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Purity Profiles of Pteroylglutamate Reference Substances by High-Performance Liquid Chromatography

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Abstract □ High-performance liquid chromatography (HPLC) in the reversed-phase mode was used for the purity analysis of three pteroylglutamic acid-type reference substances (folic acid, leucovorin calcium, and methotrexate). The influence of the pH of the mobile phase on the separation of an artificial mixture of six pteroylglutamic acid derivatives and three potential impurities was studied. Results of purity analysis of current lots of USP reference standards are reported. A better separation of methotrexate from its major impurities was achieved by using a standard buffer, rather than an ion-pairing mobile phase. A separation of methotrexate and its biologically inactive 7-isomer is reported.

Keyphrases □ Pteroylglutamate—purity profiles, reference substances, high-performance liquid chromatography, methotrexate □ High-performance liquid chromatography—pteroylglutamate, purity profiles, reference substances, methotrexate □ Methotrexate—pteroylglutamate, purity profiles, reference substances, high-performance liquid chromatography

Three USP drug substances, available as USP reference standards, belong to the class of pteroylglutamic acid derivatives: folic acid (I), methotrexate (II), and leucovorin calcium (III) (authentic substance as calcium formyltetrahydrofolate). This class of compounds exhibits high hydrolytic and oxidative reactivity. High-performance liquid chromatography (HPLC) has proved to be the method of choice for purity analysis in this laboratory because of similarities of structure within this series. Additional purity profile data were gained from 5 to 8 collateral determinations, but are not reported here as these results are independent of chromatographic purity.

Almost all the HPLC techniques have been used for the separation and analysis of this class of compounds. Anion-exchange columns were used first in 1973 for the separation of folic acid from a mixture of water-soluble vitamins (1) and then for the separation of folic acid and its reduced and N^5- and N^{10}-substituted derivatives (2). Anion-exchange chromatography alone, or coupled with an amine column operated in the reversed-phase mode, has also been used for the analysis and quantitative determination of methotrexate (3).

An extensive study (4) resulted in the development of a reversed-phase HPLC assay and purity analysis method for folic acid. The assay was made the object of a collaborative study (5), and it has been adopted into the folic acid monograph in the USP (6).

For the reversed-phase chromatographic analysis of methotrexate, mixtures of methanol or acetonitrile with the following buffers have been suggested as mobile phases: pH 3.5, 0.005 *M* ammonium formate (7); pH 5, 0.005 *M* ammonium acetate (7); pH 6.7, 0.1 *M* KH_2PO_4 (8); pH 6.8, 2-amino-2-hydroxymethyl-1,3-propanediol (tris) (9); pH 7.2, 0.05 *M* KH_2PO_4[1]; pH 6, 0.1 *M* citric acid–0.2 *M* Na_2HPO_4[2]. The latter is used in the assay of methotrexate in USP XX–NF XV (10). Mobile phases consisting of pH 5 phosphate buffer–acetonitrile (9) and pH 4 citrate buffer–dioxane (11) were recommended for the analysis and assay of leucovorin calcium. The effect of pH on the retention behavior of pteroyloligo-glutamates in reversed-phase HPLC has also been reported (12).

A third technique, ion-pair chromatography, separated 21 UV-absorbing impurities in a clinical sample of methotrexate (13) and has also been utilized in the separation of folic acid and its dihydro- and tetrahydro derivatives (14).

The object of this investigation was to study the influence of the pH of the mobile phase on the chromatographic separation of some pteryolglutamic acid derivatives and to determine optimum conditions for the purity analysis of the USP reference standards by HPLC.

EXPERIMENTAL

Apparatus—An isocratic high-performance liquid chromatograph[3] with a 254-nm detector and a gradient chromatograph[4] equipped with a variable wavelength UV detector[5] were used. The instruments were

[1] United States Pharmacopeia, Drug Research and Testing Laboratory, unpublished research.
[2] Lederle Laboratories, private communication.
[3] Altex Model 110, Altex Scientific Inc., Berkeley, Calif.
[4] Model 3500B, SpectraPhysics, Santa Clara, Calif.
[5] Model 770 Spectrophotometric detector, SpectraPhysics, Santa Clara, Calif.

