The results are shown in Table II.

Figure 2 shows a plot of the log $H_{i,j}$ values (Table III) *versus* the carbon number for the alcohols, amines, and alkanes in heptane. The free energy of the methylene group was about 730 cal/mole for the transfer of a methylene group from organic to vapor phases. The hydroxyl and amino group contributions also may be obtained from Fig. 2 by subtracting the alkane in heptane values for each corresponding carbon number as in Eq. 7. These values were 1320 cal/mole for the hydroxyl group and 1490 cal/mole for the amino group from organic to vapor phases (Table II).

The group contribution to the free energy of transfer for the methylene group from water to organic phases was −851 cal/mole based on the data for the amines. The value found for the methylene group in the alcohol studies was −907 cal/mole. Both of these values agree with reported literature values for the methylene group (Table II).

The group contribution to the free energy of transfer for the amino group from water to the organic phase was 4980 cal/mole. Pescar and Martin (20) used GLC to study the thermodynamic solution properties of two-component volatile nonelectrolyte solutions at infinite dilution. Their method for the determination of γ_i^∞ was based on chromatographic retention volumes, and the value reported for the free energy contribution of transfer of the amino group from water to the organic phase was 5140 cal/mole.

The group contribution to the free energy change for the hydroxyl group from water to the organic phase was 5570 cal/mole, which is in reasonable agreement with the literature values shown in Table II.

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Spectrophotometric Determinations of 3-Dimethylaminomethylkhellin Hydrochloride and Khellin

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Received October 7, 1976, from the **Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt*, and the ‡*Stability Research Department, The Nile Company for Pharmaceuticals and Chemical Industries, El-Ameria, Cairo, A.R. Egypt.*
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Abstract □ Spectrophotometric assays are proposed for the determination of 3-dimethylaminomethylkhellin hydrochloride and khellin in bulk chemical and dosage forms. The acid dye method, using methyl orange at pH 5, is applied to assay the amine in the form of an ion-pair extractable in chloroform with maximum absorbance at 420 nm. The perchloric acid method, depending on formation and extraction of the oxonium salts of both compounds, is used to assay the amine and khellin at 333 or 430 nm and at 325 or 410 nm, respectively. The reineckate method can be used to assay the amine as the reineckate derivative in acetone with maximum absorbance at 530 nm. However, small amounts of the amine (1.5–3 mg) can be determined as the reineckate in methanol with maximum absorbance at 245 nm. Stability determination of the two compounds can be done by the acid dye and perchloric acid methods. The three methods are sufficiently accurate, sensitive, and precise.

Keyphrases □ 3-Dimethylaminomethylkhellin—spectrophotometric analyses in bulk drug and dosage forms, various methods compared □ Khellin—spectrophotometric analyses in bulk drug and dosage forms, various methods compared □ Spectrophotometry—analyses, 3-dimethylaminomethylkhellin and khellin in bulk drug and dosage forms, various methods compared □ Spasmolytic agents—3-dimethylaminomethylkhellin and khellin, spectrophotometric analyses in bulk drug and dosage forms, various methods compared

3-Dimethylaminomethylkhellin hydrochloride (I) possesses approximately three times the spasmolytic activity of khellin and is only half as toxic (1). Clinical trials showed that I is well tolerated for the treatment of acute renal colics with negligible side effects (2).

For the quantitative determination of I, either the basic

Table I—Effect of pH on Extraction of I–Dye Complexes[a] in Chloroform

pH of Buffer Solution[b]	Absorbance (1 cm) of Complex Using Methyl Orange (1.28×10^{-3} M)	Bromthymol Blue (6.4×10^{-4} M)
3.0	0.340	0.465
4.0	0.470	0.485
4.6	0.535	—
5.0	0.590	0.545
5.5	0.580	—
6.0	0.360	0.555
7.0	0.100	0.555
7.5	—	0.525
8.0	—	0.290

[a] Obtained from 1.0 ml (200 μg) of I solution with 3.0 ml of the dye solution and 6 ml of the required buffer extracted with 20.0 ml of chloroform. [b] Phthalate buffers were pH 3–5.5, and phosphate buffers were pH 6–8.

tertiary amino group or the chromone moiety can be used. Methods generally applied for basic amino group determinations include titration in nonaqueous solvents or with anionic surfactants and precipitation with picric acid, silicotungstic acid, tetraphenylboron, reineckate, and acid dyes. The furanochromone moiety exhibits strong absorption bands in the UV region amenable to spectrophotometric determination (3, 4). Many color reactions were described for the qualitative identification of khellin (5–9), and some are applicable to its spectrophotometric assay (5, 9–11).

