Identification and Quantitation of Impurities in Methotrexate

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Abstract □ The impurities in bulk, commercial, and investigational methotrexate samples were chromatographically separated by gradient elution from a diethylaminoethylcellulose column. Besides the already known impurities in the bulk and commercial samples, two other impurities, 4-amino-N^{10}-methylpteroyl-*N*-methylglutamine and 2,4-diamino-N^{10}-methylpteramide, were identified. A new batch of methotrexate, synthesized for the National Cancer Institute for investigational use only, showed a different spectrum of impurities. The purity of all methotrexate samples studied was about 86% calculated on an anhydrous basis.

Keyphrases □ Methotrexate—chromatographic separation, UV, NMR, and mass spectral analyses of impurities in various formulations □ Impurities—in various formulations of methotrexate, chromatographic separation, UV, NMR, and mass spectral analyses □ Antineoplastic agents—methotrexate, chromatographic separation, UV, NMR, and mass spectral analyses of impurities in various formulations

Like many multisubstituted pteridines, pure methotrexate is difficult to prepare. Although methotrexate has been used as a folic acid antagonist for a long time and several reports discussed the detection of its impurities (1, 2), details of the quantitation and identification of impurities have not appeared. Recent use of 10–20-g quantities of methotrexate to treat certain neoplasias (3) renewed interest in this subject because significant amounts of impurities could be administered. Since some contaminants may have significant pharmacological activity, a systematic investigation to identify and quantitate these impurities was undertaken.

EXPERIMENTAL

Chromatographic Separation Procedure—Chromatography on a diethylaminoethylcellulose column as described previously (4, 5) was used as a separation technique. The procedures for preparing the column and buffer and for monitoring the peaks were as reported (4, 5), except that a linear gradient of 0.01–0.4 *M* ammonia–ammonium bicarbonate buffer (pH 8.3) was used as the eluant.

Identification and Quantitation of Impurities in Methotrexate—Between 10 and 25 mg of methotrexate USP reference substance was chromatographed in each batch. The peak tubes of different fractions, as indicated by the percent transmittance on the chromatogram, were then analyzed by UV spectrophotometry at various pH values. For further identification, similar fractions from several runs were pooled, quickly evaporated to a smaller volume in a flash evaporator, and then freeze dried. The freeze-dried samples were then analyzed by NMR and mass spectrometry.

NMR spectra[1] were obtained at 100 MHz using deuterium oxide as the solvent. Mass spectrometry was carried out on a computerized gas–liquid chromatograph–mass spectrometer[2] with a source temperature of 290°. The ionization potential for peak III was 70 ev, and the probe temperature was 190°. For peak II, the ionization potential was 9.2 ev and the probe temperature was 210°.

For quantitation, about 20 mg of methotrexate USP reference substance[3] was accurately weighed, loaded on the column, and chromatographed. The different fractions were then quantitatively collected, brought to a specific volume, and analyzed by UV spectrophotometry. Unless specified otherwise, all absorbance values reported were determined in pH 8.3 ammonia–ammonium bicarbonate buffer.

Similar studies to identify and quantitate impurities were conducted using commercially available methotrexate injection[4] and "for investigational use" methotrexate injection[5].

RESULTS AND DISCUSSION

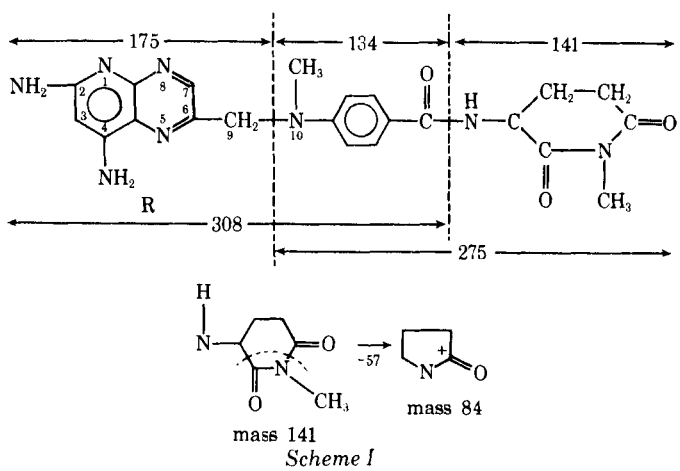
The chromatographic separation of methotrexate USP reference substance is shown in Fig. 1; the percent transmittance was plotted against the volume of ammonia–ammonium bicarbonate buffer eluant. Besides the major peak (methotrexate), the transmittance of six other peaks is illustrated.

Identification of Impurities in Methotrexate USP—*Peak VII*—The UV spectrum of the major impurity, peak VII, was identical to that of an authentic sample of N^{10}-methylpteroylglutamic acid. The relative retention times of peak VII and of an authentic sample of N^{10}-methylpteroylglutamic acid also were found to be identical in a high-pressure liquid chromatographic system reported previously (6). The identity of the impurity was reported previously (1, 2).

