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Journal of Chromatography B, 679 (1996) 185–192

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of mitoxantrone in mouse whole blood and different tissues by high-performance liquid chromatography

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Received 27 October 1995; revised 8 January 1996; accepted 8 January 1996

Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the specific determination of mitoxantrone (MTO) in whole blood and different tissues of mice (liver, heart, spleen, kidneys). MTO was extracted into dichloromethane with ametantrone (AMT) as internal standard. The different tissues were homogenised in citrate buffer (pH 3.0) containing 20% ascorbic acid. Separation of MTO and AMT was carried out using a Nucleosil C_{18} column. The mobile phase consisted of acetonitrile (33%) and 0.16 *M* ammonium formate buffer, pH 2.7. UV detection was used at 658 nm. Baseline separation of AMT and MTO was achieved in all matrices. The calibration curves were linear in all matrices ($r>0.999$) in the concentration range of 2–200 μg/l for whole blood and 2–700 μg/l for tissue homogenates, respectively. The within-day and between-day precision studies showed good reproducibility with coefficients of variation below 4.5% for whole blood and below 10% for tissue homogenates, respectively. The extraction efficiencies of MTO are 60% in whole blood and 38% in tissue homogenates. The method described is suitable for pharmacokinetic studies on the distribution of MTO in different tissues of mice.

Keywords: Mitoxantrone

1. Introduction

Mitoxantrone (MTO, Novantrone) or 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)-amino]ethyl}-amino}-9,10-anthracenedione dihydrochloride (Fig. 1) is a synthetic anthraquinone derivative which is used as an antineoplastic agent. It is active against lymphomas, breast cancer, acute leukemias and other malignancies. The dose-limiting toxicity of MTO is myelosuppression but cardiotoxicity may also occur. The risk of cardiomyopathy increases parallel to the total cumulative dose of MTO but the overall risk is considerably lower compared to the structurally-related anthracyclines. An overall incidence of MTO-associated cardiac effects of 3% in adults and 6% in children was reported [1]. The estimated worst case incidence of congestive heart failure being 1.3% compared to 2.2% with doxorubicin. In order to improve the antitumor activity and to reduce toxicity of several anthracyclines, liposomal formulations were prepared. With liposomal formulations of doxorubicin [2,3], daunorubicin [4] and epirubicin [5] it has been demonstrated that due to altered pharmacokinetic behaviour [2] and tissue distribution [4], the overall therapeutic index of the drugs could be improved. In accordance with these encouraging

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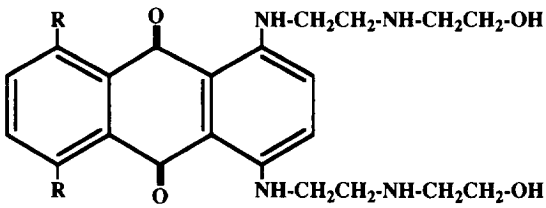
0378-4347/96/$15.00
PII S0378-4347(96)00023-0



R =	Name	Abbreviation
OH	Mitoxantrone	MTO
H	Ametantrone (internal standard)	AMT

Fig. 1. Structural formulae of mitoxantrone and ametantrone.

results, a liposomal formulation of MTO was developed [6].

Drug monitoring of cytostatic drugs becomes more and more important either in the evaluation of new drugs or new pharmaceutical formulations of drugs or in the treatment of individual patients [7]. One of the main problems with the monitoring of anticancer drugs in patients is to find an easily accessible physiological fluid which allows for estimation of the concentration of the cytostatic drug in the tumour. Usually plasma is not suitable and either blood cells or whole blood have to be analysed. To compare the pharmacokinetic behaviour of new pharmaceutical formulations of drugs, concentrations of the cytostatic drugs have also to be determined in various tissues.