Previous methods for khellin require either relatively long procedures (10, 11), with low sensitivity in the color reactions (5, 9), or some preliminary separation to eliminate possible interferences with UV spectrophotometry (3).

This report describes some sensitive and rapid spectrophotometric methods for the determination of I and khellin. These methods can be adopted for their pharmaceutical formulations.

EXPERIMENTAL[1]

Materials and Reagents—The following were used: I[2]; khellin[3]; methyl orange[4] aqueous solutions ($0.642–6.4 \times 10^{-3}$ M); bromthymol blue[5] in pH 7 buffers ($2.4–12 \times 10^{-4}$ M); 0.05 M phthalate buffer (BP 1973), pH 3–5.5; 0.05 M phosphate buffer (BP 1973), pH 6–8; I standard solutions in pH 5 and 7 buffers (10 mg of I/250 ml of the required buffer); I standard aqueous solution (20 mg of I/100 ml of water); khellin standard aqueous solution (25 mg of khellin dissolved in 2.5 ml of acetic acid and diluted with water to 250 ml); I standard chloroform solution (20 mg of I/100 ml of chloroform); khellin standard chloroform solution (25 mg of khellin/250 ml of chloroform); perchloric acid solution (~60% w/w) (60 g of perchloric acid[6] diluted to 70 g with water); acetic acid[5]; chloroform[5]; ammonium reineckate solution (2 g of reineckate[7]/100 ml of methanol); methanol[4]; and acetone[4].

Sample Preparations—*Bulk Chemical and Dosage Forms of I*—Dissolve or dilute and filter, if necessary, a weighed quantity or measured volume of the sample in a suitable quantity of water. Make successive dilutions to reach approximately 40 or 200 μg or 3.3 mg of I/ml in the acid dye, perchloric acid, or reineckate methods, respectively. Perform dilutions with water, but use pH 5 buffer in the acid dye method.

Khellin Bulk Chemical and Dosage Forms—Dissolve a weighed quantity of the solid sample in the minimum amount of acetic acid, dilute with water, and filter, if necessary. Alternatively, dilute a measured volume of the liquid sample with water to obtain approximately 100 μg of khellin/ml.

[1] Spectrophotometric measurements were done on a Pye Unicam SP 500.
[2] Recrystallized.
[3] Memphis Chemical Co., Cairo, Egypt.
[4] El Nasr Co., Cairo, Egypt.
[5] E. Merck, Darmstadt, West Germany.
[6] Hopkin & Williams (71% w/w), Essex, England.
[7] Rhone Poulenc, Prolabo, France.

