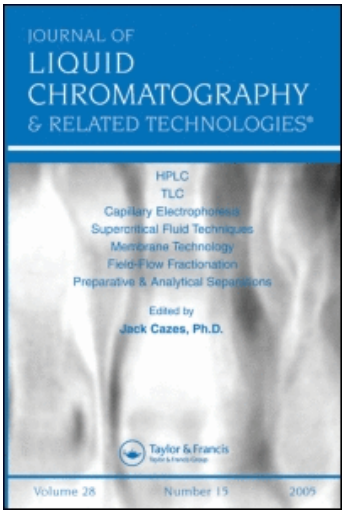

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE CARDIOPROTECTIVE AGENT DEXRAZOXANE IN HUMAN PLASMA AND URINE

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ABSTRACT

For the purpose of a pharmacokinetic study in the comparison of two intravenous pharmaceutical formulations of the cardioprotective agent dexrazoxane, we have developed an High Performance Liquid Chromatographic (HPLC) assay to quantify the drug in human plasma and urine. The plasma sample pretreatment involved a protein precipitation step with acetonitrile followed by an extraction with 10% 2-methyl-2-propanol in chloroform (v/v). Urine samples were diluted in distilled water and subsequently extracted with 10% 2-methyl-2-propanol in chloroform (v/v). After evaporation of the organic solvents, the residues were dissolved and analysed on a μBondapak Phenyl column with a mobile phase consisting of 0.01 M potassium phosphate pH 4.7 and methanol (8:2, v/v). Detection was performed at 208 nm. The lower limit of

quantitation was 0.1 μg/mL and 10 μg/mL for plasma and urine respectively, using 1.0 mL sample volumes. The usefulness of the method has been demonstrated in clinical samples originating from patients treated with dexrazoxane.

Dexrazoxane is not stable in plasma at ambient temperature: after 6.5 hours the initial concentration was 42.6 ± 1.0% (n=3) of the original concentration of 100 μg/mL. After sampling in the clinic, plasma samples should be stored immediately at -30°C. Under these conditions dexrazoxane is stable for at least 5 months. In urine the drug is stable for 24 hours when stored at 4-8°C. An aliquot of the voided urine sample can be stored at -30°C for at least 4 months without drug decomposition.

INTRODUCTION

A major impediment in the clinical use of anthracycline antitumour drugs *e.g.* doxorubicin, is a cumulative dose-related cardiac toxicity that manifests itself as congestive heart failure. Many strategies have been pursued in an attempt to prevent this serious complication. In particular, the development of a treatment with the protective agent dexrazoxane (ICRF-187, ADR-529, NCS-169780, (+)-1,2-bis(3,5 dioxopiperazinyl-1-yl)propane) seems an attractive approach. Dexrazoxane is the S(+) enantiomer of the racemic compound razoxane (ICRF-159). The (+) enantiomer was developed further because it appeared about five times more water soluble than the racemic mixture.[1]

It is hypothesized that dexrazoxane hydrolyses intracellularly into a metabolite (Figure 1) which complexes iron ions by which the generation of hydroxyl radicals through the Fenton reaction is prevented.[2] Generation of hydroxyl radicals by anthracyclines in the heart muscle may cause the cardiomyopathy.

The cardioprotective effects of dexrazoxane have been demonstrated in animals[3-11] and in patients.[12-19] The timing of drug administration appears to be very important whereby simultaneous administration of doxorubicin and dexrazoxane resulted in a better protection than when dexrazoxane is administered 2 hours after doxorubicin.[20] More pharmacokinetic data of this combination therapy are necessary.

