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Note

Improved analysis of cyclophosphamide by capillary gas chromatography with thermionic (nitrogen—phosphorus) specific detection and silica sample purification

ADNAN EL-YAZIGI* and CAZEMIRO R. MARTIN

Pharmacokinetic Laboratory, Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Centre, Riyadh 11211 (Saudi Arabia)

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Cyclophosphamide is an antineoplastic agent commonly used in the treatment of a wide variety of human malignancies. Because of its immunosuppressive properties, this drug has been shown to be valuable in the treatment of non-neoplastic diseases such as rheumatoid arthritis [1], Wegeners' granulomatosis [2] and lupus erythematosus [3]. It is also frequently used prior to bone marrow transplantation.

Cyclophosphamide has been analysed by various techniques including radioactivity measurement of labelled CP [4], mass spectrometry [5], high-performance liquid chromatography [6], gas—liquid chromatography [7—9], gas chromatography—mass spectrometry [10—12], colorimetry [13] and bioassay [14]. However, these methods are either non-specific, insensitive or require highly specialized instrumentation. Most of the gas chromatographic (GC) methods involve tedious pre-chromatographic derivatization or use relatively insensitive flame ionization detection. A capillary GC method was recently described by Van den Bosch et al. [15]; however, this assay involves a multistep conventional extraction procedure, and the detection limit is only 50 ng/ml.

The use of a silica sample clean-up in the analysis of cyclophosphamide has not been previously reported. The use of this liquid chromatographic technique prior to capillary GC of underivatized cyclophosphamide in conjunction with highly sensitive thermionic (nitrogen—phosphorus) specific detection provide the ease, rapidity and sensitivity desired in an analytical method suitable for clinical monitoring of this drug in cancer patients.

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EXPERIMENTAL

Chemicals

Analytical samples of cyclophosphamide were a gift from Mead Johnson, Evansville, IN, U.S.A. N-Nitrosodiphenylamine, the internal standard (Chem Service, West Chester, PA, U.S.A.), was > 99% purity. Methotrexate (American Cyanamid, Pearl River, NY, U.S.A.), adriamycin (Farmitalia Carlo Erba, Milan, Italy), vincristine sulphate (Eli Lilly, Indianapolis, IN, U.S.A.), bleomycin sulphate (Bristol Labs., Syracuse, NY, U.S.A.), *cis*-platinum (Laboratoire Roger Bellon, Neuilly-Paris, France), etoposide (Sandoz, Basle, Switzerland), acetaminophen (McNeil Labs., Fort Washington, PA, U.S.A.), cytarabine (Upjohn, Kalamazoo, MI, U.S.A.) and prednisone (Philips Roxane Labs., Columbus, OH, U.S.A.) were either reagent or pharmaceutical grade. Anhydrous calcium chloride and ethyl acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.) were analytical grade.

Apparatus and column

The gas chromatograph used was a Varian (Palo Alto, CA, U.S.A.) Model Vista® 6000 equipped with a thermionic (nitrogen—phosphorus) specific detector, a split/splitless capillary injector and a Vista 402 data module. A 25 m × 0.33 mm I.D. fused-silica SE-30 capillary column (Varian) was employed for the chromatography.

Gas chromatographic conditions

Temperature programming was used for separation of the compounds. For each run, the oven temperature was initially held at 115°C for 1.5 min, then raised to 200°C at a rate of 10°C/min. The temperature was then held at 200°C for 5 min. The injection port temperature was 240°C and the detector temperature was 300°C. Nitrogen at a flow-rate of 1.5 ml/min was used as the carrier gas, and the detector gas flow-rates were 4 ml/min (hydrogen) and 175 ml/min (air).

Purification of plasma sample

Silica cartridges (Sep-Pak®, Waters Assoc., Milford, MA, U.S.A.) were employed for sample purification and concentration. The cartridge was prewashed with 10 ml of ethyl acetate, then vacuum-dried for 1 min by connecting it to a 20-p.s.i. vacuum outlet. The plasma sample (1 ml) was slowly injected into the cartridge and vacuum dried for 1 min. The compounds were eluted with 6 ml of ethyl acetate, received in a centrifuge tube containing 100 mg of anhydrous calcium chloride. After vortexing for 15 s and centrifugation for 5 min at 1746 *g*, the supernatant was removed and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 125 μl of ethyl acetate and 1 μl of the solution was injected.