Different HPLC methods have been published for the determination of MTO in serum and plasma. Several authors used external standardisation [8–11], a procedure which was not suited for the determination of MTO in tissue extracts. Three groups have reported on the determination of MTO in tissues [12–14]. However, none of these methods were suitable for routine determination of nonradioactive MTO in different tissues. The method which is presented here was developed to determine the concentration of MTO in whole blood, liver, spleen, heart and kidneys of mice. The extraction procedure and the chromatographic conditions are based on the HPLC method for MTO in physiological fluids which has been described earlier [15]. This method has been shown to be accurate and reproducible for the determination of MTO in serum and urine but since we were interested in the tissue distribution, the method had to be adapted to the complex matrices of whole blood and of tissue homogenates.

The method described in this paper was used to compare a new liposomal formulation of MTO [6] with the aqueous solution which is currently being used clinically (Novantrone). The concentrations of MTO had to be measured in blood and different tissues of mice after administration of the two formulations.

2. Experimental

2.1. Chemicals

Laboratory grade ammonium formate, formic acid, sodium tetraborate·$10H_2O$, sodium dioxide, ascorbic acid and hexanesulphonic acid (sodium salt) were obtained from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile and dichloromethane were obtained from Merck (Dietikon, Switzerland).

Mitoxantrone was kindly provided by Cyanamid (Schweiz), Lederle Pharmaceuticals (Adliswil, Switzerland) and the internal standard, ametantrone (AMT) was a generous gift of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Rockville, MD, USA). For silanization of the glassware, Sylon CT (5% dimethyldichlorosilane in toluene) from Supelco (Bellefonte, PA, USA) was used.

2.2. HPLC

The HPLC system consisted of a 9010 pump, a 9100 autosampler and a 9050 UV-Vis detector (Varian, Sunnyvale, CA, USA). The eluate was monitored at 658 nm and the autosampler was equipped with a 92-μl loop. Separation of mitoxantrone and the internal standard was carried out using a 250×4 mm Nucleosil C_{18} column (Macherey Nagel, Oensingen, Switzerland) with a particle size of 5 μm. A guard column (11×4 mm) packed with the same material was used to protect the analytical column. The mobile phase consisted of a mixture of acetonitrile (33%) and 0.16 *M* ammonium formate buffer

(67%), pH 2.7 as described earlier [15]. Hexanesulphonic acid was added at a concentration of 0.25 *M*. The acidic conditions of the mobile phase prevent the degradation of MTO. To minimize the background noise, the solvent mixture was filtered through 0.22-μm modified polyvinylidene difluoride filters (Millipore, Bedford, MA, USA). The flow-rate of the mobile phase was set to 1.0 ml/min.

2.3. Sample preparation

ICR mice (Labor für Zuchthygiene, University Zürich, Zürich, Switzerland) were killed by heart puncture under diethyl ether anesthesia and blood, liver, spleen, heart and kidneys were removed and immediately frozen. To prevent oxidative degradation of mitoxantrone, 20 μl of a solution containing ascorbic acid (100 mg/ml in 0.1 *M* citrate buffer pH 3.0) was added to each tube before collecting the blood samples. Homogenization of tissues (liver, spleen, heart, kidneys) was performed with a potter on ice in a solution of 20% ascorbic acid in 0.1 *M* citrate buffer, pH 3.0 (1 ml of buffer was added to 50 mg of tissue).

One milliliter of a solution containing hexanesulphonic acid (0.01 mg/ml), ascorbic acid (0.5 mg/ml) and AMT (0.008 mg/ml) as internal standard was added to 1 ml of tissue homogenate or whole blood. After vortexing for 30 s, 1 ml of 0.1 *M* borate buffer (pH 9.5) and 300 μl of a 1 *M* sodium hydroxide solution were added and vortexed again for 30 s. Extraction was performed with 5 ml of dichloromethane on a horizontal linear shaker (Infors HT, Infors, Bottmingen, Switzerland) for 60 min at 150 rpm. After centrifugation for 15 min at 2800 *g*, the organic layer was separated and dried by evaporation (Rotavapor, Büchi, Flawil, Switzerland). The residue was dissolved in 150 μl of mobile phase.

Due to adsorption of MTO to glass and plastic material [16], all glassware was silanized and siliconized pipette tips were used.

