Table II—Assay Results on Commercial Antiseptic (Percent Concentration of Hexylresorcinol) [a]

Assay[b]	Percent Found	Percent Reported[c]
1	0.097	0.1
2	0.107	0.1
3	0.106	0.1
4	0.107	0.1

[a] Average deviation = 0.004, *SD* = 0.005, and coefficient of variation = 4.8%. [b] Replicate assays of same sample. [c] Data from "Handbook of Nonprescription Drugs," 5th ed., American Pharmaceutical Association, Washington, D.C., 1977, p. 263.

DISCUSSION

The results (Table II) show that a commercial antiseptic solution can be assayed for hexylresorcinol by using a simple HPLC method. No interfering peaks were observed from the other listed component (glycerin) in the commercial product analyzed. Recovery studies were performed on the aqueous hexylresorcinol solution (0.05%), and the extraction efficiency was 74.9%. The extraction and HPLC analyses of aqueous standard solutions gave a linear response with hexylresorcinol concentrations between 0.025 and 0.125%.

This analysis utilized an aqueous standard carried through the exact procedure as the samples. Since the aqueous standard closely simulated the samples, the concentrations were calculated by comparing peak heights of the aqueous standard and the unknown sample. Extraction efficiency affects both samples and standards equally and, therefore, need not be considered in the calculation of concentrations.

A control also was run with each batch to monitor instrument conditions. This procedure minimizes assay errors due to technique. The total analysis time was less than 10 min/sample.

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High-Pressure Liquid Chromatographic Assay of Folic Acid: A Collaborative Study

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Abstract □ A collaborative study of the USP high-pressure liquid chromatographic assay for folic acid was performed. Two samples were analyzed in duplicate by 14 participating laboratories. Relative standard deviations for a single determination (*RSDS*) ranged from ±0.40 to ±2.39%. Based on an analysis of variance, it was concluded that the method of peak measurement was a major determinant of reproducibility and that graphical measurement was associated with a high standard deviation. Adequate resolution was obtained using a variety of columns and operating conditions. The interlaboratory *RSDS* was ±1.8%.

Keyphrases □ Folic acid—high-pressure liquid chromatographic analysis in prepared samples, collaborative study □ High-pressure liquid chromatography—analysis, folic acid in prepared samples, collaborative study □ Vitamins—folic acid, high-pressure liquid chromatographic analysis in prepared samples, collaborative study

A recently developed high-pressure liquid chromatographic (HPLC) method (1) for the assay of folic acid bulk substance was introduced into the USP (2) to replace the spectrophotometric method (3). In a subsequent supplement (4), the new method was revised slightly.

The HPLC method is more specific and accurate than the previous one. A collaborative study[1] was undertaken to validate the precision and practical applicability of this HPLC method on a wide scale.

EXPERIMENTAL

Four vials containing 100 mg each of two folic acid test samples (A and B), 75 mg of folic acid USP reference standard, and 20 mg of leucovorin calcium[2] (calcium formyltetrahydrofolate) were sent to 19 collaborators, along with a copy of the method (5) and auxiliary instructions for performing the determination and reporting results. Collaborators were requested to make duplicate injections of each of two weighings of Samples A and B and the folic acid reference standard and to report raw data and results, calculated on the dry basis, using the average response from the duplicate injections in the calculation.

The method as written allowed the individual collaborator considerable latitude in choice of column and operating conditions. However, a system suitability test required that the operator obtain a resolution factor of at least 3.6 between folic acid and leucovorin and a relative standard deviation no greater than ±2% in the responses from six to 10 replicate injections of the standard preparation.

The amounts of folic acid provided were sufficient for two 30-mg weighings by each collaborator plus equipment optimization and checkout. The water content of the folic acid was determined (duplicate 50-mg samples in methanol) before subdivision by the titrimetric method and reported to the collaborators (reference standard, 7.7%; Sample A, 7.6%; and Sample B, 7.8%). Collaborators were requested to report, in addition to raw data and assay results, results of the system suitability test and certain information about instrument and operating conditions.

Raw data from all collaborators were entered into a time-shared computer, and results were calculated by a user-written program.