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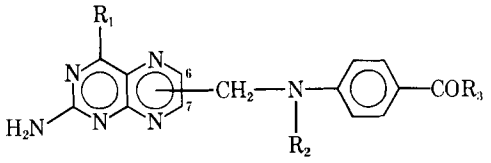
Table I—Chromatographic Separation of Some Pteroylglutamic Acid Derivatives and Potential Contaminants

Mobile Phase[a]	3.0–Methanol (100:18)		4.0–Methanol (100:14)		5.0–Methanol (100:14)		6.0–Methanol (100:13)		7.0–Methanol (100:15)		7.5–Methanol (100:13)	
Flow Rate, ml/min	2.0		2.2		2.0		1.8		2.0		1.8	
Compound	$t_{R,i}$, min	α_{II}[b]	$t_{R,i}$, min	α_{II}	$t_{R,i}$, min	α_{II}	$t_{R,i}$, min	α_{II}	$t_{r,i}$, min	α_{II}	$t_{R,i}$, min	α_{II}
IX	1.5	0.16	1.25	0.13	1.2	0.12	1.06	0.12	1.1	0.12	1.15	0.12
VIII	—	—	0.9	0.09	1.0	0.10	—	—	—	—	—	—
Leucovorin (III)	4.4	0.46	3.0	0.31	2.0	0.21	1.57	0.17	1.45	0.16	1.55	0.17
Folic acid (I)	7.9	0.82	6.0	0.63	3.45	0.36	2.1	0.23	1.8	0.20	1.8	0.19
Aminopterin (V)	5.9	0.61	5.1	0.53	4.0	0.41	3.45	0.38	3.1	0.34	3.45	0.37
Methopterin (VI)	12.2	1.27	9.55	1	5.85	0.61	3.65	0.40	3.25	0.36	3.05	0.33
7-Methotrexate (IV)	10.3	1.03	9.9	1.03	8.8	0.91	8.1	0.88	8.15	0.90	8.25	0.88
Methotrexate (II)	9.6	1	9.55	1	9.65	1	9.17	1	9.05	1	9.35	1
VII	15.7	1.64	25.2	2.64	26.8	2.78	19.0	2.07	14.35	1.59	13.35	1.43

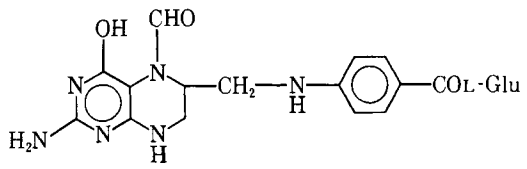
[a] 0.05 *M* KH_2PO_4 brought to either pH 3.0 or 4.0 with 85% H_3PO_4 and to pH 5.0, 6.0, 7.0, 7.2, and 7.5 with 1 *N* NaOH (Buffers pH 3–7.5, respectively). [b] $t_{R,i}$ = individual retention time; $\alpha_{II} = t_{R,i}/t_{R,II}$.

fitted with a recorder[6] and an electronic integrator[7]. Stainless steel columns containing reversed-phase packing material[8] were operated at room temperature. The samples were introduced by means of a loop injector[9] with a fixed volume of 10 or 20 μl. A digital pH meter[10] was used to measure the pH value of the buffers.

