Khellin Determination in Human Serum and Urine by High-Performance Liquid Chromatography

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Abstract □ A rapid, sensitive, and specific high-performance liquid chromatographic method was developed for the determination of khellin in serum and urine using theophylline as the internal standard. Chromatography was performed on a reversed-phase C_{18} column with 65% (v/v) methanol as the mobile phase. The assay is capable of measuring khellin concentrations down to 100 ng/ml with a total procedure time of <20 min. Oral administration of 60 mg of khellin tablets to two healthy human males was carried out, and the serum samples were analyzed by the described method. Serum concentration–time profiles are presented.

Keyphrases □ Khellin—high-performance liquid chromatographic analysis in human serum and urine □ High-performance liquid chromatography—analysis, khellin in human serum and urine □ Vasodilators—khellin, high-performance liquid chromatographic analysis in human serum and urine

Khellin (4,9-dimethoxy-7-methyl-5*H*-furo[3,2-*g*][1]-benzopyran-5-one, I), the active ingredient of *Ammi visnaga*, is used mainly as a vasodilator in the treatment of renal colic and renal stones.

Gravimetry (1), colorimetry (2, 3), and TLC–colorimetry (4) are used for the determination of I in formulations and *A. visnaga* extracts. The gravimetric assay is tedious and lacks the desired sensitivity (5), while the colorimetric methods suffer from color instability and inefficiency (4). An analytical method for the determination of this drug in biological fluids has not been available, and no information has been presented on its elimination and distribution patterns in humans.

This report describes a rapid, specific, accurate, and sensitive high-performance liquid chromatographic (HPLC) method for the determination of I in serum and urine. The procedure involves a simple, one-step solvent extraction followed by HPLC using theophylline (II) as an internal standard.

EXPERIMENTAL

Reagents and Solvents—Khellin[1] (I) was recrystallized from isopropanol[1]. Theophylline[2] (II) was used as supplied. Chloroform[3] and methanol[3] were analytical reagent grade and were used without further purification. Double-distilled water was employed in the mobile phase.

Apparatus and Operating Conditions—The analysis was performed using a high-performance liquid chromatograph[4] with a 254-nm UV detector[5], an injection system[6], and a 30-cm × 4-mm i.d. reversed-phase, high-efficiency C_{18} column[7]. The mobile phase was methanol–water (65:35), which was filtered twice through a 0.45-μm pore filter[8] and degassed before use. The flow rate was 0.8 ml/min but occasionally varied between 0.6 and 1 ml/min.

[1] Koch-Light Laboratories, Colnbrook-Bucks, England.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] E. Merck, Darmstadt, West Germany.
[4] Model 6000, Waters Associates, Milford, Mass.
[5] Model 440, Waters Associates, Milford, Mass.
[6] Model U6K, Waters Associates, Milford, Mass.
[7] μBondapak C_{18}, Waters Associates, Milford, Mass.
[8] Millipore Corp., Bedford, Mass.

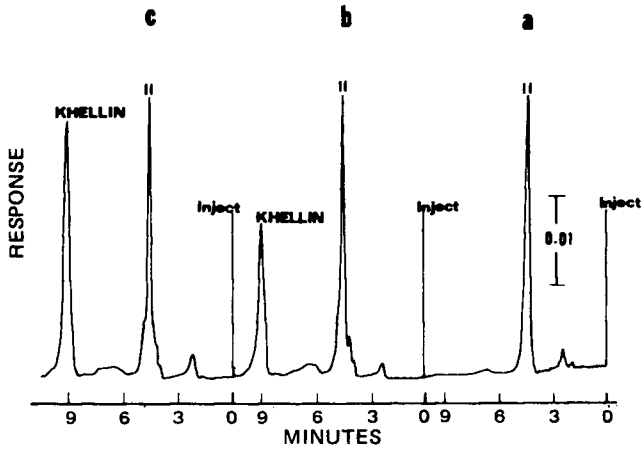

Figure 1—*Chromatograms of 1-ml human serum samples containing 5 μg of theophylline (internal standard). Key: a, taken prior to khellin administration; b, taken 6.5 hr after oral administration of 60-mg khellin tablets; and c, spiked with 1 μg of khellin. The mobile phase was 65% (v/v) methanol at a flow rate of 0.8 ml/min, and the chart speed was 0.5 cm/min.*

Extraction—A 1-ml portion of water, serum, or urine was transferred to a 13-ml centrifuge tube, and 5 or 20 μg of II and 7 ml of chloroform were added. The tube was vortexed for 60 sec and centrifuged for 8 min at 2000 rpm. After the aqueous creamy phase was aspirated and discarded, 5 ml of the organic phase was transferred to a glass tube and evaporated in a water bath at 60°. The residue was reconstituted with 0.2 ml of the mobile phase by shaking it for 30 sec on a vortex mixer, and 15–25 μl was injected onto the column.