[1] A subcommittee of the PMA-QC Section, appointed by Dr. H. Hammer and chaired by Dr. J. Sheridan, was responsible for carrying out this study.

[2] Provided by Lederle Laboratories.

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Table I—Summary of Operating Information

Collaborator	Column Length × i.d., cm	Stationary Phase[a]	Mobile Phase, % methanol	Pressure, psi	Flow Rate, ml/min	Injection Device	Injection Volume, μl	Amount Injected, μg	Measurement Method[b]	Retention Time[c], min	Resolution Factor	RSD[d], %
1	30 × 0.4	B	5	590	1	20-μl loop	20	NR[e]	E	22.6	3.7	0.96
2	30 × 0.4	B	4	2300	2.0	10-μl loop	10	30	H × W	13.8	3.7	1.12
3	30 × 0.4	B	3	1000	1.5	20-μl loop	20	NR	E	18.2	4.7	0.7
4	25 × 0.4	B	3	1750	1.7	25-μl syringe	5	NR	H × W	11.0	3.1	2.0
5	30 × 0.4	B	6	1300	1	20-μl loop	20	10	E	9.7	3.7	0.21
6	25 × 0.5	P	10	600	1	20-μl loop	20	30	E	14.0	3.9	0.95
7	30 × 0.4	B	4	1500	1.0	10-μl loop	10	NR	H	15.0	3.0	1.17
8	25 × 0.5	P	6.5	1690	2.5	25-μl loop	25	6	E	9.5	3.8	0.58
9	25 × 0.5	P	2	1000	1.5	20-μl loop	20	6	H	32.7	5.0	1.22
10	25 × 0.3	S	8	2700	1.2	20-μl loop	20	1	E	7.7	3.9	0.92
11	33 × 0.4	B	5	1000	1.5	25-μl syringe	20	4	H × W	8.8	2.0	0.78
12	30 × 0.2	B	4	2500	2	10-μl loop	10	30	H	25	3.7	1.1
13	30 × 0.4	B	6	1400	1	10-μl loop	10	0.6	H	53.5	4.6	1.4
14	30 × 0.4	B	4	1500	2	10-μl loop	10	30	E	13.1	3.8	0.36

[a] B = μBondapak C_{18}, P = Partisil ODS, and S = Spherisorb ODS. [b] E means electronic, H means peak height only, and H × W means height times width. [c] Typical or average retention time of folic acid. [d] Relative standard deviation obtained in system suitability test. [e] NR means collaborator did not specifically report any deviation from the recommended amount of 30 μg.

RESULTS

Fourteen collaborators (including this laboratory) reported results. Operational information is summarized in Table I. Although a few laboratories were unable to achieve the separation and precision required, a wide range of instrumental conditions can be used to obtain adequate separation between folic acid and leucovorin.

Results for all collaborators are shown in Table II. Collaborators 4, 7, and 11 should not be considered representative of the method because the minimum resolution factor was not obtained. However, even these cases are in good agreement with the other collaborators, presumably because, with the low content of impurity present, poor separation did not change the result very much.

To determine more specifically the sources of error in the method, an analysis of variance was performed. The reproducibility of the chromatographic portion of the procedure (*i.e.*, injection and peak measurement) was evaluated for each collaborator from the relative differences between responses for duplicate injections of samples and standard (*i.e.*, six pairs of numbers). The reproducibility of the overall procedure was evaluated by comparing results obtained for the duplicate weighing of Samples A and B, using the average of instrument responses for two injections. From these data, the variance of the preparation steps alone can be calculated. The equation is derived as follows.

The formula for calculation of results is:

$$\frac{W_s/d}{W_x/d} \times \frac{R'_x/i}{R'_s/i} \times 100 = \% \qquad \text{(Eq. 1)}$$

where W is the weight of the sample (x) or standard (s); R' is the average peak response of the sample (x) or standard (s); and d and i are the dilution volume and injection volume, respectively, and are the same for the sample and standard.