Peak IV—Peak IV was identified as 2,4-diamino-N^{10}-methylpteroic acid by comparing its UV spectrum to that of an authentic sample. The identity of 2,4-diamino-N^{10}-methylpteroic acid as one impurity present in methotrexate also was reported by Tong *et al.* (1).

Peak III—The identity of peak III has not been reported. The UV spectrum of the compound was identical in all respects to that of methotrexate and suggested that the major chromophore, the 2,4-diamino-N^{10}-methylpteroyl moiety, was intact. Mass spectrometry of peak III indicated a molecular weight of 449 (methotrexate, 454) and showed peaks at *m/e* 449 (small), 308 (small), 275, 175 (base peak), 141, 134, and 84. The peak at 449 was attributed to the molecular ion peak. The peak at 175 was attributed to the 2,4-diamino-6-methylpteridine group, the peak at 134 was attributed to the *p*-*N*-methylaminobenzoyl group, and the peak at 308 was attributed to 2,4-diamino-N^{10}-methylpteroyl or the R group. Scheme I shows the possible structure and assignments for peak III. It appears that R (2,4-diamino-N^{10}-methylpteroyl, $C_{15}H_{14}N_7O$) has a substituent mass of 141.

Hydrolysis of peak III by boiling with sodium hydroxide yielded glutamic acid, which was identified on an amino acid analyzer by comparison with marker compounds. Results indicated the fragment (substituent on R) to be a glutamic acid derivative. Since the fragment mass was odd



Scheme I

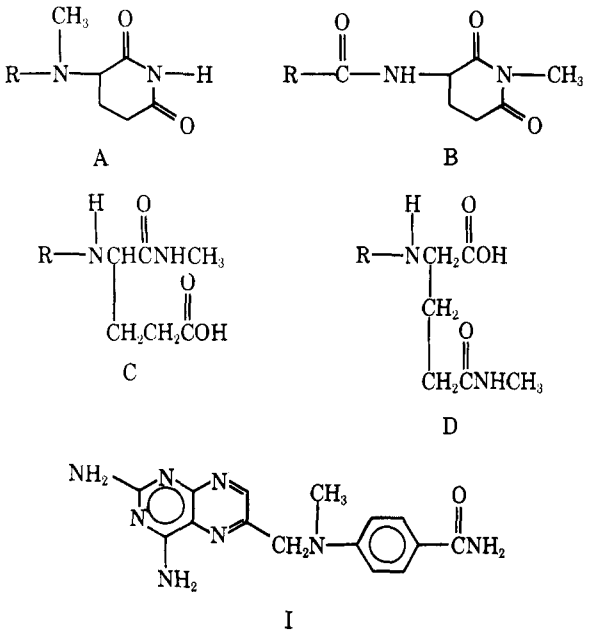
[1] Model XL-100-15 spectrometer, Varian Associates, Palo Alto, Calif.

[2] LKB 9000, LKB, Stockholm, Sweden.

[3] Lot 1260X8105, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.

[4] Lot 473-232, methotrexate sodium parenteral, 50 mg of methotrexate/vial, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.

[5] Lot BV-76-221, Ben Venue Laboratories, Bedford, Ohio, for National Cancer Institute as methotrexate for injection, preservative free, 1.0 g of methotrexate sodium/vial, for parenteral administration.



A B C D I

numbered, two nitrogens and the formula $C_8H_9N_2O_2$ (mass 141) were assigned to it.

Two structures were considered. Structure A, on hydrolysis, should yield *N*-methylglutamic acid and, therefore, was unlikely. Structure B should yield glutamic acid on hydrolysis and could account for the prominent peak at *m/e* 84 (Scheme I). Furthermore, to obtain a mass of 84 from Structure A, the route shown in Scheme II would have to be followed, which appears unlikely. This situation provided additional support for ruling out Structure A. Structure B could be the parent compound or could have resulted from dehydration and cyclization of the open chain amide, C or D, during mass spectrometry.

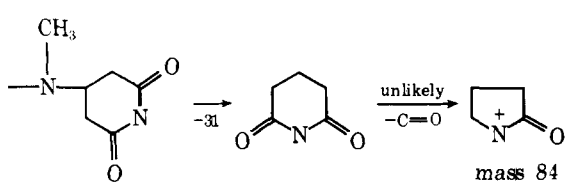
Cyclic Structure B was ruled out on the following basis.