Standard Curves—Calculations were carried out using standard curves constructed by analyzing 1-ml samples of the respective fluid spiked with 5 or 20 μg of II and known amounts of I and plotting the peak height ratio (I/II) *versus* the corresponding concentration of I. The standard curve data were subjected to least-squares linear regression analysis, and the resulting equation was utilized for the calculation of the drug concentration in the unknown samples.

Serum Level Study in Humans—Two healthy human males were given three 20-mg tablets of khellin[9] after fasting for 12 hr. Blood samples (5 ml) were removed from the median cubital vein at the indicated time intervals and placed in centrifuge tubes. After allowing the blood to clot, serum was cultivated and stored frozen until analysis. The assay was performed in duplicate.

Urine Collection—Urine was collected from one subject at various intervals after drug administration, and the volume was recorded. Samples (1 ml) were transferred into stoppered centrifuge tubes and stored frozen until assay. Urine collection was stopped 11 hr after drug administration.

RESULTS

Figure 1 shows typical chromatograms of a blank serum sample with a sample spiked with 1 μg of khellin (I) and another sample obtained from a healthy human volunteer 6.5 hr after oral administration of 60 mg of I. All three samples contained 5 μg of theophylline (the internal standard, II). The specificity of this method is clearly demonstrated by the absence of interfering peaks. With 65% methanol in water as the eluent at a flow rate of 0.8 ml/min, the retention times for I and II were 9 and 4.4 min, respectively.

[9] Lynamine, Memphis Chemical Co., Cairo, Egypt.

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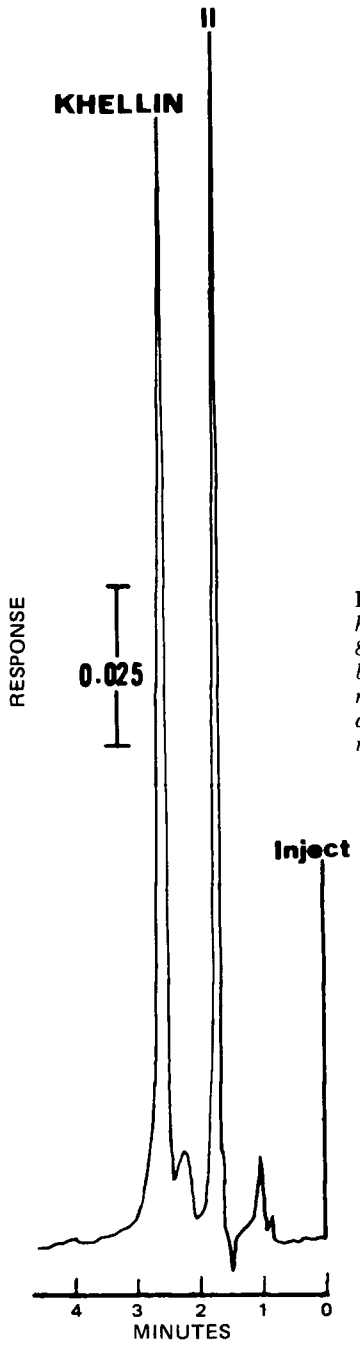

Figure 2—*Chromatogram of 1-ml human serum sample spiked with 8 μg of I and 20 μg of II. The mobile phase was 70% (v/v) methanol at a flow rate of 1.0 ml/min, and the chart speed was 0.5 cm/min.*

Figure 2 represents a chromatogram of a urine sample spiked with 8 and 20 μg of I and II, respectively. The retention times for I and II were 5 and 3.3 min, respectively, using 70% methanol as the mobile phase at a flow rate of 1.0 ml/min. The small peak preceding the drug peak was due to an impurity, which appeared to be adequately separated from the drug. Complete resolution was obtained by decreasing the flow rate and the methanol content of the mobile phase.

Recovery—The described procedure for the extraction of I from serum and urine is simple, rapid, and efficient. There was no back-extraction involved, and the extraction was carried out in <10 min. The relative serum to water recovery of I as measured by the ratio of the slopes of the standard curves of the peak height ratio *versus* concentration obtained for I in serum and water, respectively, was 0.88. Addition of 0.1 ml of 0.1 *N* NaOH to the samples did not increase the relative recovery. There was excellent linearity between the peak height ratios and khellin concentrations in serum and urine ($r > 0.999$ for both) in the concentration range employed, *i.e.*, 0.1–5 μg/ml for serum and 1–20 μg/ml for urine.