In our hospital patients with breast cancer were treated according to the FDC-regimen: 500 mg/m^2 fluorouracil, 50 mg/m^2 doxorubicin and 500 mg/m^2 cyclophosphamide given as bolus injections. The patients also received in a

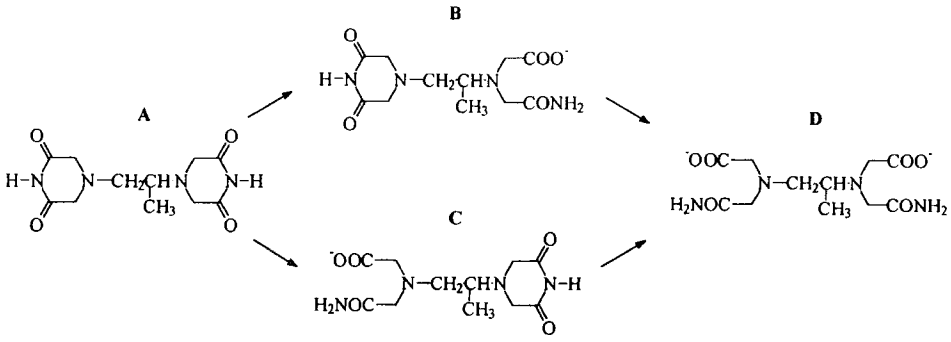


Figure 1. Chemical structure and intracellular activation of dexrazoxane (A). B, C and D are hydrolysis products.

randomized fashion two formulations of dexrazoxane (1,000 mg/m^2) as a 12 minutes intravenous infusion in normal saline in which the drug is stable for 6 hours when stored at ambient temperature.[21] To investigate the pharmacokinetic bioequivalence of the tested formulations we developed an HPLC assay for the determination of dexrazoxane in human plasma and urine.

Several analytical methods for the analysis of dexrazoxane in biological matrices have been published.[22-28] Sadee and colleagues described a method based on gas chromatography with flame ionization detection or mass fragmentography.[22,23] The mass spectrometrical equipment was not available in our laboratory at that moment, therefore, this technique could not be utilized. Flame ionization detection provides a detection limit of 5 μg/mL which was found not sufficient for our studies. Other methods all made use of HPLC, though with different sample pretreatment procedures/detection methods, including: ultrafiltration/UV-detection,[24,25] liquid-liquid extraction/UV-detection,[26] solid phase extraction/amperometric detection[27] and pre-column derivatization/fluorescence detection.[28] In our hands, the Collins method[24] was not selective: chromatograms of blank plasma samples showed a major endogenous interference after the ultrafiltration. Lewis et al.[27] developed a very sensitive (limit of quantitation: 5 ng/mL plasma) and selective method for the quantitation of dexrazoxane in biological fluids using two solid phase extraction procedures, followed by straight phase HPLC with column switching and amperometric detection. This method is, however, very laborious and a lower limit of quantitation of 0.1 μg/mL was considered sufficient for our purposes. The HPLC method with pre-column derivatization to form a

fluorescence product[28] was not supported by validation data. The method published by Earhart et al.[26] was used by us as a reference but, the assay appeared to be afflicted with several problems: interferences by endogenous components, degradation of the analyte in the eluent and the use of an internal standard which was not commercially available. In the presented assay the 'Earhart'-method has been adapted substantially in order to overcome these problems.

EXPERIMENTAL

Equipment

The chromatographic system consisted of a Model 510 solvent delivery system (Waters Assoc. Inc., Milford, MA), a Spectra 200 programmable wavelength detector, an automatic sample injection device model SP8875, and a SP4600 integrator coupled to the WINner® data system (all from Thermo Separations Products, Fremont, CA). Chromatographic separation was achieved on a μBondapak Phenyl column (internal diameter: 4.6 mm; length: 300 mm; particle size: 10μm,) (Waters Assoc. Inc., Milford, MA) protected by a guard column (3 x 10 mm) packed with reversed phase material (Chrompack, Middelburg, The Netherlands).

Chemicals

Dexrazoxane, (lot. 3354) originated from Chiron (Amsterdam, The Netherlands). Chloroform, acetonitrile and methanol were obtained from Promochem (Wesel, Germany). 2-Methyl-2-propanol, hexane, diethyl ether, ethyl acetate, potassium dihydrogen phosphate and hydrochloric acid were of analytical grade and purchased from Merck (Darmstadt, Germany). Home-made double-distilled water was used throughout. Drug-free edathamil (EDTA) human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Drug-free urine was provided from healthy volunteers.

