ELSEVIER

Journal of Chromatography B, 683 (1996) 245–249

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simple and sensitive method for the quantitative analysis of lometrexol in plasma using high-performance liquid chromatography with electrochemical detection

Timothy W. Synold[a,*], Bixin Xi[a], Edward M. Newman[b], Franco M. Muggia[c], James H. Doroshow[a]

[a]*Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010, USA*

[b]*Department of Pediatrics, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010, USA*

[c]*Division of Medical Oncology, USC-Norris Comprehensive Cancer Center, Los Angeles, CA 90033, USA*

Received 27 November 1995; revised 12 February 1996; accepted 4 March 1996

Abstract

Previously described methods for the determination of lometrexol in plasma used either fluorescence or ultraviolet detection. An alternative method for the determination of lometrexol utilizing electrochemical detection is described, having comparable sensitivity to fluorometric methods but not requiring pre-analytical oxidation. Following sample clean-up, separation is achieved on a phenyl column with a mobile-phase of 8% acetonitrile in 50 m*M* sodium acetate buffer, pH 4.0. The calibration curve in plasma is linear from 10 to 200 ng/ml, with inter- and intra-day precision of 5.4 and 5.5%, respectively. The recovery of lometrexol from plasma is 81±1.5%, and the lower limit of detection is 5 ng/ml, using a signal-to-noise ratio of 3.

Keywords: Lometrexol

1. Introduction

Lometrexol [(6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolic acid, DDATHF, Fig. 1A] is a new antifolate antimetabolite with a broad spectrum of cytotoxic activity against preclinical murine tumor model systems and human tumor xenografts [1,2]. Unlike classical antifolates, lometrexol acts by inhibiting glycinamide ribonucleotide formyltransferase (GARTF), the first folate-dependent enzyme in the *de novo* purine biosynthetic pathway [1]. In addition to its unique mechanism of action, lometrexol is an excellent substrate for folylpolyglutamate synthetase and is avidly polyglutamated [3]. It has been demonstrated previously that the polyglutamates of lometrexol are up to 100-fold more potent inhibitors of GARTF than lometrexol itself [4]. Furthermore, in addition to having a higher affinity for their target enzyme, polyglutamated anabolites are retained intracellularly for prolonged periods of time [3]. Because of promising preclinical activity, clinical trials began over five years ago; however, the utility of lometrexol has been limited by serious toxicities (thrombocytopenia, leukopenia and mucositis),

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00122-3

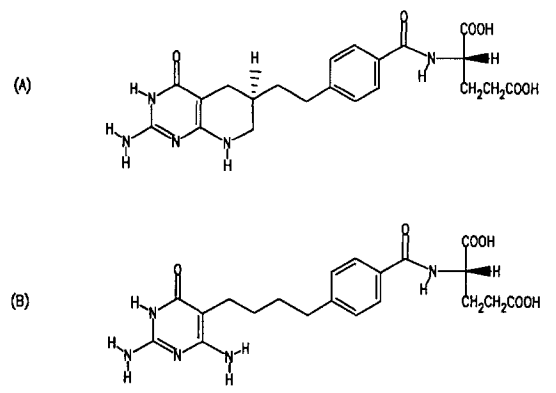

Fig. 1. Structures of lometrexol [A; (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolic acid] and internal standard (B; LY277413).

which appear to be cumulative and long-lasting [5,6]. It has been speculated that this cumulative toxicity may be related to the formation and retention of polyglutamated anabolites, resulting in longer than expected drug exposures in certain target tissues. Tissue levels of other antifolates, such as methotrexate in erythrocytes, have been used as a measure of long term drug exposure [7]. Current clinical trials of lometrexol utilize folic acid pretreatment, since folic acid modulates lometrexol toxicity in preclinical models [8]. Because of its toxicity profile and metabolism, prolonged pharmacokinetic and pharmacologic monitoring of lometrexol is currently playing an important role in the development of this interesting new anticancer agent. Attempts to improve current assay technology for lometrexol are warranted.

Previously published methods for lometrexol determination have used either ultraviolet or fluorometric detection [9–11]. Ultraviolet detection of lometrexol is relatively simple; however, it is less sensitive than fluorescence detection, despite the use of large injection volumes. While the fluorometric method is more sensitive, it requires solid-phase extraction and sample oxidation with manganese dioxide prior to analysis. Because electrochemical detection has been used successfully in the past to measure exogenous and endogenous folic acid derivatives in plasma, including tetrahydrofolic acid and 5-formyltetrahydrofolic acid [12,13], it was explored as an alternative to other published methods. Described here is a simple and sensitive HPLC assay for lometrexol in plasma using electrochemical detection.

