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DETERMINATION OF CYCLOPHOSPHAMIDE IN WHOLE BLOOD AND PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, simple, and sensitive reversed-phase high-performance liquid chromatographic determination of the cytostatic drug cyclophosphamide in whole blood and plasma has been developed. The pre-chromatography isolation of the drug involves salting-out of acetonitrile with simultaneous extraction of cyclophosphamide from whole blood and plasma. A short column packed with 5-μm reversed-phase octadecylsilane (ODS) spherical particles was used with an isocratic elution of 5 m*M* potassium phosphate (pH 6.80)-acetonitrile (80:20, v/v). The cyclophosphamide was monitored at 190 nm and 0.40–0.002 a.u.f.s. At a flow-rate of 1.0 ml/min, the retention time of cyclophosphamide was ca. 9 min. The completion time for the assay was less than 20 min and the assay had a detection limit of 0.30 μg/ml. This method was used to determine the stability of cyclophosphamide in plasma at room temperature and at $-10\,^{\circ}C$.

INTRODUCTION

Cyclophosphamide (Fig. 1) is a cytostatic drug widely used in oncology. It has been shown that cyclophosphamide is highly effective in the treatment of malignant lymphomas, multiple myeloma, leukemias, and carcinoma of the breast [1].

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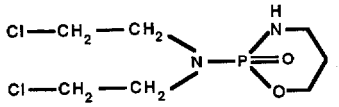

Fig. 1. Structure of cyclophosphamide.

After administration, cyclophosphamide is absorbed by the gastrointestinal tract and parental sites. The details of the metabolism of cyclophosphamide are not fully known or understood, but the metabolites were found to be distributed throughout the body including the brain [1]. A number of adverse reactions of cyclophosphamide have been reported [1]. Among these, the most serious one is the development of secondary malignancies in some patients. Cyclophosphamide is effective alone in susceptible malignancies. However, it is more effective when applied with other antineoplastic drugs [1]. Monitoring the concentration of cyclophosphamide in the blood and plasma of patients can direct dose adjustments to achieve optimum therapy with minimum adverse reactions.

After the introduction of cyclophosphamide by Arnold and Bourex [2], a number of analytical methods have been published to quantitate the drug in biological samples of patients [3–17].

All of the methods developed before 1960 utilize chemical techniques to quantitate the drug in parental and biological samples [3–6]. Gas chromatography (GC), GC–chemical ionization mass spectrometry (MS, and selective MS were used to study the pharmacokinetics of cyclophosphamide in human plasma [8–10]. The sample preparation in all of these techniques [3–10] varied from the derivatization of the parent drug to multiple-step extraction and evaporation procedures employing different organic solvents for pre-chromatography isolation of the drug. These methods are complex, or have poor selectivity and sensitivity, or are time-consuming, or all of these. Radiolabeled cyclophosphamide has also been utilized to study the pharmacokinetics of the drug [12–14]. Recently, El-Yazigi and Martin [15] published a capillary GC method to quantitate cyclophosphamide from the plasma of patients ingesting the drug. The sample preparation and pre-chromatographic isolation of cyclophosphamide is time-consuming. A capillary gas chromatograph is also not a very convenient and cost-effective instrument for routine analysis of the drug in a clinical laboratory. Two papers on reversed-phase liquid chromatography were published [16,17] to assay cyclophosphamide in raw material and in parental dosage forms. Because of poor sensitivity of these two methods, no attempt was made to determine the concentrations of cyclophosphamide in the samples of patients.

This paper describes a rapid and sensitive method of measuring cyclophosphamide from whole blood and plasma using reversed-phase high-performance liquid chromatography (HPLC) for routine analysis in a clinical laboratory.

EXPERIMENTAL

Equipment

A Waters M6000 solvent delivery pump was used for solvent delivery (Waters Assoc., Milford, MA, U.S.A.). The injector was a Rheodyne Model 7125 chro-

matographic injector, with a 200-μl injection loop (Cotati, CA, U.S.A.). A Kratos Spectroflow 773 variable-wavelength ultraviolet–visible detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) was used. A reversed-phase (100 mm $\times$ 4.6 mm) column slurry packed in our laboratory with 5-μm octadecylsilane (ODS) sorbent was used throughout the procedure. Data were recorded on a Houston Instrument Microscribe TM-4500 strip chart recorder (Bausch & Lomb, Austin, TX, U.S.A.).

