromothymol blue in $CHCl_3$ was used. Khellin solution containing 0.1 mg/ml in 50% alcohol and bergapten solution (0.1 mg/ml in ethanol) were prepared. Aqueous solutions of atropine sulfate and papaverine HCl, each containing 0.1 mg/ml of alkaloid base, were used. McIlvaine's buffer solutions (pH 3–7) were prepared from 0.1 *N* citric acid and 0.2 *M* Na_2HPO_4. Chloroform solutions of each of khellin and bergapten containing 4 μg/ml of either compound were prepared.

KHELLIN-CONTAINING DRUGS AND PREPARATIONS.—Powdered *Ammi visnaga* fruits;[4] khellin injections and tablets;[5] khellin and papaverine injections;[6] khellin and atropine injections;[7] khellin and atropine tablets;[7] pure khellin;[8] liquid extract of *A. visnaga* (28).

BERGAPTEN-CONTAINING PREPARATIONS.—Powdered *Ammi majus* fruits;[4] liquid extract of *A. majus* (28); pure bergapten.[8]

GENERAL PROCEDURE.—A sufficient quantity of either khellin or bergapten solution containing 50–400 μg of either compound was pipeted into a 100 ml-separator containing 10 ml of the buffer solution (pH 3–7 as needed). Bromothymol blue solution (10 ml) was added, and the mixture was vigorously shaken for 2 min. The organic layer was separated, and the aqueous layer was further extracted successively with 10 and 5 ml of chloroform. The combined chloroform extract was adjusted with chloroform to 25 ml in a volumetric flask. The absorbance of the solution was then measured at 420 nm against a similarly prepared reagent blank.

DETERMINATION OF BERGAPTEN IN FRUITS AND EXTRACT OF *Ammi majus*.—The powdered fruit (10 g) was exhaustively extracted with 100 ml of alcohol in a continuous extraction apparatus. A volume of 1 ml of the resulting extract was diluted to 50 ml with alcohol, and 1 ml of the latter solution was used for the determination of the the total furocoumarin content (calculated as bergapten) as in the general procedure.

DETERMINATION OF KHELLIN IN FRUITS AND EXTRACT OF *A. visnaga*.—The powdered fruit (10 g) was exhaustively extracted with 100 ml of alcohol in a continuous extraction apparatus. A volume of 1 ml of the extract was diluted to 50 ml with 50% alcohol. The furanochromone content (calculated as khellin) was determined by the general procedure using 1 ml of the latter solution.

A volume of 1 ml of *A. visnaga* extract (28) was similarly diluted to 50 ml with 50% alcohol, and the furanochromone content was determined as above.

DETERMINATION OF KHELLIN IN PHARMACEUTICAL PREPARATIONS CONTAINING ONLY KHELLIN:

a) *Injections*.—An exactly measured volume, corresponding to 10 mg of khellin, was diluted with 50% alcohol to 100 ml, and 1 ml of the resulting solution was used for the determination by the general procedure.

b) *Tablets*.—An exact weight, corresponding to 10 mg of khellin, from the pooled content of 10 powdered tablets was quantitatively extracted with 50% alcohol, and the volume of the extract was diluted with the same solvent to 100 ml. A volume of 1 ml of the resulting solution was used for the determination by the general procedure.

DETERMINATION OF KHELLIN IN PHARMACEUTICAL PREPARATIONS CONTAINING EITHER ATROPINE OR PAPAVERINE.—The same general procedure was used to measure the absorbance of the complex(es) at both pH 3 and pH 4.4. The absorbance at pH 3 represents absorbance due to either atropine or papaverine bromothymol blue complex; that at pH 4.4 represents the absorbance due to both khellin and alkaloid complexes (7). Calibration curves for atropine and papaverine at pH 3 and at pH 4.4 were determined by the same general procedure.

RESULTS AND DISCUSSION

The possibility of complex formation between each of khellin and bergapten with bromothymol blue was investigated by studying the shift in λ max of bromo-

[2]Bausch & Lomb Spectronic 700 spectrophotometer was used.
[3]B.D.H. grade.
[4]Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo Univ., Cairo, Egypt.
[5]Lynamine Ampoules & Tablets, Memphis Chemical Co., Cairo, Egypt.
[6]Lynamine Co. Ampoules, Memphis Chemical Co., Cairo, Egypt.
[7]Khellalgin Ampoules & Tablets, Misr Co., Cairo, Egypt.
[8]Memphis Chemical Co., Cairo, Egypt.

thymol blue, khellin and bergapten and their bromothymol blue complexes figure 1).

