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Reversed-phase liquid chromatography analysis of methotrexate in serum with fluorescence detection

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REVERSED-PHASE LIQUID CHROMATOGRAPHY ANALYSIS OF METHOTREXATE IN SERUM WITH FLUORESCENCE DETECTION.

by

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THESIS

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ABSTRACT

Methotrexate in human serum at concentration as low as 11 nmol/L can be assayed by this liquid chromatography method with fluorescence detection. Methotrexate is extracted from 100 ul of serum by elution from a Bond-Elut C_{18} extraction column with 1 ml of 1% diethylamine in methanol. Pterine is used as an internal standard. After evaporation, methotrexate is oxidized by potassium permanganate, and the oxidized product is then eluted with tris-phosphate buffer (pH 6.7) at a flow rate of 2.0 ml/min through a C_{18} reversed phase column. Excitation and emission wavelengths are set at 392 nm and 449 nm, respectively. The recovery of methotrexate ranged from 98% to 103% for concentrations up to 11 umol/L. Intra-day precision (CV) is 1.8% at 579 nM (n=10) and 2.6% at 2.44 uM (n=10) while inter-day precision is 11% at 579 nM (n=15) and 8.4% at 2.36 uM (n=15). The metabolites of methotrexate, citrovorum factor and the commonly used drugs do not interfere with this assay. This liquid chromatography assay was compared with enzyme multiplied immunoassay technique (EMIT) and the linear regression analysis shows good correlation (r=.997) between the two procedures. This procedure is simple, rapid and suitable for monitoring methotrexate levels in clinical laboratories.

I. INTRODUCTION

A. Antineoplastic agents

Cancer occurs in all human and animal populations. It is a disease which mainly affects dividing cells. Surgery and X-ray therapy are often used for the treatment of cancer but a number of chemotherapeutic agents are also available. These antineoplastic drugs include alkylating agents, antimetabolites, natural products, hormones, and miscellaneous agents (33).

The alkylating agents (busulfan, carmustine, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, lomustine, mechlorethamine, melphalan, pipobroman, streptozocin, thiethylenethiophosphoramide, uracil mustard) can alkylate amino, sulfhydryl, carboxyl and phosphate groups of biologically important cell constituents and thereby impair the function of the cells. When the purine base is alkylated, it may cause miscoding of the genetic message and may result in abnormal base pairing. Alkylating agents may also inhibit DNA replication, transcription of RNA and normal nucleic acid formation.

The antimetabolites are structural analogs of normal metabolites. They interfere in the synthesis of nucleic acid by competing with purines or pyrimidines in metabolic pathways. Antimetabolites may be divided into three groups: folic acid antagonists (methotrexate), purine antagonists (mercaptopurine, thioguanine) and pyrimidine antagonists (floxuridine, fluorouracil, cytarabine).

The naturally occuring chemotherapeutic agents include an enzyme (asparaginase), antibiotics (bleomycin, dactinomycin, daunorubicin, dexorubicin, mithramycin, mitomycin) and plant derivatives (vinblastine, vincristine). The asparaginase acts by depleting some of amino acid essential for the growth of tumor cells. The antibiotics work by inhibiting DNA or RNA synthesis and the plant derivatives can inhibit cell mitosis.

Hormonal agents are used to treat tumors that are sensitive to hormones. Hormonal agents are not curative, because most lack a cytotoxic action. The sex hormones (androgens, estrogens, and progestins) and the corticosteroids are used.

The exact mechanism of miscellaneous agents (hydroxyurea, procarbazine) is not completely known. They may inhibit DNA, RNA or protein synthesis.

B. Pharmacology

Methotrexate, also known as amethopterin, is 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (Figure 1). Its molecular weight is 454 and it is a bicarboxylic weak acid with pKa_s in the range of 4.8 to 5.5. Therefore, methotrexate is essentially ionized and lipid insoluble at physiological pH.

Tetrahydrofolic acid is necessaary for the conversion of deoxyuridylic acid (dUMP) to thymidylic acid (dTMP). Methotrexate

binds to the enzyme dihydrofolate reductase thereby preventing reduction of dihydrofolic acid to tetrafolic acid, thus inhibiting the synthesis of DNA since dUMP can no longer be methylated to dTMP. If exogenous tetrahydrofolic acid (folic acid, N^5-formyltetrahydrofolic acid, leucovorin) is administered, the reduction of dihydrofolic acid becomes unnecessary and the cells are spared from the effects of methotrexate (Figure 2). (3, 17 ,50)

Usually, there are three types of methotrexate protocols for the chemotherapy : first conventional low dose without citrovorum factor ; second high dose methotrexate with citrovorum factor and third intrathecal methotrexate therapy. Low doses are highly effective in treating choriocarcinoma, acute lymphocytic leukemia, breast carcinoma and in intrathecal chemotherapy.

High doses (1 to 30 g per square meter) of methotrexate followed by citrovorum factor rescue are used more and more often. The use of high doses of methotrexate leading to high plasma levels (10^{-4} to 10^{-5}M) for prolonged periods (12 to 36 hours) has several advantages based on (25) :

(1) at high plasma levels, passive entry of methotrexate into tumor cells can potentially overcome drug resistance due to defective active transport
(2) the increased free intracellular methotrexate levels achieved can overcome drug resistance secondary to increased dihydrofolate reductase or altered enzyme binding

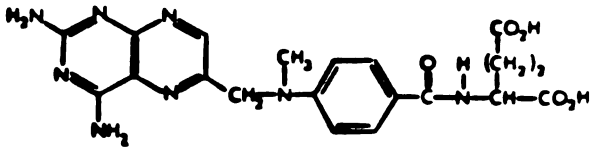


Figure 1. Methotrexate structure

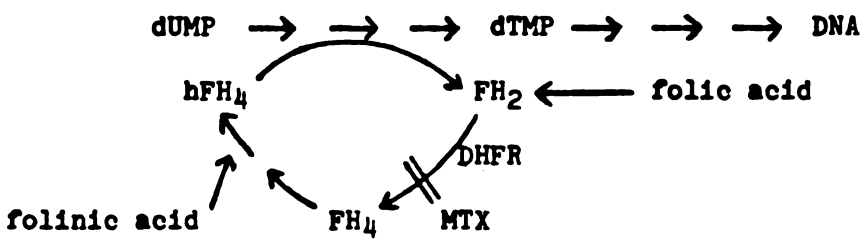


Figure 2. Methotrexate (MTX) suppression of DNA synthesis by blocking deoxyuridylic acid conversion

dUMP = deoxyuricylic acid, dTMP = thymidylic acid,

hFH_4 = N^5,N^{10}-methylene tetrahydrofolic acid,

FH_4 = tetrahydrofolic acid, FH_2= dihydrofolic acid

DHFR = dihydrofolic acid reductase,

DNA = deoxyribonucleic acid.

(3) the high and prolonged plasma drug levels can promote increased methotrexate polyglutamate formation, resulting in more prolonged drug action

(4) prolonged drug administration can expose more cells to methotrexate during DNA synthesis

(5) the subsequent rescue treatment by various agents may selectively enhance the recovery of normal but not tumor cells.

By these mechanisms high-dose methotrexate can overcome an inherent methotrexate resistance of the tumor, for example, osteogenic sarcoma is resistant to small doses of methotrexate but can be effectively treated with high-dose methotrexate (41).

According to the study of Stoller et al. (44), the 48-hour drug concentration above $9x10^{-7}M$ after 50 to 250 mg methotrexate/Kg over 6-hour infusion were considered to be toxic, therefore, in these cases continued citrovorum factor injections may be necessary in reducing methotrexate toxicity. Rescue treatment should be continued until a relatively safe methotrexate level has been achieved (e.g. $< 5x10^{-8}M$). (41)

The establishment of alkaline diuresis during treatment is essential to prevent nephrotoxicity, which probably results from the precipitation of methotrexate or 7-hydroxymethotrexate in the renal tubules, and monitoring of plasma methotrexate levels during therapy is necessary to identify patients at high risk for toxicity, who require higher doses of citrovorum factor.

However, high doses of methotrexate with citrovorum factor are used effectively against osteogenic sarcoma, pulmonary and epidermoid carcinoma and non-Hodgkin's lymphoma (10,11,22,26). Methotrexate is also useful in bone marrow transplantation, severe psoriasis, rheumatoid arthritis, dermatomyositis, Wegener's granulomatosis and sarcoidosis (3).

C. Pharmacokinetics

1. Absorption

The absorption of methotrexate following intramuscular injection is rapid and complete. Gastro-intestinal absorption of methotrexate is dose dependent. (3,42) When doses are less than 30 mg/m^2 body surface area, methotrexate is well absorbed. With dosage of 80 mg/m^2 or more, the extent of absorption is reduced to 50 - 70 % (15). Therefore, parenteral route is preferred over oral administration when large doses are given.