Materials

An ultrasonic bath was used to degas the mobile phase (S & R Manufacturing Industry, Kearney, NJ, U.S.A.). A Sorvall GLC-1 centrifuge (Ivan Sorvall, Newton, CT, U.S.A.) was used. The vortex mixer was purchased from American Scientific Products (IL, U.S.A.). An Eppendorf digital pipette (100–1000 μl) was used for all quantitative sampling (Cole-Parmer, Chicago, IL, U.S.A.).

Cyclophosphamide was obtained from Mead Johnson, (Evansville, IN, U.S.A.) was used as received. The reversed-phase 5-μm ODS sorbent were purchased from Alltech Assoc., (Deerfield, IL, U.S.A.). All the solvents used in these experiments were Gold label and were purchased from Aldrich (Milwaukee, WI, U.S.A.). All of the salts used were obtained from Alpha Products, (Danvers, MA, U.S.A.). Deionized ultrapure water was used for all purposes during the experiment. A Sybron-Barnstead 60209 nanopore water purification system was used for collecting the deionized water. The pooled plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). The zinc sulfate and anhydrous potassium carbonate obtained from Alpha Products were further purified (first washed with methanol and then with spectrograde acetonitrile) before being used in the experiment. All other reagents were used without any further purification.

Chromatographic conditions

The mobile phase consisted of 20% acetonitrile in 10 m*M* monobasic potassium phosphate buffer. The pH of the mobile phase was adjusted to 6.80 by using a 0.50 *M* sodium hydroxide solution. The flow-rate was 1.0 ml/min. Cyclophosphamide was monitored at 190 nm by a UV–visible HPLC detector with sensitivity from 0.40 to 0.002 a.u.f.s.. Quantification was done by comparing the peak height of an unknown with a standard calibration curve.

Procedure and extraction of cyclophosphamide from whole blood and plasma

The frozen, aged blood and plasma ($-10°C$) were thawed at room temperature. Thawed blood or plasma (1 ml) was pipetted into a borosilicate disposable test tube and was spiked with an aliquot of a standard stock solution of cyclophosphamide. This was vortex-mixed for 20 s, and 1.0 ml of acetonitrile was added to the mixture and vortex-mixed for another 30 s. To this mixture, 50 mg of zinc sulfate were added and vortex-mixed for 1 min, followed by 2 min of centrifugation at 3500 *g*. The supernatant was decanted into a fresh culture test tube and was saturated with anhydrous potassium carbonate. This was again vortex-mixed for 30 s and centrifuged for 1 min at 3000 *g*. The salted-out acetonitrile layer was

transferred to another fresh borosilicate test tube with a disposable micro-pipette. A 30-μl aliquot of the acetonitrile solution was injected into the chromatographic system.

Calibration

A stock solution containing approximately 0.35 mg/ml cyclophosphamide in deionized water was freshly prepared. Eight whole blood, plasma, and water solutions were prepared by adding stock solution to give concentrations of about 2.0, 5.0, 10.0, 20.0, 45.0, 55.0, 75.0, and 100 μg/ml. If plasma or whole blood samples that had concentrations much higher than 100 μg/ml were obtained, then additional higher concentration solutions were used to construct the calibration curve because the calibration curves were linear to at least 400 μg/ml. Cyclophosphamide from the calibration solutions was extracted in a manner identical to that described in the previous section. Linear calibration curves were obtained for whole blood, plasma, and water when peak height was plotted against concentration.

Stability of cyclophosphamide in plasma

A plasma solution containing 25 μg/ml cyclophosphamide was transferred in 2.0-ml volumes into 25 borosilicate disposable test tubes. Cyclophosphamide from one test tube was analyzed immediately after preparation. The concentration that was determined in this test tube was considered to be the cyclophosphamide concentration at time zero. The rest of the test tubes were randomized and each test tube content was analyzed periodically. A similar procedure was used for samples stored at $-10°$C. For the frozen samples, about 3 h were necessary for the samples to thaw at room temperature. The time required for thawing was not included in the calculation of the percentage loss of cyclophosphamide with time.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of cyclophosphamide that was obtained from a standard cyclophosphamide solution in water. Additional peaks in the chromatogram are due to impurities in the reagents used before chromatography. Chromatograms for the control plasma and whole blood were obtained using the chromatographic procedure described (Figs. 3a and 4a). At the time at which cyclophosphamide eluted the chromatogram was free of interfering peaks. Typical chromatograms that were obtained following the extraction of plasma and whole blood from the patients that ingested cyclophosphamide are shown in Figs. 3b and 4b. The total time, starting from the pipetting of whole blood or plasma until the elution of cyclophosphamide, was less than 20 min.

