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Note

High-performance liquid chromatographic assay for methotrexate utilizing a cold acetonitrile purification and separation of plasma or cerebrospinal fluid

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Methotrexate (MTX, 4-amino-N^{10}-methylpteroglutamic acid) has been used in the treatment of acute lymphocytic leukemia (ALL) either alone or in combination with other antineoplastic agents for over thirty years [1]. MTX is not only a potent cytotoxic agent that attacks leukemic cells but also non-selectively causes toxic damage to normal tissue, especially the epithelial tissues of the gut, liver and kidney [2—4]. MTX has been shown to be involved in or be the toxic cause of myelosuppression, gastrointestinal mucositis, hepatitis, acute desquamative dermatitis, and renal dysfunction [5—7]. The occurrence and severity of toxicity is dependent on the MTX concentration and duration of exposure. Since both of these are important determinants of toxicity, monitoring of plasma concentration is essential to ensure that drug levels are reduced within an appropriate period of time to minimize toxic effects.

While there are several analytical methods currently employed to assay plasma MTX, including fluorometry [8], high-performance liquid chromatography (HPLC), ultraviolet techniques [9—12], radioimmunoassays (RIA) [13], enzyme inhibition assays (EIA) [14], and enzyme-multiplied immunoassays (EMIT) [15], these methodologies possess inherent difficulties that limit their applicability. Immunochemical methods lack specificity since metabolites, such as 7-hydroxymethotrexate (7-OHMTX) and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA) can cross react with the antibody to produce spuriously high results [16]. Enzyme inhibition techniques are susceptible to interference by commonly used antibiotics such as trimethoprim [17]. The EMIT assay provides limited sensitivity. HPLC techniques utilizing either UV or

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fluorescence detection methods offer a means whereby both specificity and sensitivity can be simultaneously achieved.

Sample preparation techniques for the HPLC measurement of MTX in serum or plasma fall into two distinct categories. The first utilizes pre-column clean-up of a plasma or serum sample, does not require the precipitation of plasma or serum proteins and, therefore, serum or plasma may be directly injected. These non-extraction techniques have a rapid total analysis time (10—20 min) [18] but suffer from a decrease in column efficiency, the cost of replacement of pre-columns, lengthy washings of pre-columns, and variable recovery of the drug (80—110%) [19].

In the second category of preparation techniques, the drug is extracted in a more purified and concentrated state before analysis by HPLC. These extraction techniques also suffer from several serious disadvantages such as time required for extraction (up to 1 h), chromatography time (10—40 min) [20], low recoveries (40—50% [9] and detection limits generally greater than 50 ng/ml [18]. Most, if not all, extraction techniques utilize a protein precipitant (acid or acetonitrile) followed by a salt-saturated extraction into a volatile organic solvent such as ethyl acetate or diethyl ether. The technique described in this report eliminates the need for salt solvent extraction, yet allows for the purification and concentration of the drug by a cold separation of acetonitrile from serum, plasma, or cerebrospinal fluid (CSF). This allows for superior recovery with increased sensitivity while utilizing a small sample size, thus making it ideally suited for analysis of pediatric samples.

EXPERIMENTAL

Methotrexate was provided by the National Cancer Institute (Washington, DC, U.S.A.) as manufactured by Lederle Labs. (American Cyanamid, Pearl River, NY, U.S.A.). Monobasic sodium phosphate was purchased from Fisher (Fairlawn, NJ, U.S.A.). Tris(hydroxymethyl)aminomethane base, phosphoric acid, HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). *p*-Aminoacetophenone (PAAP) was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). Water was deionized and doubly distilled for HPLC use. All other reagents, equipment, and glassware were of standard laboratory quality.

The mobile phase consisted of 0.1 *M* monobasic sodium phosphate and 10 m*M* Tris phosphate (pH to 5.75 by the addition of a few drops of concentrated phosphoric acid), methanol, and acetonitrile (82:13:5). After filtration, the mobile phase was degassed under reduced pressure with continual sonication.

