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Measurement of 5,10-dideaza-5,6,7,8-tetrahydrofolate (lometrexol) in human plasma and urine by high-performance liquid chromatography

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Abstract

Three high-performance liquid chromatographic methods are described for the detection of the novel antifolate anticancer drug (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolate (lometrexol): one with fluorometric detection and two with detection by UV absorbance. An assay for plasma lometrexol using UV detection (288 nm) and reversed-phase chromatography was developed, with a quantitation limit of 0.2 μg/ml and linearity up to 10 μg/ml. This assay was modified for measurement of lometrexol in urine, with a quantitation limit of 2 μg/ml and linearity up to 25 μg/ml. An alternative assay for plasma lometrexol using derivatization and fluorescence detection (excitation at 325 nm, emission at 450 nm) was also developed, which proved twenty-fold more sensitive (quantitation limit of 10 ng/ml) than the UV assay, and which was linear up to 250 ng/ml. The fluorometric method requires sample oxidation with manganese dioxide prior to analysis, and uses ion-pair chromatography with tetramethylammonium hydrogensulphate as an ion-pair reagent. All assays use a similar preliminary solid-phase extraction method (recovery as assessed by UV absorption >73%), with C10-desmethylene lometrexol added for internal standardisation. Each assay is highly reproducible (inter-assay precision in each assay is <10%). Applicability of the fluorescence-based assay to lometrexol in plasma and the UV-based assay to lometrexol in urine is demonstrated by pharmacokinetic studies in patients treated as part of a Phase I clinical evaluation of the drug.

1. Introduction

(6*R*)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (lometrexol) (Fig. 1A) is a novel antifolate which exerts an antimetabolite effect via inhibition of glycinamide ribonucleotide formyltransferase (GARFT, EC 2.1.2.1) [1,2], the first folate dependent enzyme of de-novo purine biosynthesis. Lometrexol was found to exhibit a remarkable and broad spectrum antitumour activity in vivo, which exceeded that of the established antifolate methotrexate, in a number of preclinical murine and human xenograft tumour models [2,3]. Unfortunately, serious dose limiting cumulative toxicities (thrombocytopenia, leucopenia and mucositis), evident in subsequent Phase I studies [4–6], have limited its clinical utility. In an attempt to alleviate these toxicities a new Phase I study has been initiated involving administration of lometrexol to patients receiv-

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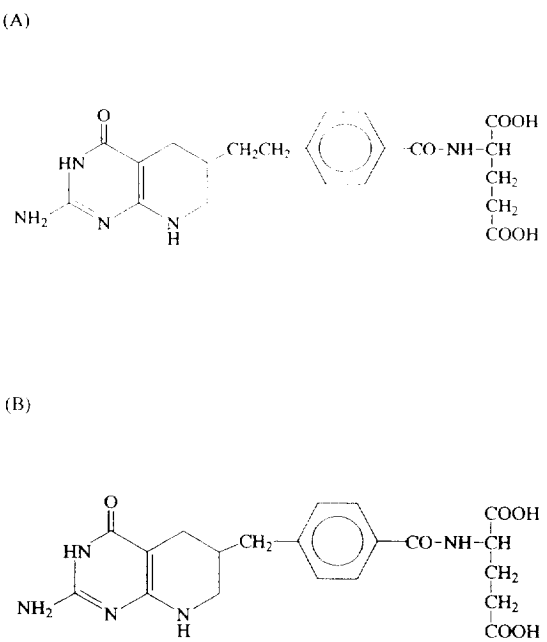

Fig. 1. Chemical structure of (A) lometrexol and (B) C10-desmethylene lometrexol.

ing an oral folic acid supplement [7], since folic acid has been found to modulate lometrexol toxicity in preclinical models [8]. In order to relate pharmacokinetic and pharmacodynamic parameters to lometrexol antitumour activity and toxicity, appropriate analytical methodology was sought for determining the concentration of lometrexol in urine and plasma. The only sensitive method available for lometrexol measurement was, until recently, a competitive particle concentration fluorescence immunoassay [9], a labor-intensive technique requiring lometrexol-specific antisera and b-phycoerythrin labelled lometrexol. Furthermore, such a method does not lend itself to the analysis of potential metabolites. A method using high-performance liquid chromatography (HPLC) with UV detection has since been reported [10], which has a recovery of approximately 58% and a detection limit of 10 ng/ml. In the present paper three methods are described for the analysis of lometrexol in human plasma and urine. In particular, a derivatization and fluorescence detection assay is described which combines sensitivity (limit of quantitation at 10 ng/ml) with specificity.