2. Distribution

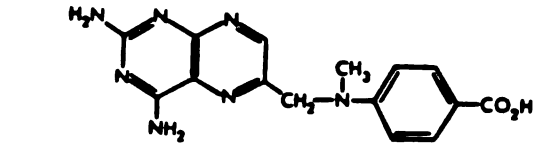
After intravenous injection, methotrexate disappears from plasma into three exponential phases (18). The initial half-life is 0.75 $\pm$ 0.11 hours and is probably due to distribution. The second phase has a half-life of 3.49 $\pm$ 0.55 hours which is primarily elimination of the drug via biliary excretion and renal clearance. The terminal half-life is much slower ranging from 6 - 69 hours with a mean of about 27 hours. This may reflect an enterohepatic circulation of the drug.

In humans, 50-70 % of the drug is bound to plasma proteins mainly albumin. The "blood-brain barrier" slows entry of systemically-administered methotrexate into the central nerve system. Methotrexate is usually given intrathecally for treatment of cancers which have metastasised into the central nerve system.

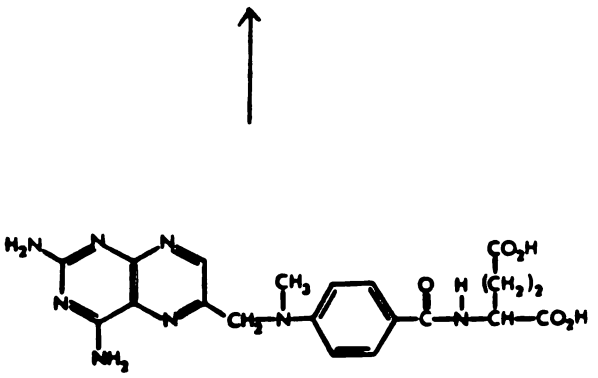
3. Metabolism

Methotrexate is metabolized into 4-amino-4-deoxy-N^{10}-methylpteroic acid and several unconjugated 2,4-diaminopteridines during its enterohepatic circulation by bacterial organism residing within the intestinal tract(51). These metabolites are less potent inhibitors of dihydrofolic acid reductase (49,51). In addition, polyglutamate derivatives of methotrexate may serve as an intracellular reservoir of methotrexate (42).

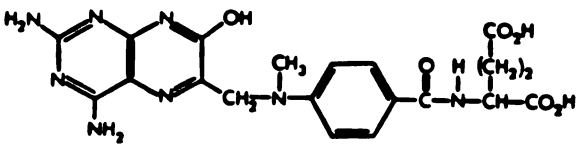
7-hydroxymethotrexate has been found in the urine of patients treated with high dose methotrexate (Figure 3). It is produced from methotrexate, probably in the liver, by the enzyme aldehyde oxidase. 7-hydroxymethotrexate is about half as potent as its parent compound as an inhibitor of dihydrofolic acid reductase. The solubility of 7-hydroxy-methotrexate is three to five-fold less than methotrexate, a fact which may be responsible for the renal toxicity during high dose methotrexate therapy.



4-amino-4-deoxy-N^{10}-methylpteroic acid



methotrexate



7-hydroxymethotrexate

Figure 3. Metabolic products of methotrexate

4. Excretion

The major route of elimination for methotrexate is renal excretion (-80%). (4,18) It is not only filtered but also actively secreted by the renal tubules. Methotrexate renal tubular secretion utilizes the general organic acid transport mechanism since concomitant administration of salicylate and para aminohippurate reduced methotrexate clearance well below the glomerular filtration rate (31). Intratubular precipitation of methotrexate may cause nephrotoxicity during high dose threapy (45). About 0-9% of an intravenous dose of ^{3}H-methotrexate appears in the feces (15 ,18). In some studies, methotrexate was also found in saliva and breast milk (18,23)

D. Adverse effects

The most frequently encountered toxic effects are myelosuppression, oral and gastrointestinal ulcers, hepatitis, and a syndrome consisting of severe dyspnea, a nonproductive cough, hypoxemia, and bilateral pulmonary infiltrates (18). The methotrexate-induced renal toxicity may cause an unexpected delay in its elimination and further toxicity may ensue. Precipitation of methotrexate in the urine may be partially responsible for the kidney damage. Thus toxicity can be prevented by hydration and urine alkalinization with sodium bicarbonate (4,41).

The maximum tolerated dose of systemic methotrexate in man varies markedly from 80-900mg/m^2 without citrovorum rescue and from 900-

30,000mg/m^2 when citrovorum factor rescue is given (3). Thus dose varies according to the differences in the levels of dihydrofolate reductase, its inducibility, variations in the affinity of the enzyme for substrate or inhibitor, or differences in cell membrane permeability, and variation in the pharmacokinetics of methotrexate (18).

The severity of toxicity is determined more by the duration of methotrexate exposure than the magnitude of methotrexate concentration above the threshhold (3). If the plasma methotrexate concentration remains > 10^{-5}M at 24 hours or $5x10^{-7}$M at 48 hours, significant toxicity is likely to occur(20). So, plasma methotrexate concentration specifies when citrovorum factor rescue can be safely discontinued.

E. Review of analytical methods

The methods available for measuring methotrexate include spectrophotofluorometry (7,27), competitive protein binding assay (35), direct ligand-binding radioassay (2), radioimmunoassay (14,32,40), enzyme inhibition assay (12,39,43), enzyme immunoassay (38,39), and liquid chromatography (6,8,9,29,36,43,46,48).

1. Spectrophotofluorometry

The method reported by Chakrabarti and Bernstein (7) involved measuring fluorescence before and after oxidation of methotrexate with permanganate. The difference between the two

fluorescence readings was proportional to the concentration of methotrexate present in the sample.

Using this method , a variable amount of methotrexate is adsorbed on TCA-precipitated plasma proteins. Therefore, heating and stirring are necessary to release the adsorbed methotrexate into the supernatant solution. At pH 6 and above a significant amount of precipitate appeared upon the addition of permanganate solution. The fluorescence readings increased with increasing the pH up to 5. A plateau was obtained between pH 5 and pH 6. This method determined plasma methotrexate levels as little as 11umol/L.

The method described by Kinkade et al. (27) used the extraction of methotrexate into n-butanol in the presence of TCA. Following reextraction into an aqueous phase, the drug was oxidized using potassium permanganate to yield products whose fluorescence was linear over a wide range of plasma concentration. The readings were based on a direct measurement rather than an increment and the lower limit of reliable measurement was 220nmol/L of methotrexate in plasma. However, the spectrophotofluorometry assays are interfered by other pteridines (e.g. folic acid, citrovorum factor) and some drugs which can undergo similar reactions. Therefore, these methods are not the best choice for the clinical monitoring of methotrexate therapy.

2. Radioimmunoassay

The radioimmunoassay for methotrexate has been developed using antibody induced in rabbits with a conjugate of methotrexate and methylated bovine serum albumin, tritium-labeled methotrexate, and a nitrocellulose membrane seperation technique (40).

Specific antibody and [^{3}H]methotrexate form a complex that can be detected upon passing through the nitrocellulose filter. Any decrease in the amount of radioactivity bound to the filter reflected competitve replacement of [^{3}H]methotrexate at the antibody-combining site and was expressed as the percentage of displacement. Inhibition of binding was determined as a function of the amount of unlabeled compound added.

Other separation techniques were reported. Loeffler et al. (32) described a radioimmunoassay using dextran-coated charcoal adsorption to remove unbound drug. The assay of Hendel et al. (14) separates antibody-bound methotrexate with polyethylene glycol.

The system measured as little as 0.1 to 1 pmol of methotrexate. Folic acid and folinic acid are required at 40,000- and 80,000- fold higher concentrations, respectively , to produce comparable displacement of [^{3}H]methotrexate. These assays are generally more sensitive than liquid chromatography. There is 5 to 10 % cross reactivity with 7-hydroxymethotrexate in radioimmunoassays (16). The procedures involved in the preparation of samples for liquid

scintillation counting and the actual counting time were the main time consuming steps. The speed and capacity of the assay can be enhanced with the replacement of the beta-emitting [^{3}H]methotrexate with a gamma-emitter such as an ^{125}I or ^{75}Se derivative of methotrexate which can be semiautomated (37).

3. Competitive protein binding assay and Direct ligand-binding radioassay

The assay described by Myers et al. (35) was based on competition between [^{3}H]methotrexate and unlabeled methotrexate for binding to dihydrofolate reductase with subsequent removal of unbound drug by charcoal adsorption. Charcoal, a known adsorbent of folates, was used to separate bound from free methotrexate. The charcoal was treated with dextran to improve sedimentation and with bovine serum albumin to eliminate partial adsorption of the binding protein.

