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Note

Plasma cyclophosphamide assay by selective ion monitoring

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Cyclophosphamide (CPH) is a cytostatic drug widely used in oncology. Reported work on this agent deals mainly with its pharmacokinetic behaviour [1—3] and metabolism [4,5] when administered alone. Clinically, other drugs may be associated with CPH, particularly other antineoplastics, analgesics, hypnotics, corticoids and antiemetics, but the influence of these associations on the pharmacokinetics of CPH has not been extensively studied [6,7]. A highly specific analytical method for cyclophosphamide assay is required, as the associated drugs are liable to interfere. To date, assay methods have involved radiolabelling [1], which is clinically inconvenient, gas chromatography (GC), either simple [2,8] or coupled with mass spectrometry (MS) [4,9], or high-performance liquid chromatography [5]. For gas—liquid chromatographic analysis, samples must be derivatized to avoid decomposition of CPH. We describe here a method of assay of CPH in plasma by selected monitoring of two characteristic ions, $m/z = 307$ and $m/z = 309$. This method is highly specific and very sensitive.

EXPERIMENTAL

Assays were carried out using a Hewlett-Packard 5710 A gas chromatograph connected to a Hewlett-Packard 5970 A selective mass detector set up for selected ion monitoring. Separation was carried out on a fused-silica capillary column (Hewlett-Packard SP-2100, 25 m × 0.2 mm I.D., film thickness 0.2 μm) at 240°C. Helium was used as carrier gas. The injection port temperature was 250°C. Plasma samples (0.2 ml) were spiked with isophosphamide (IPH) (structure given in Fig. 1) as internal standard (20 μg/ml) and analysed by the

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procedure described by Van den Bosch and De Vos [8], leaving out the purification of the extracts with hexane which proved unnecessary given the high specificity of the selected ion monitoring method. The plasma aliquots were extracted three times with 1 ml of ethyl acetate after addition of 1 ml of 0.6 *M* sodium hydroxide, by shaking on a vortex shaker for 3 min and spinning at 1600 *g* for 5 min. The extracts were evaporated to dryness in a stream of nitrogen at ambient temperature. The residue was taken up in 100 μl of ethyl acetate and 50 μl of trifluoroacetic anhydride and left for 30 min at 60°C [8]. The solution was evaporated to dryness under nitrogen and the residue taken up in 200 μl of ethyl acetate; 1 μl of this solution was injected into the chromatograph using a Ros capillary injector.

Mass spectra

Mass spectra of the trifluoroacetyl derivatives of CPH and IPH were recorded using a Hewlett-Packard 5985 B GC—MS system fitted with an SE-30 fused-silica capillary column (25 m × 0.30 mm I.D.) and a Ros capillary injector. These spectra are shown in Fig. 1. The base peak at m/z = 307 and the ion at m/z = 309 of relative intensity 30% are common to both derivatives. These were chosen for quantitative analysis. Selected ion monitoring recordings of plasma extracts are given in Fig. 2.