2. Experimental

2.1. Materials

Lometrexol and C10-desmethylene lometrexol were obtained from Eli Lilly (Indianapolis, IN, USA). Analytical grade acetontrile and methanol were purchased from FSA Laboratory Supplies (Loughborough, UK) and ammonia and analytical grade glacial acetic acid from May & Baker (Dagenham, UK). Sodium hydroxide pellets, Analar grade formic acid and 'HiPer Solv' tetramethylammonium hydrogen sulphate were purchased from BDH (Dorset, UK). Activated manganese dioxide was obtained from Sigma (Dorset, UK). Deionized water was produced using an Aquatron A4D system (Elga, Bucks., UK). Drug free control human plasma was kindly provided by the Red Cross transfusion service (Newcastle-upon-Tyne, UK) as outdated plasma, and control human urine was obtained from healthy volunteers.

2.2. Chromatography

The chromatographic equipment consisted of Waters 510 pumps (Millipore, Harrow, UK), an ISS-101 autosampler (Perkin-Elmer, Uberligen, Germany), a PU4021 multichannel detector (Pye-Unicam, Cambridge, UK) and a Model 980 variable-wavelength fluorescence detector (Applied Biosystems, Cheshire, UK). The fluorescence detector excitation wavelength was set at 325 nm (5 nm bandwidth) and used with a 450 nm bandpass emission filter (25 nm bandwidth). Data were recorded and processed using a Minichrom Chromatography Data Capture System (VG Data systems, Altringham, UK). All analytical columns were purchased from Jones Chromatography (Glamorgan, UK). All HPLC solvents were passed through 5-μm filters (Whatman, Maidstone, UK); organic solutions through a PTFE membrane and aqueous solu-

tions through a nitrocellulose membrane, prior to mixing, degassing under vacuum, and usage.

Between experiments, columns were washed (10 min, 1 ml/min) and stored in methanol. A linear gradient of 20 min was used to alternate between 100% methanol and 100% analytical mobile phase. A solution of 20% (v/v) acetonitrile in water was used for autosampler flushing. Processed samples were transferred to 250-μl glass autosampler vials (Chromacol, London, UK), which were immediately placed in a pre-cooled (4°C) autosampler.

2.3. Quantitation

Lometrexol was prepared as a 1 mg/ml solution in water and stored at 4°C. Prior to use in each experiment the concentration was verified spectrophotometrically, following dilution (1:20 in 10 m*M* NaOH), at a predetermined absorbance maximum of 277 nm using an extinction coefficient of 11 000 M^{-1} cm^{-1}. In each experiment an appropriate calibration curve was prepared in control plasma or urine (see section 2.9). Quality assurance (QA) samples were prepared in bulk, in advance, at three relevant concentrations (low, medium and high) across the linear range of calibration, in either control plasma or urine. These were aliquoted and stored at −20°C, and were routinely replaced every 2–3 months, though there was no evidence of sample deterioration within such a period. QA samples were assayed in triplicate during each experiment. Quantitation of lometrexol was achieved using the internal standard C-10-desmethylene lometrexol (Fig. 1B), which was added to all calibration standards, QA samples and patient samples, prior to analysis.

2.4. Solid-phase extraction

All assays utilised solid-phase extraction methodology, the efficiency of which was determined by comparing the recovery of lometrexol standards ($n = 2$) from plasma (at 100 ng/ml) or urine (at 15 μg/ml), with the recovery of standards of an equivalent concentration in deionized water ($n = 3$), all samples being evaporated and resuspended prior to analysis. Sample recoveries were measured by UV absorption (using the methods described herein), in three independent experiments. The recovery by solid-phase extraction was thus determined to be 79 ± 6% from plasma and 82 ± 3% from urine.