The increasing concentrations of unlabeled methotrexate caused a progressive decrease in binding of [^{3}H]methotrexate to dihydrofolate reductase. The sensitivity was at least 1.5 pmol of methotrexate.

A direct ligand-binding radioassay for methotrexate described by Arons et al. (2) measures directly the effect of unlabeled methotrexate on the binding of [^{3}H]methotrexate using dihydrofolate reductase isolated from L1210 leukemia cells. This assay is not truly a competitive inhibition assay system because the binding lysate is not

added to a mixture of labeled and unlabeled methotrexate. The procedure is a two-phase reaction system where standard methotrexate concentrations of the sample being assayed is incubated with the reagent lysate in the first phase and [^{3}H]methotrexate is then added in the second phase to titrate the remaining unoccupied binding sites on the enzyme. The lower limit of this radioassay is approximately 22 pmoles.

These assays are equal to the radioimmunoassay for methotrexate, except using dihydrofolate reductase instead of antibody.

4. Enzyme inhibition assay and Enzyme immunoassay

The enzyme inhibition assay (5,12) measured methotrexate by exploiting its ability to inhibit dihydrofolate reductase. The inhibition of dihydrofolate reductase activity increased with increasing methotrexate concentation. The method is based on the conversion of NADPH to $NADP^{+}$ during reduction of dihydrofolate reductase. The lower detection limit in serum is 22 nmol/L. Hemoglobin concentrations as great as 1g/L and bilirubin concentrations up to 100 mg/L do not affect results. Moderate lipemic samples lower the results obtained with this method (39). The enzyme inhibition assay has a 10% cross reactivity with 4-amino-4-deoxy-N^{10}-methylpteroic acid and 1% with 7-hydroxymethotrexate (16).

The enzyme-multiplied immunoassay technique (EMIT) described by Pesce (38) is based on the following principles : Serum containing the drug to be measured is mixed with an antibody to the drug, with

glucose-6-phosphate, and with NAD^+. The drug in the serum binds to the active sites of the antibody. Next, a reagent complex of glucose-6-phosphate dehydrogenase (G-6-PD) coupled with methotrexate is mixed with the serum and methotrexate antibody and competes with the drug in the serum for the remaining free antibody binding sites. Binding of the enzyme-drug complex to the antibody reduces the enzymatic activity of the G-6-PD. The free enzyme-drug complex reacts with glucose-6-phosphate to form 6-phosphogluconate, with the concurrent reduction of NAD^+ to NADH.

$$\text{glucose-6-phosphate} \xrightarrow[NAD^+ \quad NADH+H^+]{\text{G-6-PD}} \text{6-phosphogluconate}$$

The amount of NADH formed is directly proportional to the concentration of methotrexate in the samples. The linear range of EMIT is 0.2 to 2.0 umol/L. Sample concentration higher than this range needs to be diluted in 10-fold steps with water. The results are not affected by bilirubin in concentration up to 100 mg/L and moderate lipemia. The samples containing hemoglobin in concentrations exceeding 750 mg/L will lower methotrexate values in this method (39).

These enzymatic assays can be automated (38) and permit the use of small sample volumes, derived from blood drawn by skin puncture. They suffer from interference from severely icteric, lipemic, or hemolyzed samples and cross reactivity with the metabolites of methotrexate.

5. Microbiological assay

A microbiological assay reported by Icke et al.(19) was based on the bacteriostatic effects of methotrexate. This assay was able to measure methotrexate in the presence of large doses of trimethoprim which has cross reactivity with the protein binding assay. The method was capable of detecting as little as 0.5nmol of methotrexate. However, the microbiological assay requires overnight incubation and interfered by certain antibiotics and folates.

6. Liquid chromatography (LC)

LC is a highly specific and sensitive assay for monitoring the methotrexte concentrations of clinical samples. It has least cross-reactivity with methotrexate-related compounds and yielded lowest values for methotrexate when compared with enzyme-inhibition assay and radioimmunoassay (16).

Using the method described by Nelson et al. (36), methotrexate is oxidized to a fluorescent product (2,4-diamino-pteridine-6-carboxylic acid) which can be separated from other fluorescent materials in plasma with the use of an octadecylsilane (reversed phase) column. Protein was precipitated by adding trichloroacetic acid to plasma. Following centrifugation, the acid extract was oxidized by potassium permanganate. The oxidized product along with 2-hydroxyfolic acid as an internal standard were measured with a fluorescence detector at excitation wavelength of 275 nm and

emission wavelength 410 nm. Neither folic acid nor citrovorum factor interfered with the methotrexate analysis. The linear range of fluorescence response is from $2.2x10^{-8}M$ to $2.2x10^{-5}M$.

Watson et al. (48) reported an ion-pair extraction of methotrexate from plasma with chromatography on a microparticulate anion-exchange column using ultraviolet detection at either 254 nm or 315 nm. The plasma standard curves for methotrexte were linear from $2x10^{-7}M$ to $2x10^{-5}M$. The sensitivity limit was $2x10^{-7}M$ at 254 nm and increased to $1x10^{-7}M$ at 315 nm due to decreasing background absorption. Cohen et al. (9) markely improved column life by replacing the anion-exchange column with a RP-8 column.

Kankelma et al. (29) reported a liquid chromatography assay for methotrexate combined with an on-column concentration procedure. Samples are deproteinized with trichloroacetic acid solution followed by centrifugation. The clear supernatant is injected directly onto the concentration column. The first elution was aimed at high retention of methotrexate. By switching over to an eluent that flows reversely through the concentration column, methotrexate is transported directly on to an analytical column on which methotrexate can be separated from other compounds by an anion exchange resin. During concentration the methotrexate is separated from non-retained components by washing them off the column. In this way the analytical column can be kept free of the many potential plasma contaminants. The lower detection limit is $2x10^{-8}M$ and the measurable concentrations range up to $10^{-5}M$.

The method described by Tong et al.(46) utilizes a precolumn of CO:Pell ODS resin to protect the analytical u-Bondapak C_{18} column. The precolumn also provides a bed for extraction of methotrexate from the plasma proteins which are trapped on it. Therefore, plasma samples were analyzed directly without deproteinizing and extraction procedures. The precolumn was repacked after every 15 samples. The column effluent was monitored at 315 nm for methotrexte and linear range is from 10^{-6}M to 10^{-4}M.

Chen and Chiou (8) described an LC assay to monitor methotrexate in plasma, saliva, and urine. Those samples were deproteinized with acetonitrile. After centrifugation, the supernatant is extracted with isoamyl alcohol and ethyl acetate. An aliquot of the resultant aqueous solution is injected onto a cation-exchange column and the drug and its metabolites are eluted with an acetonitrile-phosphate buffer mobile phase. The effluent is monitored by UV detector at 313 nm. For urine and plasma samples containing higher concenration of methotrexate and its metabolites, only simple deproteinization is needed prior to the chromatography. The extraction efficiency of methotrexate for plasma is 70%. The linear concentration range is from 2.2×10^{-7}M to 2.2×10^{-5}M .

An automated liquid chromatographic system for separation and quantitation of the methotrexate and metabolites and the antibiotic sulfamethoxazole in body fluids was reported by Cairnes et al. (6). This method employed reversed-phase LC on a uBondapak C_{18} column and gradient elution with UV detection at 308 nm. The minimum detectable value was 48nmol/L for methotrexate.

The reversed phase LC method using UV absorbance detection at 313 nm for measuring methotrexate in serum was described by So et al. (43). This assay involves deproteinizing the serum sample on a Sep-Pak C_{18} cartridge, followed by seperation on a C_{18} column and detection at 313 nm. The extraction efficiency of free methotrexate from serum is 70% and minimum sensitivity is 22 nmol/L. This method is useful for the seperation of methotrexate and 7-hydroxymethotrexate.

The LC assays which are sensitive, specific and have broad linear concentration range and appear to be the method of choice for therapeutic monitoring of methotrexate.

F. Rationale for development of our LC assay

The analytical difficulties of a clinically useful methotrexate assay include: a) the wide range of methotrexate concentrations over five orders of magnitude present in serum, b) sensitivity requirements of approximately 20 nmol/L and c) the presence of variable levels of the metabolites, 7-hydroxymethotrexate, citrovorum factor and endogenous folates. The radioimmunoassays (14,16,32,40) , competitive protein binding assay (35), and enzyme immunoassay (38) meet the general criteria for the clinical methotrexate analysis, except for the metabolites and other folates do interfere with those assays and serum samples have to be serially diluted because of the limited dynamic assay range. In contrast, LC methods offer a higher degree of specificity for the measurement of methotrexate.

The previous published extraction methods include trichloroacetic acid precipitation (36), acetonitrile deproteinization followed by isomyl alcohol and ethyl acetate extraction (8), perchlorate deproteinization plus ethyl acetate-isopropanol (10:1) extraction (9) and solid-phase extraction using a Sep-Pak C_{18} cartridge (43).