1. The NMR spectrum of the compound did not show the expected peaks of an imide methyl as present in B; *i.e.*, there was no three-proton singlet at δ 4–5 ppm.
2. Cyclic Structure B would be nonionic in nature and should elute almost with the solvent front in the ion-exchange column.
3. The only difference between the NMR spectrum of peak III and that of methotrexate was a singlet at δ 2.6 ppm. This singlet integrated for three protons. The imide methyl (in B) would have a chemical shift much more downfield. However, the chemical shift is consistent with amide methyl structure of C or D.
4. The elution of the peak not with the solvent front, but still quite early, suggested a monocarboxylic acid structure.

Since the exact position of the methyl group (in C and D) was not confirmed, peak III was tentatively called a "glutamine derivative" and given Structure C or D.

Peak II—Peak II had a UV spectrum very close to methotrexate, indicating the presence of a 2,4-diamino-N^{10}-methylpteroyl group. Very early elution from the column also suggested the compound to be nonionic at pH 8.3 (pH of the eluting buffer). The mass spectrum of peak II (9.2 ev, probe temperature 210°) gave *m/e* 324 (molecular ion), 306, 175 (base peak), and 149. As for peak III, mass 175 was assigned to the 2,4-diamino-6-methylpteridine portion. Mass 149 was attributed to the *p*-*N*-methylaminobenzamide group. The peak at *m/e* 306 might have resulted from the dehydration of the amide to the corresponding nitrile at the high temperature of the probe. Therefore, peak II was assigned the structure 2,4-diamino-N^{10}-methylpteramide (I).

Peaks I and VI—Peak I was not identified. However, almost identical elution with the solvent front suggested that it is nonionic. The UV



Scheme II

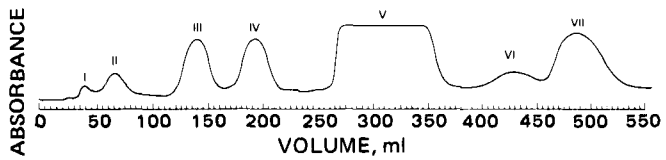


Figure 1—*Chromatogram of methotrexate USP reference substance from a cellulose column by linear gradient elution from 0.01 to 0.4* M *ammonia–ammonium bicarbonate buffer, pH 8.3.*

spectrum of the compound (pH 8.3) showed no peak around 300 nm, suggesting that the *p*-aminobenzoyl moiety was not present.

Peak VI also was not identified. However, delayed elution from a cellulose column suggested that the compound may have more than one anionic site at pH 8.3. Figure 2 shows UV spectra of peaks I and VI at pH 8.3.

Impurities in Methotrexate Injections—Commercial methotrexate injection[4], when chromatographed as described, appeared essentially similar to methotrexate USP regarding its impurities, except that the relative amounts were slightly different (Table I). The major impurities were the glutamine derivative (peak III), 2,4-diamino-N^{10}-methylpteroic acid (peak IV), and N^{10}-methylpteroylglutamic acid (peak VII). "For investigational use" methotrexate injection[3], however, had a different spectrum of impurities (Fig. 3). The only impurity common to both USP and investigational methotrexate appeared to be the glutamine derivative (peak III). All other impurities were new and have not been identified (Figs. 1 and 3).

Quantitation of Impurities in Methotrexate—The impurities in methotrexate were quantitated by determining their absorbance values at 257 nm for peak IV and at 302 nm for peaks II, III, and VII. The molar absorptivity, ϵ, of N^{10}-methylpteroylglutamic acid (peak VII) at 302 nm was the same as that of methotrexate at 302 nm (ϵ 24,500, based on anhydrous methotrexate) and was used to calculate the amount of peak VII in the samples. A molar absorptivity value of 21,700 at 257 nm for peak IV, as reported previously (7), was used to calculate the amount of peak IV in the samples.

The only other major impurity was peak III (glutamine derivative); for its calculation, its molar absorptivity at 302 nm was assumed to be the same as that of methotrexate at 302 nm and pH 8.3. The assumption was made because the UV spectrum at pH 8.3 of peak III and that of methotrexate are identical in every respect and the molar absorptivities of a wide range of substituted pteroyl groups (8, 9) are very similar (including that of N^{10}-methylpteroylglutamic acid, peak VII). However, because of the assumption, the percent impurity value reported for peak III, although correct for comparative purposes, may have some error on an absolute basis. Using similar arguments, the molar absorptivity of peak II was assumed to be the same as that of methotrexate at 302 nm (pH 8.3).

