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Specific and sensitive high-performance liquid chromatographic method with fluorescence detection for measurement of lometrexol and its polyglutamates in biologic samples☆

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) assay is described for the quantitative determination of lometrexol in biological samples; the assay is rapid, simple, specific, and highly sensitive. The method requires the dissociation of lometrexol from folate-binding proteins present in blood and formation of a fluorescent oxidized derivative of the compound. The dissociation of lometrexol from folate-binding proteins was achieved by acidification to pH 3.5 using ammonium formate, followed by serum protein precipitation with perchloric acid. The protein-free lometrexol was subsequently oxidized by MnO_2 at 90°C for 10 min. Chromatographic separation of lometrexol without interference was achieved on a C_{18} reversed-phase column with a convex gradient, using acetonitrile–0.1% ammonium formate, pH 7.0, as the mobile phase. In human serum and urine the calibration curve was linear between 5 and 300 n*M*. The lower limit of quantification was 5 n*M*. The method has been applied successfully to measure serum and urinary levels of lometrexol in patients.

INTRODUCTION

Lometrexol (the 6*R*-stereoisomer of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid, 6*R*-DDATHF) is a novel antifolate synthesized by Taylor and his co-workers that is now undergoing extensive clinical trial [1]. The drug is a potent and a specific inhibitor of glycinamide ribonucleotide formyltransferase (GARFT), the enzyme that catalyzes the first folate-dependent obligatory step in the *de novo* synthesis of purines [2,3]. Unlike the traditional antifolates, lometrexol inhibits neither dihydrofolate reductase, nor thymidylate synthase [1–3]. Recent studies have shown that the polyglutamate anabolites of lometrexol are even more potent inhibitors of GARFT than is the parent compound [3,4].

Lometrexol has demonstrated antitumor activity in a broad spectrum of solid murine and human xenograft tumors, including B-6 melanoma, Lewis lung carcinoma, X5563 plasma cell myeloma, 6C3HED lymphosarcoma, colon 26, LX-1

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lung carcinoma, GC3 and VCR colon carcinomas [5,6]. In the clinical studies, lometrexol has shown antitumor activity in ovarian and head and neck cancers [7–9]. The dose-limiting side effects of this drug were delayed and life-threatening myelosuppression and gastrointestinal toxicity [7–9]. These late side effects have been attributed to the drug's extremely prolonged terminal phase in serum, which may reflect enterohepatic re-circulation and/or the tight binding of the drug to serum folate-binding proteins. The drug's toxic effects can be ameliorated by the administration of either folic acid or folinic acid [8–10]. Although it is conceptually reasonable to monitor lometrexol serum levels to identify patients at risk for delayed toxic effects, this approach has been hampered by the lack of a simple, quick and inexpensive assay for determining lometrexol concentrations in biological samples.

Taber *et al.* have developed a particle concentration fluorescence immunoassay to measure lometrexol in biological samples, and have used this as the reference assay in support of Phase I studies done at multiple centers [11]. This assay is highly sensitive and does not react with normal circulating folate molecules; it has been useful in providing a method of record for regulatory purposes. However, it requires an expensive, dedicated facility that is not always available at the multiple centers working with lometrexol. Moreover, it probably cannot distinguish the parent drug from its metabolites. Recently an HPLC method with UV detection has been described [12] by van Tellingen *et al.* Although their method could be used widely, it is somewhat insensitive, having a lower limit of detection of 20 n*M* despite the use of a large injection volume.

To develop a more sensitive chromatographic assay for lometrexol we have made use of the fact that oxidized pteridines with a carbon at position 10 are intensely fluorescent, although that fluorescence is lost when the molecule is extensively reduced, *e.g.* biopterin *versus* tetrahydrobiopterin. Our laboratory had previously employed this characteristic to develop a satisfactory fluorescence HPLC assay for 10-deazaaminopterin derivatives [13]. Lometrexol, 5,10-dideazatetrahydrofolate, is non-fluorescent; however, two chemically synthesized oxidized analogues of lometrexol, *compound I*, N-[4-[2-(2-amino-4-oxopyrido[2,3-d]pyrimidin-6-yl)ethynyl]benzoyl]-L-glutamic acid, and *compound II*, N-[4-[2-(2-amino-4-oxopyrido[2,3-d]pyrimidin-6-yl)ethenyl]benzoyl]-L-glutamic acid, are highly fluorescent. The chemical structures of lometrexol and compounds I and II are presented in Fig. 1. The overall strategy for an assay was to oxidize lometrexol without extensive degradation, in the expectation that a fluorescent molecule would result. The initial step in methods development was to define optimum conditions for oxidation of lometrexol, and to demonstrate by the subsequent chromatographic separation that a single fluorescent species had been generated without major sample losses. Thereafter, the methods were adapted to assaying the drug in protein-depleted serum and urine and defining the limits and precision of the assay in the usual manner. A preliminary abstract of this work was presented at the