Plasma samples were initially diluted 1:1 with aqueous formic acid [1% (v/v), pH 3.7] containing C10-desmethylene lometrexol (at a concentration of 1 μg/ml for the UV assay, and 100 ng/ml for the fluorescence assay). Urine samples were also diluted 1:1 but with aqueous sodium formate [1% (v/v), pH 3.7] containing 15 μg/ml C10-desmethylene lometrexol. All samples were vortex-mixed and centrifuged (1000 *g*, 4°C, 20 min) prior to solid-phase extraction.

C_8 (1 cm) Bond-Elut cartridges (Analytichem International, Harbor City, CA, USA) were used to recover lometrexol (and added internal standard) from plasma or urine. A Vac Elut SPS 24 apparatus (Analytichem International) was used with an applied vacuum of 70–80 kPa, each sample being extracted individually using a separate column. Aqueous formic acid (1% v/v) used throughout the extraction procedure was adjusted to pH 3.7 with 5 *M* NaOH. Each sample (2 ml) was applied to a preconditioned column (washed with 5 ml of methanol followed by 5 ml of aqueous formate), which was then washed with aqueous formate (5 ml) to remove unwanted biological material. The analytes were eluted using 1.5 ml of 20% (v/v) acetonitrile in aqueous formate. Collected samples were centrifuged (1000 *g*, 4°C, 5 min) and transferred to 2-ml plastic conical microcentrifuge tubes without lids. Samples were then evaporated to dryness overnight using a Speedvac concentrator (Savant, Farmingdale, NY, USA) attached to a Model E1M5 high-vacuum pump with a Micro Modulo freeze dryer (both from Edwards High Vacuum, Crawley, UK).

2.5. HPLC assays

Initially a UV detection-based method for the measurement of plasma lometrexol was developed, which had a quantitation limit of 200 ng/ml of plasma. In order to improve the sen-

sitivity of this assay the production of fluorescent species by chemical derivatization, i.e. oxidation of lometrexol with permanganate, was attempted. However, only mild oxidation with manganese dioxide proved successful: a procedure first reported by Muindi et al. [11] who oxidised lometrexol to a fluorescent derivative following perchloric acid (PCA) treatment of plasma to remove interfering proteins. However, the extent of oxidation in PCA-treated plasma was variable (J. Muindi, personal communication). In contrast, removal of plasma components by the solid-phase extraction method described here, allowed the development of a reproducible assay.

2.6. HPLC assay for plasma lometrexol with fluorometric detection

Evaporated samples were reconstituted in 20 μl of 13% (v/v) aqueous formic acid and vortex-mixed. A suspension of manganese dioxide (0.2 mg/ml) in deionized water was freshly prepared, and rapidly stirred by a magnetic stirrer. A 100-μl volume of this suspension was added to each sample at a timed interval. Once added, each sample was vortex-mixed and incubated in a water bath at 37°C for 90 min. This period was found to be optimal for the generation of the lometrexol or C10-desmethylene lometrexol fluorescent species, i.e. maximum yield of the fluorescent products without over-oxidation and degradation. Samples were sequentially removed from the water bath and the oxidation stopped by addition of 30 μl of a 1:1 mixture of NaOH (5 M) and ammonium carbonate [1% (w/v), pH 5] at 4°C. They were then placed on ice before being collectively centrifuged (13 000 *g*, 10 min, ambient temperature). Each supernatant was carefully removed and 100 μl were analyzed chromatographically.

The analytical conditions developed used ion-pair chromatography at ambient temperature with an Apex II (C_{18}, 3 μm, 150 × 4.6 mm I.D.) analytical column. The mobile phase consisted of 12% (w/v) acetonitrile in 1% (v/v) aqueous acetic acid adjusted to pH 5 with strong ammonia, containing 0.171 g/l tetramethylammonium hydrogen sulphate. Isocratic elution was achieved using a flow-rate of 1.0 ml/min, and chromatographic data from each injection were recorded for 9 min.