The recovery of methotrexate from trichloroacetic acid precipitation method was only 40%. However,by applying a trichloroacetic acid deproteinization procedure to five fold diluted plasma samples the recovery was increased to 85-97 % (27). The loss of methotrexate was due to the adsorption on the precipitated proteins, as no loss was observed in a solution that did not contain proteins (30). Generally, the recovery was higher by solid-phase extraction techniques than the liquid-liquid extraction procedures. In addition to low recovery, the liquid-liquid extraction procedures are more tedious and time consuming.

The previous published LC methods for methotrexate suffer from low recovery, poor sensitivity to detect low concentration, time consuming extraction, and limited dynamic assay range. The assay reported here satisfies the major requirements for clinical laboratory monitoring of methotrexate by achieving a simple, rapid solid-phase extraction with high recovery and sensitivity to 11 nmol/L methotrexate with the ability to quantitate it over the broad concentration range of 22 nmol/L to 1100 umol/L by simply run the clinical sample and its hundred fold diluted sample at the same time.

II. MATERIALS and METHODS

A. Instrumentation

A Series 3 liquid chromatograph equipped with a Model 7105 fixed loop Rheodyne injector (Cotati, CA 94928) , a Model 650-10LC variable wavelength fluorescence spectrophotometer and a Model Sigma 10 recorder (all from Perkin-Elmer Corp., Norwalk, CT 06856) were used. The 250 x 4.6 mm octadecyl analytical column (Ultrasphere-ODS, particle size 5 um, Beckman Scientific Inc., Berkeley, CA 94710) along with a C_{18} Spheri-5 guard column (30 x 4.6 mm i.d., Brownlee Labs Inc., Santa Clara, CA 95050) were maintained at ambient temperature. The 83 x 4.6 mm C_{18} cartridge column (Pecosphere, 3 um, Perkin-Elmer Corp.) was also used as an analytical column. The 1 ml Bond-Elute C_{18} extraction columns were purchased form J.T. Baker chemical Co., Phillipsburg, NJ 08865 or from Analytichem International, Inc., Harbor City, CA 90710. The Vac Elut^{TM} vacuum chamber was from Analytichem International, Inc. and Buchler Evapomix (Buchler Instruments, Fort Lee, NJ) was used for evaporation of extracted samples.

B. Reagents

All inorganic chemicals were of analytical grade. The mobile phase was prepared by dissolving 12.11g of tris(hydroxymethyl)-aminomethane (Trizma Base, Sigma, St. Louis, MO 63178) in one liter of deionized water with or without 2% acetonitrile and then adjusting PH to 6.7 with phosphoric acid. The 5M acetic acid-sodium acetate buffer was

prepared by diluting 31 ml of acetic acid in 100 ml volumetric flask and adjusting the pH to 5 with sodium acetate powder. ($NaC_2H_3O_2$, Mallinckrodt Inc., Paris, Kentucky 40361). A 5% $KMnO_4$ (Sigma) solution was prepared by dissolving 5g of $KMnO_4$ in a 100 ml volumetric flask with distilled deionized water. A 3% solution of hydrogen peroxide was prepared by diluting a 30% hydrogen peroxide solution 10 fold with deionized water.

C. Drug standards

Methotrexate ((+)-Amethopterin) and pterine were obtained from Sigma. The stock methotrexte and stock internal standard (pterine), 10 mg in 100 ml methanol, are stable for at least 3 months at 4°C if wrapped with aluminum foil in order to minimize losses due to light. The working methotrexate, standard for calibration, was prepared by adding 0.1 ml of the stock methotrexate in 9.9 ml pooled drug-free serum. It is stable for at least 2 weeks at room temperature. The working internal standard (2.2 umol/L) was made by diluting 1 ml of the stock internal standard to 100 ml with methanol.

D. Synthesis of 2-hydroxyfolic acid

Folic acid (2.25g), (pteroylglutamic acid, Sigma), was partially dissolved in a solution of 40 ml of water, 20 ml of acetic acid and 8 ml of concentrated sulfuric acid (1). This solution was cooled to 10°C and 5g of solid sodium nitrite (Na_2NO_2) was added in portions. A clear solution was obtained. The solution was heated to 50°C

and an additional 2g of sodium nitrite was added. The temperature was kept between 40°C to 50°C for one hour and the mixture then cooled to 10°C for several hours. The crystalline product was collected, washed with water and dried. This material was recrystallized from 75 ml of water to give a white crystalline product (I) and dried in 37°C oven; yield 1.15g.

0.75g of I was suspended and partially dissolved in 4 ml of concentrated hydrochloric acid. A 0.5g of phenol was added to this solution and the mixture allowed to react for 15 minutes with occasional stirring. It was then treated with 0.4g charcoal, filtered and the filtered cake was washed with 2 ml of concentrated hydrochloric acid. The filtrates were combined and poured into 50 ml of water preheated to 60°C. Upon cooling the product crystallized. The solid was collected, washed with water, several times with ethanol and with ether. The ethanol removes the nitrosophenol and some phenol oxidation products. The yellow needle-like crystals (II) was dried in 37°C oven.

Stock solution of 2-hydroxyfolic acid, 10 mg of product II in 100 ml methanol, was stored at 4°C. The working solution of 2-hydroxyfolic acid, was prepared by diluting the stock solution 100 fold with water.

E. Procedure

A 100ul of patient serum is diluted to 10 ml with water. Both the 100 fold diluted and undiluted samples are run simultaneously. For

each sample, a C_{18} extraction column is activated by washing with 2 ml of methanol followed by 1 ml of water and 1 ml of 0.1M H_3PO_4. A 500 ul of 0.1M H_3PO_4 is added to each column followed by 100 ul serum standard, control or patient sample. The samples are forced through the column by vacuum and then washed with 1 ml of 0.1M H_3PO_4. A rack of labeled 10 x 75 mm glass tubes containing 500 ul of the 1ug/ml internal standard solution (pterine) is placed in the Vac-Elut chamber. The extraction column is eluted with 1.0 ml of methanol containing 1% diethylamine . The eluate is vortex-mixed and then transfered to the special glass tubes for evaporation and evaporated on Buchler Evapomix (below 40°C). The residue in each tube is mixed with 100 ul of 5M acetic acid-sodium acetate buffer (pH 5.0) followed by 100 ul of 5% potassium permanganate and allowed to react for 10 minutes at room temperature. The permaganate is decolorized by adding 100ul of 3% H_2O_2. An aliquot of this solution is injected onto the liquid chromatograph and the C_{18} analytical column is eluted with the mobile phase (0.1 M Tris-PO_4 containing 2% acetonitrile) at flow rate of 2.0 ml/min. The excitation and emission wavelengths are set on 392 nm and 449 nm, respectively. Quantification of methotrexate is based on the peak-area ratio of methotrexate oxidized product to pterine.

Quantitation

$$\text{Response factor (R.F.)} = \frac{\text{Peak area of internal standard}}{\text{Peak area of methotrexate standard}} \times \text{concentration of methotrexate standard}$$

$$\text{Concentration of unknown} = \frac{\text{Peak area of unknown}}{\text{Peak area of internal standard}} \times \text{R.F.}$$

III. RESULTS

A. Optimum condition of extraction and derivatization

I tried Octadecyl, strong cation-exchange (SCX) and weak cation-exchange (CBA) Bond Elute columns for the isolation of methotrexate. The SCX and CBA Bond Elute columns are both ion-exchange columns. Methotrexate and internal standard (pterine) were not retained on CBA Bond Elute columns. By using 1M or 2.56M of patassium citrate containing 20% of methanol or 20% of acetonitrile as an eluent for SCX Bond Elute column, methotrexate was still present in the third 500 ul fraction of eluate. It's hard to concentrate the eluate since the percentage of organic solvent is very low. Since methotrexate did not retain on the CBA Bond Elute column and was not eluted completely from the SCX Bond Elute columne, we decided to try octadecyl Bond Elute columns and used methanol containing 1% diethylamine as an eluent. This eluent (compared to the method reported by So et al. (43)) gave us almost complete recovery of methotrexate. The pH of Bond Elute column was adjusted with 0.1M H_3PO_4 to keep the pH low enough for retaining methotrexate on the C_{18} Bond Elute column.

The oxidation procedure was modified from the assay described by Nelson et al. (36). We used 10 minutes of reaction time and 100 ul of the oxidation reagent for the reaction. The methanol in the eluate interfered with oxidation of methotrexate, therefore methanol was evaporated before the oxidation reaction.

Although Nelson et al. reported that 2-hydroxyfolic acid can be used as an internal standard and do not interfere with that assay, however we found it eluted at the same retention time as 7-hydroxymethotrexate and interfered with other folate compounds. Even by changing the pH and composition of mobile phase, this problem still could not be resolved. Therefore, we decided to use pterine as the internal standard for our assay.