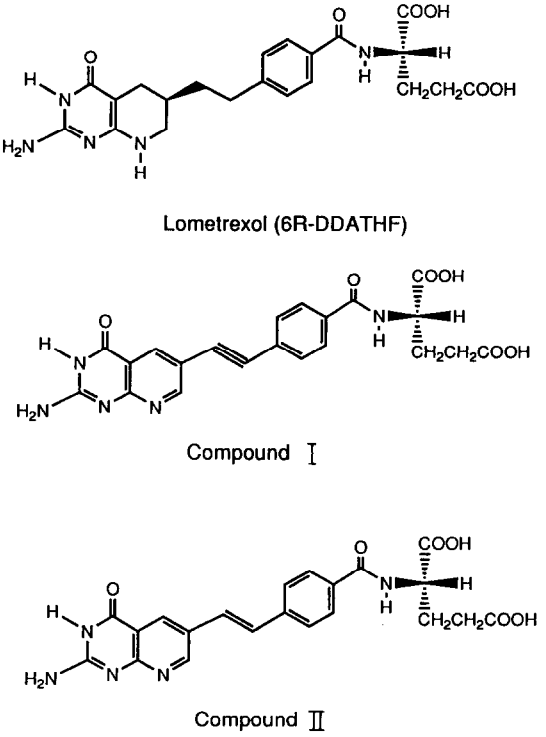

Fig. 1. Chemical structures.

83rd Annual Meeting of the American Association for Cancer Research in San Diego, CA in May 1992 [14].

EXPERIMENTAL

Apparatus

Chromatographic analyses were performed with a Waters HPLC system (Waters, Milford, MA, USA), consisting of a Model 721 programmable system controller, Waters Model 710B WISP automatic injector, two Waters 6000A solvent delivery systems and Waters Model 730 Data module. Fluorescence was quantitated by a Perkin-Elmer 650 10S fluorescence detector (Norwalk, CT, USA) operated at 330 nm excitation and 425 nm emission with slit widths of 5 and 10 nm, respectively.

Reagents and solvents

Lometrexol and its di- and tri-glutamate metabolites and two oxidized reference analogs of lometrexol (compounds I and II), and [^{14}C]lometrexol, universally labeled in the glutamate moiety, were supplied by Eli Lilly and Co. (Dr. Shih, Indianapolis, IN, USA). Stock solutions (1 m*M*) of these compounds were prepared in 5% sodium bicarbonate and stored at −20° C. Subsequent dilutions were made in distilled water. Compound I was used as the internal standard for HPLC analysis. HPLC grade acetonitrile was obtained from J.T. Baker Chemical (Phillipsburg, NJ, USA). Manganese dioxide was purchased from EM Science (Cherry Hill, NJ, USA). All other chemicals were of analytical grade and were obtained from either Fisher Scientific (Fair Lawn, NJ, USA) or J.T. Baker Chemical.

Chromatographic conditions.

Chromatographic separations were carried out on a C_{18} μ-Bondapak column (30 cm × 3.9 mm I.D.; particle size 10 μm; Waters Assoc., Milford, MA, USA). The analytical column was protected by a 7 μm C_8 NewGuard cartridge (1.5 cm × 3.2 mm I.D.) pre-column (Applied Biosystems, Forster City, CA, USA). Lometrexol was eluted from the column using two mobile phases. Mobile phase A consisted of 0.1% ammonium formate, pH 7, while mobile phase B consisted of 0.1% ammonium formate (pH 7)–15% acetonitrile. Ammonium hydroxide was used to adjust the pH of the mobile phases. Initial conditions for lometrexol analysis consisted of A–B (25:75, v/v); the final conditions were A–B (10:90, v/v); they were reached in 10 min using a convex gradient (Waters Curve 3) and were maintained for 10 min. The column was subsequently re-equilibrated at the initial conditions for 8 min prior to the next injection. The flow-rate was 1 ml/min. For lometrexol polyglutamate analyses, the initial conditions were changed to A–B (50:50, v/v), in order to separate the peaks of interest from the solvent front.