2.7. HPLC assay for plasma lometrexol with UV detection

The UV-based HPLC assay for plasma lometrexol made use of reverse-phase chromatography with binary gradient elution. Following solid-phase extraction, samples evaporated to dryness were reconstituted in 150 μl of 7.4% (w/v) acetonitrile in aqueous formic acid 1% (v/v) adjusted to pH 3.7 with NaOH (5 *M*), and 100 μl analyzed chromatographically. Two mobile phases were prepared containing different concentrations of acetonitrile; mobile phase A at 7.4% (w/v) and mobile phase B at 15.3% (w/v) acetonitrile, both in 1% (v/v) aqueous formic acid (adjusted to pH 3.7). Samples were eluted using a 5-min linear gradient of 100% A to 100% B, followed by isocratic elution for a further 5 min in 100% B. The column was equilibrated between injections (100% B to 100% A in 8 min, followed by a further 2 min in 100% A). Mobile phases were run at 1.5 ml/min at ambient temperature, using a C_6 Spherisorb column (5 μm, 150 × 4.6 mm I.D.) preceded by a guard column (20 × 2.1 mm I.D.) containing pellicular C_{18} (Whatman Int., Maidstone, UK), and UV absorbance was measured at 288 nm.

2.8. HPLC assay for lometrexol in urine with UV detection

The assay for detection of lometrexol in urine was similar to that developed for its detection in plasma by UV absorption, in so much as the same reversed-phase column (Spherisorb C_6; 5 μm; 150 × 4.6 mm I.D.) and guard column (pellicular C_{18}; 20 × 2 mm I.D.) were used, with UV detection at 288 nm. However, it was possible to separate lometrexol from urinary components using simple isocratic elution with 13% (w/v) acetonitrile in aqueous phosphoric acid [1% (v/v) adjusted to pH 3.2 with 5 *M* NaOH], run at 1.5 ml/min. Evaporated samples were

resuspended in 150 μl of mobile phase, and 50 μl injected for chromatographic analysis, with data acquisition for a period of 16 min.

2.9. Calibration

Each assay was calibrated using a five-point calibration curve (duplicate samples), with quality assurance (QA) samples selected at three relevant concentrations and assayed in triplicate. Calibration curves were constructed using peak-height ratios: values from calibration samples were plotted against nominal concentration, and linear regression analysis used to determine the slope of the line (i.e. the response factor) which would best fit the data with the intercept fixed at zero.

Preliminary experiments were used to establish the limit of quantitation (LOQ) for each assay; a concentration which would be anticipated to be reproducibly accurate and precise (see section 3.2). The LOQ was, for each assay, determined at a signal-to-noise ratio of approximately 5. For the plasma lometrexol assay using fluorescence detection the calibration standards were 10, 25, 50, 100 and 250 ng/ml, with QA samples of 10, 50 and 250 ng/ml. The UV plasma lometrexol assay was calibrated using standards of 0.2, 1, 2, 4 and 10 μg/ml with QA samples of 0.2, 2 and 10 μg/ml, and the UV assay for lometrexol in urine with calibration standards of 2, 5, 10, 15 and 25 μg/ml and QA samples of 2, 10 and 25 μg/ml, respectively.

2.10. Pharmacokinetic study

The fluorescence-based HPLC assay for plasma lometrexol and the UV-based assay for lometrexol in urine were applied to the monitoring of lometrexol pharmacokinetics in an ongoing Phase I study of the drug given together with folic acid. This study was conducted in patients with advanced cancer, for which no effective or standard therapy existed. Lometrexol (reconstituted in 0.9% saline) was administered at a concentration of 1 mg/ml as a rapid i.v. bolus over 0.5–1.0 min. Folic acid was given orally as a daily 5-mg tablet for 7 days prior to the administration of lometrexol and continued for 7 days afterwards. Plasma was obtained at various time intervals by collection of blood via venipuncture into vacutainer tubes (containing the sodium salt of ethylenediaminetetraacetic acid as an anticoagulant), centrifugation (1000 *g*, 8 min, 4°C), and separation using a pasteur pipette with storage at −20°C prior to analysis. Urine was collected in 6-h aliquots over the first 24 h after lometrexol administration, the total volume of each aliquot recorded, and a sample of each stored frozen at −20°C prior to analysis.

Because of the sensitivity of the fluorescence-based plasma assay, those patient plasma samples collected between 5 min and 12 h had to be diluted appropriately in control plasma, in order to be within the defined region of quantitation (10–250 ng/ml). Adapt II software, kindly provided by Drs. D'Argenio and Schumitzky (Biomedical Simulations Resource, USC, CA, USA), was used to fit a biexponential equation to the plasma concentration–time data.