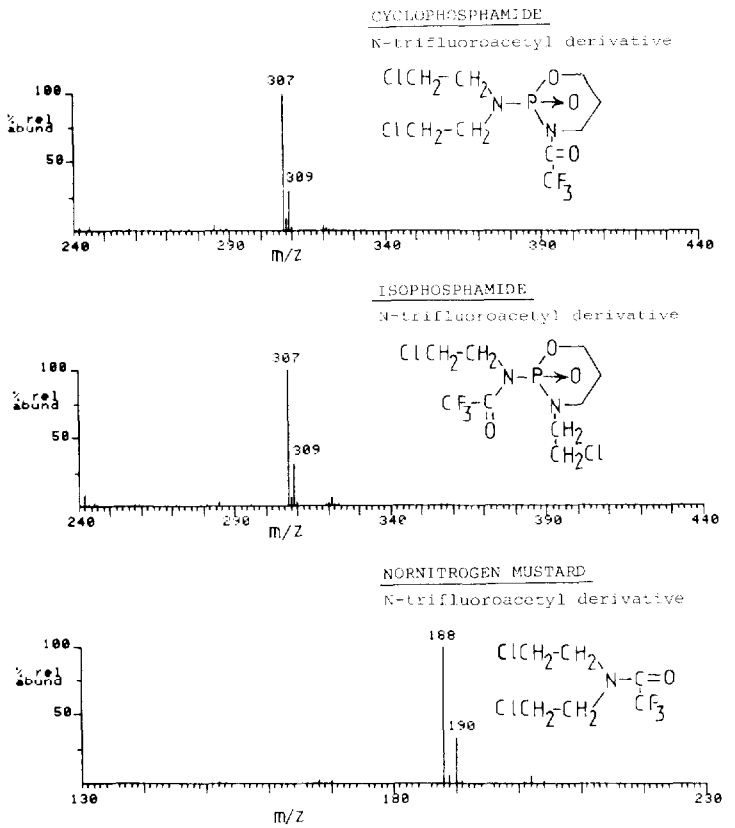

Fig. 1. Structures and electron-impact mass spectra of N-trifluoroacetyl derivatives of cyclophosphamide, isophosphamide and nornitrogen mustard.

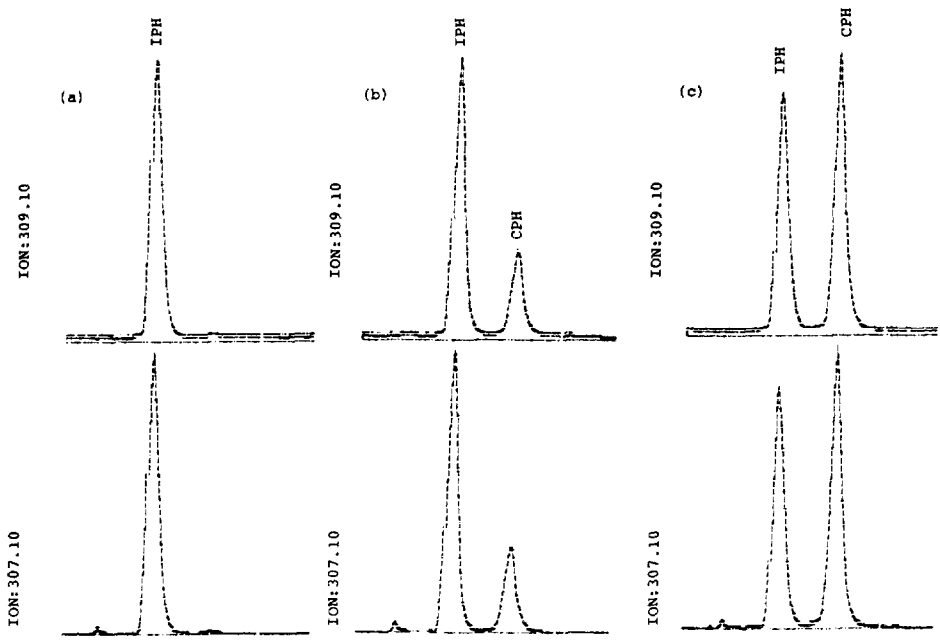

Fig. 2. Selected ion monitoring records for plasma extracts: (a) blank plasma, (b) 2.5 μg/ml cyclophosphamide, (c) 10.0 μg/ml cyclophosphamide.

Calibration

A calibration plot was obtained for each series of assays by adding varying amounts of CPH (0.2—50.0 μg/ml) and a constant amount of IPH (20.0 μg/ml), to control plasma aliquots, and analysing them as above.

RESULTS AND DISCUSSION

Assessment of the method

Extraction yield. CPH extraction efficiency was determined using control plasma samples spiked with IPH (20.0 μg/ml). CPH was added either before or after extraction with ethyl acetate. Comparison of the peak area ratios of unextracted CPH to extracted CPH gave CPH extraction yields of 89.7 ± 3.6% at 2.5 μg/ml ($n=5$), 96.3 ± 1.9% at 10.0 μg/ml ($n=5$) and 98.4 ± 6.0% at 25.0 μg/ml ($n=5$). The IPH extraction yield, similarly determined (unextracted IPH/extracted IPH), was 94.2 ± 5.7% at 20.0 μg/ml.

Sensitivity. Under the operating conditions described above, the sensitivity threshold was about 0.2 μg/ml. This threshold can, however, be lowered by calibrating over a lower concentration range, 0.05—1.00 μg/ml, and adding IPH as internal standard at 0.5 μg/ml.