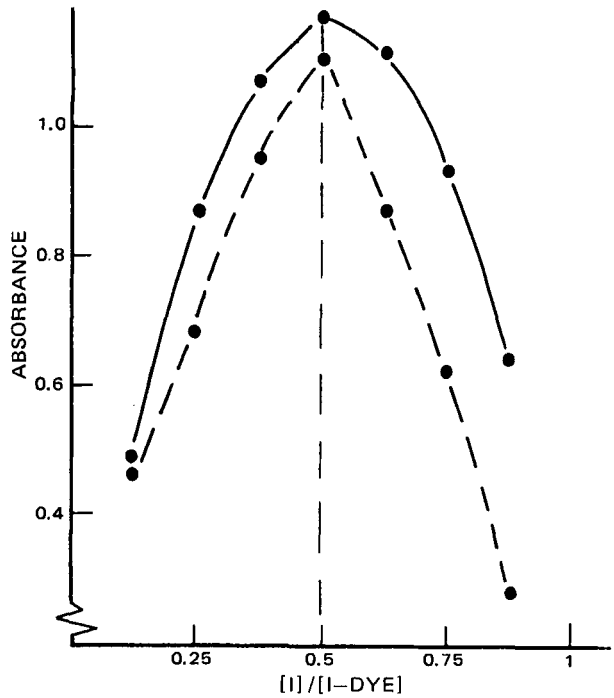

Figure 1—*Continuous variation curves obtained from 1.28×10^{-3} M solution of I with methyl orange (—) and from 8.4×10^{-4} M solution of I with bromthymol blue (– –).*

Acid Dye Assay—Pipet 3.0 ml of the I sample preparation into a 50-ml separator and dilute with 3.0 ml of the pH 5 buffer. Add 3.0 ml of methyl orange solution (3.2×10^{-3} M) and shake the mixture thoroughly for 2 min with 20.0 ml of chloroform. Separate and centrifuge the chloroform layer for 2 min at 2000 rpm and measure the absorbance at 420 nm against a chloroform blank made from the dye solution and the buffer.

Repeat the experiment three times and determine the exact amount of I from a calibration curve.

Perchloric Acid Assay—Pipet 2.0 ml of the I or khellin sample preparation into a 50-ml separator, saturate with sodium chloride, and shake the mixture thoroughly for 2 min with 15.0 ml of chloroform. Allow layers to separate. Transfer 10.0 ml of the chloroform layer into a dry 50-ml separator and add, with swirling, 2.0 ml of glacial acetic acid and 5.0 ml of perchloric acid solution. Shake gently and then separate and centrifuge the upper layer for 2 min at 2000 rpm.

Measure the absorbance of the separated layer at either 333 or 430 nm for I and at 325 or 410 nm for khellin against a blank of acetic acid. Determine the exact amount of I or khellin from constructed calibration curves at the specified wavelengths.

Reineckate Assay—Pipet 15.0 ml of the I sample preparation into a 100-ml beaker containing 10 ml of dilute hydrochloric acid (BP 1973). Precipitate I by adding, with continuous stirring, 25 ml of the ammonium reineckate methanolic solution and leave for 1 hr in the refrigerator.

Filter the I reineckate onto a sintered-glass crucible (porosity 4). Then wash with 2 × 5 ml of cold diluted ammonium reineckate solution (1 ml of the methanolic reineckate in 50 ml of water) and with 2 × 5 ml of ice cold water. Dissolve the I reineckate in 25.0 ml of acetone and measure the absorbance at 530 nm against an acetone blank. Determine the exact amount of I from a calibration curve.

RESULTS AND DISCUSSION

Compound I–Dye Complexes—Methyl orange and bromthymol blue formed complexes with I, with maximum absorbances in chloroform at 420 and 410 nm, respectively. Table I shows that optimum extraction of the dye complexes in chloroform occurred at about pH 5 with methyl orange and at pH 6–7 with bromthymol blue. Dye concentrations of 1.065×10^{-3} M methyl orange and 2.8×10^{-4} M bromthymol blue were required to reach maximum absorbance values at a fixed I concentration of 6.286×10^{-5} M in the aqueous phase. The color intensities of the yellow complexes in chloroform were stable for 2 hr at room temperature.

Stoichiometric Balance—Figure 1 illustrates a stoichiometric relationship of 1:1 between I and the acid dyes.

Adherence to Beer's Law—The yellow complexes of I (40–200 μg) with

Table II—Assay Results[a] of I in Pharmaceutical Preparations following the Acid Dye, Perchloric Acid, and Reineckate Spectrophotometric Methods

