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Claire Joqueviel · Véronique Gilard · Robert Martino
Myriam Malet-Martino · Ulf Niemeyer

Urinary stability of carboxycyclophosphamide and carboxyifosfamide, two major metabolites of the anticancer drugs cyclophosphamide and ifosfamide

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Abstract Phosphorus-31 nuclear magnetic resonance spectroscopy was used to evaluate the stability of carboxycyclophosphamide (CXCP) and carboxyifosfamide (CXIF) in human urine at pH 7.0 and 5.5 at 25°, 8°, −20°, and −80 °C. At 25 °C and pH 7.0, CXCP and CXIF are relatively stable (≈10% degradation in 24 h). In contrast, they are much less stable at pH 5.5 (≈80% degradation of CXIF and ≈50% degradation of CXCP in 24 h). The rate of degradation of CXCP and CXIF was a function of the storage temperature of the urine samples but, even at −80 °C, was not negligible: ≈30% degradation for CXCP irrespective of pH and ≈40% and 50% degradation for CXIF at pH 7.0 and 5.5, respectively, after storage for 6 months. CXCP was more stable than CXIF at either pH (7.0 or 5.5) and at all storage temperatures (8°, −20°, or −80 °C) of the urine samples. CXCP and CXIF were more stable at pH 7.0 than at pH 5.5, although this difference fell with decreasing temperatures to be almost negligible at −80 °C. To ensure a true estimate of CXCP and CXIF levels, urine samples must be frozen and stored at −80 °C within a few hours of micturition. CXCP and CXIF assays should also be carried out within 2 months and 1 month of storage, respectively.

Key words Carboxycyclophosphamide · Carboxyifosfamide · Urinary stability · Phosphorus-31 NMR

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C. Joqueviel · V. Gilard · R. Martino · M. Malet-Martino
Groupe de RMN Biomédicale, Laboratoire des IMRCP,
Université Paul Sabatier, 118, route de Narbonne,
F-31062 Toulouse, France
Tel: +33 0561 55 62 71; Fax: +33 0561 25 17 33;
e-mail: rmnbio@ramses.ups-tlse.fr

U. Niemeyer
ASTA Medica AG, Weismüllerstraße 45,
D-60314 Frankfurt am Main, Germany

Introduction

Cyclophosphamide (CP) and ifosfamide (IF; Fig. 1), the most widely used antitumor alkylating agents, are therapeutically inactive prodrugs that must be activated to express their cytotoxicity [21, 26, 28]. The metabolic pathway of CP and IF (Fig. 2 depicts an overview of that of IF) is mediated by a hepatic cytochrome P450 reaction that leads to N-dechloroethylation (detoxication pathway) [25, 29], liberating chloroacetaldehyde, a compound that may be responsible for the oxazaphosphorine-induced neurotoxicity and urotoxicity [11, 24]. This reaction also induces hydroxylation at the carbon-4 of the oxazaphosphorine ring (activation pathway) [25, 29], producing 4-hydroxy-CP (or -IF; OHCP or OHIF), which exists in equilibrium with its ring-opened tautomer ALDOCP or ALDOIF. OHCP or OHIF may be partially deactivated by an alcohol dehydrogenase into KETOCP or KETOIF. ALDOCP or ALDOIF undergoes spontaneous β-elimination of urotoxic acrolein to yield phosphoramide mustard (PM) or isophosphoramide mustard (IPM), the ultimate alkylating species. Alternatively, ALDOCP or ALDOIF can be deactivated into carboxycyclophosphamide (CXCP) or carboxyifosfamide (CXIF; Fig. 1) by an aldehyde dehydrogenase (ALDH) or into ALCOCP or ALCOIF by an aldehyde reductase (Fig. 2).

Large interpatient differences in CP and IF metabolism have been noted in both adults [2, 4, 7, 9, 12, 13, 16, 23] and children [3, 5, 6, 10, 22, 27, 31], although the clinical consequences of these differences remain to be determined [3, 31]. It has been suggested that the marked interpatient variation in CXCP and CXIF production is the result of phenotypic variation in ALDH activity as demonstrated by the bimodal distribution of the amount of CXCP or CXIF excreted in the urine of adults treated with CP or IF [2, 12, 16]. However, no genetic polymorphism in the metabolism to CXCP and CXIF was evidenced from the urinary excretion of children treated with CP [31] or adults or children treated with IF [3, 13].