The chromatographic system consisted of a M-45 solvent delivery system, a Model 480 UV/VIS variable-wavelength detector, a Z module radial compression unit, and a 5-μm reversed-phase C_{18} Radial Pak column (all from Waters Assoc., Milford, MA, U.S.A.). The injector was a Reodyne Model 7125 (Berkeley, CA, U.S.A.) equipped with a 100-μl loop. A flow-rate of 2.3 ml/min at a pressure of 7 mPa (1000 p.s.i.) was used for all chromatography. Chromatography was carried out at ambient temperature. The column eluent was monitored for UV absorbance at 313 nm. Detector output was recorded and integrated by a Perkin-Elmer Model 15 (Perkin-Elmer, Norwalk, CT, U.S.A.) data station.

Stock solutions of internal standard (PAAP) and MTX were prepared in water at concentrations of $2.5 \cdot 10^{-4}$ *M* and $2.5 \cdot 10^{-5}$ *M*, respectively. MTX analytical and quality-control standards were prepared over a concentration range of $5 \cdot 10^{-8}$ *M* to $1 \cdot 10^{-5}$ *M* by adding 20 μl of an appropriate dilution of stock solution to 460 μl freshly pooled drug-free plasma obtained from healthy volunteers. Analytical standards for CSF assays were similarly prepared in Elliotts B solution.

Patient blood, 1 ml, was collected in tubes containing ethylenediaminetetra-acetic acid (EDTA) as the anticoagulant. Plasma was transferred to tubes covered with aluminum foil and stored on ice or refrigerated until assayed. A 20-μl aliquot of internal standard stock solution was added to 480 μl plasma or CSF to yield a final concentration of 1352 ng/ml. Each tube was vortexed for 5 sec. Protein was precipitated by the slow dropwise addition of acetonitrile during vortexing. The supernatant and precipitate were transferred into a 1.5-ml Eppendorf microfuge tube with a wide-bore transfer pipet and centrifuged at room temperature for 5 min at 13 000 *g*. The supernatant was removed, placed into a new 75 $\times$ 12 mm disposable culture tube that was pre-cooled on ice for 5 min, and kept on ice for at least an additional 10 min. The tubes were then centrifuged at 2175 *g* at 0°C for 5 min, after which they were returned to ice for an additional 5 min. The top layer of the acetonitrile supernatant was aspirated with a Pasteur pipette and discarded. Of the lower aqueous layer 360 μl were removed and transferred to a clean 75 $\times$ 10 mm disposable culture tube and taken to dryness at 50°C under nitrogen. The dried material was reconstituted in 100 μl of mobile phase and approximately 40 μl were injected onto the column.

In order to assess the linearity, precision, and accuracy of this procedure, standard curves were constructed in plasma or serum over a concentration range of $1 \cdot 10^{-5}$ *M* (4540 ng/ml) to $5 \cdot 10^{-8}$ *M* (22 ng/ml). Precision was determined with a medium range concentration of $1 \cdot 10^{-6}$ *M* (454 ng/ml) for both intra- and inter-assay variation. Accuracy was assessed by the daily determination of a plasma control known to contain $1 \cdot 10^{-6}$ *M* (454 ng/ml) MTX over a period of ten days.

RESULTS AND DISCUSSION

At ambient temperature acetonitrile is water miscible and separation of the organic and aqueous phases does not occur. However, at 0°C phase separation occurs allowing differential separation of MTX from interfering plasma constituents. This phenomenon provides the basis for the purification and concentration procedure used in this method.

Fig. 1 demonstrates typical chromatograms obtained from a plasma sample containing MTX at $1 \cdot 10^{-6}$ *M* (454 ng/ml) after the separation of the two phases. The upper acetonitrile layer (Fig. 1A) of the two-phase system is devoid of MTX (retention time = 5.00 min) while MTX remains exclusively in the lower aqueous phase (Fig. 1B).

While it can be seen that the internal standard separates between the two phases, this separation is reproducible and consistent so that a constant amount of the internal standard is always present in the phase containing MTX. The