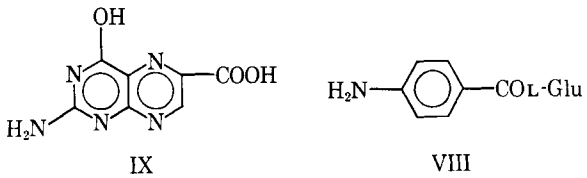
Samples, Reagents, and Solvents—Three methotrexate samples (a candidate lot for USP reference standard referred to as Lot A and two old lots kept at room temperature, referred to as Lots B and C), USP folic acid reference standard Lot J, and USP leucovorin calcium reference standard Lot F were analyzed for chromatographic purity. The following



	Position side chain	R_1	R_2	R_3
I:	6	OH	H	L-Glu
II:	6	NH_2	CH_3	L-Glu
IV:	7	NH_2	CH_3	L-Glu
V:	6	NH_2	H	L-Glu
VI:	6	OH	CH_3	L-Glu
VII:	6	NH_2	CH_3	OH



III



IX VIII

L-Glu = —NH—CH(COOH)—CH_2—CH_2—COOH (L-form)

[6] Linear model 261, Linear Instruments Corp., Irvine, Calif.
[7] System I Computing Integrator, SpectraPhysics, Santa Clara, Calif.
[8] 250 × 4.0-mm RP-18, Chromanetics, Baltimore, Md.; (b) 300 × 3.9-mm μ-Bondapak C-18, Waters Associates, Milford, Mass.; (c) 250 × 4.6-mm Zorbax ODS, DuPont, Wilmington, Del.; (d) 250 × 4.6-mm Partisil-10 ODS Whatman, Inc., Clifton, N.J.
[9] Altex 210, Altex Scientific Inc., Berkeley, Calif. or Valco sample injector CV-6-UHPa-N60.
[10] Model 701A, Orion Research Inc., Cambridge, Mass.

materials were used as received: 7-methotrexate (IV)[11], aminopterin (V)[12], methopterin (VI)[13], 4-amino-4-deoxy-N^{10}-methylpteroic acid (VII)[12], *N*-(*p*-aminobenzoyl)-L-glutamic acid (VIII)[13], 2-amino-4-hydroxypteridine-6-carboxylic acid (IX)[13]. Solvents were chromatographic grade. Tetrabutylammonium bromide and the materials used in the preparation of the buffers were reagent grade.

Mobile Phases—Premixed, degassed solvents were used. The following buffers were used as the aqueous component of the mobile phase:

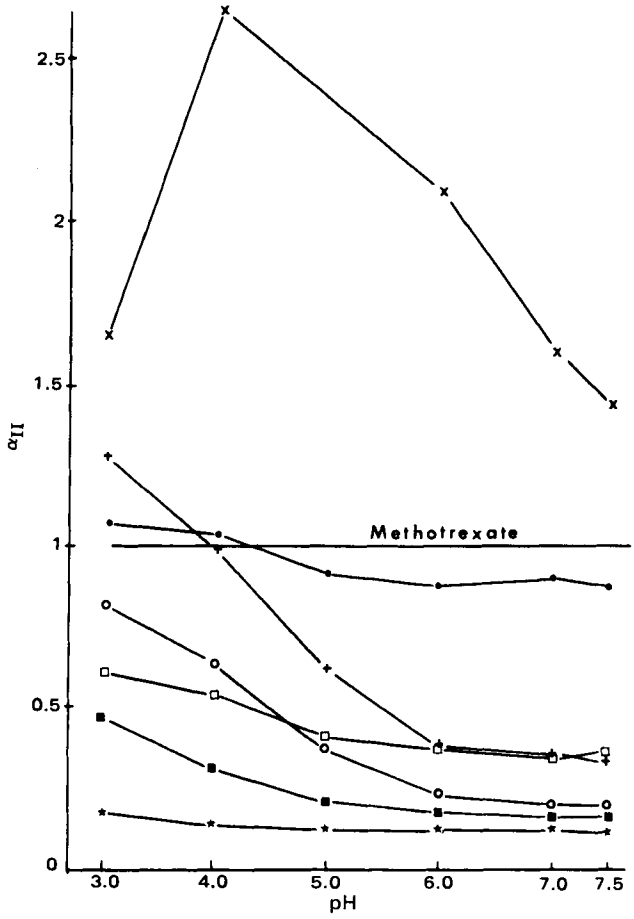


Figure 1—*Order of elution and separation as a function of the mobile phase pH. Key: (×) VII; (+) methopterin; (●) 7-methotrexate; (○) folic acid; (□) aminopterin; (■) leucovorin; (★) VIII.*

[11] Received on a complimentary basis from the Oncological Institute, Bucharest, Romania.
[12] Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.
[13] Sigma Chemical Company, St. Louis, Mo.