Stock solutions

A stock solution of dexrazoxane (1 mg/mL) was prepared by dissolving the appropriate amount of drug, accurately weighed, in distilled water. Typical

amounts of the stock solution were diluted with distilled water to give standard solutions with concentrations of 500 μg/mL, 100 μg/mL and 10 μg/mL. The solutions were stored at -30°C and were found to be stable for at least 2 months under these conditions.

Calibration Samples

Plasma

Blank human plasma samples (1,000 μL), in 10 mL brown glass containers, were spiked with 10 to 100 μL of a dexrazoxane standard solution with appropriate concentration to obtain calibration samples in the range of 0.1 - 1 μg/mL, 1 - 10 μg/mL and 10 - 100 μg/mL. Distilled water was then added to the plasma samples to achieve a total volume of 1.1 mL. To precipitate the plasma proteins 2.0 mL of acetonitrile was added. The containers were shaken for 10 minutes and centrifugated at 2,500 g for 10 minutes. The clear supernatant was transferred to a clean glass container of 30 mL. Next, 20.0 mL of extraction fluid (chloroform - 2-methyl-2-propanol; 9:1, v/v) was added and the flasks were shaken for 30 minutes and subsequently centrifugated for 10 minutes at 2,500 g. The water phase was discarded and 15.0 mL of the organic extract was transferred into clean glass container and evaporated to dryness under a stream of nitrogen at 40°C. The residues were redissolved in 500 μL 0.1 M hydrochloric acid - methanol (8:2, v/v) and subjected to HPLC analysis.

Urine

Blank human urine samples (1,000 μL) were transferred to 100.0 mL volumetric flasks and appropriate volumes (100 - 1,000 μL) of dexrazoxane standard solutions were added to obtain calibration samples in the range of 10 - 100 μg/mL and 100 - 1,000 μg/mL. Distilled water was then added to achieve a total volume of 100.0 mL. From each diluted urine solution 1.00 mL was pipetted into a clean glass container of 30 mL. Next, 20.0 mL of extraction fluid (chloroform - 2-methyl-2-propanol; 9:1, v/v) was added and the sample pretreatment was followed as described for the plasma samples.

Clinical samples

Blood samples (5 mL) of patients were collected in EDTA-tubes. Plasma was immediately isolated by centrifugation (10 minutes at 2,500 g) and subsequently stored at -30°C prior to analysis.

Before treatment, an aliquot of the patient's urine sample was stored at -30°C and was used as a blank. During treatment, urine voided over 24-hours was pooled and stored in a refrigerator at 4-8°C.

The total volume was recorded and approximately 20 mL of the homogenized sample was taken and stored at -30°C prior to analysis.

Chromatography

Chromatography was performed at ambient temperature with the mobile phase composed of 0.01 M potassium phosphate pH 4.7 and methanol (8:2, v/v). Prior to use, the mobile phase was filtered under reduced pressure through a 0.2 μm cellulose acetate filter (Sartorius, Spruyt-Hillen, Utrecht, The Netherlands).

The absorbance of the effluent was monitored at 208 nm. The flow rate was maintained at 1.0 mL/min and a volume of 50 μL was injected into the chromatograph.

Validation Parameters

A validation programme was completed for the determination of dexrazoxane in plasma and urine. The following parameters were determined: linearity, precision, accuracy, specificity, selectivity, absolute recovery and stability.

Linearity

For each calibration range five standards were prepared and analysed in duplicate, producing calibration curves in plasma ranging from 0.1 - 1 μg/mL, 1 - 10 μg/mL and 10 - 100 μg/mL and in urine ranging from 10 - 100 μg/mL and 100 - 1,000 μg/mL. Unweighted linear regression analysis of the peak area of dexrazoxane versus the nominal concentration was applied.

A split-curve approach was used to improve accuracy at the low concentration levels. The lack of fit test (α=0.05) was used to evaluate the linearity of the calibration curves.

Accuracy and precision

Quality control samples were prepared from a second, fresh stock solution with analyte concentrations of 0.5, 5 and 50 μg/mL in plasma and 50 and 500 μg/mL in urine. Six replicates of each quality control sample were processed and analysed with calibration standards to determine the accuracy and precision of the method.