2. Experimental

2.1. Materials and reagents

Lometrexol and internal standard (LY277413, Fig. 1B) were supplied by Eli Lilly and Co. (Indianapolis, IN, USA). SPE-aromatic sulfonic acid columns for solid-phase extractions and analytical-grade acetic acid were from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile and analytical-grade hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was first purified using the Milli-Q system (Millipore, Bedford, MA, USA) and then filtered through 3-ml Sep-Pak C_{18} cartridges (Millipore) to remove all trace organic material. All other chemicals were of analytical grade and were obtained from Mallinckrodt (Paris, KY, USA).

2.2. Liquid chromatography

The HPLC system consisted of a Shimadzu Model LC-10AS solvent delivery pump (Shimadzu, Columbia, MD, USA) fitted with a Rheodyne Model 7725 injector valve (Rheodyne, Cotati, CA, USA). Detection was performed using an ESA Model 5100A electrochemical detector equipped with a 5020 guard cell and a 5011 high-sensitivity analytical cell (ESA Inc., Bedford, MA, USA). The electrochemical detector potentials were set at +1.0 V for the guard cell, +0.40 V for the screening electrode and +0.90 V for detection. Separation was achieved using a SPS-phenyl column (5 μm, 250×4.6 mm I.D.) preceded by a phenyl guard cartridge (both from Regis Technologies, Morton Grove, IL, USA). The mobile-phase consisted of 8% acetonitrile in a 50 m*M* sodium acetate buffer, pH 4.0. Elution was performed at 1.8 ml/min and ambient temperature. Data acquisition and integration was performed using the EZ Chrom Chromatography Data System (Shimadzu).

2.3. Hydrodynamic voltammograms

To optimize the electrochemical detector conditions for lometrexol and the internal standard (I.S.), hydrodynamic voltammograms were generated over the voltage range of +0.40 to +1.0 V in 0.05 V increments. At each different applied potential, a fixed concentration of lometrexol and internal standard were injected in duplicate. The maximum response, defined by peak area, was recorded and all other responses were expressed as a percentage of maximum. Voltammograms were generated by plotting the percentage of maximum response versus the applied voltage at the electrode.

2.4. Sample pretreatment

To 1 ml of plasma, 40 μl of I.S. (10 μg/ml in water) was added along with 1 ml of 0.5 *M* acetic acid. The sample was then applied to a SPE-aromatic sulfonic acid cartridge that had been preconditioned with 3 ml of methanol and 3 ml of 1% (v/v) acetic acid in water. After the sample was applied to the cartridge, it was rinsed sequentially with 2 ml of 1% acetic acid and 1 ml of water. Finally, both lometrexol and internal standard were eluted with 1 ml of a 0.5 *M* phosphate buffer, pH 7.0, containing 20% acetonitrile. The pH of the eluate was adjusted to 4.0 with 6 *M* HCl, and 100 μl was injected onto the column.

2.5. Calibration and validation

A stock solution of lometrexol in water was prepared and aliquots stored at −70°C until use. A fresh aliquot of stock solution was thawed just prior to use and further diluted in water to obtain the desired standard solutions. For the generation of the standard curve, drug-free human plasma was spiked with the necessary volume of the appropriate lometrexol standard solution to give calibrator concentrations in the range of 10–200 ng/ml. The calibrators were extracted as previously described and analyzed by HPLC. At the same time that the calibrators were prepared, a sufficient number of low and high plasma controls (20 and 180 ng/ml, respectively) were prepared, extracted and frozen at −70°C until validation was performed. The standard curve was established by injecting known concentrations of lometrexol, in duplicate, and then plotting the ratio of the peak heights for lometrexol to I.S. vs. the concentration of lometrexol. Once the standard curve was generated, the assay was validated by injecting ten low and ten high controls on one day followed by three low and three high controls on subsequent days to determine both the intra- and inter-day accuracy and precision. Accuracy is defined as the percentage of target value (i.e. mean of controls/target concentration ×100%), while the precision is expressed as the coefficient of variation.

3. Results

3.1. Electrochemical properties

Hydrodynamic voltammograms for lometrexol and I.S. in extracted plasma are shown in Fig. 2. The voltammograms for lometrexol and I.S. in water and extracted plasma were identical (data not shown). Under the mobile-phase conditions of this assay, both lometrexol and I.S. had similar electrochemical properties, with maximum responses occurring at applied potentials above +0.90 V. No responses for lometrexol or I.S. were obtained at applied potentials of less than +0.45 V. Therefore, a screening potential of +0.40 V was chosen for the first set of electrodes, while a potential of +0.90 V was chosen for detection at the second set. Due to the relatively high potentials required for detection, a guard cell set