It was found that both khellin and bergapten, similar to alkaloids (3–8), are capable of forming chloroform-soluble complexes with bromothymol blue. The complex formation was tested at different pH values from pH 3–7. Khellin and bergapten bromothymol blue complexes show maximum absorption at 420 nm in $CHCl_3$ (figure 1).

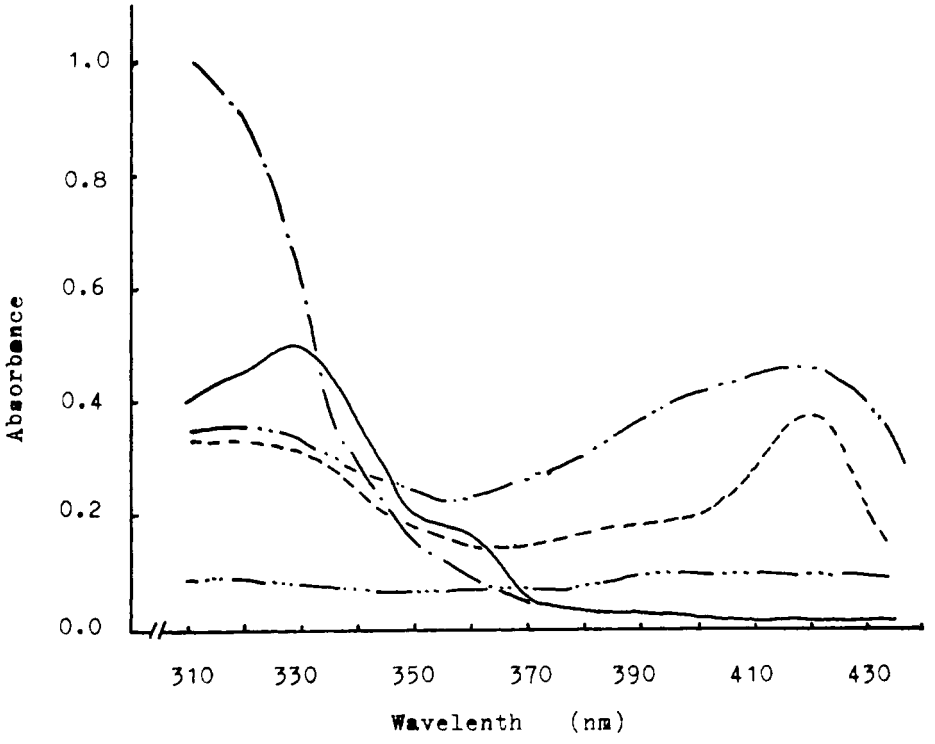

FIG. 1. *Absorbance of khellin* (————————), *bergapten* (—·—·—·—), *bromothymol blue* (—·····—·····—), *khellin bromothymol blue complex* (— — — — — — —) *and bergapten bromothymol blue complex* (—·····—·····—) *in* $CHCl_3$.

The optimum complexation was observed at pH 4.4 for both complexes (figure 2). This pH value is therefore recommended for khellin or begapten determination by this acid-dye complexation technique and was used in their estimation in this report.

BEER'S LAW, RANGE AND PRECISION.—The optimum concentration range for the measurements which conforms to Beer's law, at 420 nm and a 1.0 cm optical path, was 2–16 μg/ml in case of khellin and 2–14 μg/ml in case of bergapten (figure 3). The relative standard deviation of the calculated absorptivities in the optimum pH and concentration range was $\pm 1.0\%$.

Light absorption of khellin and of bergapten bromothymol blue complex was observed over the pH 3.4–7 range with the maximum absorption at pH 4.4. Both complexes show no light absorption at pH 3.