The first term in this expression represents the preparation of material for injection while the second term represents the injection and peak measurements. In multiplication and division, the squares of the relative standard deviations are additive; therefore:

$$2RSD_p^2 + 2RSD_{c'}^2 = RSD_o^2 \qquad \text{(Eq. 2)}$$

where p, c', and o refer to preparative, chromatographic, and overall relative standard deviation, respectively. Since responses from duplicate injections were averaged to obtain R', the $RSD_{c'}^2$ is equal to half of RSD_c^2 (RSD for a single injection) and the expression reduces to:

$$0.5(RSD)_o^2 - RSD_c^2) = RSD_p^2 \qquad \text{(Eq. 3)}$$

Table II—Summary of Results

Collaborator	A_1	A_2	$\bar{A}$	B_1	B_2	$\bar{B}$
1	100.5	99.6	100.1	100.8	99.2	100.0
2	98.5	101.3	99.9	98.2	99.1	98.7
3	100.5	98.6	99.6	97.9	99.1	98.5
4	96.9	101.5	99.2	97.2	98.4	97.8
5	99.9	97.6	98.8	100.4	100.5	100.5
6	99.3	98.9	99.1	99.7	98.5	99.1
7	98.4	96.3	97.3	95.9	94.5	95.2
8	96.0	95.2	95.6	97.9	96.3	97.1
9	99.4	98.3	98.9	96.4	98.8	97.6
10	101.5	99.3	100.4	98.3	98.3	98.3
11	101.8	99.8	100.8	100.3	99.7	100.0
12	100.0	101.1	100.6	103.9	100.0	101.9
13	98.5	99.0	98.8	100.5	99.9	100.2
14	98.8	100.3	99.5	97.9	99.1	98.5
Average	99.3	99.0	99.2	98.9	98.7	98.8
±*RSD*, %	1.7	1.8	1.4	2.1	1.6	1.7

Table III—Precision of the Method

Collaborator	Overall *RSD* (σ_o)	Chromatographic *RSD* (σ_c)	Preparation Relative Variance (σ_p^2)
1	0.93	0.25	0.40
2	1.47	0.51	0.95
3	1.17	0.79	0.37
4	2.39	2.50	−0.26
5	1.15	0.32	0.61
6	0.67	0.36	0.16
7	1.30	1.28	0.03
8	0.91	0.88	0.03
9	1.34	2.17	−1.46
10	1.11	0.86	0.24
11	1.07	0.58	0.41
12	1.99	1.87	0.24
13	0.40	0.60	−0.10
14	0.96	0.36	0.39

The variance of the preparation step for each collaborator was calculated with this equation (Table III). Except for the extreme negative value for Collaborator 9, the results are more uniform than the chromatographic variance. The average variance for the preparation step (excluding Collaborator 9) is 0.27, equivalent to an *RSD* of ±0.5%.

A possible explanation for the poorer chromatographic precision by some collaborators is the apparent correlation between a high *RSD* and graphical peak measurements (Table I). This correlation suggests that electronic area measurements are more precise than either height or height × width measurements.

One collaborator arranged for biological assays of the folic acid samples and reported 10 results on each sample. The average and standard deviation of these results were calculated and compared to the average results obtained by the chromatographic method, using the t test for significance of difference between two averages.

The results seem to indicate that the biological assay result for Sample A differ significantly from both the biological result for Sample B and the chromatographic result for Sample A. However, in view of the limited nature of the biological testing, the real significance of this difference is not known. In any case, the question was not pursued further since it was not the purpose of this collaborative study.

Upon discussion with collaborators, some recommendations concerning the description of the method were formulated:

1. The exact manner of preparation and degassing of the mobile phase should be left to the operator as long as the final solution has the proper pH.

2. The preparation and injection of folic acid solutions should be made more flexible to allow for smaller amounts of folic acid and larger injection volumes. The former change may be necessary to prevent overloading of some detectors while the latter change permits better reproducibility of manual injections.

3. The particle base may be nonspherical since its shape is not critical to the determination.

These recommendations have been incorporated in the latest USP supplement (4).

The majority of collaborators were able to achieve acceptable separation and precision with the chromatographic method in spite of the variety of instrumental and operating conditions used, indicating that the method should be widely applicable.

