yield values equivalent to the flow rate although the latter is not distribution rate limiting.

REFERENCES

(1) Nagashima, R., and Levy, G., *J. Pharm. Sci.*, **57**, 1991 (1968).
(2) Nagashima, R., and Levy, G., unpublished data.
(3) Rescigno, A., and Segre, G., "Drug and Tracer Kinetics," Blaisdell, Waltham, Mass., 1966, p. 84.
(4) Nagashima, R., Levy, G., and Sarcione, E. J., *J. Pharm. Sci.*, **57**, 1881(1968).
(5) Riegelman, S., Loo, J., and Rowland, M., *ibid.*, **57**, 128 (1968).
(6) *Ibid.*, **57**, 117(1968).
(7) Price, H. L., in "Uptake and Distribution of Anesthetic Agents," Papper, E. M., and Kitz, R. J., Eds., McGraw-Hill, New York, N. Y., 1963, p. 123.
(8) "Blood and Other Body Fluids," Altman, P. L., and Dittmer, D. S., Eds., Fed. Am. Soc. Exptl. Biol., Washington, D. C., 1961, p. 1.
(9) Rackow, H., Salanitre, E., Epstein, R. M., Wolf, G. L., and Perl, W., *J. Appl. Physiol.*, **20**, 611(1965).
(10) Perl, W., Rackow, H., Salanitre, E., Wolf, G. L., and Epstein, R. M., *ibid.*, **20**, 621(1965).

Keyphrases

Drug distribution—perfusate to rat liver
Flow rate, perfusate—drug-distribution effect
Pharmacokinetics—flow rate-drug distribution

Rapid Assay Method for the Determination of Methotrexate

By M. K. BALAZS, C. A. ANDERSON, and P. LIM

A rapid procedure for the assay of methotrexate samples (bulk or formulated) has been developed in these laboratories. This procedure, which employs quantitative paper chromatography, makes use of relatively inexpensive equipment and is simple to execute.

METHOTREXATE (MTX),[1] a clinically useful anticancer agent, is without exception an impure preparation consisting of MTX, contaminants related to MTX, and water. Efforts to assay MTX preparations have led to the development of column chromatographic procedures[2] that utilize adsorbents such as DEAE cellulose (1, 2) and diatomaceous earth.[3] These elegant procedures are adequate, but they require elaborate equipment and considerable time. The paper chromatographic procedure, which constitutes the basis of this note, requires relatively inexpensive equipment, is rapid and simple to execute, and affords a better separation of MTX from its contaminants.

The sensitivity of the method is limited only to the amount of MTX needed to give a useful UV spectrum. In this laboratory, a sample size of 1 mg. has been found to be convenient. The results of this procedure are comparable to those obtained by column methods.

Procedure—One milligram of methotrexate is dissolved in 0.2–0.3 ml. of 0.1 *N* sodium hydroxide and applied as a narrow band on Whatman No. 1 chromatographic paper 22.8 × 48.2 cm. (9 × 19 in.). The chromatogram is developed by descending chromatography with 0.5% aqueous sodium carbonate solution for 2.5 hr. The solvent front travels approximately 43 cm. (17 in.) during this time. The chromatogram is dried (in a fume hood) and the major band (MTX) is located ($R_f \simeq 0.6$–0.8) by illumination of the paper with shortwave UV light. This band is cut from the chromatogram and divided into 6 to 8 pieces, which are placed in a glass-stoppered, 125-ml. conical flask. Fifty milliliters of 0.1 *N* sodium hydroxide solution is added to the flask, which is then shaken vigorously until the paper is reduced to a pulp. A portion of the pulpy mixture is centrifuged at high speed for 3 min. or until a clear supernatant is obtained. The UV absorption of the supernatant solution is measured at 303 mμ, and the amount of methotrexate in the sample is calculated, using as a standard the molar absorptivity, 2.24×10^4.[4] A blank is prepared by the procedure cited above, and the slight increase in the base line is subtracted from the absorption of each sample. Due to the photo-instability of MTX, the entire analysis is carried out under minimal illumination.

Results and Discussion—The examples presented in Table I illustrate the precision of this method. The authors have observed that this procedure generally yields values as much as 2% lower than those obtained when the column chromatographic assay is used. The higher values obtained by column method are due to the inclusion of materials which contribute to the absorbance of the MTX but are in reality impurities. These impurities, which are absent in purified[5] samples of MTX, travel similarly to it but fluoresce brightly under UV light in contrast to the MTX, which absorbs. This visible difference permits excellent mechanical separation

Received May 21, 1968, from Stanford Research Institute, Menlo Park, CA 94025
Accepted for publication July 17, 1968.
This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U.S. Public Health Service, contract No. PH-43-64-500. The opinions expressed in this paper are those of the authors and not necessarily those of the Cancer Chemotherapy National Service Center.

[1] *N*-{*p*-[(2,4-Diamino-6-pterdinyl)methyl]methylamino}-benzoylglutamic acid, Lederle Laboratories.
[2] Unpublished procedure from Lederle Laboratories.
[3] Celite, Johns-Manville, New York, N. Y.
[4] The average ε value of four recent MTX bulk samples. Since the chromophores in the impurities are indistinguishable from those in MTX, the percent of MTX is calculated from this value.
[5] Purified by the Procedure described by Noble, E. P., *Biochem. Prep.*, **8**, 20(1961).