B. Optimization of chromatography

The octadecyl analytical columns we tested include 5 um particle size Ultrasphere^{TM}-ODS (Beckman Scientific Inc.,25 Cm x 4.6 mm ID) and 3 um particle size cartridge column (Perkin-Elmer Corp., 8.3 Cm x 4.6 mm ID). With cartridge column, the retention times were shorter and peaks were sharper. The major disadvantage of the cartridge column was the very short useful life of the column. It only lasted for a couple of weeks with no more than 100 analyses. The useful life of the 5 um octadecyl analytical column is at least 1000 injections or more with guard column. The octadecyl guard column was replaced after every 150-200 injections. This protected the main analytical column.

The mobile phase (0.1M Tris-PO_4 containing 2% acetonitrile, pH 6.7) became turbid after 3 to 4 days. This might be due to microbial growth in the mobile phase. The mobile phase was replaced once turbidity was found. The analytical column was washed with 50% methanol for 5 to 10 minutes at the end of every working day to prevent microbial growth and increase the useful life of the column.

The excitation and emission wavelength of 392 nm and 449 nm for methotrexate were determined by scanning the oxidized product. The rentention times of methotrexate and internal standard were 8 min and 10 min using the mobile phase without acetonitrile and were approximately 3 min and 4.5 min using the mobile phase containing 2% acetonitrile.

Figure 4 and Figure 5 show the chromatograms of methotrexate standard and patient samples using 0.1M Tris-PO_4 as mobile phase. Figure 6 and Figure 7 show the chromatograms of TDM control sera and patients samples using 0.1M Tris-PO_4 containing 2% acetonitrile as mobile phase.

The retention time of methotrexate was shortened and the peaks were sharpened when the mobile phase containing 2% acetonitrile was used. We found that the peak shape affects the linear range. The sharper the peak, the narrower the linear range.

C. Sensitivity

The lower detection limit for this assay is 11 nmol/L when 100 ul of serum is extracted. The signal/noise ratio was > 3.5 at 11 nmol/L of methotrexate in serum.

D. Precision

The results shown on table I are from repeated analysis of OMEGA Therapeutic Drug Monitoring (TDM) Control Sera containing methotrexate at two different concentrations (TDM 1 and TDM 3). Intra-day CVs ranged from 1.8 to 2.6%, inter-day from 8.4 to 11%.

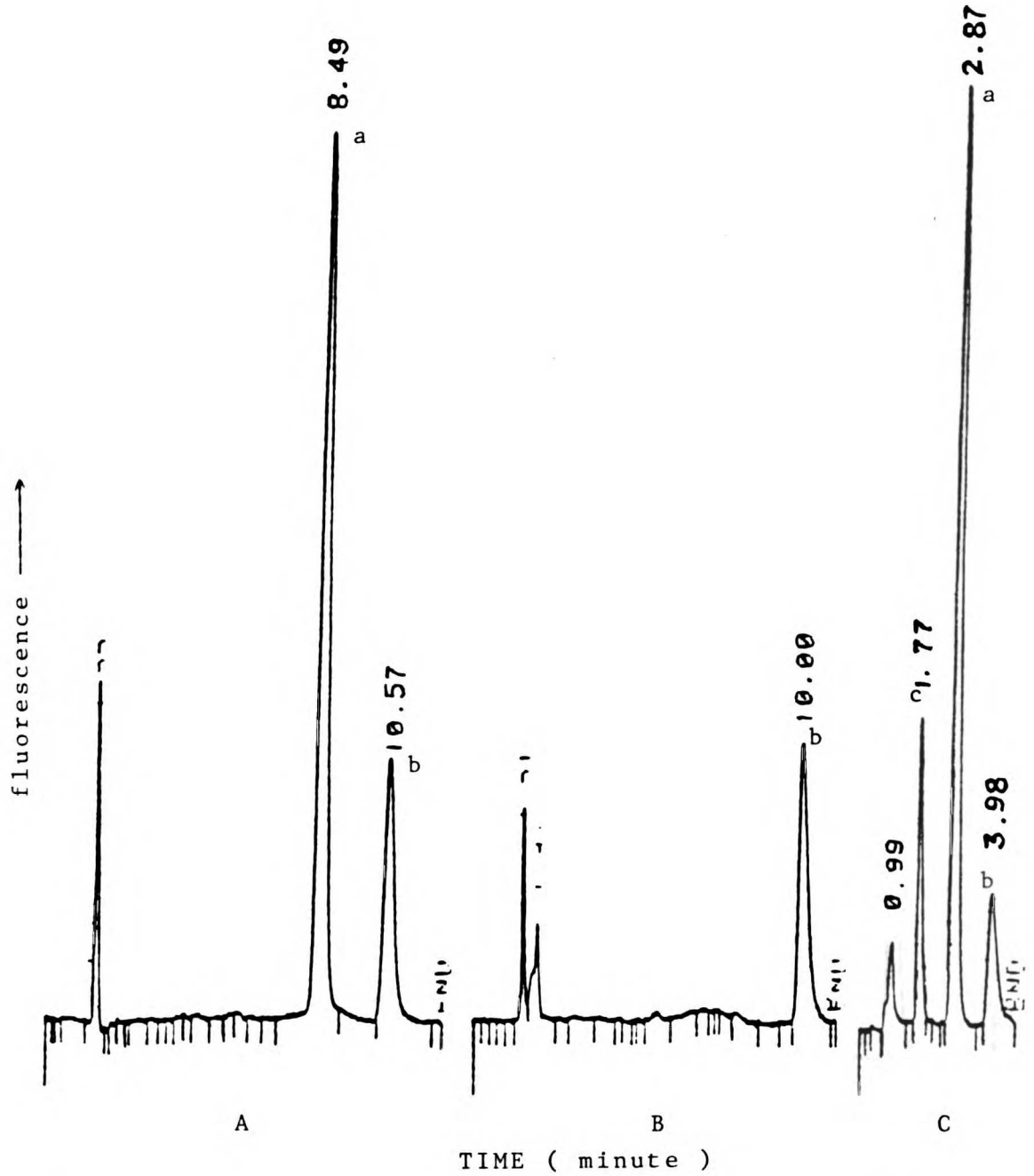


Figure 4. Chromatograms of (A) serum standard containing 2.2 umol/L methotrexate (20ul injected), (B) blank serum (20ul injected), (C) serum standard containing 2.2 umol/L methotrexate and 10.6 umol/L 7-hydroxymethotrexate (10ul injected).

Mobile phase : 0.1M Tris-PO_4, pH 6.7
peak a : methotrexate
peak b : internal standard (pterine)
peak c : 7-hydroxymethotrexate
(A) and (B) using 5um Ultrasphere-ODS column,
(C) using 3um Pecosphere-ODS cartridge column.