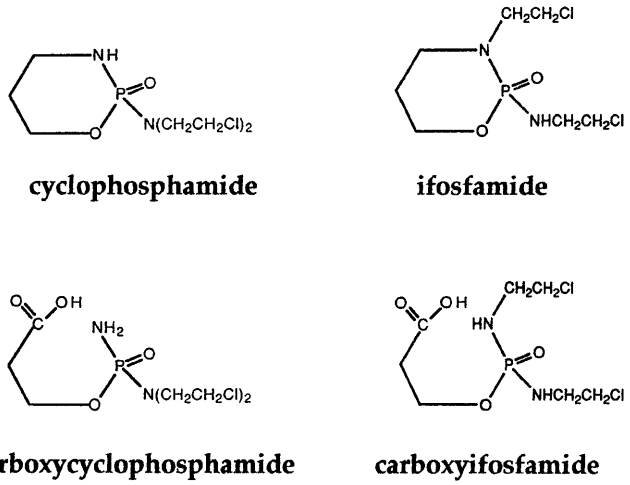

Fig. 1 Structures of CP and IF and their respective metabolites CXCP and CXIF

Two lines of evidence indicate that factors other than a deficiency in ALDH 1, the major isoenzyme responsible for CXCP (and, probably, CXIF) production [8], contribute to the high degree of interpatient variations in urinary excretion of CXCP and CXIF. First, ALDH 1 variants have been found in both Oriental and Caucasian populations at a low frequency ($\ll 10\%$) [30], which is considerably lower than the frequency reported (35%) for patients deficient in the formation of CXCP [2, 12]. Second, a patient receiving IF by continuous infusion over 3 days produced very little CXIF in the 1st day, whereas the amount was considerably increased by the 3rd day [13]. Since both CXCP [14, 15, 17, 31] and CXIF [9, 13, 18] are unstable, especially in acidic medium, excretion of these metabolites is likely to be underestimated. This could also help account for the marked interpatient variation in the amounts detected.

In an attempt to evaluate the instability of CXCP and CXIF, we used phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) to study the degradation of these two compounds in human urine as a function of time, pH (7.0 and 5.5), and temperature of storage (25°, 8°, −20°, and −80 °C). The pH values were chosen to reflect the extreme conditions of urinary pH observed in our previous studies [9, 18]. ^{31}P-NMR enables direct quantitation of CXCP or CXIF in intact urine samples, avoiding the extraction and chemical derivatization procedures required in chromatography methods (combined thin-layer chromatography-photography-densitometry [1, 2] or gas chromatography/mass spectrometry [19, 20]).

Materials and methods

Materials

CXCP and CXIF cyclohexylamine salt were generously supplied by ASTA Medica AG (Frankfurt, Germany). Ifosfamide and