Dissociation of lometrexol from serum and urinary proteins

Lometrexol in 0.5 ml of sample was dissociated from folate-binding protein by acidification to pH 3.5 using 50 μl of 1% ammonium formate, pH 2.0, and vortex-mixing for 1 min. Serum samples with high lometrexol concentrations were diluted with double-distilled water prior to acidification; urinary samples were routinely diluted at least × 5. The internal standard was added to a final concentration of 100 n*M* prior to acidification. The dissociated folate-binding proteins and other serum proteins were precipitated by the addition of 15 μl of 70% perchloric acid. The mixture was then micro-centrifuged at room temperature for 3 min and the supernatant was oxidized with manganese dioxide.

Manganese dioxide oxidation

A 280-μl aliquot of a 1 g/ml MnO_2 slurry in water was added to 420 μl of protein-free sample, vortex-mixed and heated in a boiling water bath for 10 min to oxidize lometrexol. The oxidation mixture was cooled and then micro-centrifuged for 3 min at room temperature to remove the MnO_2 slurry. The clear supernatant was assayed for lometrexol content by HPLC with fluorescence detection. The injection volume was 150 μl.

Yield of the dissociation and oxidation reactions

Assessment of the dissociation yield and the efficiency of MnO_2 oxidation was determined using serum samples spiked with [^{14}C]lometrexol to achieve sample concentrations of 100 and 300 n*M*. Radioactivity in the spiked serum and in the clear supernatant after PCA protein precipitation was used to assess sample loss during protein precipitation. Oxidation efficiency was assessed by collecting and determining the radioactivity associated with the oxidized product peak on HPLC analysis, compared with the counts in the un-oxidized aliquot. The overall efficiency of the procedure was determined using the radioactivity associated with the HPLC peak of interest with that in a spiked but otherwise unprocessed serum sample. Appropriate adjustments for volume changes were taken into account in yield and efficiency calculations.

Peak identification

Peaks of the lometrexol oxidized product were identified by chromatographic retention time, coelution with the oxidized [^{14}C]lometrexol and fluorescence spectrum of the peak of interest.

RESULTS AND DISCUSSION

Fluorescence spectra

As noted previously, lometrexol itself is nonfluorescent; however, significant fluorescence develops when the drug is heated in the presence of MnO_2. In Fig. 2 the fluorescence excitation and emission spectra of the MnO_2-oxidized lometrexol product ("C" spectra) are contrasted with those of the ethynyl-containing compound I (the chromatographic internal standard) ("A" spectra), and the ethenyl-containing compound II ("B" spectra). The excitation and emission maxi-