Peak-height measurements were used in all quantitative calculations. The relative standard deviation (R.S.D.) of the method was 5% or less ($n=8$) when

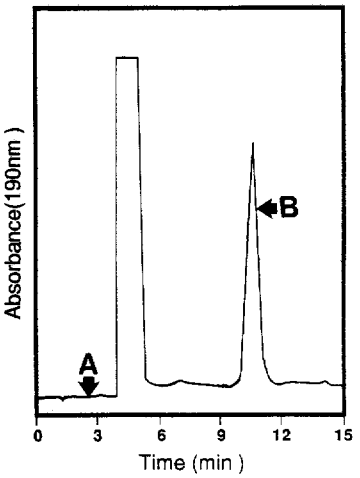

Fig. 2. Chromatogram of cyclophosphamide in water solution. Detector conditions were 0.005 a.u.f.s. and 190 nm. A = Injection point; B = cyclophosphamide.

peak heights were used for quantitation. However, the R.S.D. was approximately 10% ($n=8$) or higher when peak area was used for quantitation. The discrepancy in R.S.D. was probably due to poor peak shape and symmetry. At concentration levels of 1.0 μg/ml and below, the R.S.D. was even higher than 10% ($n=8$) when peak area was used for quantitation. However, when peak height was used for quantitation, the R.S.D. was 6% or less ($n=8$) for the 1.0 μg/ml concentration. An internal standard was not used in the method. Instead, a linear calibration curve of peak height versus concentration was used ($r=0.999$).

From statistical calculations at the 98% confidence interval, the slopes of the calibration curves that are obtained after extraction from whole blood, plasma or

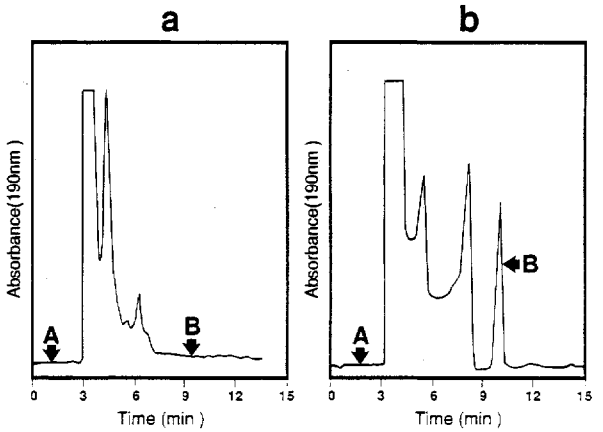

Fig. 3. (a) Chromatogram of an extract of plasma containing no cyclophosphamide; detector conditions were the same as in Fig. 2. (b) Typical chromatogram of an extract of plasma from a patient treated with cyclophosphamide; the concentration found was 6.3 μg/ml; detector conditions were 0.004 a.u.f.s. and 190 nm. A = Injection point; B = cyclophosphamide.

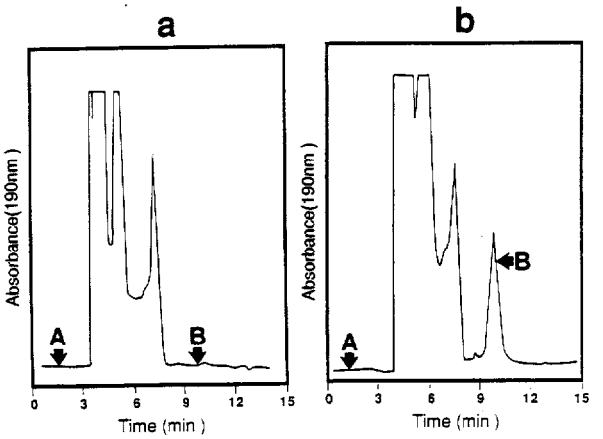

Fig. 4. (a) Chromatogram of an extract of whole blood containing no cyclophosphamide; detector conditions were the same as in Fig. 2. (b) Typical chromatogram of an extract of whole blood from a patient treated with cyclophosphamide; the concentration found was 5.5 μg/ml; detector conditions were the same as in Fig. 2. A = Injection point; B = cyclophosphamide.

water are identical. Therefore, a calibration curve obtained by extraction of standards in water can be used to quantitate cyclophosphamide in whole blood or plasma patients.