Reproducibility. The intra-assay reproducibility was determined by extracting and analysing ten replicate human plasma samples spiked with CPH at 2.5, 15.0, 25.0 and 50.0 μg/ml. It is expressed by the coefficient of variation (%) in Table I. The inter-assay reproducibility was determined by analysing two series of ten replicates containing 2.5 and 25.0 μg/ml CPH. It is expressed by the coefficient of variation (%) between the two series, i.e. 7.5% at 2.5 μg/ml and 8.3% at 25.0 μg/ml.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ASSAY

$n = 10$.

Theoretical (μg/ml)	Calculated (μg/ml ± S.D.)	Coefficient of variation (%)	Mean error (%)
2.5	2.56 ± 0.11	4.4	4.0
15.0	15.45 ± 0.50	3.2	3.7
25.0	24.80 ± 1.70	6.8	5.4
50.0	52.50 ± 2.70	5.1	6.4

Accuracy. Accuracy was assessed in terms of mean relative errors for the concentrations studied (Table I).

Linearity. Linearity is satisfactory over the range 0.2—50.0 μg/ml as shown by linear regression analysis of four calibration curves plotted on different days over a period of one month for both the ion at $m/z = 307$ ($Y = 0.110X + 0.041$, $r = 0.9993$) and that at $m/z = 309$ ($Y = 0.109X + 0.039$, $r = 0.9991$).

Specificity. Two ions characteristic of CPH were monitored, namely $m/z = 307$ and $m/z = 309$. Identity of the 307 CPH/307 IPH and 309 CPH/309 IPH ratios is consistent with absence of interference. In addition, we tested under the same analytical conditions various drugs likely to be administered along with CPH. No interference was observed in different control plasma samples spiked to maximum in vivo concentrations with adriblastin, teniposide, methotrexate, hydrocortisone, dexamethasone, ketoprofen, amphotericin B, allopurinol, metopimazine or clonidine, or after administration of 5-fluorouracil, adriamycin or vincristine to patients.

Sample stability

Jardine et al. [9] in a previous study of CPH and its metabolites reported the decomposition of CPH (11%), 4-keto-CPH (19%) and carboxyphosphamide (58%) into nornitrogen mustard in the course of work-up of biological samples. The extraction and chromatography procedures used by these authors were different from ours. We tested for the presence of this breakdown product in our plasma extracts and in CPH pure solutions (0.2, 1, 2, 5 and 10 μg) after trifluoroacetylation. Assay of nornitrogen mustard can be performed under the same conditions as that described for CPH if the chromatography is carried out at 130°C. The mass spectrum of the N-trifluoroacetyl derivative of nornitrogen mustard is given in Fig. 1. The ions monitored for quantitative analysis were those with $m/z = 188$ and $m/z = 190$. Under the conditions described, decomposition of CPH to nornitrogen mustard stayed below 0.1%.

Jardine et al. [9] report that, though breakdown of CPH and its metabolites occurs mainly during extraction, some decomposition also occurs during freezing. Analysis of our plasma samples after storage for one month at −20°C and comparison of results with those obtained from fresh plasma samples (Table II) failed to reveal any significant differences in assay values. Hence freezing apparently does not account to any great extent for the breakdown of CPH, though it may affect its metabolites, which were not assayed here.

TABLE II

STABILITY OF CYCLOPHOSPHAMIDE IN DEEP-FROZEN PLASMA SAMPLES AFTER STORAGE FOR ONE MONTH AT −20°C

Patient (dose injected)	Time of sampling after administration	Concentration of cyclophosphamide (μg/ml)		Coefficient of variation (%)
		Analysis after sampling	Analysis after one month at −20°C	
No. 1	5 min	6.1	5.8	4.9
(200 mg i.v.*)	1 h	5.0	4.5	10.0
	6 h	2.8	2.4	10.0
No. 2	5 min	6.7	7.1	6.0
(200 mg i.v.)	1 h	4.8	4.4	8.3
	6 h	2.4	2.6	8.3
No. 3	5 min	12.3	12.4	0.8
(300 mg i.v.)	1 h	4.4	4.6	4.6
	6 h	3.2	3.1	3.1
No. 4	5 min	38.9	37.0	4.9
(400 mg i.v.)	1 h	10.4	9.4	9.6
	6 h	5.5	5.4	1.8

*i.v. = intravenously.

To conclude, the method described here should provide a means of reliably monitoring plasma levels of CPH in patients, and thereby allow the influence of coadministered drugs on its pharmacokinetics to be accurately studied.

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