Table II—Purity Analysis of Methotrexate [a]

Lot A, 4 mg/ml						Lot B, 4 mg/ml					
Ion-Pairing Mobile Phase[b]			Standard Buffer[c]			Ion-Pairing Mobile Phase[b]			Standard Buffer[d]		
Peak No.	t_R, min	%[e]	Peak No.	t_R, min	%[e]	Peak No.	t_R, min	%[e]	Peak No.	t_R, min	%[e]
1	4.8	0.01	1	2.6	0.1	1	3.9	0.03	1	2.3	0.09
2	5.1	0.03	2[f]	4.7	0.45	2	4.5	0.09	2	2.5	0.10
3	5.8	0.08	3[g]	5.1	0.20	3	4.8	0.09	3	3.1	0.37
4	6.6	0.15	4	6.7	0.02	4	5.5	0.22	4[f]	4.1	6.18
5[f]	7.1	0.72	5	7.1	0.01	5[f]	6.7	7.67	5[g]	5.9	0.16
II	9.0	98.53	6	8.2	0.03	II	8.6	90.16[i]	6	6.8	0.11
6	11.2	0.10	II	9.7	98.84	6	9.8	0.38	II	8.0	90.64
7	13.7	0.04	7	12.9	0.03	7	12.9	0.02	7	9.5	0.03
8	14.7	0.02	8	13.5	0.05	8	13.3	0.01	8	11.1	0.06
9	15.7	0.02	9	20.1	0.04	9	14.4	0.22	9	15.6	0.53
10	18.4	0.30	10	21.0	0.05	10	17.9	0.07	10[h]	18.9	1.44
			11[h]	23.5	0.26	11	21.3	0.04	11	35.8	0.3 (est)
						12	25.6	0.07			
						13	29.2	0.93			

[a] Instrument—SpectraPhysics 3500B, column-μ-Bondapak C-18 300 × 3.9 mm, detector 302 nm-0.1 aufs. [b] 0.005 *M* Tetrabutylammonium bromide in pH 7.5 0.05 *M* KH_2PO_4-acetonitrile (82:18), flow rate 0.8 ml/min. [c] Buffer A-acetonitrile (10:1), flow rate 1 ml/min. [d] Buffer A-acetonitrile (10:1), flow rate 1.2 ml/min. [e] By area normalization. [f] Eluted at the same retention time as an authentic sample of methopterin. [g] Eluted at the same retention time as an authentic sample of aminopterin. [h] Eluted at the same retention time as an authentic sample of 4-amino-4-deoxy-N^{10}-methylpteroic acid. [i] Poorly resolved from Peak 5.

pH 6.0, 0.1 *M* citric acid–0.2 *M* Na_2HPO_4 (37:63, Buffer A); 0.05 *M* KH_2PO_4 brought to either pH 3.0 or 4.0 with 85% H_3PO_4 and to pH 5.0, 6.0, 7.0, 7.2, and 7.5 with 1 *N* NaOH (Buffers P-3–P-7.5, respectively); and pH 7.2, 0.05 *M* KH_2PO_4–0.25 *M* $NaClO_4$ (Buffer B).

As an ion-pairing mobile phase a 0.005 *M* solution of tetrabutylammonium bromide in pH 7.5, 0.05 *M* KH_2PO_4 buffer was used.

Sample Solutions—Mixtures of 0.25–4 mg of compound/ml of mobile phase were sonicated for 15–20 min and filtered through a 5-μm membrane filter prior to injection[14]. Fresh solutions were prepared daily.

RESULTS AND DISCUSSION

Analysis—In Table I and Fig. 1 the results are presented both as absolute values of the retention times (obtained from chromatograms of individual compounds) and as their ratios to the retention time of methotrexate (α_{II}). The quantitative data were calculated by area normalization. Unless otherwise mentioned, all the analyses were run in duplicate.