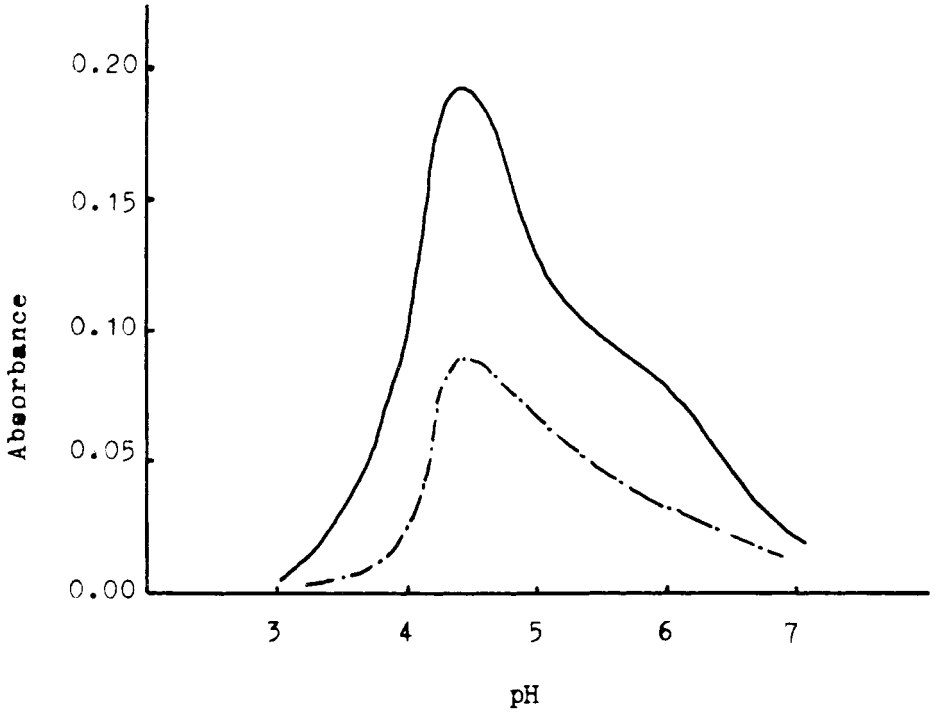

FIG. 2. *Absorbance of bromothymol blue complexes each of khellin (————————) and bergapten (— — — — — —) prepared over the pH 3–7 range, in $CHCl_3$ and at 420 nm.*

DETERMINATION OF KHELLIN IN PHARMACEUTICAL PREPARATIONS:

a) *Preparations containing khellin only.*—When khellin is the only compound capable of forming a complex with bromothymol blue, the determination is straightforward and gives a rapid and precise determination of the khellin content with no interference. The method can be applied for the determination of the total furanochromone content (calculated as khellin) in crude extracts without prior separation of the pure compounds. Individual furanochromones may be estimated by this technique after their separation by preparative thin-layer chromatography.

b) *Preparations containing atropine or papaverine in addition to khellin.*—Pharmaceutical preparations containing khellin together with either atropine or papaverine are available as smooth muscle relaxant preparations. Papaverine and atropine were found to form complexes with acid-dyes suitable for their determination (7). It was found that the bromothymol blue complexes with both alkaloids are extracted with chloroform at pH 3 as well as at pH 4.4. At pH 3, khellin does not interfere with the determination of either alkaloid. Calibration curves were, therefore, constructed for both atropine and papaverine at pH 3 and pH 4.4. The absorbance at pH 4.4 is due to the sum of absorbances of the khellin complex and the complex of the respective alkaloid.

Beer's law was found to hold over the concentration range of 2–14 μg/ml in the case of atropine complex at pH 3 and in the case of papaverine complexes at both