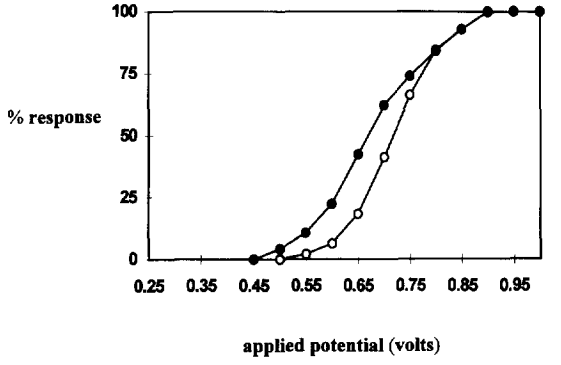

Fig. 2. Hydrodynamic voltammograms for lometrexol (closed circles) and internal standard (open circles).

to +1.0 V was used between the solvent delivery pump and the injector to reduce baseline noise.

3.2. Chromatography

Chromatograms for blank human plasma and plasma spiked with lometrexol and internal standard are shown in Fig. 3. Previously reported HPLC methods for the determination of lometrexol in plasma have used reversed-phase separation with C_{18} analytical columns. For the current assay, several different packing materials were tested. The SPS-phenyl column was found to be the best column tested, as judged by separation characteristics and peak shape. Under optimized assay conditions, the retention times for lometrexol and internal standard were 9.9 and 7.7 min, respectively (Fig. 3B). Extracted blank plasma had no interfering peaks at the retention times of lometrexol or internal standard (Fig. 3A). A total of ten different blank plasma samples were tested and all were found to be free of interfering peaks.

3.3. Sample pretreatment

Several sample pretreatment methods were investigated including protein precipitation and solid-phase extraction. Precipitation of plasma proteins with trichloroacetic acid, perchloric acid or methanol resulted in chromatograms with multiple interfering peaks. Although solid-phase extraction with C_8 or C_{18} cartridges gave reasonable recoveries, extracted plasma samples again contained multiple interfering peaks. Cation-exchange solid-phase extraction using aromatic sulfonic acid cartridges provided the cleanest blank plasma chromatogram (Fig. 3A) and the best percentage recovery for lometrexol and I.S. The recoveries of lometrexol and I.S. in plasma were 81±1.5 and 83±2.1%, respectively.

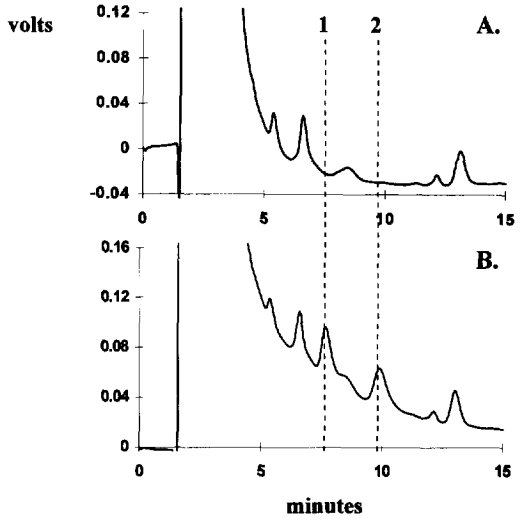

Fig. 3. Chromatograms of extracted human plasma samples. (A) Blank plasma; (B) plasma spiked with 180 ng/ml of lometrexol and internal standard. Retention times for internal standard (1) and lometrexol (2) are 7.7 and 9.9 min, respectively.

Table 1

Sample	Concentration (ng/ml)	n[a]	Accuracy[b] (%)	Precision[c] (%)
Intra-day				
Low control	20	10	100.5	5.4
High control	180	10	106.3	2.1
Inter-day				
Low control	20	9	99.0	5.5
High control	180	9	104.6	2.2

[a]Number of replicates
[b]Mean percentage of target concentration.
[c]Percent coefficient of variation.

3.4. Assay performance

The standard curve, as determined by linear regression, displayed good linearity over the range tested, with a slope of 4.98×10^2 and an intercept of 2.015 ($r^2=0.999$). Inter- and intra-assay performance was determined over four days, and the results are shown in Table 1. The assay was accurate over the range of concentrations measured, with an inter- and intra-day accuracy ranging from 99.0 to 106.3%. In addition, the quantitation of both high and low controls was precise, with inter- and intra-day coefficients of variation of ≤5.5%.