2.4. Linearity

Five MTO standards in the concentration range of 5–200 μg/l in whole blood or 5–700 μg/l in homogenates were prepared by adding the appropriate amount of an MTO solution in 0.1 *M* citrate buffer (pH 3.0) containing 10% ascorbic acid either to whole blood or to the different tissue homogenates of untreated mice. These standard samples were extracted as described above and the standard curves plotted as the peak-area ratio of MTO to the internal standard. To assess linearity, the line of best fit was determined by least square regression.

2.5. Precision and accuracy

To determine the within-day precision of the method, two samples of blood and of each tissue were analysed three times (heart, kidneys) or four times (liver, spleen, whole blood) on the same day. To determine the between-day precision and the accuracy, an appropriate amount of an MTO solution in 0.1 *M* citrate buffer (pH 3.0) containing 10% ascorbic acid was added to the whole blood, liver, heart, spleen and kidney homogenates of untreated mice. At three or four different days a calibration curve for whole blood and the different tissue homogenates was run and the single determinations of sample concentrations were made once.

To obtain the within-day and between-day coefficients of variation, mean and standard deviations were calculated for each series of analyses.

The accuracy of the method was assessed by expressing the mean of the assayed concentration for the precision samples as percent of the nominal concentration.

2.6. Recovery

The recovery was determined in whole blood and liver tissue. Liver tissue was chosen as a representative tissue because of its biological composition which represents an average of the other organs studied [17]. The same amount of MTO was added either to 1 ml of whole blood or liver homogenate ('sample') or to 150 μl of mobile phase ('standard'), respectively. The concentrations of MTO in whole blood or homogenate were 200 μg/l or 75 μg/l. The samples were extracted as described. These analyses were performed in triplicate and the mean peak area of each compound in the samples was compared to the corresponding peak area of the standard.

2.7. Application

In order to study the distribution of MTO after the administration of two different pharmaceutical formulations, 56 μg MTO as aqueous solution or 67.5 μg MTO in a liposomal preparation [6] were injected intravenously into the tail vein of female ICR mice. Three animals per time point were sacrificed after 5, 30, 60 min and 2, 6 and 24 h, respectively. Blood was collected in heparin-coated tubes which contained 20 μl of a 10% solution of ascorbic acid. Liver, spleen, heart and kidneys were removed from each mouse and all samples were frozen at −20°C until analysis.

3. Results and discussion

The absorbance of MTO at 658 nm could be used for the detection and quantitation of the drug. Because there are no endogenous substances and only a few drugs which show a significant absorption at 658 nm, the chromatograms were free of interferences of other compounds after the extraction of tissue homogenates and whole blood. The peaks of MTO and the internal standard were symmetrical and baseline separation was obtained in all matrices (Fig. 2). Day-to-day differences in the retention times of MTO and AMT could be attributed to column-to-column variability and changes in the ambient temperature which influenced the actual column pressure. The calibration curves for MTO were linear in the range of 5–200 μg/l whole blood or 5–700 μg/l tissue homogenate (Table 1). The sensitivity of the procedure was determined to be 2 μg MTO/l in whole blood as well as tissue homogenates. The result of the precision and accuracy experiments are summarized in Table 2. The validation data of the extraction and HPLC procedure in the different tissues demonstrate that the method is accurate and precise with coefficients of variation within-day and between-day below 4.5% for whole blood and below 10% for tissue homogenates, respectively. Comparing these results with the data obtained for plasma samples [15] it is evident that they are equivalent despite the fact that the sample material used here is more complex.

The relative recoveries were 60% for whole blood at a level of 200 and 75 μg/l and 38% for liver homogenate at the corresponding concentrations,