TABLE I—ASSAY PROCEDURE FOR MTX

Lot[a]	Paper Chromatography	Column Chromatography
	In Bulk Material	
	% MTX	% MTX
A0301	88.8, 87.9, 88.5, 87.5, 88.3	88.8, 91.7, 89.7
	Av. %, 88.2	Av. %, 90.1
8105	91.5, 90.2	91.5
	In Formulated Samples	
250-mg. lyo vials[b]		
Vial No.	mg. MTX/vial, av.	mg. MTX/vial, av.
1	227, 225, 230 (227)	240, 226 (233)
2	230, 225 (228)	228, 236 (232)
3	225, 229 (227)	228
50-mg. lyo vials[c]		
		UV Assay, mg.
1	47.2, 48.0, 47.8 (47.7)	47.1
2	48.3, 46.7, 47.2 (47.4)	47.0
3	47.2, 49.2, 46.6 (47.7)	47.4
4	47.2, 48.5, 48.4 (48.0)	47.8

[a] Received from Cancer Chemotherapy National Service Center. [b] The lyo vials contained MTX as the sodium salt, 86 mg. of sodium chloride, and a small amount of sodium hydroxide. [c] The lyo vials contained MTX as the sodium salt, 34.4 mg. sodium chloride, 3.2 mg. methylparaben, 0.8 mg. propylparaben, and a small amount of sodium hydroxide.

of MTX from these contaminants and as a result the accuracy of the assay is enhanced. Agreement between the paper and column method is obtained if the first and last tube of the MTX band eluted from the column are eliminated. These tubes contain the fluorescent contaminants seen on the papers.

The total recovery of MTX from the chromatogram and evidence that no deterioration occurs during a run is illustrated by the analysis of a chromatographically homogeneous sample.[5] The comparison of the UV of the bulk with the UV of the band removed from the paper showed a 99.7% recovery (average of four runs).

REFERENCES

(1) Oliverio, V. T., *Anal. Chem.*, 33, 263(1961).
(2) Gallelli, J. F., and Yokoyama, G., *J. Pharm. Sci.*, 56, 387(1967).

Keyphrases

Methotrexate—analysis
Paper chromatography—analysis
UV light—methotrexate spot detection
UV spectrophotometry—analysis

Heterocyclic Amines V. Electrophilic Substitution in Some Carbamate Derivatives of 3-Aminothiophene

By EMERY W. BRUNETT* and WALTER C. McCARTHY

t-Butyl N-(3-thienyl)carbamate has been brominated, and neopentyl N-(3-thienyl) carbamate has been chlorinated, brominated, iodinated, acetylated, nitrated, and coupled with a diazonium salt. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

VARIOUS ELECTROPHILIC substitution reactions were investigated for some carbamate derivatives of 3-aminothiophene in order to determine suitable conditions for such reactions with these sensitive compounds, and to confirm the orientation of the incoming group.

t-Butyl *N*-(3-thienyl)carbamate (1) was smoothly brominated in the 2-position with *N*-bromosuccinimide. Similarly, the neopentyl ester (1) could be chlorinated, brominated, or iodinated with the corresponding *N*-halosuccinimides. The neopentyl ester was acetylated under mild conditions using only acetic anhydride and acetic acid. Attempted nitration of the neopentyl ester with nitric acid in acetic anhydride, as used by Campaigne (2) to nitrate the similar 3-acetamidothiophene, gave only tar, but the nitration could be effected in good yield by Anderson's (3) reagent, cupric nitrate in acetic anhydride. The neopentyl ester also coupled readily with sodium *p*-nitrobenzenediazotate. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

Received May 3, 1968, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication July 16, 1968.
* Pharmaceutical Manufacturers Association Fellow of the American Foundation for Pharmaceutical Education, 1965–1966.

EXPERIMENTAL[1]

t-Butyl N-(2-bromo-3-thienyl)carbamate—From 4.0 g. (0.02 mole) of *t*-butyl *N*-(3-thienyl)carbamate (1) and 3.6 g. (0.02 mole) of *N*-bromosuccinimide refluxed in carbon tetrachloride for 2 hr., after the usual workup of filtration, extraction with water to remove succinimide, evaporation of solvent, decolorization with carbon, and recrystallization from dilute alcohol, there was obtained a yield of 3.54 g. (64%), m.p. 69.5–71°, NMR spectrum in CCl_4: τ = 8.46 (s, CH_3, 9H), 3.46 (broad peak, NH, 1H), 2.81 (d, 4-H of ring, 1H), 2.37 (d, 5-H of ring, 1H) J_{45} = 5.8 c.p.s.

Anal.—Calcd. for $C_9H_{12}BrNO_2S$: C, 38.86; H, 4.35; Br, 28.73; N, 5.04; O, 11.50; S, 11.54.

[1] All of the analyses reported in this paper were performed in the laboratories of Dr. Alfred Bernhardt, Mülheim, Germany.