Assay of Methotrexate in the Presence of Its Decomposition Products and Other Folic Acid Analogs

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Extensive studies with the folic acid antagonist methotrexate in cancer chemotherapy prompted the development of an assay for methotrexate in the presence of its decomposition products and other folic acid analogs. This was accomplished by quantitative chromatographic separation and spectrophotometric analysis of intact methotrexate and its decomposition products. Methotrexate U.S.P. samples were assayed and found to be at best approximately 90 per cent pure.

THE PRESENT U.S.P. method of assay for methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) is based solely on the characteristic ultraviolet absorption of the compound. However, spectrophotometric studies of this type are subject to several limitations. Methotrexate is known to be contaminated with its decomposition products and other closely related folic acid analogs, often differing by as little as one functional group. The similarity of the ultraviolet absorption characteristics of methotrexate, its decomposition products, and other folic acid analogs requires that some type of quantitative separation and purification technique be utilized prior to a spectrophotometric assay of the intact methotrexate in the ultraviolet region.

Although paper chromatography (1) has been used for the separation and identification of closely related derivatives of pteroylglutamic acid, it is not suitable for purification and resolution of folic acid analogs on the preparative scale of milligram or gram quantities. Furthermore, compounds in the folic acid series have been reported (2, 3) to be degraded when subjected to variations of paper chromatography due to photolytic breakdown and transformation due to extreme pH changes.

Column chromatography of proteins on a cellulose anion exchange adsorbent has been described by Sober and Peterson (4). Mixtures of proteins were separated using gradients of pH and salt concentrations for elution. Heinrich *et al.* (5) have employed a strongly basic anion-exchange resin[1] for the resolution of mixtures of certain folic acid analogs.

Oliverio (6) applied diethylaminoethyl (DEAE) cellulose and linear gradient elution by means of phosphate buffers to separate folic acid analogs in milligram quantities. The present study involved an adaptation of this method. A specific method of assay was developed for methotrexate in the presence of its decomposition products and other folic acid analogs, utilizing in part modified techniques and methods of separation and purification.

EXPERIMENTAL

Reagents—Methotrexate[2] U.S.P., m.p. 182–189°, and sodium methotrexate for injection,[3] 50 mg. methotrexate per vial. One of the decomposition products of methotrexate, N^{10}-methylpteroylglutamic acid, had m.p. 224–228°. Stock solutions of 0.1 *M* and 0.4 *M* ammonium bicarbonate were prepared and adjusted to pH 8.3 with ammonium hydroxide U.S.P. Diethylaminoethyl (DEAE) cellulose,[4] 0.75 meq./Gm., was passed through a 100-mesh screen and prepared batch-wise by successive washings with 1 *N* sodium hydroxide, water, 1 *N* hydrochloric acid, water, until pH 5.6 to 6.5. The cellulose was finally washed with 0.1 *M* ammonia–ammonium bicarbonate buffer until pH was approximately 8.3 and then stored in the refrigerator. All other chemicals used were of reagent grade.

Apparatus—A Gilson Medical Electronics (GME) volumetric fractionator model V 15-square[5] was used for fractionations. The eluent was monitored by a GME ultraviolet absorption meter, model UV-265 1F[5] and transmittance recorded on a Texas Rectilinear recording milliammeter.[6] A Sigmamotor pump[7] was employed to monitor rates of approximately 2 ml. per minute. Borosilicate glass containers, 1-cm. inner diameter and 25-cm. in length, held the cellulose adsorbent. A Beckman DU spectrophotometer was used for spectrophotometric determinations.

Chromatographic Separation Procedure—A dilute suspension of the washed DEAE cellulose in 0.1 *M* ammonia–ammonium bicarbonate buffer was added to a column and packed by gradually increasing air

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[1] Marketed as Dowex 1 (chloride form) by the Dow Chemical Co., Midland, Mich.

[2] Obtained from Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y.

[3] Marketed as Methotrexate Sodium Parenteral by Lederle Laboratories.

[4] Marketed as Cellex-D by California Corporation for Biochemical Research, Los Angeles, Calif.

[5] Gilson Medical Electronics, Middleton, Wis.

[6] Texas Instruments, Inc., Houston, Tex.