Dosage Form	Acid Dye Method	Perchloric Acid Method		Reineckate Method
		At 430 nm	At 333 nm	
Tablet (30 mg)	29.6	30.0	29.5	29.64
	29.6 29.73	29.5 29.66	29.5 29.50	29.64 29.45
	30.0 ± 0.2364	29.5 ± 0.2955	—	29.07 ± 0.3368
Tablet (50 mg)	53.83	54.75 54.40	53.44 53.72	—
	55.51 54.19	54.06 ± 0.6113	54.01 ± 0.499	—
	53.25 ± 1.335	—	—	—
Ampul, 2 ml im, A (50 mg)	48.00	47.95 48.16	—	48.0 48.0
	48.00 47.9	47.95	—	48.0
	47.71 ± 0.1714	48.60 ± 0.3841	—	—
B (50 mg)	51.63	51.25 51.25	50.88 51.06	53.50
	54.16 53.08	51.25	51.25 ± 0.3278	—
	53.37 ± 1.495	—	—	—
Ampul, 5 ml iv (30 mg)	29.16	29.12	30.0	29.00
	28.12 28.67	28.89 29.15	29.57 29.71	—
	28.75 ± 0.6146	29.45 ± 0.3309	29.57 ± 0.2541	—

[a] Shown in milligrams.

the acid dyes obeyed Beer's law. A 1.25 times increase in the sensitivity determination of I was obtained with methyl orange compared to bromthymol blue, as indicated by a more steep slope of color formation with the former.

Analysis of I in Pharmaceutical Preparations—Microamounts of I could be determined with methyl orange as a complex-forming agent. Table II shows good recovery and precision. Khellin, ω-acetylkhellinone, and dimethylamine, involved in the synthesis of I, did not interfere with the assay. However, when bromthymol blue was used, only dimethylamine interfered.

Effect of Decomposition Products—Aqueous acid solutions of I, autoclaved at 115° for 30 min, showed complete recovery of I by the acid dye method. Heating the material (50 mg) with 10% sodium hydroxide in 50% ethyl alcohol (6 ml) at the boiling point of the water bath for the same period resulted in a very low recovery (6.5%) of I by the same method. Accordingly, the acid dye method with methyl orange was considered valid for determining I in the presence of its decomposition products.

Perchloric Acid Oxonium Salt of I and Khellin—Khellin forms colored oxonium salts with concentrated sulfuric and phosphoric acids. The oxonium phosphate of khellin (7) readily hydrolyzes to khellin on addition of water. Likewise, khellin and I formed the corresponding oxonium salts with perchloric acid. On addition of water, however, I oxonium perchlorate underwent hydrolysis to I perchlorate (mp 202–203°), an amine.

Absorption of I and Khellin Oxonium Perchlorates in UV–Visible Region—The oxonium salts of I and khellin showed two maxima at 333 and 430 nm and at 325 and 410 nm, respectively. The absorption spectra of both compounds in the perchloric–acetic acid mixture as compared to those in methanol showed bathochromic shifts, attributed to the formation of new chromophoric entities (maxima at 430 and 410 nm) and hyperchromic shifts due to increases in absorption bands (maxima at 333 and 325 nm). Accordingly, the calculated $E^{1\%}_{1\,cm}$ values of I at 333 nm and of khellin at 325 nm in the acid mixture were ~143 and 252 instead of ~125 and 180 in methanol, respectively.

The absorption ratio for I oxonium salt at 333 nm compared to that at 430 nm was 2.026 ± 0.0208; for khellin oxonium salt, the absorption ratio at 325 nm compared to that at 410 nm is 2.021 ± 0.0513 (Table III).

Optimum Conditions for Extraction of Oxonium Salts—In the presence of small amounts of water, oxonium salts undergo hydrolysis. Accordingly, it was essential to extract I and khellin from water into chloroform before adding perchloric acid to form a stable oxonium salt. This extraction was completely accomplished by shaking sodium chloride-saturated I and khellin preparations (2.0–5.0 ml) with chloroform (15.0 ml). Complete recovery of I and khellin into chloroform was ascertained by the perchloric acid assay when different amounts of I and khellin standard aqueous solutions were extracted and compared with the standard chloroform solutions.

The optimum conditions for extraction of the oxonium salts were obtained by shaking I and khellin chloroform solutions (10.0 ml) with 60% (w/w) perchloric acid (5.0 ml) in the presence of acetic acid (2.0 ml). The presence of less than 2.0 ml of acetic acid did not allow good separation of perchloric acid from chloroform, and the use of higher concentrations of perchloric acid neither altered the color intensity nor contributed to the ease of separation of the two phases. Replacement of perchloric acid with syrupy phosphoric acid produced no change in the color intensity of the formed oxonium but caused difficulty in pipetting and withdrawal because of the high viscosity of the acid.