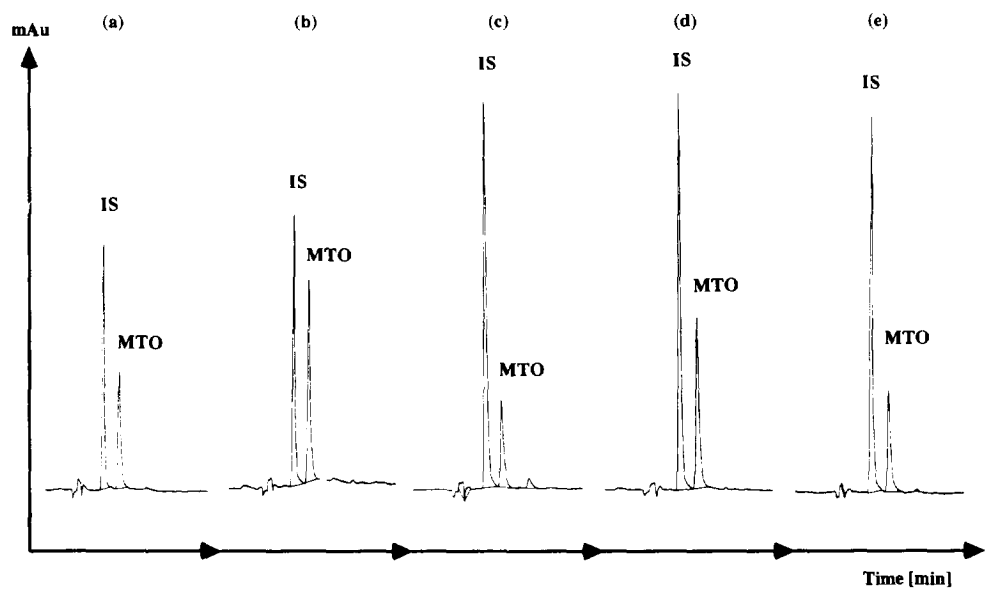


Fig. 2. Chromatograms of standards in the different matrices. IS=internal standard (AMT, 800 ng). The retention time is 5.4 to 6.7 min for MTO and 6.9 to 8.3 min for the IS. (a) Whole blood, 200 ng MTO; (b) liver, 596 ng MTO; (c) heart, 98.1 ng MTO; (d) spleen, 190 ng MTO; (e) kidney, 157 ng MTO.

Table 1
Calibration curves for MTO in whole blood, liver, heart, spleen and kidney

Sample matrix	Concentration (μg/l)	Slope	Intercept (μg/l)	Correlation coefficient (r)
Whole blood	4.74–187	0.0034	0.0061	0.9952
Liver homogenate	5.95–606	0.0032	−0.0303	0.9998
Heart homogenate	8.67–333	0.0025	−0.0042	0.9997
Spleen homogenate	11.8–188	0.0030	−0.0240	0.9936
Kidney homogenate	20.9–667	0.0020	0.0508	0.9996

respectively. The pK_a values of MTO are 5.99 and 8.13. The pH of the extraction solution of the tissue homogenates was 9.4 after the addition of 1 ml of 0.1 *M* borate buffer and 300 μl of 1 *M* sodium hydroxide. This corresponds to the pH of the serum extraction where a recovery rate of 91% was obtained (unpublished results). Therefore a change in pH cannot be responsible for the rather low recovery rate in tissue homogenates. These low recovery rates might be caused by the distribution of MTO in the tissues and its strong binding to proteins. In blood, 51% of MTO can be found in plasma, 23% in erythrocytes and 23% in other cells. In plasma, 90% of MTO is bound to proteins. In the different tissues, the amount of proteins given as percent of sample weight is much higher as compared to plasma. With the extraction procedure used, MTO is released quantitatively from serum proteins but it is very unlikely that a similar high release of MTO from tissue proteins can be achieved. Due to the limited

Table 2
Precision and accuracy data for MTO in whole blood, liver, heart, spleen and kidney

Sample matrix	Concentration (μg/l)	*n*	Mean (μg/l)	S.D. (μg/l)	C.V. (%)	Accuracy (%)
Whole blood	*Within-day*					
	165	4	171	6.02	3.5	104
	50.0	4	46.9	1.27	2.7	93.8
	Between-day					
	141	4	144	5.90	4.1	102
	47.4	4	42.9	1.88	4.4	90.5
Liver homogenate	*Within-day*					
	946	4	917	58.3	6.4	96.9
	Between-day					
	946	4	906	67.6	7.5	95.8
Heart homogenate	*Within-day*					
	98.1	3	93.1	5.22	5.6	94.9
	Between-day					
	90.1	3	85.6	4.77	5.6	95.0
Spleen homogenate	*Within-day*					
	72.3	4	72.4	5.67	7.8	100
	Between-day					
	69.3	4	69.4	5.43	7.8	100
Kidney homogenate	*Within-day*					
	259	3	261	24.4	9.3	101
	Between-day					
	214	3	222	19.9	9.0	104