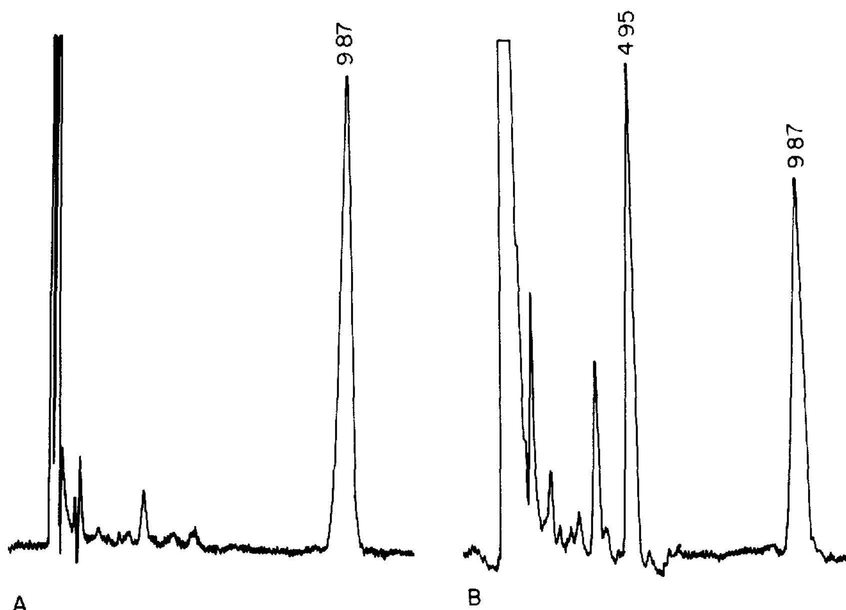

Fig. 1. Chromatograms of a control plasma sample. Methotrexate (MTX) was present at $1 \cdot 10^{-6}$ M (454 ng/ml). (A) Acetonitrile upper phase devoid of MTX with the internal standard, *p*-aminoacetophenone (PAAP), eluting at approximately 10 min; (B) aqueous lower phase with MTX eluting at approximately 5.00 min and PAAP at 10 min. Chromatographic conditions are detailed in the text.

TABLE I

REPRODUCIBILITY DATA FOR *p*-AMINOACETOPHENONE, INTERNAL STANDARD, IN AQUEOUS PHASE

Values represent the mean actual area counts for the internal standard peaks using day-to-day comparisons.

Integrator type	Group*	Mean ± S.D.	*n*	Coefficient of variation (%)
Waters Model 700	A	378874.3 ± 15314.7	28	4.10
	B	389392.1 ± 15603.2	14	4.01
(8-84)**	C	369593.9	50	4.20
Perkin-Elmer Model Sigma 15 data system	A	1.453 ± 0.0750	29	5.20
	B	1.4825 ± 0.1298	24	8.73
(5-84)**	C	1.3948 ± 0.0902	79	6.50

*Groups are designated as follows: A, calibration standards; B, quality controls, C, unknown patient samples.
**Month assayed.

actual area counts of the internal standard peaks of calibration standards, quality-control, and patient plasma samples from two different integrators during two different months are shown in Table I. It is apparent that the concentration of internal standard in the aqueous phase which contains MTX

is extremely reproducible and stable. Coefficients of variation (C.V.) on day-to-day assays were 4–6% throughout both months irrespective of the type of integrator used or the type of sample assayed (calibration standard, quality control, or patient sample).

Since the area counts directly reflect the concentration of a sample, these results indicate that although the internal standard does not fully partition into the aqueous phase, its concentration in that phase is constant.

The recovery of MTX was determined to be 96%. MTX had a retention time of 5.00 min, while that of the internal standard, PAAP, was approximately 10 min. A standard curve prepared in normal plasma was linear (linear regression correlation coefficient $r = 0.99$) between $1 \cdot 10^{-5}$ *M* (4450 ng/ml) and $5 \cdot 10^{-8}$ *M* (22 ng/ml). The linear dynamic range was $2 \cdot 10^{2}$. Within-assay precision was excellent (C.V. = 1.73%, $n = 6$) at a concentration of $1 \cdot 10^{-6}$ *M* (454 ng/ml) as was day-to-day precision for the same concentration (C.V. = 3.80%, $n = 10$) (Table II). The sensitivity limit was found to be $4.40 \cdot 10^{-8}$ *M* (20 ng/ml). This concentration yielded a response with a signal-to-noise ratio of at least 2.

TABLE II

REPRODUCIBILITY DATA FOR METHOTREXATE IN HUMAN PLASMA

Values are based on a midrange concentration of 454 ng/ml. Approximately 40 μl were injected at a sensitivity setting of 0.005 a.u.f.s.