Influence of Buffer pH—Only a few of the impurities typical of commercial production of compounds in this class have been identified. To gain information on the influence of buffer pH on the chromatographic behavior, a synthetic mixture of Compounds I–IX was prepared and the separation has been studied over a pH range limited by the stability of the packing material (3.0–7.5). Buffers of 0.05 *M* KH_2PO_4 were prepared in this range in increments of whole pH units, and the amount of methanol in the mobile phase was adjusted to keep the retention time of the methotrexate peak between 9 and 10 min at a flow rate of 2 ± 0.2 ml/min.

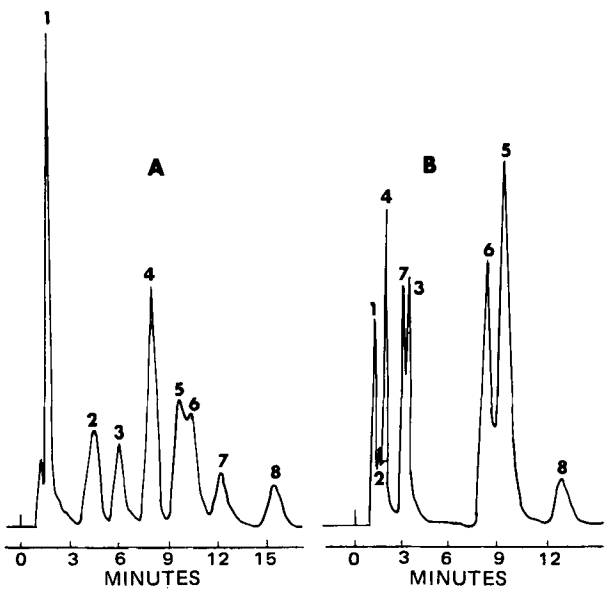


Figure 2—*High-pressure liquid chromatograms of a synthetic mixture. Key: (A) mobile phase pH 3.0; (B) mobile phase pH 7.5; peak identity: (1) VIII; (2) leucovorin; (3) aminopterin; (4) folic acid; (5) 7-methotrexate; (6) methotrexate; (7) methopterin; (8) VII.*

[14] Fluoropore membrane filter, Millipore Corp., Bedford, Mass.

No attempts were made to obtain baseline separation of all nine compounds, since their simultaneous presence in any one specimen is unlikely. The results are presented in Table I and Fig. 1.

A few aspects of the strong influence of the pH value of the buffer on the separation and order of elution of the peaks can be outlined as follows:

1. The α_{II} values for methopterin, aminopterin, folic acid, and leucovorin calcium increase with the decrease of pH value of the buffer. The most pH-sensitive is the methopterin peak. At pH 7.5 it elutes before the aminopterin peak; at pH 7.0 this order is reversed; at pH 4.0 it overlaps the methotrexate peak; and at pH 3.0 it is eluted after both methotrexate isomers.
2. At pH 3.0, methotrexate elutes before the 7-isomer; at pH 4.0 the two peaks overlap and the order of elution is reversed at all pH values above 4.0.
3. A reversal in the elution order of the folic acid and aminopterin peaks takes place at a pH value between 4.0 and 5.0.
4. The retention time of Compound VII reaches a maximum at pH 5.0. For practical reasons (long analysis time) it is probably better to avoid the use of this pH value in the analysis of mixtures containing VII.

The change in the order of elution is illustrated by the chromatograms at pH 3.0 and 7.5 (Fig. 2).

It is apparent that in this class of compounds the pH value of the buffer is the most powerful parameter in adjusting the separation of any given combination of components in a mixture, with optimization of a particular separation making use of other capabilities of HPLC such as selection of a percentage of the organic solvent or gradient elution.

Purity Analysis—*Folic Acid*—Using Buffer B and UV detection at 254 nm, the chromatographic purity of the current lot of USP folic acid RS (J) was found to be 99.8%. Four minor impurities were separated (three before the main peak, one after), none of them at the locus of formyltetrahydrofolate, which as calcium salt is used as an internal standard in the USP assay of folic acid.