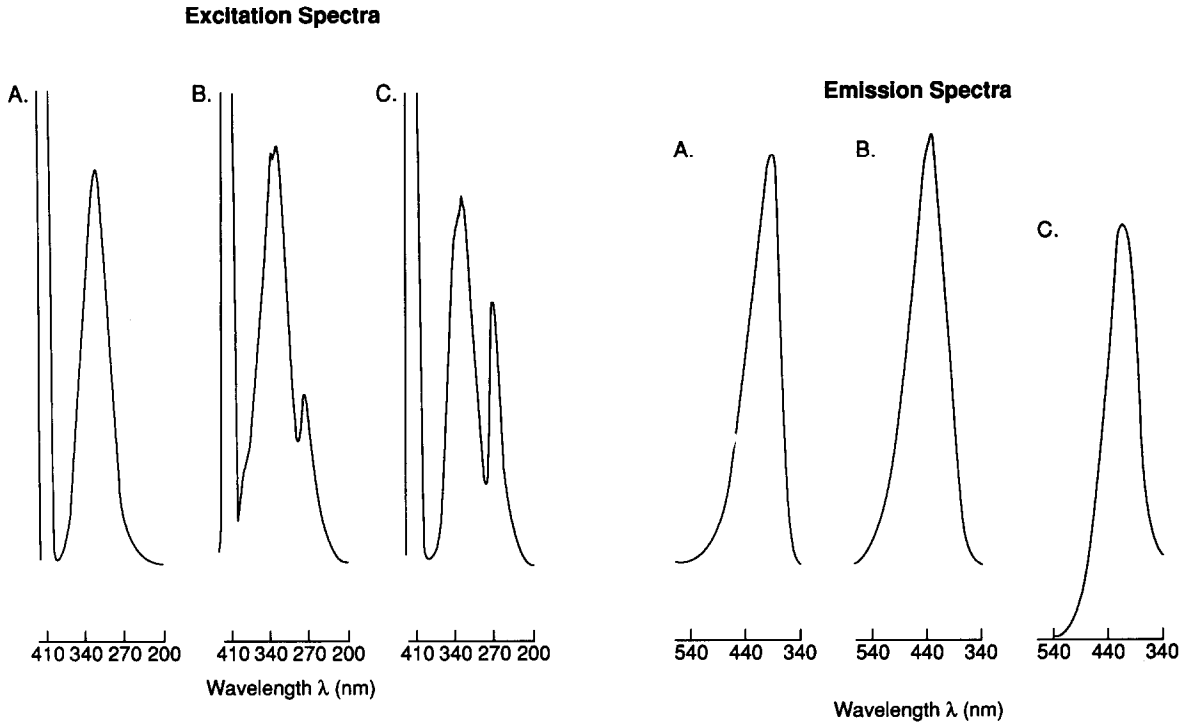

Fig. 2. Excitation and emission fluorescence spectra of compound I (spectral curve A), compound II (spectral curve B), and the MnO_2-oxidation product of lometrexol (spectral curve C).