Group	Mean ± S.D.	*n*	Coefficient of variation (%)
Within-day	484.9 ± 8.42	6	1.73
Between-day	437.1 ± 16.70	10	3.80

A comparison of the accuracy of this technique with other commonly used assays was assessed from College of American Pathologists (CAP) Therapeutic Drug Monitoring quality-control surveys obtained during the month of April, 1984. Accuracy was assessed at two target MTX concentrations, a low value of $6 \cdot 10^{-6}$ *M* and a high value of $1 \cdot 10^{-5}$ *M*. This method resulted in a relative error of –8.6 for the low concentration and +5.6% for the high concentration based on a comparison of mean assay values to the target value of the unknown samples. In contrast, mean relative errors for the assays currently in common clinical use (RIA, EIA, EMIT) were –15.4% and –13.26% for the low and high concentrations, respectively.

Fig. 2A demonstrates a control plasma sample at a MTX concentration of $1 \cdot 10^{-6}$ *M* (454 ng/ml) with the internal standard present. A chromatogram of plasma from a child with ALL is presented in Fig. 2B. Before drug administration, there is a stable baseline with no interfering peaks at the retention time of MTX. Fig. 2C illustrates a typical chromatogram for an ALL patient receiving MTX. MTX appears at its characteristic retention time along with a later eluting peak with a retention time of about 6.00 min which represents the

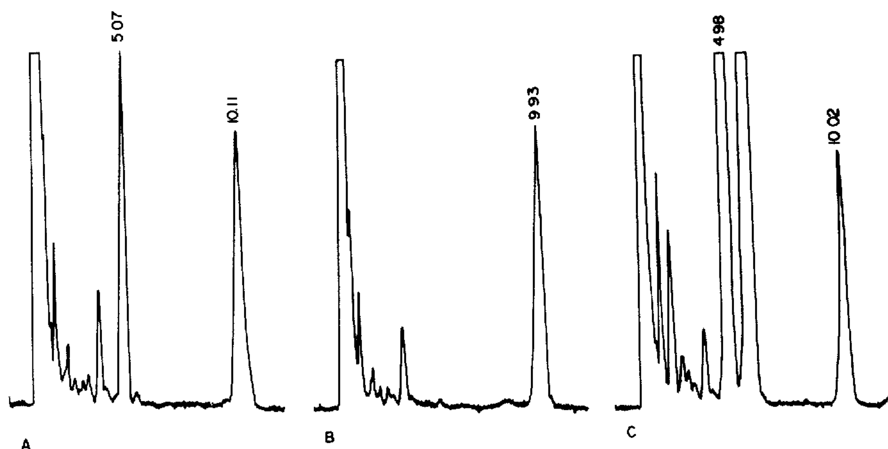

Fig. 2. Chromatograms of (A) a control plasma containing methotrexate (MTX) at $1 \cdot 10^{-6}$ M (454 ng/ml); (B) blank plasma of a typical pediatric acute lymphocytic leukemia patient before drug administration; (C) same patient as in (B) after 12 h of methotrexate infusion. Internal standard (PAAP) was present in all three cases.

7-OH metabolite of MTX. Hemolysis was not found to interfere. The assay may be used equally well with plasma, serum, and CSF samples.

An artifical CSF vehicle such as Elliott's B solution must be used to prepare standards and controls for the measurement of MTX in CSF. The acetonitrile and aqueous phases do not separate during the extraction procedure when water or serum albumin dissolved in water is used. Therefore, it appears that inorganic salts are essential for separation of the two phases.

Commonly used antineoplastic agents and adjuvants, such as cytosine arabinoside, hydrocortisone, cyclophosphamide, vinblastine, antibiotics, citrovorum factor, and adriamycin, do not interfere with the chromatography of MTX or PAAP in this system.

The method described here employs a simple cold acetonitrile separation of interfering plasma constituents from MTX, is highly sensitive, and relatively quick when compared to other extraction methods. It has several additional advantages over existing techniques. It allows for isocratic separation with a relatively short overall chromatography time (11 min), the injection volume is low (40 μl or less), sensitivity is high ($4.4 \cdot 10^{-8}$ M; 20 ng/ml), and small sample volumes (as little as 240 μl) are required. Recovery by this process is nearly complete, i.e. 96%. Dilutions for higher concentrations (10^{-4} or 10^{-5} M) are not required as in RIA or non-isotope immunoassays. Finally, the precision and accuracy is better than that of many of the previously existing techniques because of the use of an internal standard.