Specificity and selectivity

Six batches control human plasma and urine were tested whether endogenous constituents co-eluted with dexrazoxane.

The medication used by patients treated according to the FDC-regimen were investigated for interference with the analytical method: fluorouracil, doxorubicin, cyclophosphamide, codeine, paracetamol and indometacin.

Absolute recovery

The extraction efficiencies were determined at 5 and 50 μg/mL in plasma and at 50 and 500 μg/mL in urine by comparing the response of three processed quality control samples to the mean response of two unprocessed samples of dexrazoxane in 0.1 M hydrochloric acid in methanol (8:2, v/v).

Stability

The stability of dexrazoxane at ambient temperature, has been studied at a concentration of 5 μg/mL in the following solutions: 0.01 M phosphate buffer pH 6.1 - methanol (8:2, v/v), 0.01 M potassium phosphate pH=4.7 - methanol (8:2, v/v) and 0.1 M hydrochloric acid - methanol (8:2, v/v).

The stability of dexrazoxane in human plasma at a concentration of 100 μg/mL has been studied during a period of 6.5 hours at ambient temperature. Triplicates were analysed at every time point.

Five plasma samples from patients treated with dexrazoxane containing about 70 μg/mL were re-analysed in duplicate after 5 months storage at -30°C. The stability of dexrazoxane has been investigated at a concentration of 500 μg/mL in human urine during a period of 29 hours at 4°C. Three replicates were analysed at every time point.

Four urine samples from patients treated with dexrazoxane containing about 900 μg/mL were re-analysed in duplicate after 4 months storage at -30°C. The stability of the drug in the organic extract originating from plasma spiked with dexrazoxane at a concentration of 100 μg/mL was evaluated after 24 hours at -30°C while the stability of the dry extract was investigated after storage at -30°C for one week.

The stability of the drug extracted from plasma and reconstituted in 0.1 M hydrochloric acid - methanol (8:2, v/v) was evaluated during storage at ambient temperature for 16 hours in the autosampler. Triplicates were analysed at every time point.

Statistical Quality Control

Quality control samples were prepared from a second, fresh stock solution at concentrations of 0.5, 5 and 50 μg/mL in plasma (see 'Accuracy and precision') and were stored together with the clinical samples. Three replicates of each quality control sample were processed and analysed with each calibration curve for statistical process control during the study. Exponentially weighted moving-average (EWMA) control charts were constructed using Statgraphics® version 7.0 (Manugistics Inc., MA).

Pharmacokinetics

To demonstrate the applicability of the assay for pharmacokinetic studies an example of a treated patient is presented. Serial blood samples and urine were taken from a patient suffering from breast cancer treated with dexrazoxane (1,000 mg/m^2) given as a 12 minute intravenous infusion prior to the FDC-regimen: 500 mg/m^2 fluorouracil, 50 mg/m^2 doxorubicin and 500 mg/m^2 cyclophosphamide given as bolus injections. The plasma concentration time curve was analysed using MW\PHARM[29] (MEDI\WARE, Groningen, Netherlands).

RESULTS AND DISCUSSION

The extraction properties of dexrazoxane with different organic solvents were investigated because the bio-analysis of the drug is hampered by poor extractability. Chloroform, 2-methyl-2-propanol in chloroform (10%, v/v), hexane, diethyl ether and ethyl acetate were tested and blank plasma samples were injected to examine the sample clean-up. All extractions were performed