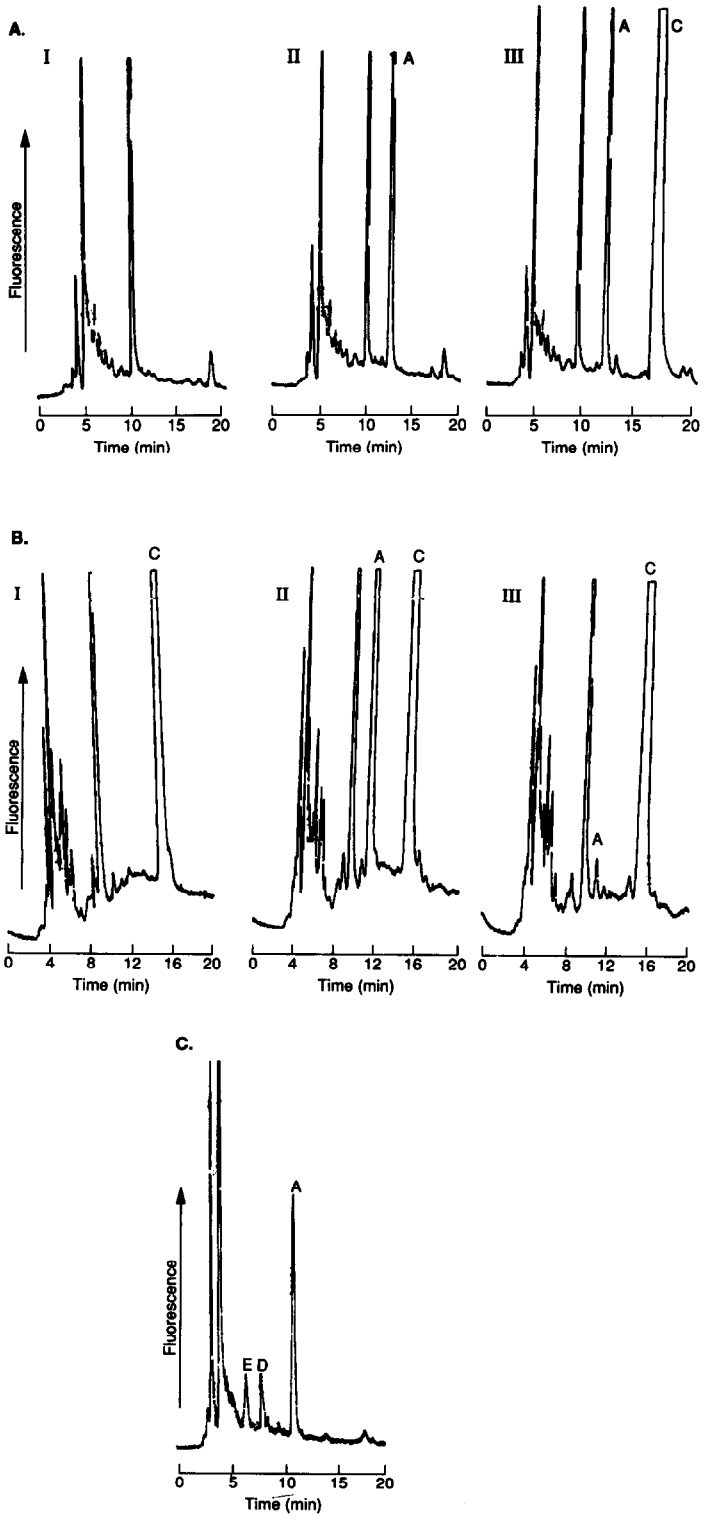

Fig. 3. Chromatographic separation of lometrexol.
(A) Serum: I, unspiked, II. spiked with 300 n*M* lometrexol (peak A), and III. spiked with lometrexol and internal standard (peak C).
(B) Urine from patient: I. pretreatment, II. 0–24 h, and III. 24–48 h urine collection. All samples had been spiked with the internal standard (peak C).
(C) Homogenate of a cell pellet from the L1210 leukemia cell line homogenate spiked with 100 ng/ml of lometrexol (peak A) and its diglutamate and triglutamate metabolites (peaks D and E respectively).

ma of "oxidized lometrexol" were 330 and 420 nm respectively. The emission spectra of the three compounds were quite similar; however, on a molar basis, the triple-bonded compound I had a fluorescence intensity that was over five times as great as that of the other compounds. Folic acid and its analogues have two UV absorption maxima, consistent with the *para*-aminobenzoic acid and the pteridine moieties. We have previously shown that the fluorescence excitation spectrum of 10-ethyl-10-deazaaminopterin displayed two maxima that reflected the characteristics of the UV absorption spectrum; however, excitation at the pteridine wavelength produced 2.5 fold greater fluorescence than did excitation at the PABA wavelength [13]. Similarly, the excitation spectrum of the lometrexol oxidation product shows two well defined maxima, at 270 and 330, compatible with separate PABA and pteridine absorption. In contrast, the excitation spectrum of the highly fluorescent triple-bond ethynyl-containing compound I shows a single maximum at 315 nm, suggesting a free resonance of electrons between the two UV absorbant moieties. The excitation spectrum of the double-bond ethenyl-containing compound II has a greatly attenuated PABA-related maximum, occupying an intermediate position between the other two. These observations are consistent with the oxidized lometrexol product having an intact pteridine ring and the presence of an ethane linkage between C9 and C10. The chromatographic behavior of the three entities is quite similar; however, with our standard assay conditions there is baseline separation between them; the order of elution from the column is as follows: compound II, lometrexol oxidized product, and compound I (data not shown).

Chromatographic separation of the oxidized product of lometrexol and its polyglutamates in biologic samples

Chromatograms of unspiked serum and urine and samples spiked with lometrexol and the internal standard (compound I) are shown in Figs. 3A and 3B. Depending upon the gradient used, the retention times for lometrexol and the internal standard were 11 to 12.5 min and 15 to 17.5 min, respectively. Of the more than 200 patient samples analyzed to-date, an interfering peak has been observed in only one patient. Furthermore, by modifying the initial conditions to A–B (50:50, v/v), lometrexol eluted at 11.3 min and was separated from the oxidized products of the di- and the tri-polyglutamates of lometrexol added to L1210 cellular homogenate. The polyglutamate retention times were 6.75 and 8.25 min respectively (Fig. 3C).

Studies to optimize the dissociation and oxidation conditions

Lometrexol is not readily extracted from serum by C_{18} absorption columns that successfully remove methotrexate or edatrexate; this may be due in part to the compound's tight binding to folate-binding proteins that are present in varying quantities in blood. Direct acidic precipitation of proteins with perchloric or trichloroacetic acids, or by means of methanol and acetonitrile, was associated with excessive sample losses. Reasoning that the folate-binding protein interaction with folic acid or 5-methyl-THF is sensitive to mildly acidic pH, we adjusted the serum sample to pH 3.5 by addition of ammonium formate (pH 2.0), vortex-mixed briefly, then precipitated the proteins with perchloric acid. This technique produced yields of 97%, 85%, and 77% at serum lometrexol concentrations of 100 n*M*, 300 n*M*, and 1000 n*M* respectively (Table I).