At present, clinical monitoring of serum MTX is essential to ensure that serious toxicity does not occur. This assay is applicable to both routine clinical and basic research work. Its sensitivity is well within the values required for clinical monitoring. MTX serum levels at 48 h of less than $9 \cdot 10^{-7}$ M and 72-h values of less than $1 \cdot 10^{-7}$ M have been found to correlate well with a lack of serious clinical toxicity [21—27]. While not as sensitive as the dihydrofolate reductase enzyme binding assay [28], this assay provides superior sensitivity to other published HPLC assays and offers greater specificity than RIA or other immunoassays currently available.

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REFERENCES

1 S. Farber, L.K. Diamond, R.D. Mercer, R.F. Sylvester and V.A. Wolff, N. Engl. J. Med., 238 (1948) 787.
2 P.T. Condit, R.E. Chanes and W. Joel, Cancer, 23 (1969) 126.
3 N. Jaffe and D. Traggis, Cancer Chemother. Rep., 6 (1975) 31.
4 M. Nesbit, W. Krivit and R. Heyn, Cancer, 27 (1976) 1048.
5 E. Frei, Med. Pediatr. Oncol., 2 (1976) 227.
6 S.W. Pitmay, L.M. Parker, M.H. Tatersal, N. Jaffe and E. Frei, Cancer Chemother. Rep., 6 (1975) 43.
7 E.D. Thomas and R. Storb, Ann. N.Y. Acad. Sci., 186 (1971) 467.
8 J.A. Nelson, B.A. Harris, W.J. Decker and D. Farquhar, Cancer Res., 37 (1977) 3970.
9 E. Watson, J.L. Cohen and K.K. Cow, Cancer Treat. Rep., 62 (1978) 381
10 J.L. Wisnicki, W.P. Tong and D.B. Ludlum, Cancer Treat. Rep., 62 (1978) 529.
11 C. Canfell and W. Sadee, Cancer Treat. Rep., 64 (1980) 165.
12 D.A. Cairnes and W.E. Evans, J. Chromatogr., 231 (1982) 103.
13 C. Bohuon, F. Duprey and C. Boudene, Clin. Chim. Acta, 57 (1974) 263.
14 L.C. Falk, D.R. Clark, S.M. Kalman and T.F. Long, Clin. Chem., 22 (1976) 785.
15 R.G. Buice, W.E. Evans, J. Karas, C.A. Nicholas, P. Sidhu, A.B. Straughn, M.C. Meyer and W.R. Crom, Clin. Chem., 26 (1980) 1902.
16 G.W. Aherne and M. Quinton, Cancer Treat. Rep., 65 (1981) 55.
17 J.L. Bock and R. Pierce, Clin. Chem., 26 (1980) 1510.
18 H. Breithaupt, E. Kuenzlen and G. Goebel, Anal. Biochem., 121 (1982) 103.
19 G.J. Lawson and P.F. Dixon, J. Chromatogr., 223 (1981) 225.
20 W.M. Deen, P.F. Leoy, J. Wei and R.D. Partridge, Anal. Biochem., 119 (1981) 335.
21 J. Jolivet, K.H. Cowan, G.A. Curt, N.J. Clendininn and B.A. Chabener, N. Engl. J. Med., 309 (1983) 1094.
22 J.R. Bertino, Med. Pediatr. Oncol., 10 (1982) 401.
23 C. Perez, W.W. Sutow, Y.M. Wang and J. Herson, Med. Pediatr. Oncol., 6 (1979) 219.
24 R.G. Stoller, K.R. Hande, S.A. Jacobs, S.A. Rosenberg and B.A. Chabener, N. Engl. J. Med., 297 (1977) 630.
25 A. Nirenberg, C. Mosende, B.M. Mehta, A.L. Gisolfi and G. Rosen, Cancer Treat. Rep., 61 (1977) 779.
26 S.W. Pitman, L.M. Parker, M.H.N. Tattersall, N Jaffer and E Frei, III, Cancer Chemother. Rep., 6 (1975) 43.
27 W.E. Evans, C.B. Pratt, R.H. Taylor, L.F. Barker and W.R. Crom, Cancer Chemother. Pharmacol., 3 (1979) 161.
28 J.R. Bertino and G.A. Fischer, Methods Med. Res., 10 (1964) 297.