[7] Sigmamotor, Inc., Middleport, N. Y.

pressure from 2 to 10 p.s.i. Five milliliters of a sample solution representing theoretically 5 mg. of methotrexate in 0.1 *M* ammonia–ammonium bicarbonate buffer was allowed to run slowly into the prepared column of washed DEAE cellulose. The methotrexate is separated from its decomposition products and other folic acid analogs by a linear gradient with pH 8.3 ammonia–ammonium bicarbonate buffer of increasing molarity. The mixing chamber contained 500 ml. of 0.1 *M* ammonia–ammonium bicarbonate buffer, and the reservoir contained 500 ml. of 0.4 *M* ammonia–ammonium bicarbonate buffer. The column was attached *via* polyethylene tubing to the automatic fraction collector. The transmittance of the eluent was continually monitored by a 265 mμ ultraviolet light source and per cent transmittance plotted on a rectilinear recorder. Five-milliliter fractions were collected at the rate of 2 ml. per minute. All chromatographic operations were carried out at room temperature in subdued light to protect the sample from any possible photochemical effects. The peak tubes, as indicated by the per cent transmittance on the chromatogram, were then pooled and the ultraviolet absorption spectrum (230 to 340 mμ) of each peak was measured on a Beckman DU spectrophotometer.

Determination of the Purity of Methotrexate—The sample solution of 1 mg. methotrexate per ml. before fractionation was diluted to 1 mg. per 100 ml. with ammonia–ammonium bicarbonate buffer, pH 8.3, and its absorbance at a wavelength of 302 mμ was recorded on a Beckman DU spectrophotometer. The sample solution was then fractionated as described under *Chromatographic Separation Procedure*. The major peak (methotrexate) tubes were pooled, and a dilution to a theoretical 1 mg./100 ml. solution was prepared and its absorbance recorded at a wavelength of 302 mμ on a spectrophotometer. The purity of methotrexate was determined by calculating the per cent of its absorbance at a wavelength of 302 mμ recovered following chromatographic separation. Further confirmation of the purity of the methotrexate peak was obtained by taking the pooled fractionated solution from the major peak (methotrexate) and refractionating it by first concentrating the methotrexate fractions *via* freeze-drying, followed by reloading on a fresh column utilizing the chromatographic separation procedure previously described.

Identification of the Decomposition Products—In addition to the major peak (methotrexate) on the chromatogram (Fig. 1), three other peaks were obtained. The tubes of each peak were pooled and the ultraviolet absorption spectra (230 to 340 mμ) were recorded on a Beckman DU spectrophotometer. (See Fig. 2.) The identities of the single peaks were then obtained by comparing the ultraviolet curve with that of the authentic sample.

RESULTS AND DISCUSSION

Chromatographic separation of methotrexate U.S.P. is shown in Fig. 1, in which the per cent transmittance has been plotted against the volume of ammonia–ammonium bicarbonate buffer (eluent). Figure 1 shows, in addition to the major peak (methotrexate), the transmittance of three other peaks. These indicated that contaminants or hydrolysis products were present in the methotrexate U.S.P. sample. The hydrolysis products appearing as peaks 1, 2, and 4 on the chromatogram all absorbed at the wavelength of maximum absorbance, about 302 mμ, for methotrexate (see Fig. 2) and accounted for approximately 10% of the methotrexate sample. When the methotrexate fraction was collected, freeze-dried, reconstituted, and refractionated, none of the possible decomposition products showed up in the chromatogram seen in Fig. 3.

The spectrum of the major decomposition product of methotrexate U.S.P. was identical to an authentic sample of N^{10}-methylpteroylglutamic acid. This hydrolysis product appearing as the last peak (No. 4) on the chromatogram accounts for approximately one-half of the total impurities. The molar ab-

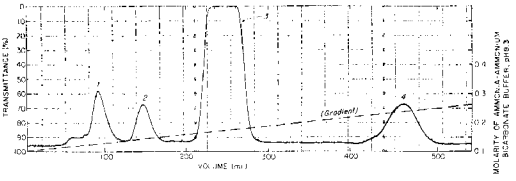

Fig. 1—Chromatogram of methotrexate U.S.P. from DEAE ion-exchange column by linear gradient elution from 0.1 M *to 0.4* M *ammonia–ammonium bicarbonate buffer, pH 8.3. Gradient is represented by dashed line.*

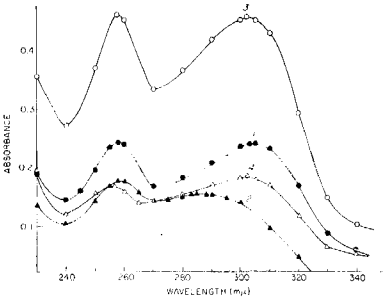

Fig. 2—Ultraviolet spectra of peaks 1, 2, 3, and 4 obtained from chromatographed methotrexate U.S.P. in ammonia–ammonium bicarbonate buffer, pH 8.3. Key: ○, peak 3 (methotrexate); ●, peak 1; △, peak 4 (N^{10}-methylpteroylglutamic acid); ▲, peak 2.