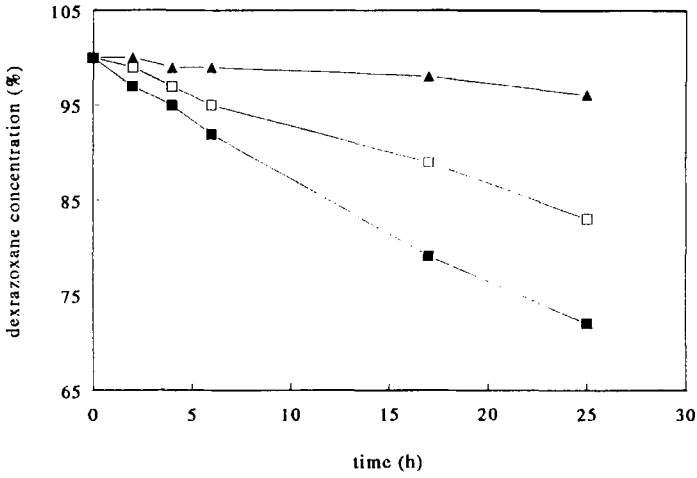


Figure 2. The stability of dexrazoxane during storage at ambient temperature at a concentration of 5 μg/mL in 0.01 M phosphate buffer pH 6.1 - methanol (8:2, v/v, -■-), 0.01 M potassium phosphate pH 4.7 - methanol (8:2, v/v, -□-) and 0.1 M hydrochloric acid - methanol (8:2, v/v, -▲-).

in glass containers. Polypropylene could not be used in consequence of additional peaks in the chromatograms originating from the container material used. 2-Methyl-2-propanol in chloroform (10%, v/v) gave the best results (recovery >60%), however the sample pretreatment method in combination with the chromatographic system was not very selective: there was still an interfering endogenous component present. Using a μBondapak Phenyl column, dexrazoxane had a significantly longer retention time compared with the μBondapak C-18 column and the drug was well separated from all endogenous compounds.

Earhart et al.[26] used the analog ICRF-192 as the internal standard of the assay and residues were reconstituted in the eluent: 20% methanol (v/v) in 0.01 M sodium-potassium phosphate buffer pH 6.1. In this solvent dexrazoxane is not stable (Figure 2) but the peak ratio with ICRF-192 was stable over at least 24-hours.[26] Unfortunately this compound was not available to us and we decided to reconstitute the residues in 0.1 M hydrochloric acid - methanol (8:2, v/v) in which dexrazoxane is stable for at least 24 hours at ambient temperature (Figure 2). This allows the use of an autosampler injection device with samples stored for 24 hours. In addition we have tested a range of compounds as potential internal standards for the bio-analytical assay (Table 1). These

Table 1

Compounds Tested as Potential Internal Standards for the Bio-analytical Assay and the Medication used by Patients Treated according to the FDC-Regimen

Compound	Concentration (μg/mL)	Retention Time (min)
Dexrazoxane	50	6.2
5-Bromouracil	10	4.5
5-Chlorouracil	10	4.5
8-Chlorotheophylline	5	16.0
Caffeine	5	19.8
5-Fluorocytosine	6	3.4
Methylparaben	5	N.D.
Phenazone	5	N.D.
Phenacetine	5	N.D.
Phenylethyleneglycol	10	7.3
Prednisone	25	N.D.
Theophylline	5	10.7
Tiamcinolonacetate	0.5	N.D.
Triamcinolone	0.2	N.D.
Codeine	10	N.D.
Cyclophosphamide	200	N.D.
Doxorubicin	200	N.D.
5-Fluorouracil	500	4.0
Indomethacin	10	N.D.
Paracetamol	5	front

N.D.: not detected; 50μL injections.

compounds were eluted with a mobile phase composed of methanol/0.01 M phosphate buffer pH 4.7 to minimize on-column degradation of dexrazoxane. 8-Chlorotheophylline was added as an internal standard during initial studies at a concentration of 25 μg/mL in plasma and 250 μg/mL in urine.

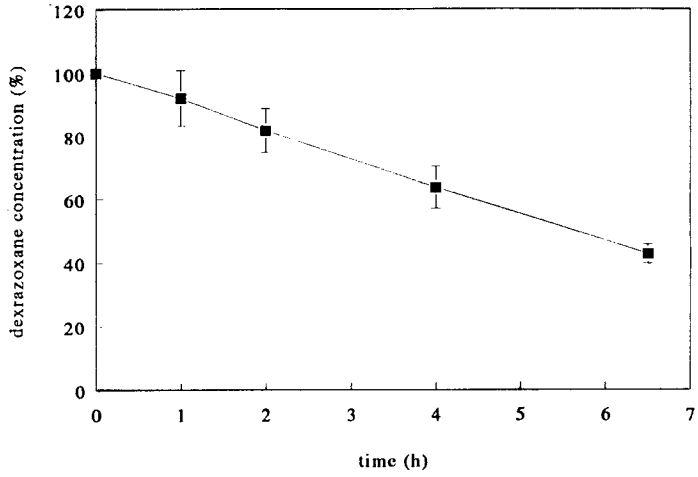


Figure 3. The stability of dexrazoxane in human plasma at a concentration of 100 μg/mL, measured during a period of 6.5 hours at ambient temperature. Percentages of the initial dexrazoxane concentrations are plotted (± the standard deviation).