3. Results and discussion

3.1. Chromatographic profiles

For the three assays developed chromatographic elution profiles of lometrexol and the internal standard, C10-desmethylene lometrexol at the LOQ are shown in Fig. 2I–III. Fig. 2I shows the separation of oxidised lometrexol and C10-desmethylene lometrexol with fluorescence detection, the retention times (t_R) being 4.2 and 5.3 min, respectively. Since this chromatogram represents 10 ng/ml of lometrexol and 100 ng/ml of internal standard, their oxidation results in products with similar fluorescent yields. It is also noteworthy that baseline interference from plasma components is minimal. Fig. 2II and 2III show chromatographs obtained with UV detection; the t_R values of lometrexol and C10-desmethylene lometrexol being, respectively, 7.2 and 7.8 min in the method devised for detection in plasma (Fig. 2II), and 6.8 and 8.7 min in that developed for urine analysis (Fig. 2III). Evidence of each method's specificity is obtained

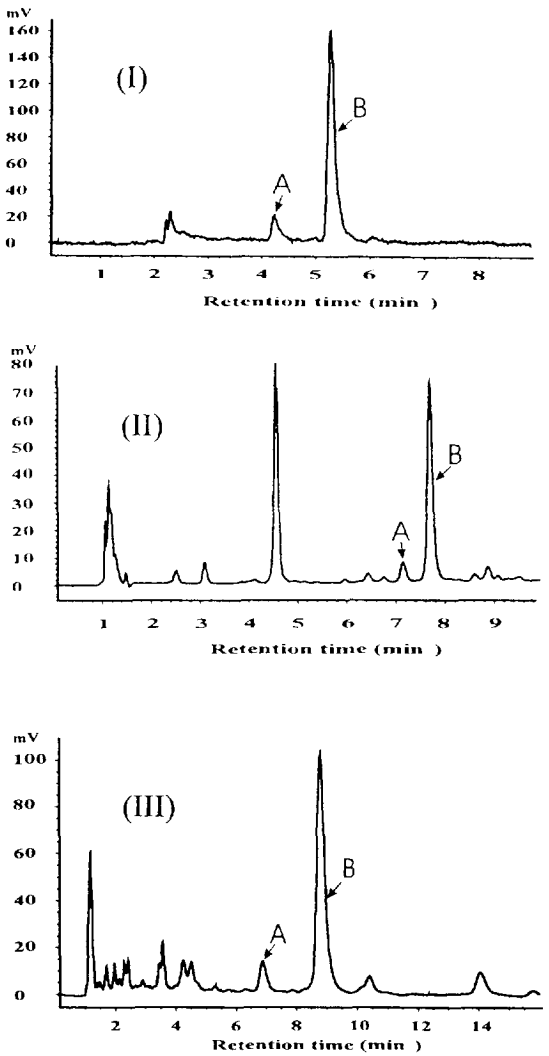

Fig. 2. Chromatographic separation of (A) lometrexol and (B) C10-desmethylene lometrexol internal standard. (I) Extract from human plasma (A = 10 ng/ml, B = 100 ng/ml), oxidised then analyzed by fluorometric detection; (II) extract from human plasma (A = 0.2 μg/ml, B = 1 μg/ml) and analyzed using UV detection; and (III) extract from human urine (A = 2 μg/ml, B = 15 μg/ml) and analyzed using UV detection.

from the fact that many patients, receiving a variety of concomitant medication, have been successfully analyzed by these methods without any deterioration in chromatographic resolution.

3.2. Linearity, accuracy and precision

Good linearity was observed for all three methods, the square of the correlation coefficient (r) for every calibration curve always being higher than 0.997. Assay variability was calculated using QA samples (analyzed in triplicate in each experimental run). The relative error (R.E.) calculated for each mean interpolated concentration, by comparison with the relevant nominal concentration, was taken as an indication of accuracy, while the coefficient of variation (C.V.) was used as a measurement of precision. Intra-assay accuracy and precision were calculated from a typical analytical run, while mean interpolated values from a number of experiments were used to calculate inter-assay parameters. Inter-assay variation was calculated for the derivatization/fluorescence assay, for the UV-absorbance assay for detection in plasma and for the UV-absorbance assay for detection in urine using 7, 3 and 5 independent experiments, respectively. The results for each method are shown in Tables 1–3. Both assays for the detection of lometrexol in plasma, i.e. the derivatization/fluorescence assay (Table 1) and the UV-absorbance assay (Table 2), had an intra- and inter-assay accuracy of ≤5% and an intra- and inter-assay precision of <10%. The UV absorbance assay for detection of lometrexol in urine