Preparation of standard curves

Separate stock solutions of different concentrations of cyclophosphamide and the internal standard were prepared in methanol. Aliquots of the above cyclophosphamide stock solutions were added to 1-ml blank plasma samples to

construct standard curves in the following ranges: 0.01—0.5, 0.5—10 and 10—100 μg/ml. To each tube, aliquots equivalent to 10, 50 or 500 μg of the internal standard were added, and the mixture was vortexed for 15 s. The solution was then subjected to the above-described clean-up procedure.

Analysis of patients' samples

Plasma samples from two patients treated with high doses of cyclophosphamide were collected at different intervals and analysed according to the described assay after the addition of the internal standard. Patient A, a 29-year-old male weighing 90.5 kg, received 1500 mg of cyclophosphamide by rapid intravenous administration; whereas patient B, a 17-year-old female weighing 48 kg, received 2100 mg of cyclophosamide at a 1-h constant rate of infusion.

RESULTS AND DISCUSSION

The use of a narrow-bore, fused-silica, SE-30 capillary column for underivatized cyclophosphamide under the described conditions yielded high chromatographic efficiency (very sharp peaks) with retention times of 11.75 and 8.08 min for cyclophosphamide and the internal standard, respectively. Typical chromatograms of spiked blanks and two patients' samples collected 48 or 20 h after completion of the cyclophosphamide administration are depicted in Fig. 1. Chromatograms of an intact blank plasma sample and a blank plasma sample spiked with 10 ng/ml cyclophosphamide (the sensitivity limit of the assay) are demonstrated in Fig. 2. The choice of this capillary column with the SE-30 liquid phase was the result of extensive investigation for optimization of the chromatographic conditions. A number of fused-silica or wide-bore glass capillary columns with different liquid phases were employed isothermally or with temperature programming, but were found unsuitable for analysis.

Sample clean-up was performed using silica cartridges. The procedure is simple and rapid and yielded clean chromatograms. Plasma samples supplemented with 1, 10 and 50 μg/ml cyclophosphamide were analysed. The extraction efficiency of cyclophosphamide was computed from the ratio of the peak area acquired for each of the above plasma samples to that obtained for an equal amount of cyclophosphamide in ethyl acetate injected directly into the capillary column. Mean extraction efficiencies (n = 3) of 95.5, 96.3 and 91.7% were obtained at the above concentrations, respectively. These values are comparable to [7, 15] or larger than [11] the recovery ratios acquired by other investigators for cyclophosphamide in plasma. The expediency and rapidity with which the sample is purified here, make the described procedure an improved approach over the relatively tedious conventional liquid—liquid extraction technique.

The plasma concentration of cyclophosphamide following high-dose treatment extends over a wide range. Therefore, standard curves in the concentration ranges 0.01—0.5, 0.5—10 and 10—100 μg/ml were constructed by plotting the peak-area ratio (CP/internal standard) against concentration. Seven curves were prepared in each range, and the correlation coefficient (r) was calculated