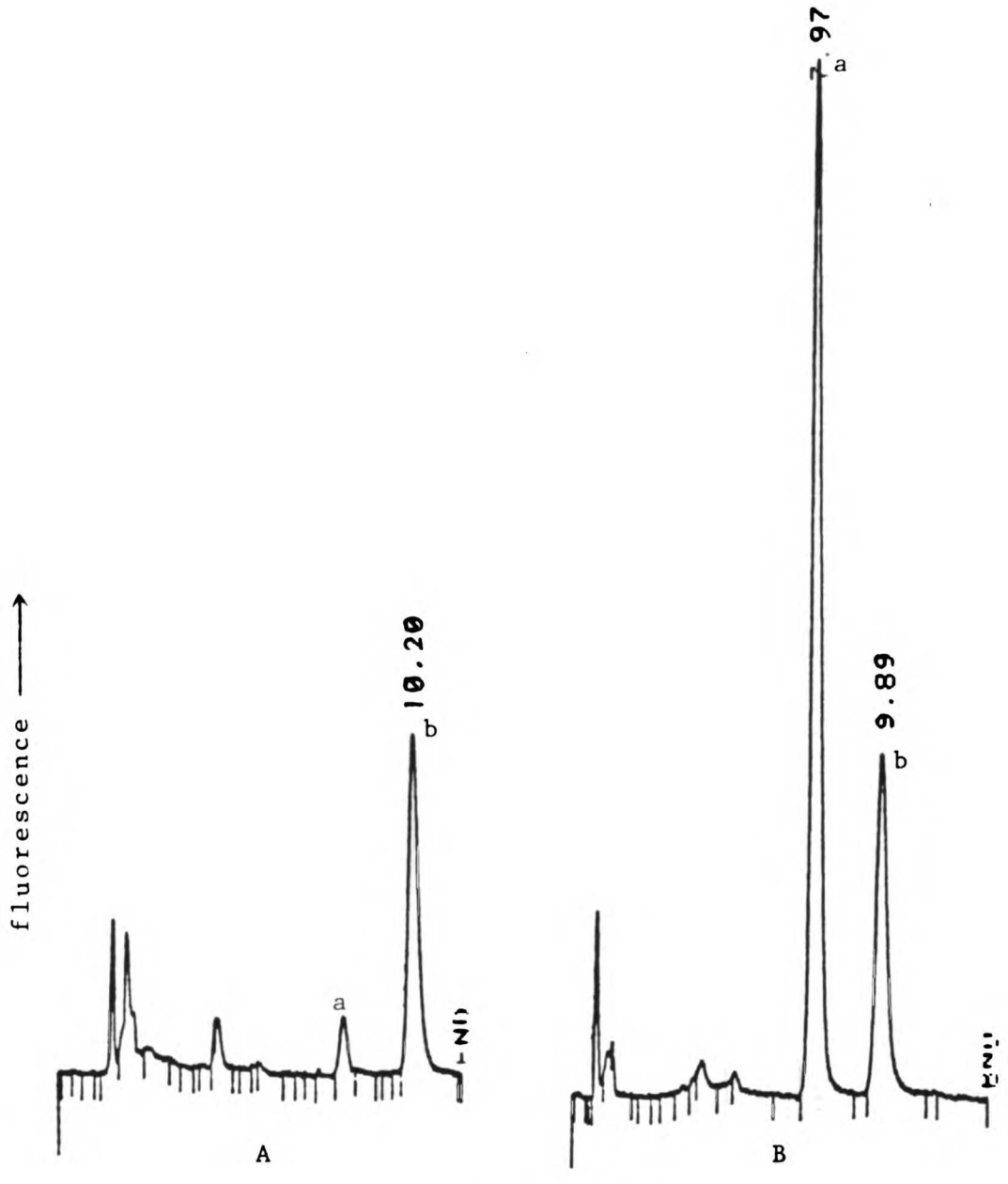


Figure 5. Chromatograms of (A) patient serum containing 0.081 umol/L methotrexate (30ul injected), (B) patient serum containing 159.5 umol/L methotrexate (100 fold dilution, 30ul injected)

Mobile phase : 0.1M Tris-PO_4, pH 6.7

peak a : methotrexate

peak b : internal standard

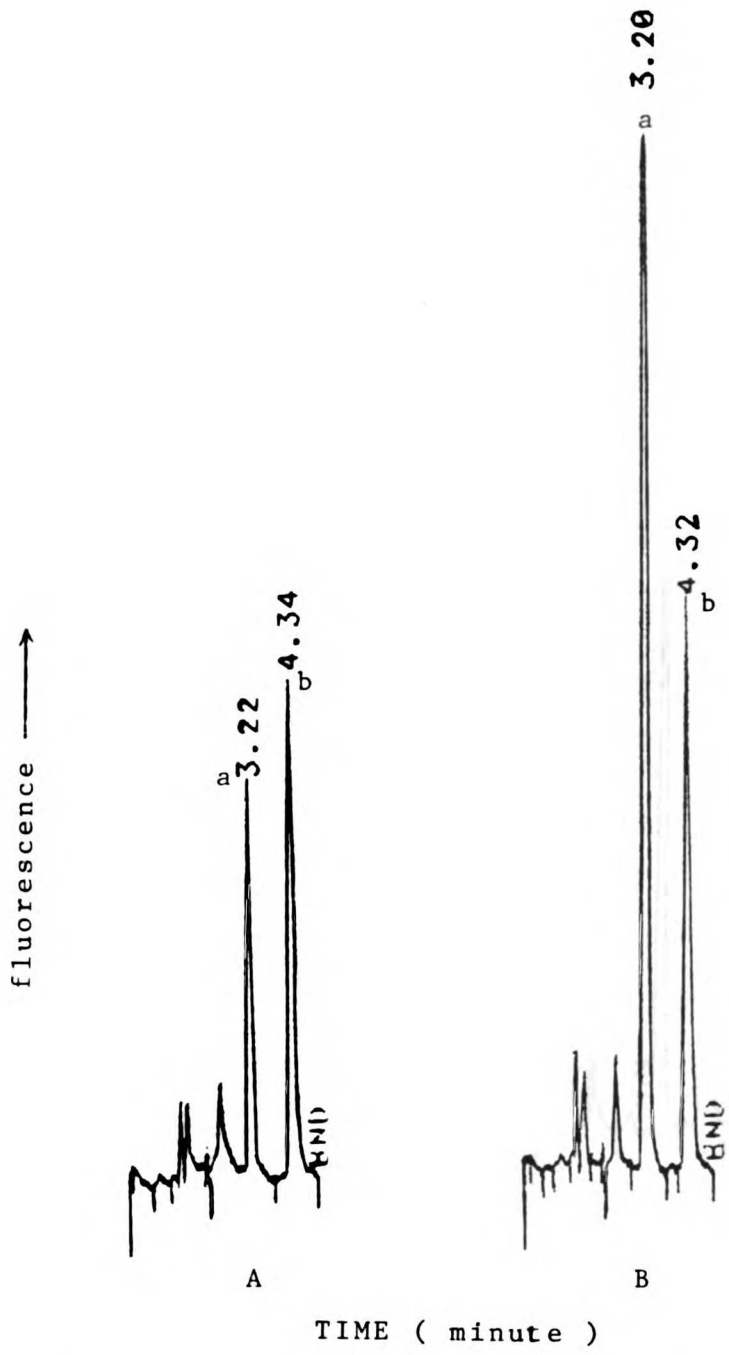


Figure 6. Chromatograms of (A) TDM I sera (10ul injected),
(B) TDM III sera (7ul injected).

Mobile phase : 0.1M Tris-PO_4 (pH 6.7) containing 2% acetonitrile
peak a : methotrexate
peak b : internal standard

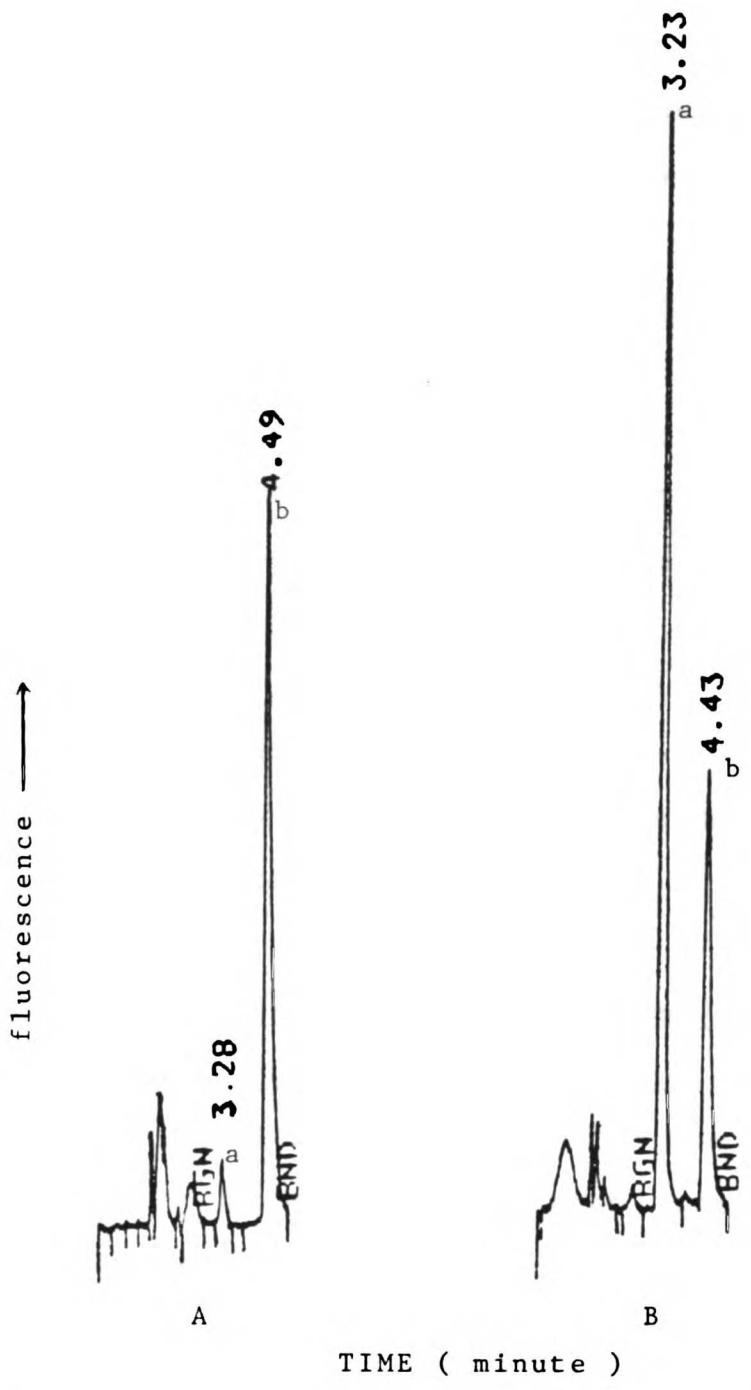


Figure 7. Chromatograms of (A) patient serum containing 0.068 umol/L methotrexate (20ul injected), (B) patient serum containing 214.9 umol/L methotrexate (100 fold dilution, 10ul injected)