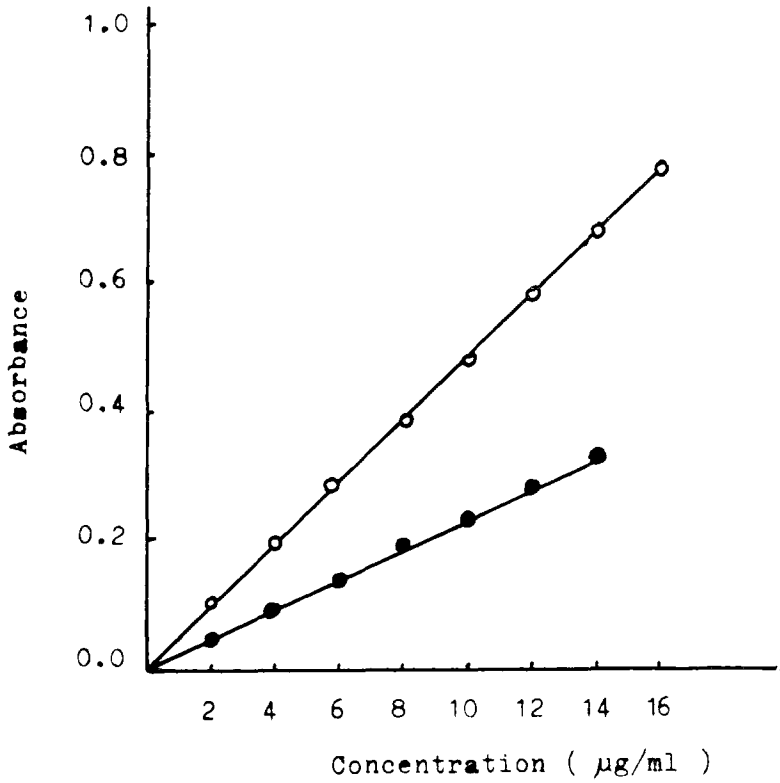

FIG. 3. *Calibration curves of khellin (——○——) and bergapten (——●——) bromothymol blue complexes prepared at pH 4.4, in $CHCl_3$ and at 420 nm.*

pH values, while the range for atropine complex at pH 4.4 was 2–12 μg/ml (figure 4). The following interrelationships were derived:

a) *Khellin and papaverine:*

1) Milligrams papaverine in mixture $= \frac{A}{3.4}$

2) Milligrams khellin in mixture $= \frac{B-(0.5\ A)}{1.7}$

where A = absorbance at 420 nm of the papaverine bromothymol blue complex at pH 3; 3.4 = slope of the curve at pH 3 (fig. 4).

B = absorbance at 420 nm of complexes of both khellin and papaverine at pH 4.4; 1.7 = slope of the curve at pH 4.4 (fig. 3) for the khellin complex.

0.5 = ratio of slopes of the calibration curves of papaverine at pH 4.4 and at pH 3.