Lometrexol has a poor UV absorbance and lacks intrinsic fluorescence, making it impossible to detect the prolonged submicromolar serum levels of the drug that may produce clinical toxicity. However, the presence of a carbon at position 10 conveys to the pteridine component of lometrexol the capacity to fluoresce, provided it can be oxidized without destruction of the pteridine moiety or production of multiple breakdown products. Attempts to oxidize lometrexol using potassium iodide, potassium permanganate, hydrogen peroxide, and Fenton's reagent (H_2O_2 and $FeSO_4$) were unsuccessful. After these approaches had failed, we were able to obtain consistent fluorescence by use of a slurry of MnO_2

TABLE I

YIELD OF [^{14}C]GLUTAMATE–LOMETREXOL FROM SERUM

Results were from 3 separate experiments. The overall yield for 300 n*M* was 86 ± 13%.

Experimental conditions	Added radioactivity recovered (%)		
	100 n*M*	300 n*M*	1000 n*M*
Formate and PCA precipitation	97 ± 5	85 ± 7	77 ± 4
Acidic MnO_2 oxidation	94 ± 11	94 ± 14	52 ± 5
Neutral MnO_2 oxidation	103 ± 12	96 ± 10	98 ± 10
Cumulative formate/PCA ppt. and MnO_2 acid pH oxidation	91 ± 13	80 ± 12	42 ± 4

under acidic conditions. In order to establish the optimum conditions for the oxidation of lometrexol in serum by MnO_2, we examined the effects of alkaline and acidic pH, temperature, oxidation time, and the concentration of the oxidizing agent. There was no oxidation of lometrexol at alkaline pH. A 3:2 ratio of sample to 1 g/ml of MnO_2 slurry was optimal for the oxidation of lometrexol in serum (Fig. 4A); the oxidation was temperature dependent but not remarkably critical between 10 and 15 min in a boiling water bath (Fig. 4B). The ratio of 3:1 (sample to 1 g/ml MnO_2 slurry) was adequate for urinary samples (data not shown). These results are applicable to serum and urine; if the assay is to be extended to other biological materials, the quantity of other oxidizable material in biological samples may be a major determinant of how much MnO_2 is required. Whereas an excess of MnO_2 may completely destroy lometrexol, excessively small amounts may lead to incomplete oxidation. This will probably need to be examined on a case by case basis.

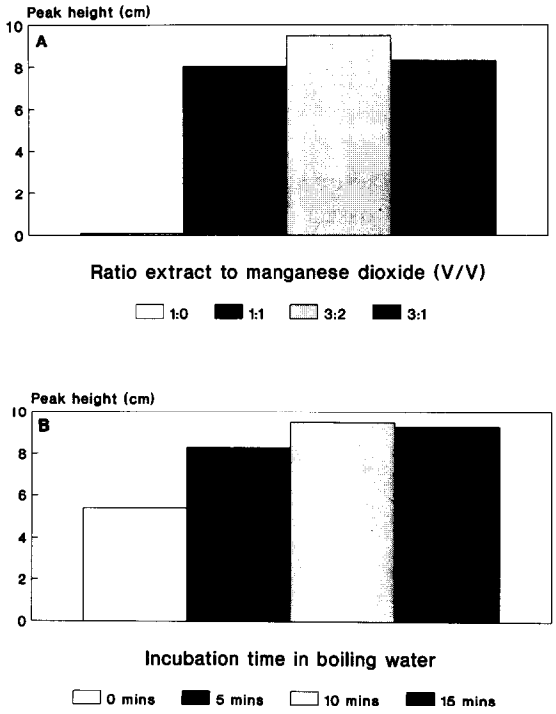

Fig. 4. Optimization of the dissociation and oxidation conditions. (A) Effect of ratio of sample to MnO_2 slurry (1 g/ml) on lometrexol oxidation. (B) Effect of time in a boiling water bath on lometrexol oxidation.