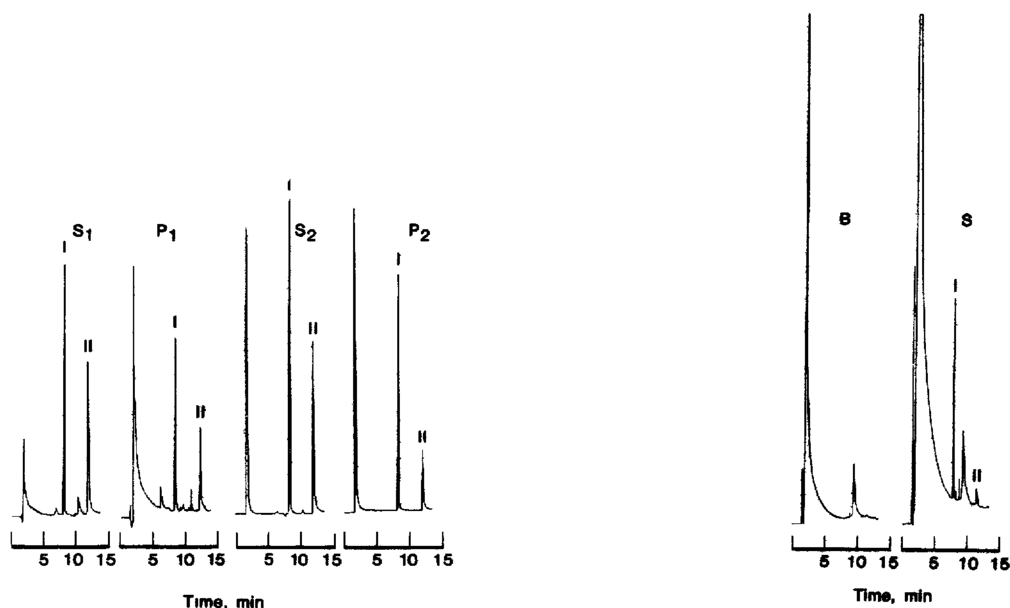

Fig. 1. Typical capillary gas chromatograms obtianed according to the described method. S_1, blank plasma sample spiked with 1 μg of cyclophosphamide (II) and 10 μg of the internal standard (I); S_2, blank plasma sample spiked with 50 μg of II and 500 μg of I; P_1, plasma sample collected 48 h after rapid administration of 1500 mg of II to patient A and spiked with 10 μg of I; P_2, plasma sample collected 20 h after the termination of a 1-h intravenous infusion of 2100 mg of II to patient B and spiked with 500 μg of I (see text).

Fig. 2. Chromatograms of an intact blank plasma sample (B) and a blank plasma sample spiked with 2 μg/ml internal standard (I) and 10 ng/ml cyclophosphamide (II), the sensitivity limit of the assay (S).

for each curve. A good assay linearity was obtained, and r was never less than 0.991 (range 0.991—0.9999; mean 0.9963). The inter-curve variability in slope and intercept was relatively small, signifying good assay reproducibility.

The intra-run precision was examined by analysing blank plasma samples supplemented with 0.1, 10 or 100 μg/ml in five replicates and the coefficients of variation (C.V.) were computed. The C.V. values were 3.3, 4 and 3.2% at the above concentrations, respectively. The inter-run precision was also studied. Plasma samples containing 0.05, 5 or 50 μg/ml were analysed on at least three different days, and the C.V. values obtained were 3.2, 4.5 and 4.5% at the above concentrations, respectively.

The interference in the assay by other drugs commonly used with cyclophosphamide in combined cancer chemotherapy was investigated. Solutions of methotrexate, etoposide, vincristine, bleomycin, *cis*-platinum, cytarabine, prednisone, adriamycin and acetaminophen in suitable organic solvents were prepared and chromatographed according to the described procedure. None of these drugs were detected nor did they interfere in the assay, indicating good assay specificity.

On examining the analytical recovery of the described method, 1-ml portions of blank plasma were supplemented with various amounts of cyclophosphamide (100—0.01 μg) and subjected to the described analysis after the

addition of internal standard. The amounts of cyclophosphamide found ranged from 96.3 to 0.0108 μg and the recovery ranged from 96 to 108%. The percentage recovery for each amount added was calculated according to the following equation: percentage recovery = 100 amount found/amount added.

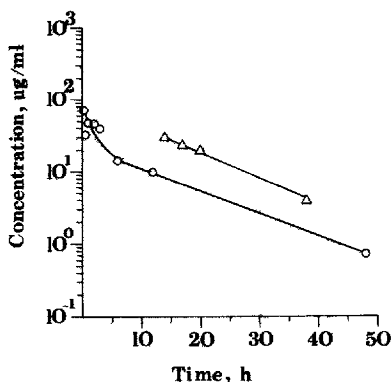

Fig. 3. Plasma concentration—time profiles of cyclophosphamide in patients treated with this drug. (○) Patient A; (△) patient B (see text).

Plasma concentration- time profiles obtained in the two patients included in this study are presented in Fig. 3. As shown in this figure, plasma concentration of cyclophosphamide as a function of time in patient A exhibited a bi-exponential decay pattern with a β-half-life of 9.7 h. The data for patient B showed a good linearity with a terminal half-life of 8.1 h. For clinical reasons, blood sampling in this patient was not initiated until 14 h after termination of the infusion.

In conclusion, the described method is an improved assay for cyclophosphamide in plasma. The use of a silica liquid chromatographic procedure for sample purification prior to capillary GC provided the expediency and rapidity needed in an analytical method. The assay is also very sensitive, with a readily analysable limit of 10 ng/ml. With this sensitivity, the concentration of cyclophosphamide in plasma can be followed reliably more than 48 h after administration of the drug. This sensitivity, combined with rapidity and ease, makes this assay of cyclophosphamide suitable not only for routine clinical monitoring but also for pharmacokinetic studies of the drug.

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