Table 2

Validation Parameters for the Determination of Dexrazoxane in Human Plasma and Urine

Matrix	Nominal Concentration (μg/mL)	Concentration Found (μg/mL)	Accuracy (%)	Precision (%)	Number of of Replicates
Plasma	0.500	0.498	99.6	4.1	6
Plasma	5.00	5.00	100.0	3.4	6
Plasma	50.0	50.3	100.6	2.9	6
Urine	50.0	47.3	96.4	5.4	6
Urine	500	493	98.6	8.2	6

Comparable accuracies and precisions were obtained when the internal standard was rejected. The recovery of 8-chlorotheophylline from plasma was very poor (< 20%) unlike the recovery from urine (79% ± 3.4%, n=6). Caffeine,

Table 3

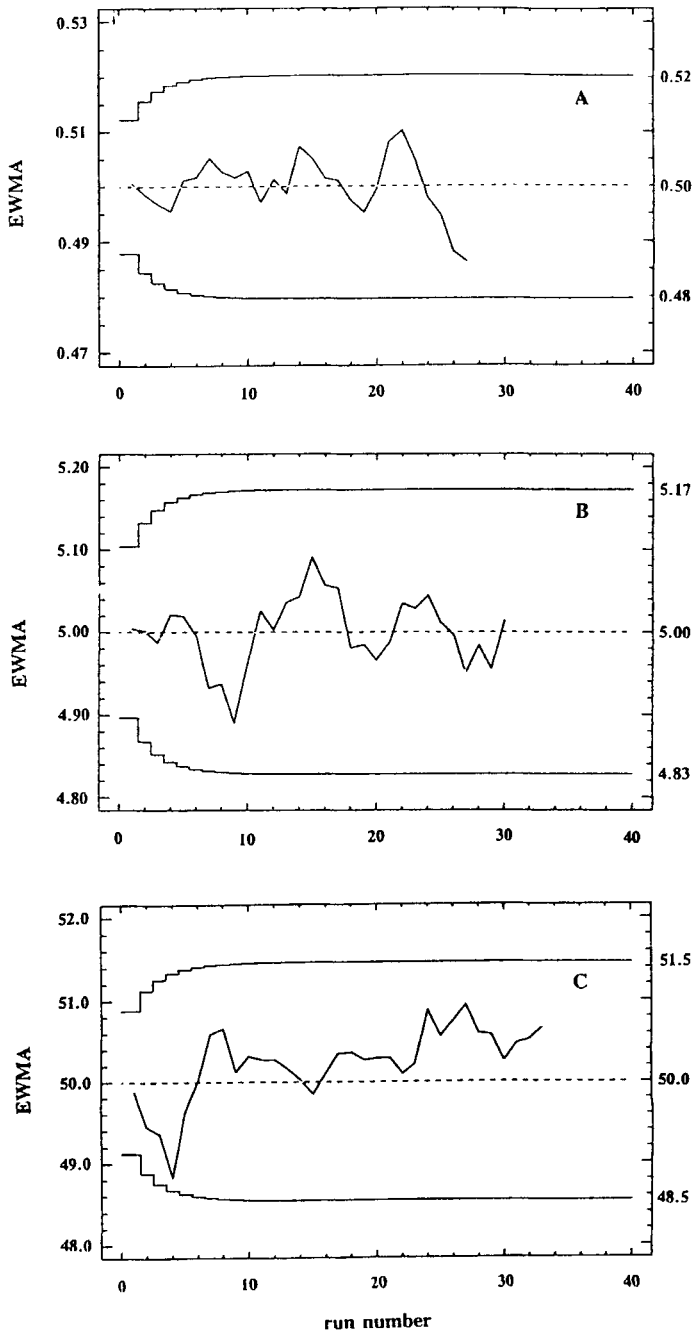
Stability Data of Dexrazoxane at Several Storage Conditions