Stability of I and Khellin Oxonium Salts—The yellow colors of the oxonium salts in 60% (w/w) perchloric–acetic acid (5:2) remained stable for 2 hr at room temperature and tolerated the presence of trace amounts of moisture.

Adherence to Beer's Law—The oxonium salts in the acid mixture obeyed Beer's law for 120–400 μg of I at 333 nm, for up to 600 μg of I at 430 nm, for 50–200 μg of khellin at 325 nm, and for up to 300 μg of khellin at 410 nm.

Analysis of I and Khellin in Pharmaceutical Preparations—Tables II and IV include the assay results of I and khellin preparations through formation of the oxonium salt with perchloric acid. Absorbance was measured at either of their maximum peak wavelengths with sufficient accuracy and precision. The common solubilizers of khellin—*viz.*, urethan, sodium salicylate, sodium benzoate, glucose, and dihydroxypropyltheophylline, when added in 100 times the concentration of khellin, did not interfere with the assay.

Assay of I and Khellin Binary Mixtures—A total assay of I and khellin mixtures could be carried out by the perchloric acid assay at the predetermined isoabsorptive wavelength, 333.5 nm, of I and khellin. The method of location of the isoabsorptive point was practically determined on the separate materials as described previously (12). Compound I was then assayed by the acid dye method, and the amount of khellin could be obtained by difference. Alternatively, I and khellin could be individ-

Table III—Absorption Ratios of I and Khellin Oxonium Perchlorate Obtained from Absorbances at Maximum Peak Values

	I Oxonium Salt ($A_{333\ nm}/A_{430\ nm}$)	Khellin Oxonium Salt ($A_{325\ nm}/A_{410\ nm}$)
	2.023	2.071
	2.000	1.999
	2.060	2.055
	2.025	1.960
	2.014	
	2.036	
Mean	2.026 ± 0.0208	2.021 ± 0.0513

Table IV—Assay Results[a] of Khellin in Pharmaceutical Preparations following the Perchloric Acid Spectrophotometric Method

Dosage Form	410 nm	325 nm
Tablet (20 mg)	19.03	18.37
	18.92 18.96	18.37 18.41
	18.92 ± 0.650	18.50 ± 0.768
Ampul[b], 2 ml (100 mg)	103.1 103.9	103.10 102.79
	104.7 ± 1.417	102.48 ± 0.549
Ampul[c], 10 ml iv (30 mg)	29.40	29.25
	29.40 29.27	29.25 28.85
	28.95 ± 0.266	28.05 ± 0.709

[a] Shown in milligrams. [b] Lynamine, Memphis Chemical Co., Cairo, Egypt. [c] Glucolynamine, Memphis Chemical Co., Cairo, Egypt.

Table V—Recovery Results of I and Khellin in Synthetic Mixture Applying the Acid Dye and Perchloric Acid Methods

Mixture[a], mg	Acid Dye		Perchloric Acid			
			Total I and Khellin		% I	% Khellin
	Found, mg[b]	% Recovery	Found, mg[c]	% Recovery	Recovery[c]	Recovery[c]
I, 66.6, and khellin, 133.4	66.95	100.50	203.28	101.64	101.50	101.71
I, 133.4, and khellin, 66.6	135.56	101.62	197.72	98.36	98.3	98.46
I, 100, and khellin, 100	98.80	98.80	197.72	98.36	98.04	98.68
Mean		100.30 ± 1.42		99.45 ± 1.88		

[a] Dissolved in the minimum amount of acetic acid and appropriately diluted with the required solvent. [b] Mean of three results. [c] Mean of two results.

ually assayed by the same method, applying the absorbance ratio procedure for binary mixtures (13) at 325 and 333.5 nm. Table V presents the recovery results on synthetic mixtures and depicts sufficient accuracy and precision.