Leucovorin Calcium—A 2-year-old sample was recently reanalyzed using two mobile phases. With Buffer B–methanol mixtures a 95.6% chromatographic purity was obtained, eight impurities being separated before the main peak and one after it.

Using a mobile phase recommended in the literature, pH 5.0, 0.05 *M* KH_2PO_4–acetonitrile (95:5) (9), a chromatographic purity of 95.8% was obtained (average of seven injections, 10 impurities separated).

Methotrexate—From the data in Table I, it appears that the best separation of methotrexate from closely related compounds takes place in the pH range of 6.0–7.5. A comparison between the pH 6.0 citrate buffer and the pH 7.5 phosphate buffer in the purity analysis of a 2-year-old sample (C) shows that the same number of impurities (13) was separated. Quantitation by area normalization indicated a chromatographic purity of 92.7% with either pH 6.0 or 7.5 mobile phases.

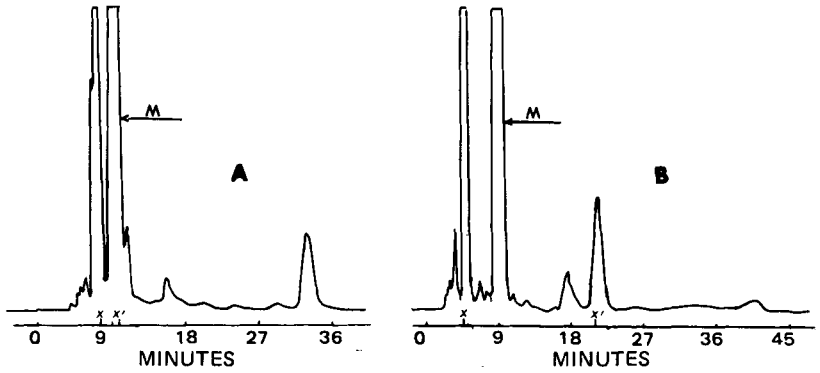


Figure 3—*High-pressure liquid chromatogram of methotrexate Sample B. Key: (A) ion-pairing mobile phase; (B) mobile phase–citrate buffer A: acetonitrile 10:1; (M) methotrexate peak; (X) retention time of an authentic sample of methopterin; (X′) retention time of an authentic sample of VII.*

To compare the pH 6.0 citrate buffer and the ion-pairing mobile phases, two methotrexate samples (A and B) were analyzed for chromatographic purity by the two procedures. The results are shown in Table II. The two methods separate the same number of major impurities, but there are significant advantages for the citrate buffer method:

1. The resolution between the peaks corresponding to methotrexate and methopterin (the major degradation product) is considerably larger using the citrate buffer. The average ratio of the retention times of the two peaks is 2.1 for the citrate buffer method and 1.3 for the ion-pair method. As a result, the resolution of the two peaks by the ion-pair method is acceptable at rather low concentrations of methopterin (V) (Sample A, <1%), but the peaks overlap at a 6% concentration (Sample B) (Fig. 3A). In Reference 13, although the methotrexate peak has a retention time of >40 min, V still elutes as a shoulder on the main peak. The better separation of the two peaks by the citrate buffer method is illustrated in the chromatogram of Sample B (Fig. 3).

2. Another potential impurity in methotrexate is 4-amino-4-deoxy-N^{10}-methylpteroic acid (VII). Again, the citrate buffer provided better separation. In the reported ion-pair chromatography[13], VII is well separated from methotrexate ($t_{R,VII}$ = 18 min, $t_{R,II}$ > 40 min). However, in conditions that, for practical purposes, bring the retention time of the methotrexate peak <10 min, the two peaks overlap ($t_{R,VII}$ = 8.4 min; $t_{R,II}$ = 8.5–8.6 min), and VII could not be identified by spiking a methotrexate sample. Using the citrate buffer mobile phase, with the methotrexate peak eluting between 8 and 10 min, the retention time of VII was ~20 min.