Standard curve calibration and linearity

Calibration of the assay was performed by analyzing 0.5 ml of serum spiked with known amounts of lometrexol (0–3000 n*M*) in the presence of a fixed amount of internal standard (100 n*M*). Standard curves were constructed by plotting the peak-area ratios (lometrexol/internal standard) *versus* the amount of lometrexol. The results showed that the standard curve was linear from 0–300 n*M* with a correlation coefficient of 0.999 (Fig. 5). This correlation coefficient dropped to 0.91 when the range is extended to 1000 n*M*. The lower limit of detection was 5 n*M*

Stability of processed samples and assay precision

No deterioration or changes were observed in

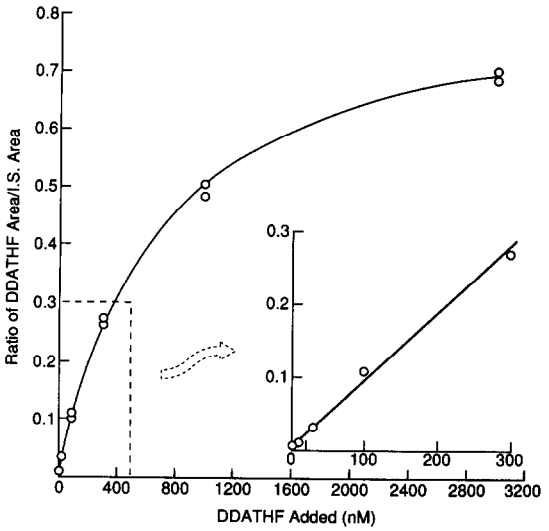

Fig. 5. Standard curve for lometrexol quantitation in serum.

oxidized lometrexol and internal standard during the 72 h storage at either room temperature or at 4°C (data not shown). Intra-day precision estimated using serum samples spiked with 100 and 300 n*M* of lometrexol yielded a coefficient of variation of 3% ($n = 6$), while the day to day coefficient of variation over a one month period was 8.9% ($n = 10$).

Yield studies

Parallel measurement of fluorescence and radioactivity deriving from [^{14}C]glutamate-labeled lometrexol demonstrated the existence of only one radiolabeled peak which exited the column coincident with the fluorescent lometrexol oxidation product (Fig. 6). Over the concentration range examined, 100, 300, and 1000 n*M*, there was a close correspondence between the total radioactivity recovered and the fluorescence peak area (Fig. 7). Under the assay conditions described, the overall yield of radiolabeled lometrexol from serum was determined to be $\geq 90\%$ at a 100 n*M* concentration and 80% at 300 n*M*, but fell to 42% at 1000 n*M* (Table I). The overall yield observed in these studies at concentrations ≤ 300 n*M* is superior to that obtained by Tellingen *et al.* using a solid-phase extraction followed by HPLC separation with UV detection [12]. The consistency of the assay is greatly enhanced by the availability of an internal standard which has similar physicochemical properties to those of the oxidized product. That consistency, coupled with the high fluorescence of the oxidized lometrexol product makes this assay useful despite the low overall yield at high serum concentrations.

Three known factors could contribute to the observed sample losses: (i) persistent binding to serum proteins; (ii) non-specific degradation un-

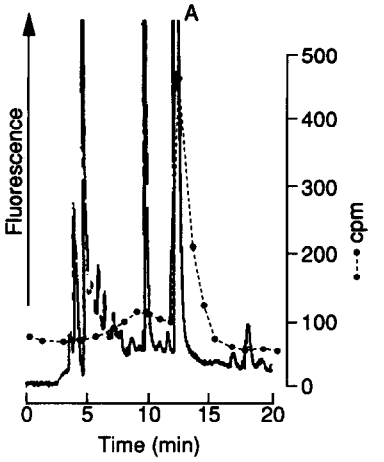

Fig. 6. HPLC separation of the MnO_2 oxidation product of [^{14}C]lometrexol in serum as examined by fluorescence and liquid scintillation counting of the column eluate.

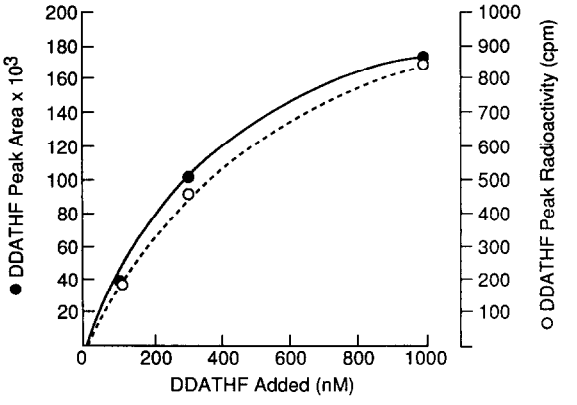

Fig. 7. Comparison of the fluorescent peak area with radioactivity of HPLC eluates of [^{14}C]lometrexol spiked serum supernatants following MnO_2-oxidation.