Accuracy—Table I shows the amounts found and the actual amounts with which 1-ml blank serum samples were spiked and analyzed according to the described method. The experiment was performed in duplicate, and the percent error for each unknown sample was calculated according to (6):

Table I—Accuracy of HPLC Assay for Khellin in Serum

Amount Added, μg	Experiment 1 Amount Found, μg	Experiment 1 Percent Error	Experiment 2 Amount Found, μg	Experiment 2 Percent Error
1.0	1.04	4	1.03	3
2.0	1.90	5	1.86	7
4.0	3.96	1	4.12	3
8.0	8.04	0.5	7.82	2.25
10.0	9.98	0.2	9.28	7.2

$$\% \text{ error} = \frac{\text{amount added} - \text{amount found}}{\text{amount added}} \times 100 \qquad \text{(Eq. 1)}$$

As can be seen in Table I, the percent error was ~0.2–5%. In no case did it exceed 7.5%, indicating the excellent accuracy and reproducibility of the described method.

Reliability—Since no other method is available for the determination of I in serum and urine, comparison of this HPLC assay with another method was impossible. However, the accuracy demonstrated for this method indicates that it is reliable for assaying I in these biological fluids.

Serum Levels and Urine Collection Data—The serum khellin concentration–time profiles are shown in Fig. 3. The total amount of the unchanged drug excreted through the urine in 11 hr was only 48.1 μg.

DISCUSSION

The lack of an analytical method for the determination of khellin (I) in biological fluids has precluded its clinical monitoring in patients. Indeed, since the therapeutic serum index for this drug has not been established, dosing is quite arbitrary. The daily dose of I ranges from 20 to 100 mg, and dosing frequency varies from once to three times daily. Furthermore, no information is available on the half-life and other pharmacokinetic parameters for this drug.

To the knowledge of the present investigators, this report is the first to deal with the determination of khellin in biological fluids and the first to give data on the oral absorption of this drug in humans. As shown in Fig. 3, the serum khellin levels following the administration of 60 mg of khellin tablets ranged from 0.100 to 1.7 μg/ml, and the half-life, as determined from the semilogarithmic plot of the three terminal data points assuming rapid absorption, was ~1.5–5.6 hr. The fraction of the dose excreted unchanged through the urine in 11 hr was only 0.8%. Therefore, it appears that the elimination of I occurs principally by metabolism, and, hence, analysis of the unchanged drug in the urine may be of little interest.

Further investigation to extend this preliminary information on the

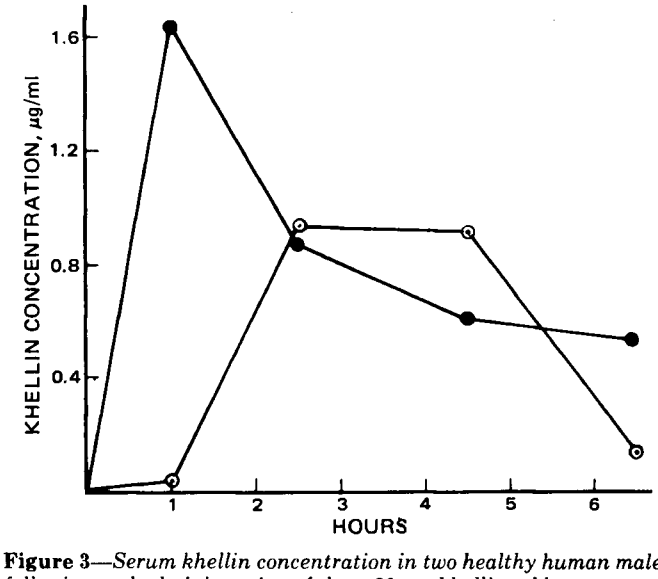

Figure 3—*Serum khellin concentration in two healthy human males following oral administration of three 20-mg khellin tablets.*

pharmacokinetics of I in humans is underway, and the results will be reported later.

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(3) M. S. Karawya, G. Nour, and A. Sina, *Planta Med.*, **19**, 367 (1971).

(4) M. S. Karawya, M. A. El-Kiey, and G. Nour, *J. Pharm. Sci.*, **59**, 1025 (1970).

(5) I. R. Fahmy, M. A. El-Kiey, and A. Shoaib, *Proc. Pharm. Soc. Egypt., Sci. Ed.*, **5**, 91 (1953).

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Differential Pulse Polarographic Determination of Colchicine

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Received December 4, 1979, from the *Food and Drug Administration, Department of Health, Education, and Welfare, 850 Third Avenue, Brooklyn, NY 11232.* Accepted for publication June 4, 1980.