3. The ion-pairing procedure did not separate methotrexate and its 7-isomer. Without giving baseline resolution, the citrate buffer method allowed the detection of 0.2% of the 7-isomer in a spiked sample of methotrexate.

The ion-pairing method proved to be an excellent tool for biochemical studies on methotrexate and its metabolites, but from the previously mentioned considerations it may be concluded that standard buffers should be used as mobile phases for the purity analysis of methotrexate.

The chromatogram of the candidate lot for USP methotrexate reference standard is presented in Fig. 4. Additional chromatograms with mobile phases containing 5–20% acetonitrile ascertained the absence of late-eluting peaks or of potential impurities overlapping the main peak in Fig. 4. The chromatographic purity by area normalization with UV detection at 302 nm was 98.8%.

Separation of the 6- and 7-Isomers of Methotrexate—The formation of 6- and 7-pteridyl isomers in the reaction between 4,5-diaminopyrimidines and polyfunctional 3-carbon compounds is well known (15, 16). Isomerically pure 6- and 7-methotrexate have been synthesized and it was found that the position of the side chain is mainly a function of the pH of the reaction mixture in the pteridine ring closing step and that 7-methotrexate is biologically inactive (17). The two isomers have similar physical chemical characteristics, differing significantly only in the NMR spectra. By TLC or paper chromatography they could be separated only after a preliminary hydrolysis and oxidation to the 2-hydroxy-pteridine-6-carboxylic acid and 2-hydroxy-pteridine-7-carboxylic acid (17).

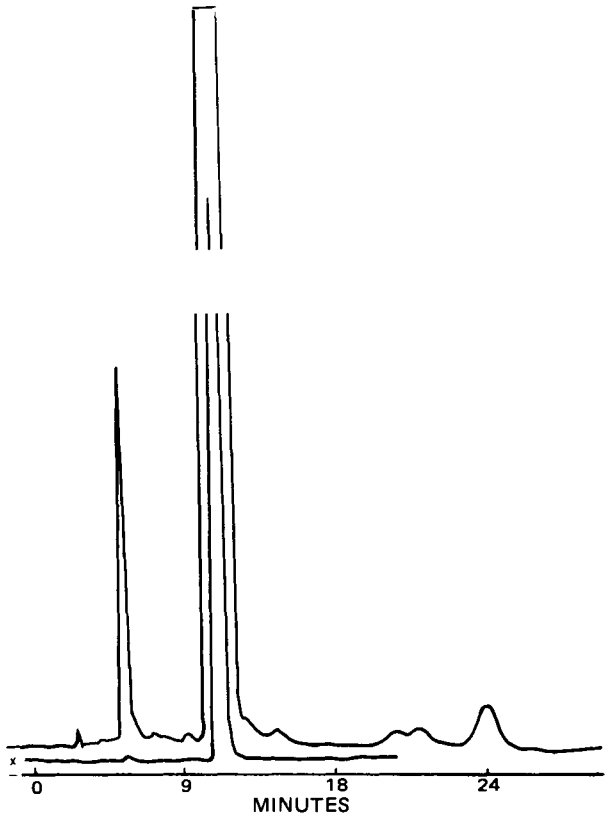


Figure 4—*High-pressure liquid chromatogram of a candidate lot for USP methotrexate reference standard. Mobile phase–Buffer A: acetonitrile 10:1, flow rate 1 ml/min. Key: (X) attenuation 32.*