b) *Khellin and atropine:*

1) Milligrams atropine in mixture $= \frac{C}{3.7}$

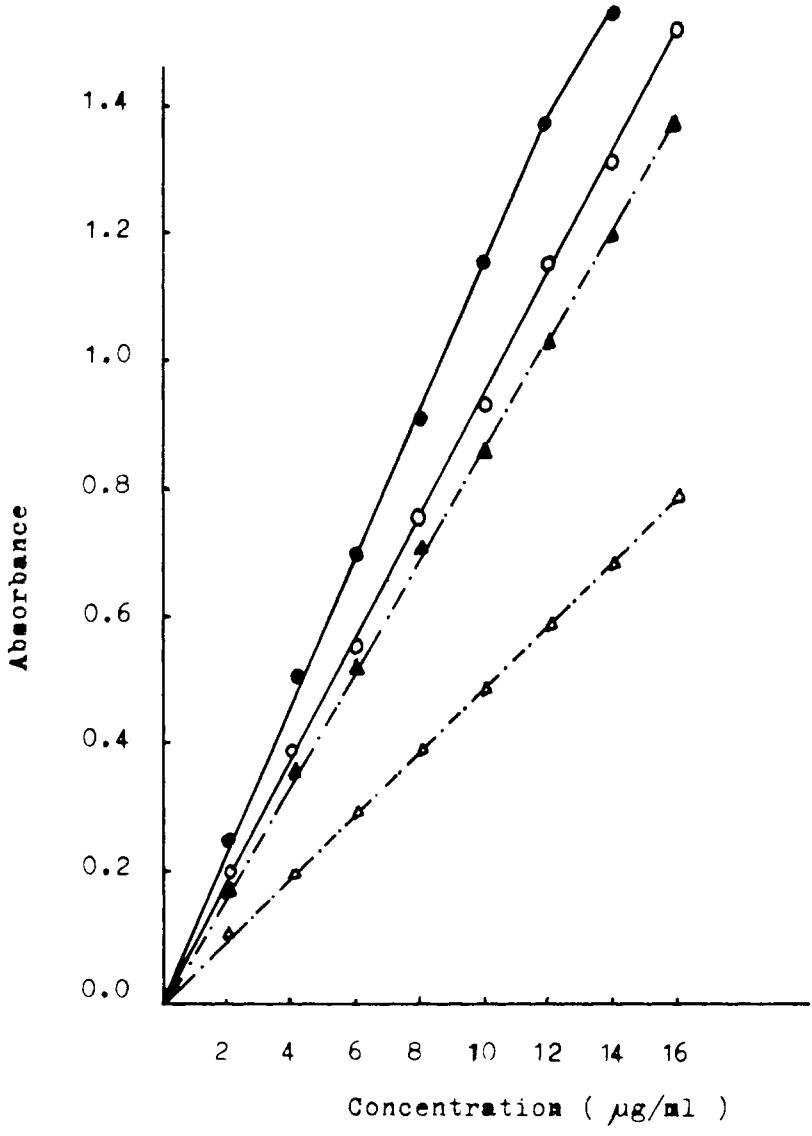

FIG. 4. *Calibration curves of atropine bromothymol blue complexes at pH 3 (———○———) and at pH 4.4 (———●———), and of papaverine bromothymol blue complexes at pH 3 (—·—·—▲—·—·—) and at pH 4.4 (—·—·—△—·—·—), in $CHCl_3$ and at 420 nm.*

2) Milligrams khellin in mixture $= \dfrac{D-(1.27\ C)}{1.7}$

where C = absorbance at 420 nm of atropine bromothymol blue complex at pH 3; 3.7 = slope of the curve (fig. 4).

D = absorbance at 420 nm of the complexes of both atropine and khellin in mixture at pH 4.4; 1.7 = slope of the curve for khellin complex at pH 4.4 (fig. 3).

1.27 = ratio of the calibration curves of atropine at pH 4.4 (slope = 4.7) to the calibration curve at pH 3 (slope = 3.7).

The results of the various analyses are shown in table 1.

A method has been reported for the estimation of khellatrine tablets containing khellin in mixture with both papaverine and atropine (29). Papaverine and atropine were estimated by the acid-dye technique using bromothymol blue, while khellin was estimated by a colorimetric method using methalonic H_2SO_4.

TABLE 1. *Determination of khellin in pharmaceutical dosage forms.*

Prep. no.	Dosage form and/or ingredients	Stated potency	Found potency	% Label strength
Extracts:				
1	Ammi visnaga extract (28)	0.5% w/w	0.495%	99
Injections:				
2	Lynamine ampoules[a]			
	khellin	50 mg/2 ml	50.5 mg/2 ml	101
3	Glucolynamine ampoules[a]			
	khellin	30 mg/10 ml	30.06 mg/10 ml	100.2
4	Lynamine Co ampoules[a]			
	khellin	50 mg/ml	50.0 mg/ml	100
	+			
	papaverine HCl	40 mg/ml	38.4 mg/ml	96
5	Khellalgin ampoules[b]			
	khellin	50 mg/2 ml	50.5 mg/2 ml	101
	+			
	atropine	0.5 mg/2 ml	0.475 mg/2 ml	95
Tablets:				
6	Lynamine tablets[a]			
	khellin	20 mg/tab.	20.2 mg/tab.	101
7	Lynamine Co tablets[a]			
	khellin	100 mg/tab.	100.0 mg/tab.	100
	+			
	papaverine HCl	50 mg/tab.	49.0 mg/tab.	98
8	Khellalgin tablets[b]			
	khellin	100 mg/tab.	101.0 mg/tab.	101
	+			
	Belladonna Extract	80 mg/tab. (corresponding to 0.8 mg/tab. of total alkaloids)	0.728 mg/tab.[c]	91

[a]Memphis Chemical Co., Cairo, Egypt.
[b]Misr Drug Co., Cairo, Egypt.
[c]Calculated as atropine.

DETERMINATION OF BERGAPTEN IN *A. majus* EXTRACT.—Estimation of bergapten through complexation with bromothymol blue can be done on the crude extract as well as on the pure compound. The results compare well with the spectrophotometric methods (18–19). When applied to crude extracts, the method determines the amount of total furocoumarins calculated as bergapten. Individual components may be estimated by this technique after their separation by preparative tlc.

In conclusion, the reported method can be successfully applied to the microdetermination of khellin and bergapten in galenicals and various dosage forms. The method compares favorably with other reported methods for these compounds in terms of speed of the process and limits of detection.

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