The concentrations of cyclophosphamide that were determined for whole blood and plasma on a given patient sample were similar (plasma, 9.9 μg/ml; whole blood, 10.3 μg/ml). From statistical calculations at the 95% confidence interval, the values obtained for plasma and whole blood samples were identical. Plasma and whole blood samples were analyzed from at least six patients ingesting cyclophosphamide. The concentration of cyclophosphamide determined in whole blood and plasma was found to be the same for all of the patients. Hence, either whole blood or plasma can be used to quantitate cyclophosphamide for adjustment of dose to obtain optimum therapy with minimum toxicity.

Extraction efficiency was studied by adding known amounts of cyclophosphamide to control whole blood or plasma at high and low concentrations. After extraction and injection into the chromatograph, the peak height obtained was compared with the peak height of a standard solution of cyclophosphamide in an acetonitrile solution. Extraction efficiencies ranged from 60 to 64% ($n=8$ for each concentration). Acetonitrile was used as the extraction solvent because, compared to methanol, ethanol, *n*-propyl alcohol, and isopropyl alcohol, it showed the best chromatographic selectivity for the parent drug from whole blood and plasma. The extraction efficiencies for methanol, ethanol, *n*-propyl alcohol, and isopropyl alcohol were 95, 74, 64, and 68%, respectively. All of these alcohols extracted whole blood and plasma constituents that interfered strongly with the cyclophosphamide peak. In addition to interference, they also absorb strongly at 190 nm and overlap the region of the chromatogram where cyclophosphamide elutes. For all quantitative calculations, the volume of acetonitrile added for extraction was used. The percentage of acetonitrile salted-out by anhydrous potassium carbonate from aqueous solution was $95 \pm 5\%$ (v/v) ($n=10$) [18]. The

TABLE I

WITHIN-DAY ASSAY REPRODUCIBILITY

Actual concentration (μg/ml)	Concentration determined (mean ± S.D., $n=8$) (μg/ml)	Relative standard deviation (%)
2.00	2.20 ± 0.10	4.5
5.00	4.90 ± 0.32	6.5
15.0	14.7 ± 0.63	4.3
25.0	25.4 ± 0.76	3.0
55.0	55.7 ± 2.3	4.0

mobile phase acetonitrile/buffer ratio was optimized to obtain maximum selectivity with minimum loss of sensitivity.

Interference of some common drugs were also studied under the experimental chromatographic conditions. Ampicillin, acetaminophen, aspirin, cimetidine, and ranitidine were tested; no interference with the cyclophosphamide peak was observed. Interference of creatinine, creatine, and caffeine was also studied and was negative.

No guard column was used in the analytical system. The inlet frit of the analytical column needed to be cleaned by a 6 *M* nitric acid solution after 50 to 100 injections of samples. No deterioration of the efficiency of the analytical column was observed after 250 injections. However, the column must be flushed with methanol–isopropyl alcohol (50:50) with at least 300 column volumes twice a week.

The precision and accuracy of this method were demonstrated by repetitive analysis of whole blood or plasma spiked with cyclophosphamide. Five aliquots of pooled whole blood spiked with cyclophosphamide at different concentrations are listed in Tables I and II. Table I shows the assays performed on the same day. The average R.S.D. was 4.4%. Table II shows the reproducibility of the assays from day to day (every other day over ten days). The average R.S.D. was 5.5%.

TABLE II

BETWEEN-DAY ASSAY REPRODUCIBILITY

Day-to-day analysis was carried out every other day for ten days.

Actual concentration (μg/ml)	Concentration determined (mean ± S.D., $n=8$) (μg/ml)	Relative standard deviation (%)
2.00	1.84 ± 0.12	6.5
5.00	4.76 ± 0.22	4.6
15.00	15.6 ± 0.86	5.5
25.00	24.4 ± 1.5	6.2
55.00	56.3 ± 2.7	4.8

TABLE III

RECOVERY OF CYCLOPHOSPHAMIDE FROM PATIENTS' WHOLE BLOOD AND PLASMA SAMPLES ($n=3$)

Sample No.*	Cyclophosphamide concentration (μg/ml)			Recovery (%)
	Detected before spiking	Expected after spiking	Detected after spiking	
Plasma				
A	9.88	19.76	20.30	103
B	2.67	5.34	5.65	106
C	6.78	13.56	13.20	97
Whole blood				
A	10.3	20.6	20.1	98
B	2.85	5.70	5.85	102
C	6.45	12.9	13.3	103

*Samples A, B, and C are from separate patients.