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Potential Antineoplastics I: Substituted 3,5-Dioxo- and 3-Thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines

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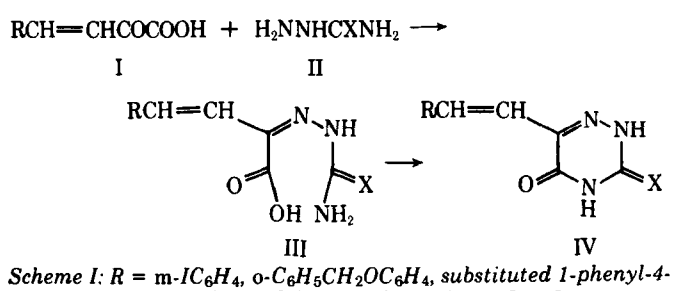
Received April 24, 1978, from the *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, A.R. Egypt.* Accepted for publication July 10, 1978.

Abstract □ The synthesis of some 6-substituted 3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines for possible antineoplastic activity is reported. The assigned structures were substantiated by IR, NMR, and mass spectral studies of representative members of the series. Four compounds were tested against P-388 lymphocytic leukemia and were inactive.

Keyphrases □ 1,2,4-Triazines, substituted—synthesized, antineoplastic activity evaluated in mice □ Antineoplastic activity—various substituted 1,2,4-triazines evaluated in mice □ Structure–activity relationships—substituted 1,2,4-triazines evaluated for antineoplastic activity in mice

Of the different 6-aza analogs of pyrimidines screened for anticancer potency, 6-(3,4-methylenedioxystyryl)-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine (IV, R = 3,4-$CH_2O_2C_6H_3$ and X = S, Scheme I) was more active than mercaptopurine and fluorouracil in animals (1, 2).

In a search for novel antineoplastic agents, it was of interest to prepare 6-styryl-3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines with new substituents



Scheme I: R = m-IC_6H_4, o-$C_6H_5CH_2OC_6H_4$, *substituted 1-phenyl-4-pyrazolyl, or* $C_6H_5CH{=}CH$ *and* X = *O or S*

on the phenyl ring (IV*a*–IV*c*, Table I). The diverse biological activities of the pyrazole nucleus suggested the synthesis of two new *as*-triazines in which a substituted 1-phenyl-4-pyrazolyl moiety replaced the phenyl ring: 6-[β-(3,5-dimethyl-1-phenyl-4-pyrazolyl)vinyl]- and 6-[β-(1,5-diphenyl-3-methyl-4-pyrazolyl)vinyl]-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (IV*d* and IV*e*, Table I). It was also of interest to prepare and screen *as*-triazines having the phenyl ring separated from position 6 by a 1,3-butadiene instead of the vinyl side chain: 6-(4-phenyl-1,3-butadienyl)-3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (IV*f* and IV*g*, Table I).

RESULTS AND DISCUSSION

Chemistry—For the synthesis of the new triazines, the reactions shown in Scheme I were followed. Condensation of arylidenepyruvic acids (I) with semicarbazide (II, X = O) hydrochloride or thiosemicarbazide (II, X = S) yielded semicarbazono- or thiosemicarbazonoarylidenepyruvic acids (III*a*–III*g*, Table I). Subsequently, III*a*–III*g* were cyclized to the corresponding 1,2,4-triazines (IV*a*–IV*g*, Table I) in the presence of sodium hydroxide. Compound IV*g* previously was synthesized by heating thiosemicarbazonocinnamylidenepyruvic acid (III*g*) with aqueous sodium carbonate for 3 hr (3). Conversion of IV*g* to IV*f* was reported to take place when IV*g* was heated with aqueous alkaline potassium permanganate solution followed by acidification of the reaction mixture (3).

The IR spectra of the triazines showed multiple bands in the 3500–2900-cm^{-1} region (NH stretching) and strong bands at 1720–1680 (C=O stretching) and 1560–1520 (amide II) cm^{-1}. In addition, the spectra of the 3-thio derivatives revealed a band of medium intensity between 1300 and 1270 cm^{-1} (C=S stretching) but lacked the band characteristic of the SH group of a thiol tautomer. These data indicate the existence of

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