stability of MTO, a stronger deproteinisation procedure could not be applied. MTO degrades by oxidation of the phenylenediamine moiety to the corresponding quinonimine which prevents the use of oxidative agents (e.g. perchloric acid). Furthermore, MTO is only stable in acidic conditions and rather unstable in alkaline solutions [18]; therefore, the use of zinc sulfate for deproteinisation was not possible.

A loss of MTO due to adsorption on glass surfaces is unlikely. According to other authors [16,19], all glass equipment used was either siliconized (Vacutainer tubes) or silanized (extraction vials). Storage containers and pipette tips used were made of polypropylene because it was shown that MTO does not bind to this particular plastic material [16].

Different HPLC methods have been published for the determination of MTO in serum and plasma. Methylene blue was used as internal standard in some reports [16,20], however, it is rapidly decolourized by ascorbic acid which had to be added to all solutions for the stabilisation of MTO. On the HPLC column, methylene blue reacts with the air dissolved in the mobile phase and then reoxidizes. Therefore, it seems inappropriate to use this substance as an internal standard for quantitative analysis because its absorption is strongly dependent on the pH of the surrounding solution. The same considerations are valid for cresyl violet used in the first report published for the determination of MTO [21]. Bisantrene, as internal standard requires electrochemical detection [22] and haloperidol absorbs only at a relatively nonspecific wavelength of 242 nm [23]. AMT has a similar structure compared to MTO [15,24–26] and can therefore be expected to behave similarly in the present extraction procedure. Three authors describe direct injection of serum combined with column switching techniques [9,10,19] but these methods lack sensitivity when using 1 ml of sample material.

Three groups have reported on the determination of MTO in tissues. Stewart et al. [12] used AMT as internal standard but a rather time-consuming double extraction procedure into chloroform–methanol (2:1) and chloroform–30% ammonium hydroxide (10:1) was employed. The authors report C.V. values of 5.5% within-day and 6.6% between-day, respectively and a recovery rate of 46%. These results correspond to the data obtained in our study despite the fact that only a single extraction step was used which made the analysis easier. Alberts and co-workers used ^{14}C-labelled MTO determining the tissue concentrations of MTO by measuring the radioactivity [13,14]. Finally, Roboz et al. used anthracenedione diacetate as internal standard and extraction of MTO into dichloromethane was performed at a pH of 11 [27]. However, no details on validation of the analytical method were given.

4. Application

The method described here was applied in a preclinical study in order to compare the tissue distribution of two pharmaceutical formulations of MTO. Mice were treated intravenously either with an aqueous solution of MTO or with a liposomal preparation. In Fig. 3, the amounts of MTO detected in the different tissues are shown. The administration of liposomal MTO resulted in a different tissue distribution as compared to aqueous MTO. With the liposomal formulation, lower amounts of MTO were found in the kidney, the spleen and the heart. In contrast, equivalent amounts of MTO were determined in liver and blood with both formulations. The accumulation of liposomal drugs in the liver is a well known phenomenon which may be useful for the treatment of liver diseases, however, it can also cause toxic effects [28]. Depending on the lipid composition used for the liposomal formulation, it has been described previously that MTO is quickly released from the liposomes, subsequently undergoing a similar distribution as aqueous MTO [29].

In conclusion, the method described here represents an accurate and reproducible procedure for the determination of MTO in whole blood and different tissue homogenates. It could be shown that this method is suitable for pharmacokinetic studies in an animal model to describe the distribution of MTO in different tissues.

Acknowledgments

We thank D. Horber for collaboration and R. Bührer for excellent technical assistance.