Mobile phase : 0.1M Tris-PO_4 (pH 6.7) containing 2% acetonitrile
peak a : methotrexate
peak b : internal standard

Table I. Precision of methotrexate analysis in serum

	Intra-day (n = 10)		Inter-day (n = 15)	
	Mean (S.D.),umol/L	CV,%	Mean (S.D.),umol/L	CV,%
TDM 1	0.579 (0.011)	1.8	0.579 (0.064)	11
TDM 3	2.436 (0.064)	2.6	2.355 (0.198)	8.4

Table II. Recovery of methotrexate in serum through Bond-Elut extraction column (n = 5)

added	direct oxidation of pure methotrexate	through Bond-Elut extraction	Recovery
umol/L	umol/L	umol/L (S.D.)	%
0.022	0.022	0.0227 (0.002)	103
0.22	0.24	0.238 (0.010)	99
2.20	2.22	2.29 (0.04)	103
11.0	11.9	11.8 (0.37)	99

E. Recovery and linearity

Methotrexate was added to the drug-free pooled serum. The final concentrations of methotrexate ranging from 22 nmol/L to 11 umol/L were analyzed using the procedure described above. Concentrations and peak area ratios were linear over this range. (Figure 8, r = 1.000, slope = 1.649 , intercept = -0.013) The recoveries of methotrexate in serum through the Bond-Elut extraction columns ranged from 98% to 103%. (Table II)

F. Comparison of LC with EMIT assay

We analyzed 50 patient serum samples by our LC method and compared the results with enzyme-multiplied immunoassay technique (EMIT) (by SKBL LAB OC, Oakland 94621). The concentrations of these serum samples ranged from 0.04 to 1458 umol/L. Generally, the results of our LC measurements were lower than EMIT assay. Because of the 10,000-fold concentration range of patient's results, it is practically impossible to compare the value on a linear graph. Instead, the results of our LC method (y) and EMIT (x) assay were compared on a log scale (Figure 9) and the results of the regression analysis were : n = 50, r (correlation coefficient) = 0.997, slope = 0.991, y-intercept =0.003. The mean value of LC method was 147.91 umol/L and the mean value of EMIT method was 160.61 umol/L.

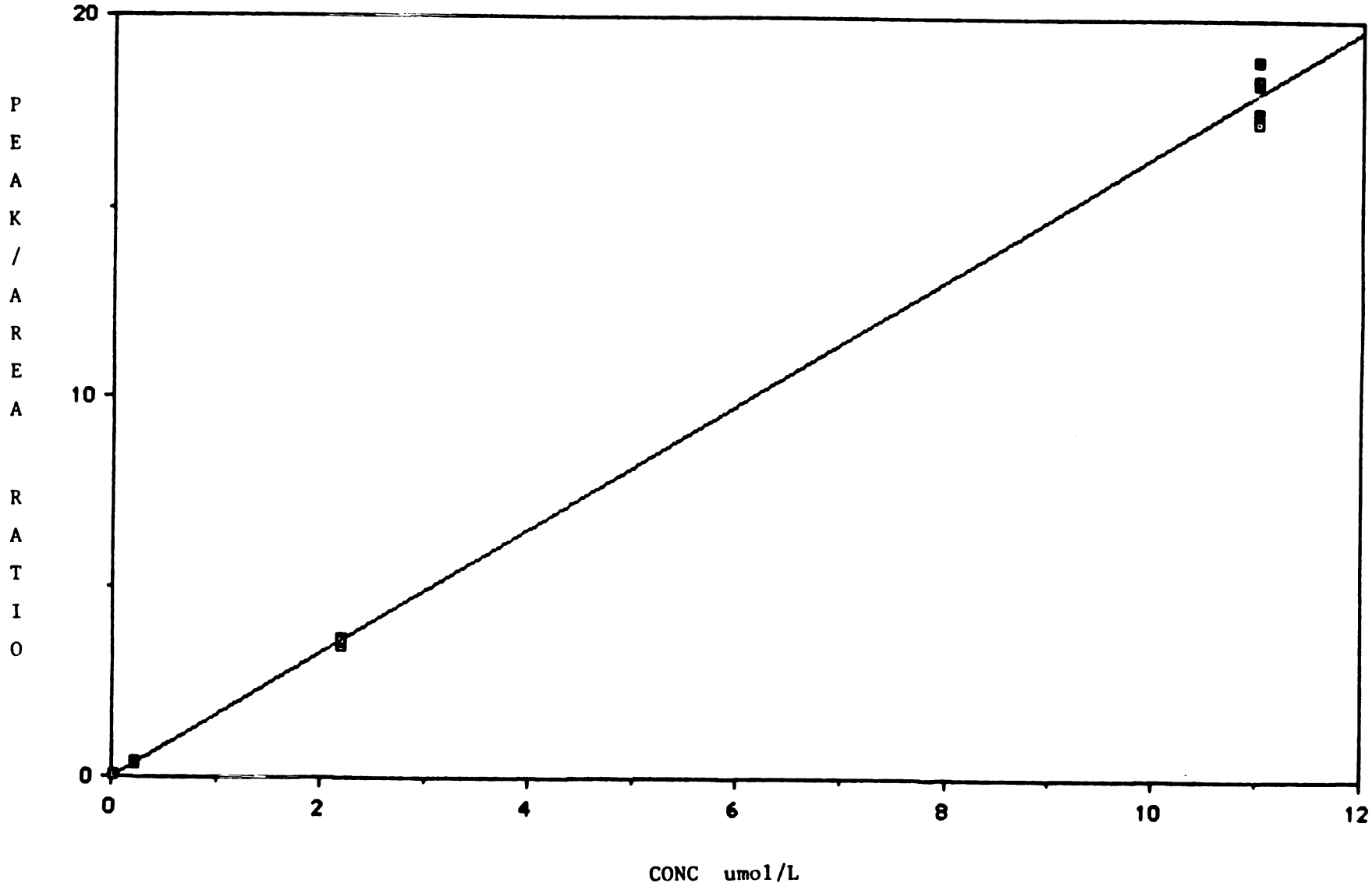


Figure 8. Linearity of a serum calibration curve. r = 1.000, equation of the linear line y = 1.649x - 0.013