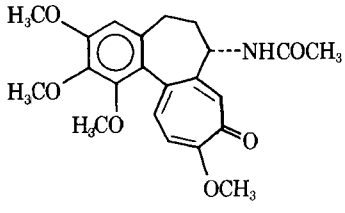
Abstract □ A differential pulse polarographic method for the analysis of colchicine-containing dosage forms is described. The reduction of the tropolone carbonyl is useful for quantitative analysis in that the relationship of the colchicine concentration to the current is linear over the concentration range of 0 to ~50 μg/ml, with a slight negative deviation at higher concentration. The procedure involves no preliminary treatment, is simple and specific, and is applicable to the assay of composite tablets, individual tablets, and injectable solutions. Polarography is conducted on a solution of colchicine in pH 1.81 Britton–Robinson buffer with 0.01% alkylphenoxy polyethoxyethanol. The quantitative analysis is achieved using the method of standard addition. A relative standard deviation of 3.2% was obtained for tablets. The results agree with those obtained using the USP XIX method.

Keyphrases □ Colchicine—analysis by differential pulse polarography □ Polarography, differential pulse—analysis of colchicine

Colchicine (I), an alkaloid obtained from various species of *Colchicum*, is used for the suppression of gout. The USP XIX assay for colchicine tablets involves extraction with chloroform followed by a spectrophotometric determination. The quantity of the drug specified to be taken for the assay is 3 mg. Since the usual tablet strength is 0.5 mg, this amount is equivalent to six tablets. For a content uniformity test, the method needs to be scaled down sixfold to accommodate a single tablet. As a result, the aqueous phase volume, as well as the organic volumes used for extractions, becomes less than optimum, so that difficulties with the procedure may be encountered.

Colchicine has a tropolone ring which bears a reducible carbonyl group, similar to an aromatic aldehyde that was determined by classical direct-current polarography in Britton–Robinson or McIlvane buffer (1–3). Reduction of colchicine at a dropping mercury electrode was studied at various pH levels by Sartori and Guadiano (4). These investigators reported that below pH 8, two reduction waves are obtained; the potential of the first wave becomes more negative with increasing pH and reaches that of the second wave at pH ~8. Above pH 8, there is only one wave, whose potential does not vary with pH and corresponds to the formation of a secondary alcohol. This reduction is irreversible (4).

However, the sensitivity of direct-current polarography is limited due to the large capacitance current contribution to the Faradaic current. This difficulty is reduced in differential pulse polarography. This paper describes the application of differential pulse polarography to the determination of colchicine in dosage forms with the goal of developing a general method applicable to the assay of composite tablets as well as individual tablets and injection solutions.



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EXPERIMENTAL

Apparatus and Polarographic Conditions—A polarographic analyzer[1] equipped with a drop timer[2] in conjunction with a three-electrode system was used for polarographic determinations. The electrodes were a dropping mercury electrode, a saturated calomel electrode, and a platinum wire auxiliary electrode. The drop timer was set at 1 sec, and the height of the mercury column was 70 cm. Other conditions were: current range, 2–5 μamp; pulse amplitude, 50 mv; and scan rate, 5 mg/sec. The potential range scan was from −0.7 to −1.2 v. All polarograms were recorded on an *x–y* recorder[3].

Reagents and Chemicals—All chemicals were reagent grade unless otherwise specified. The standard was colchicine USP[4].

Supporting Electrolyte Solution—One liter of solution was prepared to contain 0.04 *M* acetic acid, 0.04 *M* phosphoric acid, and 0.04 *M* boric acid in water (pH 1.81). Different pH buffers were prepared for the pH *versus* polarographic behavior study by mixing 100 ml of the supporting electrolyte solution with the required volumes of 0.2 *M* NaOH and checking with the pH meter[5].

Standard Colchicine Solution—Colchicine standard, 25 mg, was accurately weighed and dissolved with supporting electrolyte solution in a 25-ml volumetric flask and then diluted to volume with the same solvent.

Surfactant Solution—The surfactant solution was 0.5% alkylphenoxy polyethoxyethanol in water[6].

[1] Model 174, Princeton Applied Research Corp., Princeton, N.J.
[2] Model 174/70, Princeton Applied Research Corp., Princeton, N.J.
[3] Omnigraphic model 2200-3-3, Houston Instruments, Austin, Tex.
[4] City Chemical Corp., New York, N.Y.
[5] Zeromatic SS-3, Beckman Instruments, Fullerton, Calif.
[6] Triton X-100, Rohm & Haas, Philadelphia, Pa.

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