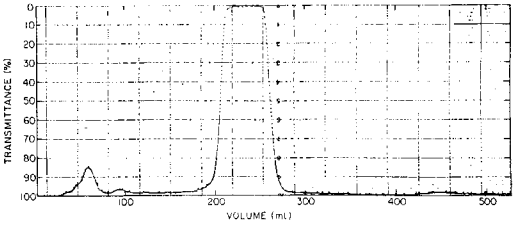

Fig. 3—Chromatogram of refractionated methotrexate U.S.P. from DEAE ion-exchange column by linear gradient elution with ammonia–ammonium bicarbonate buffer, pH 8.3.

sorptivity of N^{10}-methylpteroylglutamic acid was determined, at a wavelength of 302 mμ, and found to be the same as that of the parent methotrexate compound. The molar absorptivities of the unidentified first and second peaks were assumed because of their spectral characteristics of pteroyls to be essentially the same as that of the parent methotrexate compound. It should be noted that molar absorptivity values have been reported in the literature (7, 8) for analogs of pteroyl and pteroylglutamic compounds and found to be approximately the same as that for methotrexate at a wavelength of 302 mμ in alkaline media.

Specificity of Assay for Intact Methotrexate—The present U.S.P. XVII method of assay for methotrexate consists of determining and comparing the absorbance of both the solution of an unknown sample and a standard solution of a U.S.P. methotrexate reference substance (theoretically 100% methotrexate) at the wavelength of maximum absorbance at about 306 mμ with a suitable spectrophotometer using 0.1 *N* hydrochloric acid as the blank. This method is unsatisfactory for distinguishing intact drug from decomposition products, because all decomposition products isolated were shown (see Fig. 2) to absorb at the wavelength of maximum absorbance at about 302 mμ for methotrexate.

The quantitative chromatographic separation of a solution of a methotrexate sample followed by the determination of the absorbance of the methotrexate fraction at a wavelength of 302 mμ on a suitable spectrophotometer does provide a specific method of assay for the intact drug in the presence of its decomposition products and other folic acid analogs. Also, the assay method is such that it might not require the use of a U.S.P. methotrexate reference substance as employed in the U.S.P.

Calculation of Intact Methotrexate—The following formula would permit calculation of per cent intact methotrexate (% IM) on an anhydrous basis at a wavelength of 302 mμ in a 0.1 *M* ammonia–ammonium bicarbonate buffer, pH 8.3:

$$\% \text{ IM} = (A_u/A_s)\,(100 - \% \text{ water})$$

where A_u is the absorbance of the methotrexate solution after chromatographic separation, and A_s is the absorbance of the methotrexate solution before chromatographic separation. The absorptivity (a) of pure or intact methotrexate at a wavelength of 302 mμ in 0.1 *M* ammonia–ammonium bicarbonate buffer, pH 8.3, was calculated in terms of anhydrous methotrexate and found to be 53.9. The above formula can be further expressed as

$$\% \text{ IM} = \frac{(A_u)\,(10)\,(d)}{53.9\,(W_s)}$$

where A_u = absorbance of the chromatographed methotrexate solution whose fractions made up to 100 ml. are diluted to a theoretical 1 mg. methotrexate per 100 ml. solution, d = dilution factor required to dilute the methotrexate fractions made up to 100 ml. to a theoretical 1 mg. methotrexate per 100 ml. solution, and W_s = weight, in grams, of methotrexate sample put on the column.

SUMMARY

A chromatographic separation followed by a spectrophotometric method of analysis is reported for the determination of methotrexate in the presence of its products of hydrolytic decomposition and other folic acid analogs.

All methotrexate samples assayed were found to be at best approximately 90% pure methotrexate.

Recommendation is made for revision of the present U.S.P. method of assay for methotrexate to the one discussed in this paper.

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