The percent purity of methotrexate in the three methotrexate preparations was determined by the previously reported method (4). Briefly, the percentage purity of methotrexate is calculated as a function of A_u/A_s, where A_s is the absorbance of the methotrexate solution before chromatographic separation and A_u is the absorbance of the pure metho-

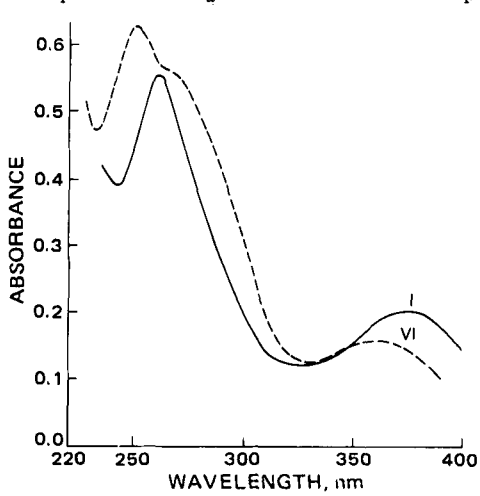


Figure 2—*UV spectra of peaks I (—) and VI (- - -), from chromatogram in Fig. 1, in ammonia–ammonium bicarbonate buffer, pH 8.3.*

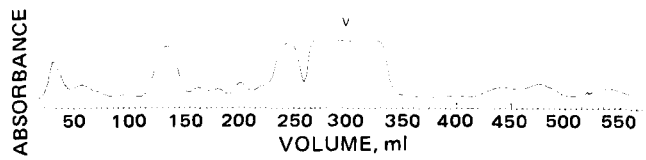


Figure 3—*Chromatogram of investigational methotrexate from a cellulose column by linear gradient elution from 0.01 to 0.4 M ammonia–ammonium bicarbonate buffer, pH 8.3.*

trexate solution after chromatographic separation. The procedure needs further explanation. The method implies that the absorptivities (on a weight basis rather than a molecular basis) of the impurities are identical to the absorptivity of methotrexate, which may not be absolutely true. However, as mentioned, the major impurities have similar UV spectra, extinction coefficients, and molecular weights. Therefore, the assumption is probably valid. Furthermore, even assuming a 25% error in the overall assumption, the absolute error in the determination of the purity of methotrexate would only be about 2% (total impurities are approximately 8%, and a 25% error would cause an absolute error of 8 × 0.25 = 2%). Therefore, this assumption, although not absolutely valid theoretically, is reasonable for practical calculations and correct for comparing various methotrexate samples if the impurities and their relative quantities are similar.

Table I—Comparative Percent Purity of Methotrexate and Quantitation of Its Impurities in Three Different Preparations

Compound	Concentration, % USP	Commercial Injection	Investigational Injection
Methotrexate (anhydrous basis)[a] (peak V)	86.0	86.2	86.7
N^{10}-Methylpteroylglutamic acid (peak VII)	4.0	5.2	—
2,4-Diamino-N^{10}-methylpteroic acid (peak IV)	1.5	2.6	—
4-Amino-N^{10}-methylpteroyl-N-methylglutamine (peak III)	1.6	1.2	—
2,4-Diamino-N^{10}-methylpteramide (peak II)	0.5	0.2	—
Unidentified impurities	1.0	0.5	—

[a] All methotrexate samples contained approximately 5% water.

Presented in Table I is the comparative percent purity of methotrexate and the identity and quantitation of its impurities in three different preparations. The percent purity of methotrexate in all three preparations was almost identical. In addition, methotrexate USP and commercial methotrexate injection had similar impurities. However, the investigational methotrexate preparation contained different impurities, which have not been identified (Figs. 1 and 3). These impurities warrant further investigation.

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Thermodynamics of Aqueous Solutions of Parabens

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Abstract □ The solubility of a related series of parabens was determined in water at four temperatures. The parabens chosen were the methyl through *n*-butyl *p*-hydroxybenzoates, and the temperature variations were 5° increments from 25 to 40°. These solutes are useful preservatives, especially combinations of the methyl and propyl ester derivatives. The chemical relationship of these compounds varied by successive linear methylene additions on the ester portion of the molecules. The thermodynamic values obtained for these aqueous systems could be related to these molecular variants since the remainder of the molecule was constant. For the overall thermodynamics, the free energy functions such as the ideal, actual, and excess were found to be smooth, nonlinear functions of the number of carbon atoms in the alkyl portion of the paraben esters. A linear relationship with the number of carbon atoms in the ester portion of these esters was found with the partial excess free energy of the solute.

Keyphrases □ Parabens, various—solubility in water at four temperatures, thermodynamic parameters related to number of carbon atoms □ Solubility—various parabens in water at four temperatures, thermodynamic parameters related to number of carbon atoms □ Thermodynamic parameters—related to solubility of various parabens in water at four temperatures □ Preservatives—various parabens, solubility in water at four temperatures, thermodynamic parameters related to number of carbon atoms

The thermodynamic parameters associated with solution phenomena were determined by studying the variation of solubility with temperature.

The well-known relationship of log mole fraction solubility *versus* reciprocal temperature allows for the determination of heats of solution, ΔH_s, and entropies of solutions, ΔS_s, from slopes and intercepts, respectively (1).

From heats of fusion, ideal mole fraction solubilities can be determined. From these basic derived quantities, mixing and excess functions also can be calculated.