4. Discussion

In light of the serious dose-limiting toxicities observed on previous trials, detailed pharmacokinetic and pharmacologic monitoring of lometrexol is important, and requires a relatively simple and sensitive assay. Previously published assay methods for lometrexol either lack sufficient sensitivity [9] or

require a complicated pre-analytical oxidation step [10]. The assay described here simplifies the pre-analytical sample processing required for fluorescence detection, while providing equivalent sensitivity. Electrochemical detection has been used successfully to determine exogenous and endogenous folate levels in plasma [12,13]. Because lometrexol is an analogue of tetrahydrofolate, it was anticipated that it would be electrochemically active. While the potential required for oxidation of lometrexol is much higher than that for tetrahydrofolate, it is similar to the potential required for detection of folic acid and leucovorin [12]. Once the electrochemical spectrum of lometrexol was determined, various sample clean-up procedures were explored. A solid-phase extraction procedure using a cation-exchange cartridge gave the cleanest plasma and the highest recovery. While this extraction procedure is similar to the one used by van Tellingen et al. [9], we observed a 20% greater recovery of lometrexol, most likely due to the addition of acetonitrile to the elution buffer. Adequate chromatographic separation was achieved using a reversed-phase phenyl column, and was consistently good using columns from within the same lot, even after several hundred injections. However, because of lot-to-lot variation in performance, columns from different lots may require small changes in the mobile-phase in order to maintain good separation.

By combining a solid-phase sample clean-up with electrochemical detection, we have developed an assay for lometrexol that is both simple and sensitive. Moreover, the assay is accurate and precise to within $\leq 5.5\%$, over the range of lometrexol concentrations studied. In addition to measuring plasma concentrations, by decreasing the mobile-phase pH to 3 and using a different I.S., we have determined lometrexol levels in whole blood samples obtained from patients. From plasma and whole blood concentrations, we are able to calculate the level of lometrexol in erythrocytes, which may provide a better indicator of cumulative drug exposure and toxicity [7]. In order to analyze lometrexol over the wide range of clinically achievable concentrations, we have explored either decreasing the gain at the detector or diluting samples so that they fall within the range of our lower standard curve. While both these methods appear to be adequate, we have found it more convenient to combine previously described UV detection methods [9,11], for low sensitivity, with electrochemical detection, for high sensitivity, enabling us to make one injection per sample, regardless of the lometrexol concentration. We are currently applying this new assay methodology to plasma and red blood cell samples obtained from patients treated on an on-going Phase I clinical trial of lometrexol.

Acknowledgments

This work was supported by NIH UO1 CA 62505 and NIH 5P30 CA 33572.

References

[1] G.P. Beardsley, B.A. Moroson, E.C. Taylor and R.G. Moran, J. Biol. Chem., 264 (1989) 328.
[2] E.C. Taylor, J.M. Hamby, C. Shih, G.B. Grindey, S.M. Rinzel, G.P. Beardsley and R.G. Moran, J. Med. Chem., 32 (1989) 1517.
[3] G. Pizzorno, J.A. Sokolosky, A.R. Cashmore, B.A. Moroson, A.D. Cross and G.P. Beardsley, Mol. Pharmacol., 39 (1991) 85.
[4] S.W. Baldwin, A. Tse, L.S. Gossett, E.C. Taylor, A. Rosowsky, C. Shih and R.G. Moran, Biochemistry, 30 (1991) 1997.
[5] S.R. Wedge, S. Laohavinij, G.A. Taylor, D.R. Newell, C.J. Charlton, M. Proctor, F. Chapman, D. Simmons, A. Oakey, L. Gumbrell and A.H. Calvert, Br. J. Cancer, 67 (1993) 17.
[6] M.S. Ray, F.M. Muggia, C.G. Leichman, S.M. Grunberg, R.L. Nelson, R.W. Dyke and R.G. Moran, J. Natl. Cancer Inst., 85 (1993) 1154.
[7] M.L. Graham, J.J. Shuster, B.A. Kamen, D.L. Cheo, M.P. Harrison, B.G. Leventhal, D.J. Pullen and V.M. Whitehead, Cancer Chemother. Pharmacol., 31 (1992) 217.
[8] G.B. Grindey, T. Alati and C. Shih, Proc. Am. Assoc. Cancer Res., 32 (1991) 1921.
[9] O. van Tellingen, J.H. Sips, J.H. Beijnen, J.H. Schornagel and W.J. Nooyen, J. Chromatogr., 576 (1992) 158.
[10] J.R. Muindi, C.W. Young and C. Shih, J. Chromatogr., 621 (1993) 55.
[11] S.R. Wedge, S. Laohavinij, G.A. Taylor and D.R. Newell, J. Chromatogr. B, 663 (1995) 327.
[12] M.D. Lucock, R. Hartley and R.W. Smithells, Biomed. Chromatogr., 3 (1989) 58.
[13] M. Kohashi, K. Inuoe, H. Sotobayashi and K. Iwai, J. Chromatogr., 382 (1986) 303.