Table 1
Fluorometric assay for the determination of lometrexol in plasma: Intra- and inter-assay accuracy and precision

Variation	n	Concentration (ng/ml)		R.E. (%)	C.V. (%)
		Nominal	Interpolated (mean ± S.D.)		
Intra-assay	3	10	10.5 ± 0.4	5.0	3.8
		50	52.1 ± 4.3	4.2	8.3
		250	256 ± 5	2.3	2.1
Inter-assay	7	10	10.1 ± 0.6	1.0	5.9
		50	50.6 ± 1.9	1.2	3.8
		250	252 ± 8	1.0	3.1

Table 2
UV assay for the determination of lometrexol in plasma: Intra- and inter-assay accuracy and precision

Variation	n	Concentration (μg/ml)		R.E. (%)	C.V. (%)
		Nominal	Interpolated (mean ± S.D.)		
Intra-assay	3	0.2	0.21 ± 0.02	5.0	7.1
		2	2.04 ± 0.04	2.0	2.0
		10	10.3 ± 0.4	3.4	3.6
Inter-assay	3	0.2	0.21 ± 0.02	5.0	9.5
		2	1.94 ± 0.09	−3.0	4.6
		10	10.2 ± 0.24	1.6	2.4

(Table 3) had an intra- and inter-accuracy of ≤9% and an intra- and inter-precision of <3%. These values indicate that each method is acceptably reproducible; the criteria for a valid analytical assay being defined by others [12] as an assay with an intra- and inter-assay accuracy and precision of ≤15% for midrange and high range QA samples, and ≤20% for QA samples at the LOQ.

3.3. Applicability

The applicability of the fluorescence-based HPLC assay for lometrexol in plasma was demonstrated in a patient receiving 12 mg/m^2 lometrexol (Fig. 3). Data collected within the first 12 h following bolus administration of lometrexol were found to fit a two-compartment model with an initial and terminal plasma half-life of 10 min and 201 min, respectively. The volume of distribution was 11.6 l/1.73 m^2, and the systemic clearance 44 ml/min/1.73 m^2, resulting in an AUC of 0.46 mg/ml min. The assay enabled quantitative determination of plasma levels up to 24 h after drug adminstration. An application of the urine assay is shown in Fig. 4 for a patient who received 45 mg/m^2. These data demonstrate that lometrexol is subject to rapid and extensive urinary elimination with essentially the entire dose present in the urine by 24 h.

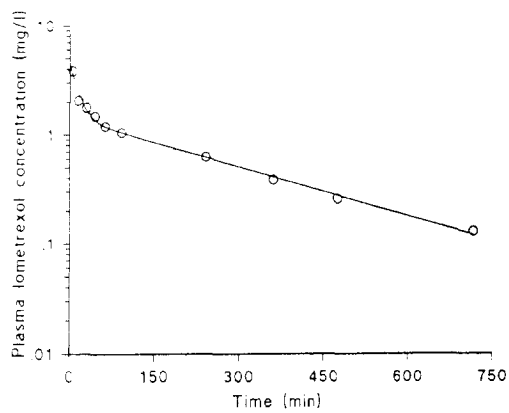

Fig. 3. Plasma concentration vs. time data for lometrexol (fitted to a biexponential curve), in a patient treated with 12 mg/m^2 lometrexol as an intravenous bolus.