der the oxidative conditions; (iii) solubility losses. Continued binding to serum proteins appears not to be a major factor; the addition of 100 μM folic acid to serum to facilitate the displacement of lometrexol from the serum folate-binding protein prior to protein precipitation did not improve the yield; moreover, although high affinity binding should be saturable, sample losses increased with an increasing sample load (Table I). Since we did not observe a large peak of radioactivity at the solvent front it is unlikely that the glutamate moiety is lost during oxidation. The fluorescent peak increased with continued incubation through 10 min; therefore, it is unlikely that there are appreciable losses as CO_2; moreover, loss of the glutamate moiety while the pteridine ring remained intact would be manifest as a late exiting fluorescent peak.

It is most likely that sample losses at high serum concentrations are due to limited aqueous solubility of the free acid of the oxidized product of lometrexol; the aqueous solubility of the free acid of the polyglutamates is probably even lower. Conversion of lometrexol, or of the oxidation product, to the sodium salt should enhance overall yield. Neutralization of the serum supernatant with potassium carbonate prior to the oxidation step increased the yield of radioactivity after the MnO_2 oxidation step and hence raised the overall yield at 1000 nM to 75%; however, HPLC analysis showed no fluorescent lometrexol oxidation peak. This observation is consistent with the previous comment that lometrexol oxidation by MnO_2 occurs only at an acidic pH. A logical extension of this approach would be to modify the solubilization procedures after the oxidation step. This would, however, complicate the assay which currently consists of simple centrifugation and injection of the supernatant. Alkalinization would have to be done in the presence of the MnO_2 slurry in small volumes which present mixing problems. An ethanolic wash/extraction step might be more reasonable; however, this would produce sample dilution and possibly require an extra concentration step. The above modification of the assay could be of use in analyses seeking to measure polyglutamates, where apparent sample losses are high; however, the gain in sensitivity would be doubtful at lometrexol serum levels ≤ 300 nM, where product solubility is unlikely to be a major problem. At serum or urinary lometrexol levels ≥ 300 nM, it is simpler to dilute the sample and rerun the assay.

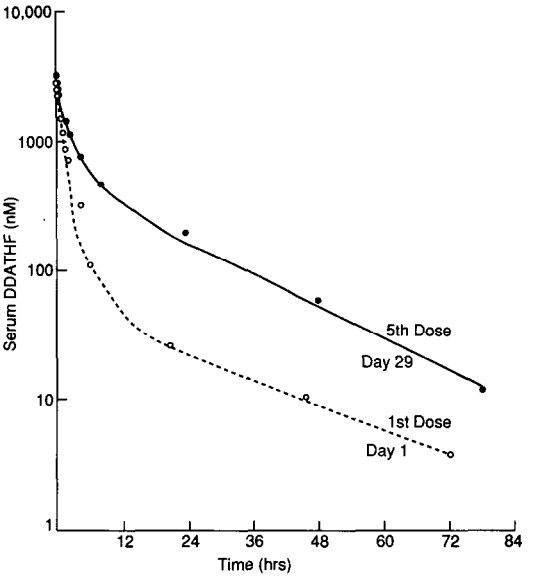

Fig. 8. Lometrexol serum concentration over time in a patient receiving multiple courses of lometrexol, 2 mg/m^2/dose × 4 doses.

Application of the method to patient serum and urine samples

The utility of this fluorescence HPLC method has been evaluated by analyzing serum and urine samples of patients in the Phase I study of lometrexol. The serum concentration *vs.* time profiles on days 1 and 29 are presented in Fig. 8 for a patient who received lometrexol (2 mg/m^2) twice weekly × 4 doses (days 1, 4, 8 and 11), then had a new cycle initiated on day 29. Lometrexol exhibits a triphasic serum elimination with a prolonged terminal phase and accumulation with repeated doses. Approximately 50% of the administered dose was excreted in urine within the first 24 hr, but less than 1% was excreted on days 2 and 3 in spite of persistent drug levels in the blood. No lometrexol metabolites have been identified in urine or serum.

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