A recovery experiment of cyclophosphamide for patients' whole blood and plasma samples was performed by spiking an amount of cyclophosphamide equal to that previously detected in the samples. The recovery was then obtained as follows: detected concentration of cyclophosphamide after spiking, divided by the expected concentration of cyclophosphamide after spiking, and multiplied by 100. The recovery of plasma samples (for three patients) ranged from 97 to 106%. Similarly, the recovery for whole blood (of three patients) ranged from 98 to 103%. The results are in Table III.

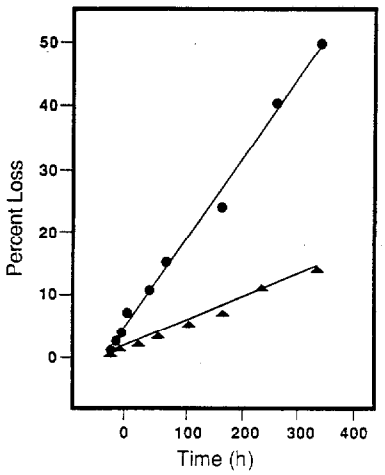

Fig. 5. Stability of cyclophosphamide in plasma at 25°C (●) and −10°C (▲) at a concentration of 25 μg/ml.

Plasma samples spiked with cyclophosphamide at 1, 0.8, and 0.6 μg/ml were also analyzed by this method to find the limit of quantification (LOQ). It was found that at 0.6 μg/ml the R.S.D. was approximately 10%, when the detector was operated at 0.002 a.u.f.s. Below 0.6 μg/ml, the uncertainty of quantification was very high (R.S.D. was greater than 15%). Therefore, the LOQ of the method in our laboratory was approximately 0.6 μg/ml at a signal-to-noise ratio of the chromatographic system of 2.

Because cyclophosphamide does not have a strong chromophore, it was necessary to monitor the drug at very low wavelengths (190 nm). It was found that the UV aborption peak for the same amount of cyclophosphamide showed a peak height two times greater when the UV absorption detector was set at 190 nm, compared to the peak height when the UV detector was set at 195 nm. The baseline noise of the UV detector used in this experiment was less than 5% of full scale at 190 nm and 0.004 a.u.f.s. with the mobile phase in the flow cell. The baseline noise can be further decreased if the grating monochromator of the detector is constantly bathed by helium gas and also by purging the mobile phase with helium gas.

By using this method, the stability study of cyclophosphamide in plasma at 25°C and −10°C was done (Fig. 5). At 25°C, ca. 50% of the parent drug disappeared in approximately two weeks (ca. 350 h). The loss of cyclophosphamide from plasma samples at 24, 48, and 72 h was approximately 3, 7, and 10%, respectively. However, at −10°C, less than 10% of the parent drug disappeared after more than two weeks. This shows that cyclophosphamide is not very stable at room temperature, but highly stable at lower temperatures. Therefore, the blood samples drawn from a patient must be analyzed on the same day or should be stored at −10°C.

Using this analytical method, one can study the in vivo pharmacokinetics of cyclophosphamide. Because of the simplicity and high sensitivity of this method, a pharmacokinetic study can be carried out for concentrations as low as 0.60 μg/ml.

CONCLUSION

The cyclophosphamide HPLC assay described in this paper is the first one that can be used to determine the levels of cyclophosphamide in the whole blood or plasma of patients ingesting the drug. Prior to this report, Kensler et al. [15] and Wantland and Hersh [16] published an HPLC method to determine cyclophosphamide in dosage and parental solutions. Because of poor analytical sensitivity and sample extraction procedures, no attempt was made to use the methods to determine cyclophosphamide in the biological samples of patients. The range of the assay is 0.60–400 μg/ml with an R.S.D. of 6% or less, except for the samples that have concentrations lower than 1.0 μg/ml. The pre-chromatography isolation of cyclophosphamide is simple and rapid. Elimination of the evaporation step from the sample preparation procedure reduced the manipulative errors and the time. The cyclophosphamide HPLC assay described here is highly reprodu-

cible and much faster than the other reported methods [5–14]. The limit of detection of the method is 0.30 μg/ml for a 30-μl injection at a signal-to-noise ratio of 2.

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