Effect of Decomposition Products—Results showed that the perchloric acid method could determine as low as 6.56 and 5.3% of I and khellin, respectively, in their alkaline hydrolysates, and the assay result of I agreed well with the result obtained by the acid dye method.

I Reineckate Derivative—A complete precipitation of I in an acid medium took place after 30 min with an aqueous solution of the reineckate reagent. However, with a 2% methanolic solution of the reineckate, an easily filtrable crystalline precipitate was quantitatively produced after 1 hr in the refrigerator. The reaction between I and reineckate proceeded in a stoichiometric relation of 1:1 (Table VI) and may be represented by Scheme I.

$$C_{16}H_{16}(CH_3)O_5N\cdot HCl + NH_4Cr(NH_3)_2(SCN)_4$$

I (0.5555 g)

$$\xrightarrow{H_2O} C_{16}H_{17}(CH_3)O_5NCr(NH_3)_2(SCN)_4 + NH_4Cl$$

I reineckate (1 g)

Scheme I

Compound I reineckate was slightly soluble in methanol and very soluble in acetone. It was not affected by heating at 105° for 1 hr.

In acetone solution, the reineckate derivative showed a maximum at 530 nm. In methanolic solution, it showed two maxima in the UV region at 245 and 310 nm.

Adherence to Beer's Law—Compound I reineckate obeyed Beer's law for I amounts of 20–70 mg/25 ml of acetone at 530 nm and for 200–500 μg/100 ml of methanol at 245 nm.

Quantitative Determination of I—Because a linear relation exists between I and amount of the precipitated reineckate, Table VI demonstrates assay results of I bulk chemical with good recovery and precision.

Spectrophotometric Analysis of I in Pharmaceutical Preparations—Results shown in Table II depict sufficient accuracy and precision.

CONCLUSION

Sensitive methods are described for the spectrophotometric determination of I and khellin in bulk chemical and pharmaceutical preparations. The applied techniques can differentiate and characterize I from khellin. Of these methods, the acid dye and perchloric acid techniques are stability indicating for I or both compounds.

Compound I, being a base, forms acid dye complexes with methyl orange at pH 5 and with bromthymol blue at pH 6–7. Accordingly, based on this ion-pair formation, optimum conditions were studied to assay I with methyl orange as a complex-forming agent between 40 and 200 μg of I at 420 nm.

Perchloric acid produced an oxonium salt with khellin, but with I it formed both the amine and oxonium salts. The UV–visible absorption spectra of I and khellin oxonium salts were compared and showed two maxima characterizing the chromone moiety of both I and khellin. The absorption ratios of the oxonium salts in the perchloric acid–acetic acid mixture at the maximum peak value in the UV region to the maximum peak value in the visible region represent fixed values.

For the spectrophotometric determination of I and khellin as the oxonium salts, optimum conditions for extraction and color development were chosen to permit the determination of 120–400 μg of I and 50–200 μg of khellin in the UV region and up to 600 μg of I and 300 μg of khellin in the visible region. Tablet excipients and solubilizers of khellin did not interfere.

Ammonium reineckate precipitated I in an acid medium in a stoichiometric relationship of 1:1. The precipitated reineckate is acetone soluble and forms the basis for a colorimetric determination of I (30–70 mg) at the maximum absorbance of 530 nm. A much more dilute solution of I reineckate in methanol (200–500 μg/100 ml) showed two maxima at 245 and 310 nm. Its high absorptivity at 245 nm was useful for determining 1.5–3 mg of I as the reineckate.

Table VI—Gravimetric Assay Results of I as the Reineckate Derivative

Sample, mg	Weight of Reineckate, mg	Found, mg	Recovery, %
52.25	96.0	53.34	102.1
	93.7 95.03	52.00 52.79	99.71 100.77
	95.4	53.03	101.50
56.0	100.9	56.04	100.07
58.5	105.9	58.82	100.55
	105.4	58.54	100.08
	105.0	58.32 58.56	99.69
	106.5 105.44	59.15	101.12 100.11
	104.4	58.00	99.13
60.0	109.0	60.66	101.10

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