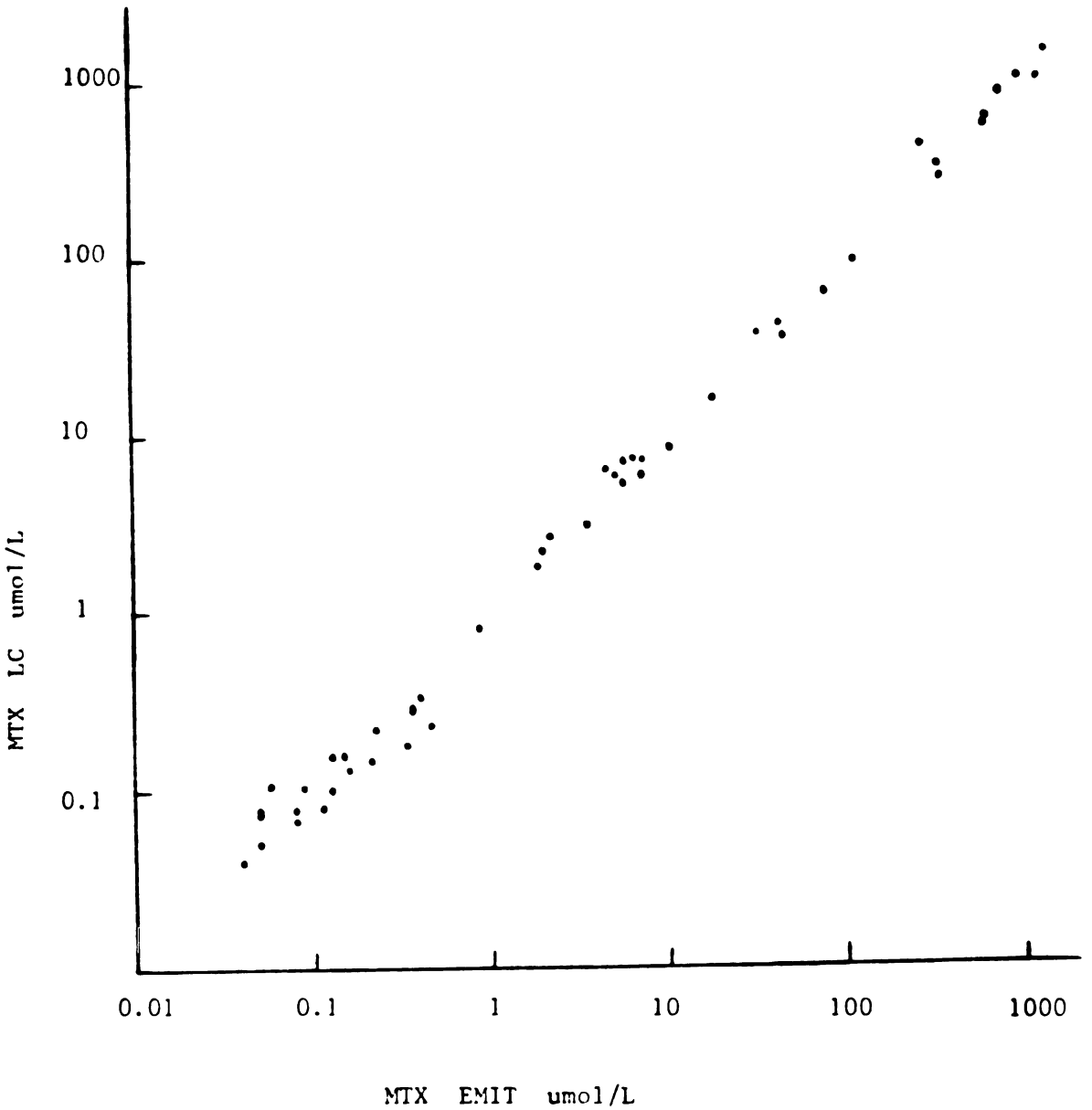


Figure 9. Comparison of EMIT and LC on 50 sera. r = 0.997, equation of the regression line y = 0.991x + 0.003

G. Interference

The drugs (Table III) present in TDM sera were not detectable by this LC assay. Similarly injecting the oxidized products of folic acid, citrovorum : folinic acid, 7-hydroxymethotrexate without extraction did not interfere with the methotrexate analysis.

H. Stability

Serum standards containing 2.2 umol/L methotrexate without aluminum foil stored at room temperature were analyzed for two weeks. The mean and standard deviation for these samples were 2.22 umol/L $\pm$ 0.11 umol/L, respectively. Coefficient of variation of those results is 5.05%. The oxidized products (in final solution) stored at room temperature and at 4°C were tested for one week. The oxidized product was stable at 4°C for about 5 days and the one stored at room temperature and exposed to light gave increased concentration after a couple of days due to breakdown of the internal standard pterine. (smaller internal standard peak)

Table III. The drug in TDM sera which showed no interference in the LC assay for methotrexate

Anticonvulsants	Antiarrhythmics
Carbamazepine	Digoxin
Ethosuximide	Disopyramide
Phenobarbital	Lidocaine
Phenytoin	Procainamide
Primidone	N-acetyl procainamide
Valproic acid	Propranolol
	Quinindine

Antibiotics
Amikacin
Gentamicin
Tobramycin
Acetaminophen

IV. DISCUSSION AND SUMMARY

One of the major difficulties in the methotrexate analysis is the wide concentration range present in the patient samples. The linear range of this method is from 22 nmol/L to 11 umol/L. By analyzing the clinical sample and its hundred fold diluted sample simultaneously, the linear range is increased up to 1100 umol/L. This range covers majority of the clinical samples. By testing two different analytical columns, we found that the sharper the peak, the narrower the linear range (Figure 4).

Pterine is used as an internal standard instead of 2-hydroxyfolic acid in the method described by Nelson et al. (36). The advantage of using pterine as an internal standard is that the pterine dose not interfere with 7-hydroxy methotrexate, folinic acid and folic acid, while the 2-hydroxyfolic acid can not resolved from the 7-hydroxy methotrexate.

By combination of solid-phase extraction and fluorescence detection, the lower limit of sensitivity in this assay is 11 nmol/L when 100 ul serum is used. Using larger volumes of serum, the sensitivity can be increased.

Although methotrexate is a light-sensitive compound, it's stable in serum for at least 2 weeks. Even if the clinical samples are not wrapped with aluminum foil, one can still obtain acceptable results. If the situation demands, the oxidized products can be stored in refrigerator for about 5 days at 4°C.

By adding 2% acetonitrile to the 0.1 M Tris-PO_4 mobile phase, the total chromatographic time was decreased from 11 minutes to 5 minutes approximately. Therefore, 6 minutes were saved for each analysis.

With the use of the Vac-Elute chamber designed for 10 columns , we can process 10 samples simultaneously. The whole procedure takes less than 30 minutes. This simple, rapid and sensitive assay ia useful in clinical laboratories for methotrexate analysis.

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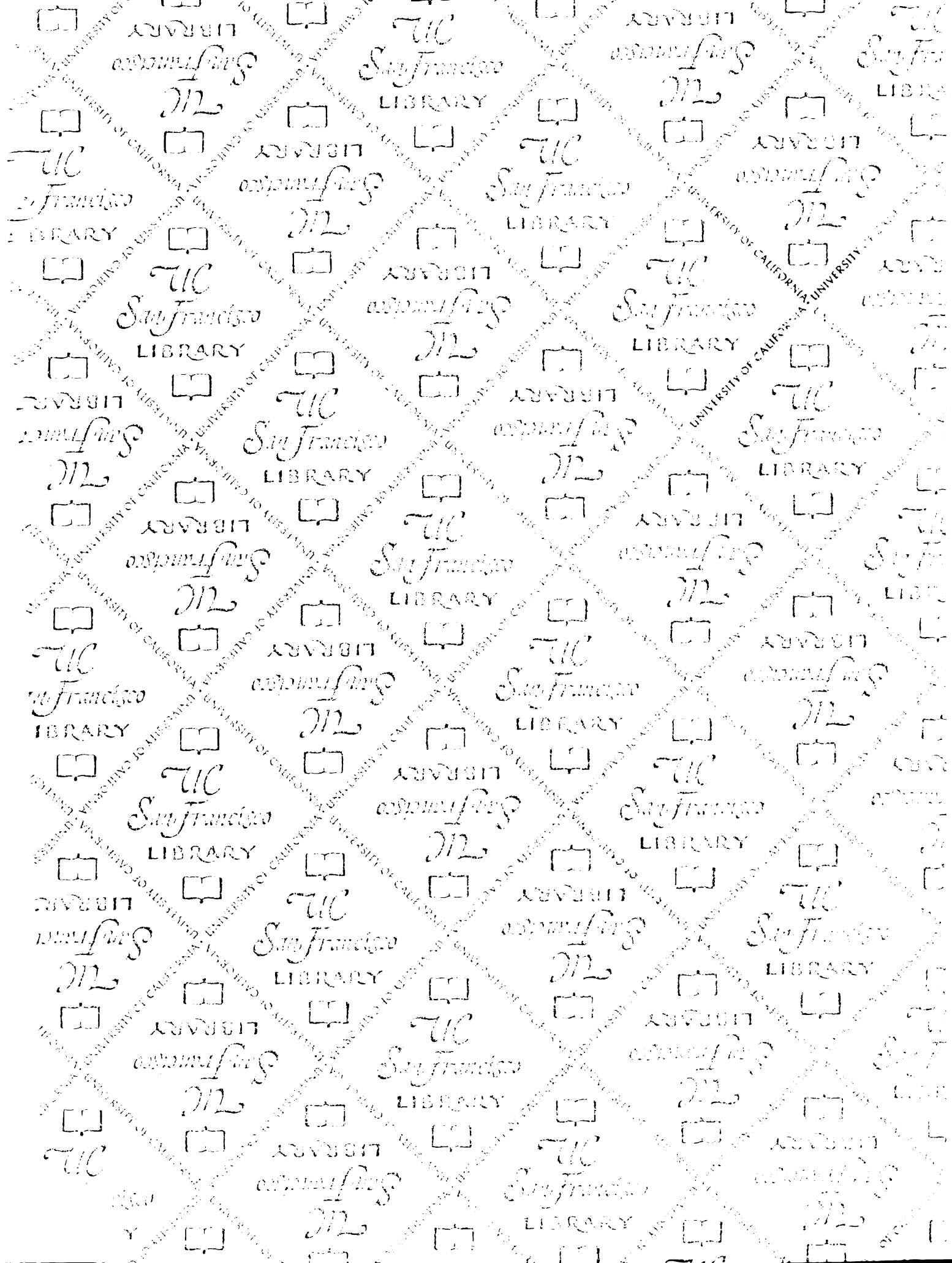
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