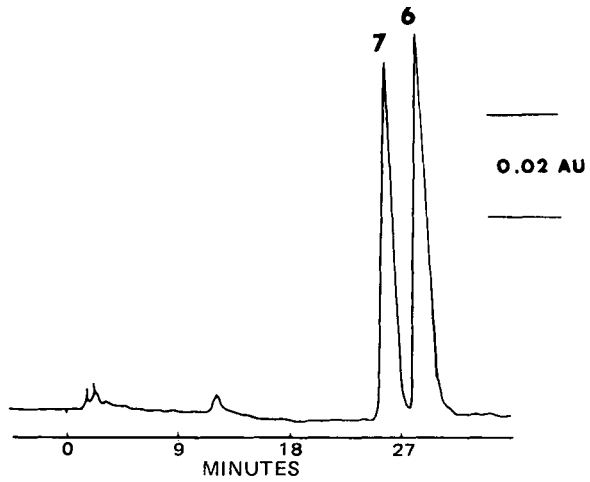


Figure 5—*HPLC separation of the 6- and 7-isomers of methotrexate. Mobile phase–Buffer P-7–CH_3OH; initial 100:5; final 100:12.5; sweep time 15 min, flow rate 2 ml/min. Sample 0.25 mg each/ml.*

Reversed-phase HPLC has been used for the separation of 6- and 7-methyl- (and-methanol-)2,4-diaminopteridines (9).

A nearly baseline separation of 6- and 7-methotrexate (R = 1.4) was obtained using a gradient elution system illustrated in Fig. 5.

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Microbial Transformations of Natural Antitumor Agents XVIII: Conversions of Vindoline with Copper Oxidases

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Abstract □ Vindoline occurs structurally intact in the clinically important *Vinca* alkaloids vinblastine and vincristine. It is oxidized by human ceruloplasmin and fungal and plant laccases into a reactive intermediate which undergoes intramolecular cyclization to an enamine which ultimately dimerizes. Transformations of vindoline by these copper oxidases are enhanced when enzyme incubations are performed with cofactors such as chlorpromazine. The role of copper oxidases in alkaloid metabolic interconversions and the possible implications of these reactions in *Vinca* alkaloid toxicity are discussed.

Keyphrases □ Vindoline—microbial transformations, natural antitumor agents, conversions with copper oxidase, *Vinca* alkaloids □ Alkaloids, *Vinca*—vindoline, microbial transformations, natural antitumor agents, conversions with copper oxidase □ Antitumor agents—vindoline, microbial transformations, natural antitumor agents, conversions with copper oxidase, *Vinca* alkaloids

Vincristine (II) and vinblastine (I) are widely used dimeric antitumor alkaloids obtained from species of *Catharanthus rosea*. These compounds differ structurally only in the oxidation state of one carbon atom which is attached to the dihydroindole nitrogen atom of the *Aspidosperma* portion of the molecule. Studies concerned with the metabolism of I and II are intended to reveal pathways of metabolic transformations which might ultimately be implicated in mechanism(s) of action and/or the toxicities associated with their use. Several attempts have been made to date (1–5) to study the metabolism of *Vinca* alkaloids, but no metabolites of these compounds have been isolated and fully identified. Low amounts of metabolites produced, very low doses of compounds employed, high molecular weights, and structural complexities probably rendered the identification of presumed metabolites difficult in previous studies.

Copper oxidases are widely occurring enzymes found in mammals, plants, and microorganisms. Enzymes from these different sources possess different physical characteristics including molecular weight, the number and oxidation states of copper, and the nature of copper ligands at presumed active sites (6, 7). Recognized similarities also exist among these enzymes, and direct comparisons between the catalytic capabilities of ceruloplasmin and laccases have been made previously (8, 9). The enzymes achieve the oxidation of substrates by the direct removal of substrate electrons and protons with the subsequent transfer of electrons to molecular oxygen *via* copper (6, 7, 10, 11). True substrates interact directly with copper oxidase enzymes to yield products, while pseudosubstrates require substances capable of interfacing between them and the enzyme during oxidations (6). The requirement for such cofactors has been noted primarily in work with ceruloplasmin in the oxidation of xenobiotics such as arylamines, phenols, and some centrally acting drugs and their analogs (6).

It was discovered that vindoline, a dihydroindole monomer found in the structure of I undergoes oxidation in the presence of copper oxidase enzymes including human ceruloplasmin, fungal, and plant laccases. This report describes the types of chemical transformations of vindoline catalyzed by the copper oxidases which result

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