Matrix	Storage Conditions	Initial Conc. (μg/mL)	Recovery (%)	C.V. (%)	N
Spiked human plasma	6.5 hours at 21°C	100	42.6	1.0	3
Plasma originating from patients	5 months at -30°C	70	99.6	3.1	5
Spiked urine (pH = 6.92)	29 hours at 4-8°C	500	91.7	2.5	3
Urine originating from patients	4 months at -30°C	900	88.8	6.1	4
Organic layer after extraction from spiked plasma	24 hours at -30°C	100	100.2	6.2	4
Dry residue after extraction from spiked plasma	1 week at -30°C	100	100.5	2.1	4
Plasma residue after reconst. in 20% (v/v) methanol in 0.1M HCL	16 hours at 21°C	10	93.0	2.0	3

C.V. = Coefficient of Variation.
N = Number of determinations.

phenylethyleneglycol and theophylline were not tested as potential internal standards as their chromatographic characteristics were not ideal in terms of symmetry. It was then decided to execute the validation programme without internal standard.

Figure 4 (right). EWMA charts of the quality control samples in human plasma at nominal concentrations of 0.5 (A), 5 (B) and 50 μg/mL (C) measured during the pharmacokinetic study.



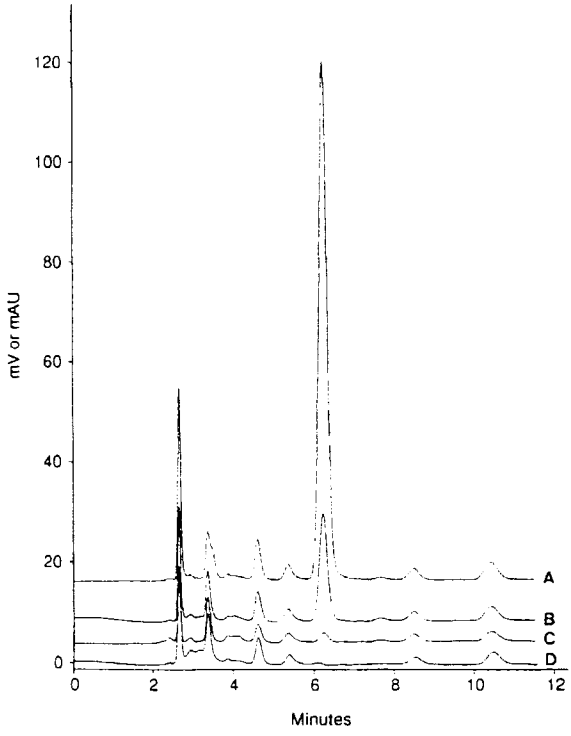


Figure 5. HPLC chromatograms of a patient treated with dexrazoxane (1,000 mg/m^2) as a 12 minutes intravenous infusion prior to adminitration of the FDC-regimen: samples were taken at 2 (A, 13.75 μg/mL), 6 (B, 2.75 μg/mL) and 12 hours (C, 0.22 μg/mL) after the end of the infusion and a blank plasma sample before treatment (D). Dexrazoxane elutes after 6.2 minutes.

The calibration curves were linear over a concentration range of 0.1 μg/mL to 100 μg/mL in human plasma and over a range of 10 to 1,000 μg/mL in urine. Assay validation parameters are summarized in Table 2.

The precision was less than 10% for all quality control samples. The average accuracies were within 95 and 105%. The chromatograms of six batches of control human plasma and urine contained no endogenous constituents co-eluting with dexrazoxane.