The methods described for the measurement of lometrexol in human plasma and urine are relatively simple, and have proven to be reproducibly selective, accurate and precise. The derivatization/fluorometric method enables measurement of lometrexol pharmacokinetics by HPLC down to a limit of quantitation of 10 ng/ml. This assay is complemented by the UV method for detection of lometrexol in plasma, which can be selected where sensitivity (<200 ng/ml) is not a prerequisite. Indeed, as shown in Fig. 3, the UV-based assay for lometrexol in

Table 3
UV assay for the determination of lometrexol in urine: Intra- and inter-assay accuracy and precision

Variation	n	Concentration (μg/ml)		R.E. (%)	C.V. (%)
		Nominal	Interpolated (mean ± S.D.)		
Intra-assay	3	2	1.82 ± 0.01	−9.0	0.6
		10	9.79 ± 0.17	−2.1	1.7
		25	25.2 ± 0.2	1.0	0.8
Inter-assay	5	2	1.82 ± 0.05	−9.0	2.8
		10	9.64 ± 0.11	−3.6	1.1
		25	24.8 ± 0.6	−1.0	2.3

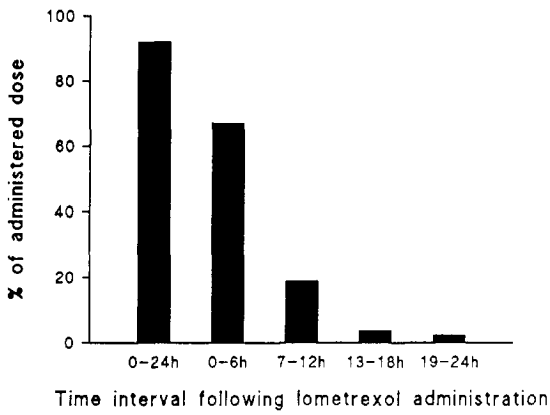

Fig. 4. Urinary excretion of intact drug in a patient treated with 45 mg/m^2 lometrexol.

plasma would have been adequate for the analysis of lometrexol concentrations for the first 6–8 h after drug administration, even at the lowest dose level in the current clinical trial [7]. The use of the simpler and more rapid UV-based assay could therefore have been used for the majority of patient samples. The measurement of lometrexol by HPLC in urine, has not been previously reported.

Previous HPLC methodology for the detection of lometrexol in plasma [10] was reported to have a detection limit of 10 ng/ml, with reproducibility data presented for 20 ng/ml. The fluorometric method described here results in a chromatographic profile with a much cleaner and flatter baseline than observed in the previously published study [10] and, in addition, the applicability of the current method to clinical samples has been proven. An immunoassay with greater sensitivity (limit of quantitation 1 ng/ml) has been described [9], but the method developed here is much easier to establish, and also lends itself to the analysis of potential drug metabolites.

Although fluorometric methodology for the determination of inherently fluorescent compounds is common, the oxidation of a reduced compound to obtain a highly fluorescent species has not been widely adopted. As long ago as 1949, a fluorometric-based assay for folic acid was described in which permanganate oxidation of the compound prior to analysis improved detection sensitivity by up to 130-fold [13], and an alternative method for the detection of folic acid and its dihydro and tetrahydro derivatives has been described which uses oxidation with chlorine [14]. There is also a method involving post-column UV irradiation of tamoxifen to produce a fluorescent phenanthrene derivative [15], but the most widely used method using derivatization and fluorescence detection, involves detection of the antifolate methotrexate following oxidation with permanganate [16,17]. Such an approach is clearly attractive, especially when applied to the detection of a compound in a biological matrix such as plasma, where background fluorescence from interfering components is unlikely to increase following oxidation, and should not, therefore, compromise sensitivity or selectivity.

As part of the current study attempts were made to synthesize the oxidized lometrexol product in significant yield, to enable structural characterisation by spectroscopic methods. However, contamination of the product with paramagnetic manganese prevented the acquisition of interpretable NMR spectra. The excitation and emission maxima of the fluorescent compound at 325 nm and 450 nm, respectively, suggest that the fluorescence originates from the deazapteridine region of the molecule [18]. In addition, lometrexol and C10-desmethylene lometrexol have similar extinction coefficients, negligible native fluorescence under the conditions stated, but near equivalent fluorescence following oxidation. One may therefore hypothesize that the major oxidation product is formed by oxidation at the C5 and/or N8 positions and not the C10 (absent in C10-desmethylene lometrexol). In particular, the fully oxidized deazapteridine bicycle might be expected to have fluorescence similar to the endogenous fluorescence of methotrexate and folic acid.

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