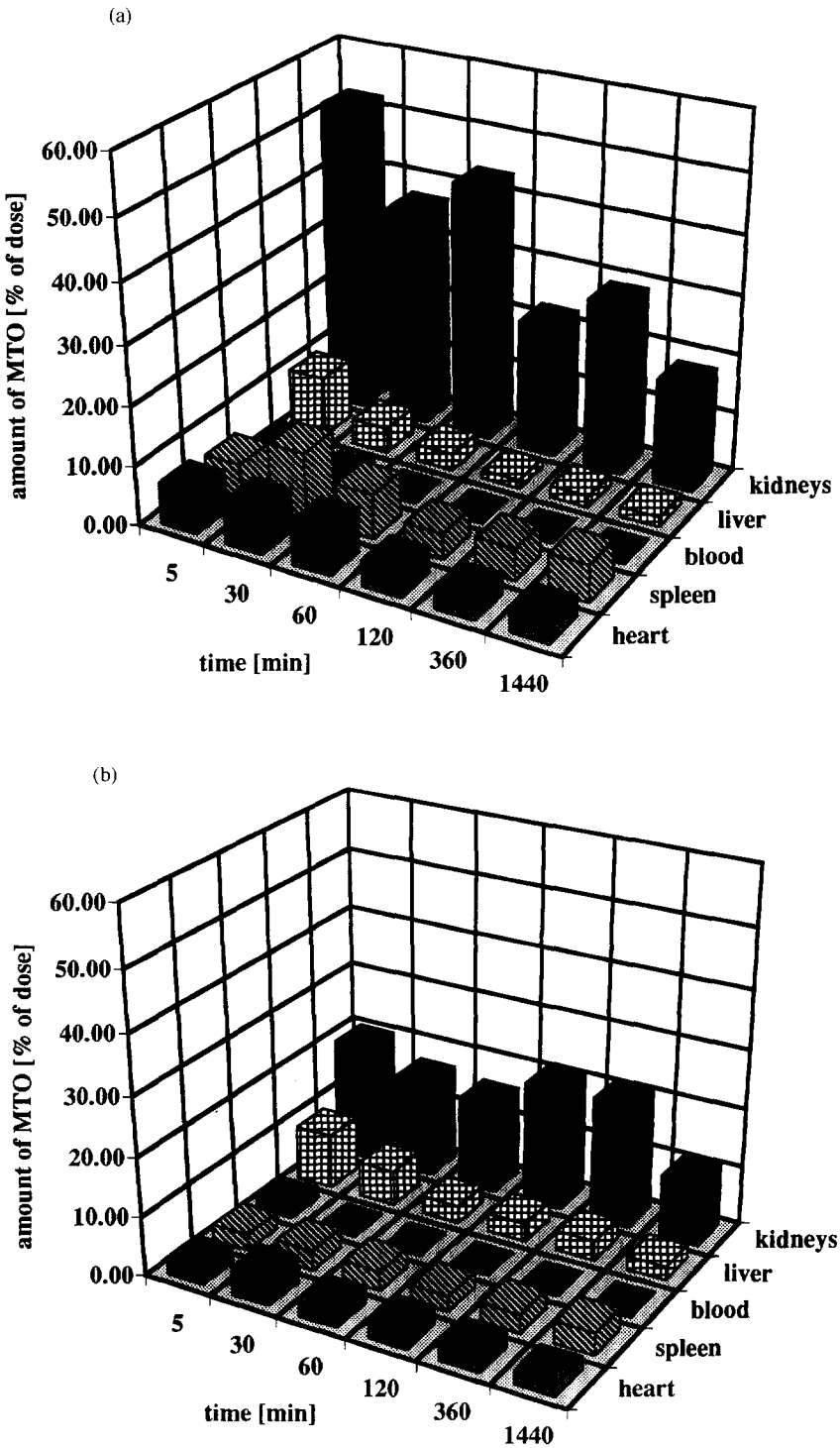


Fig. 3. Time-course of the amount of MTO (expressed as percent of dose) in the different tissues of ICR mice after intravenous application (average of 3 mice per time point) of (a) an aqueous solution and (b) a liposomal preparation.

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