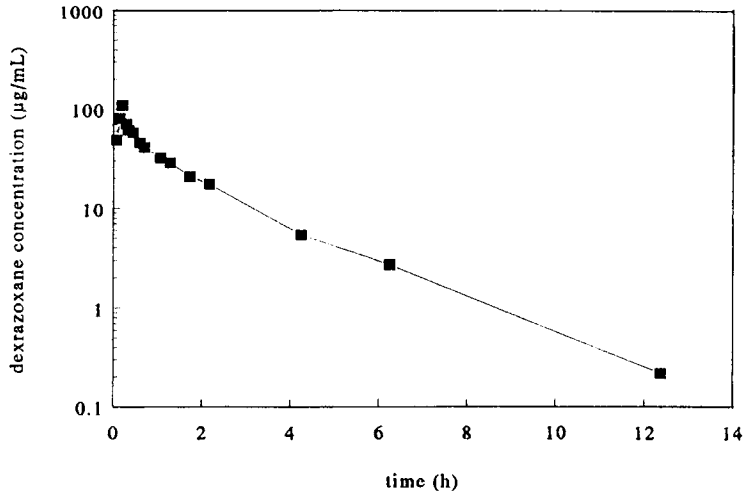


Figure 6. The plasma concentration versus time curve of a patient treated with dexrazoxane (1,000 mg/m^2) as a 12 minutes intravenous infusion prior to the FDC-regimen.

The medication used by patients treated according to the FDC-regimen (Table 1) showed no interference with the analytical method.

The extraction efficiencies in plasma were 76% ± 1.0% at 5 µg/mL and 71% ± 2.3% at 50 µg/mL (n=3). In urine comparable extraction efficiencies were found: 66% ± 1.0% at 50 µg/mL and 70% ± 0.6% at 500 µg/mL (n=3).

Stability data of dexrazoxane at several storage conditions are presented in Table 3. Dexrazoxane is not stable in plasma at ambient temperature (Figure 3): after 6.5 hours the dexrazoxane concentration was only about 40% of the original value. Consequently for the determination of dexrazoxane, plasma should be isolated immediately after sampling in the clinic and can then be stored at -30°C for at least 5 months.

A basic method for statistical process control is the Shewhart control chart.[30] A major disadvantage of a these charts, however, is that it only uses the information about the process obtained during the last analytical run, and it ignores any information gathered before. This feature makes the Shewart control chart relatively insensitive to small shifts in the process. A very effective alternative is the exponentially weighted moving-average (EWMA) control chart.[30]

Table 4

Pharmacokinetic Parameters of Dexrazoxane in a Patient Suffering from Breast Cancer Treated with Dexrazoxane (1,000 mg/m^2; Total Dose 2000 mg) as a 12 Minutes Intravenous Infusion using a Two Compartment Model

AUC (Area Under the Curve, $0 \rightarrow \infty$)	118 mg.h/L
Cl_{tot} (Total Body Clearance)	16.9 L/h
Distribution volume	38.7 L
$t_{½}$ (α)	0.16 h
$t_{½}$ (β)	1.59 h
MRT (Mean Residence Time)	1.93 h
Cl_{ren} (Renal Clearance)	6.8 L/h
Urinary excretion	40%

These charts were constructed for the quality control samples in human plasma at nominal concentrations of 0.5, 5 and 50 μg/mL with a weighting factor (λ) of 0.2 and estimations of σ determined at the validation phase (Figure 4). No 'out-of-control points' were detected indicating that the bio-analytical method was in control during the analysis of dexrazoxane for the pharmacokinetic study.

The applicability of the assay has been demonstrated in a patient with breast cancer treated with dexrazoxane (1,000 mg/m^2) as a 12 minutes intravenous infusion prior to the FDC-regimen. Typical chromatograms of extracted patient samples are presented in Figure 5 and the plasma concentration-time curve is depicted in Figure 6. An open two compartment model was used to calculate the pharmacokinetic parameters (Table 4).

In summary, a simple, validated HPLC assay for the quantitative determination of dexrazoxane in human plasma and urine is described. The assay quantifies dexrazoxane concentrations in plasma in a range of 0.1 to 100 μg/mL and in urine of 10 to 1,000 μg/mL using a 1,000 μL sample volumes.

The sensitivity of the assay is sufficient to monitor patients during 24 hours after the end of the infusion when treated with a dexrazoxane dose of 1,000 mg/m^2. The method has been succesfully used in our hospital for pharmacokinetic studies (>1000 samples).

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