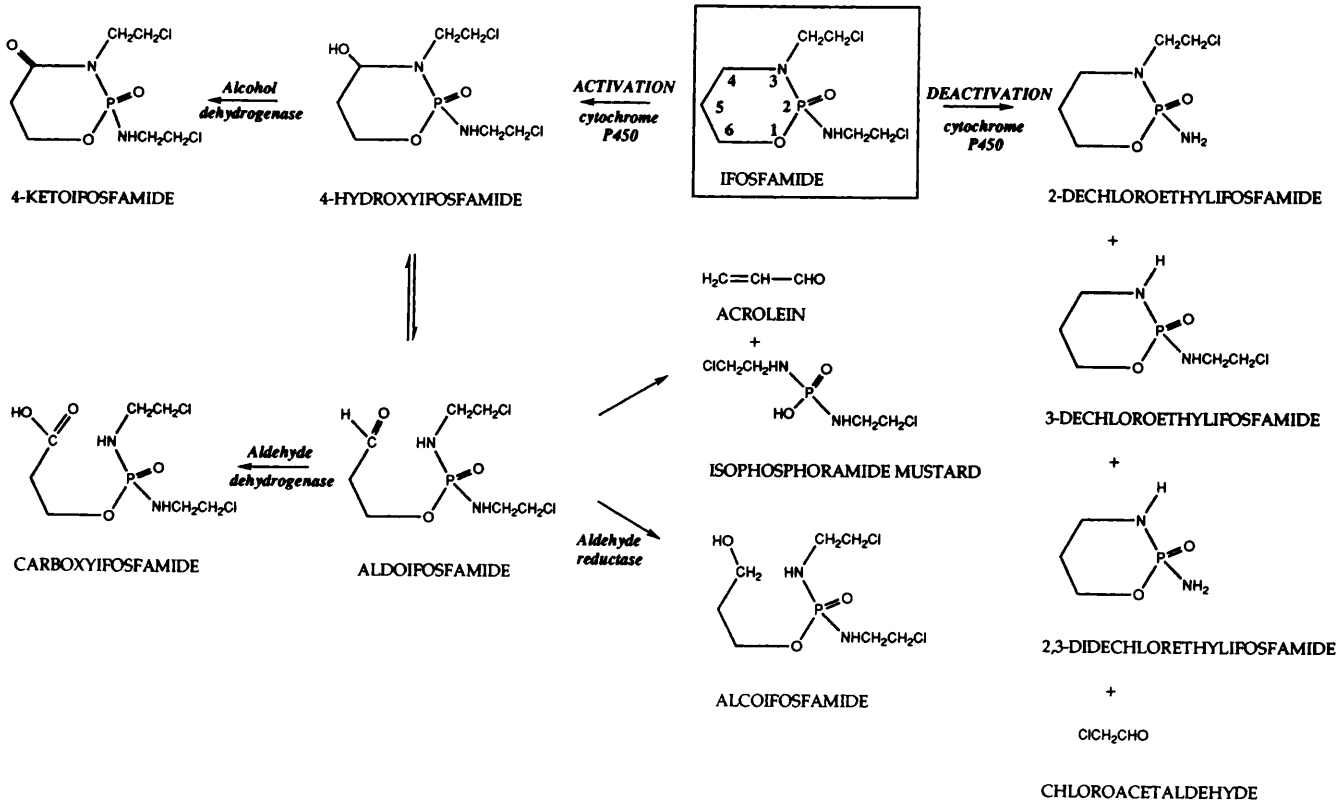

Fig. 2 Metabolic pathways of IF

chromium(III) acetylacetonate [$Cr(acac)_3$] were obtained from ASTA Medica (Bordeaux, France) and Spectométrie Spin et Techniques (Paris, France), respectively. Methyl phosphonic acid (MPA) was purchased from Aldrich (St. Quentin Fallavier, France).

The degradation compounds of CXCP and CXIF were column-chromatography-purified on a C18 reversed-phase column from (1) aqueous solutions of CXCP or CXIF maintained for 5–8 h at pH 2.5 by periodic addition of HCl solution to get $HOOC-(CH_2)_2OP(O)(OH)_2$ (2) aqueous solutions of CXCP maintained for 24 h at pH 4.7 by periodic addition of HCl solution in the presence of a 30 *M* excess of ammonia to obtain $H_2NOC(CH_2)_2OP(O)(OH)_2$ and (3) aqueous solutions of CXIF maintained at pH 2.2 for 5 h at 25 °C to yield $Cl(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$. An aqueous solution of the latter compound kept at 25 °C and pH 3.0 for 17 days led to the formation of $HO(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$. The structures of all these compounds were characterized by mass spectrometry and ^1H and ^{13}C-NMR.

Preparation of solutions of CXCP and CXIF in urine

For the study of stability at 25 °C the pH of the solutions of CXCP and CXIF cyclohexylamine salt (about 6.5×10^{-3} and 5.0×10^{-4} *M*) in human urine was adjusted to 5.5 or 7.0 with HCl or NaOH at 1 *M*. An aliquot of each of these solutions was maintained at 25 °C in a circulating water bath for 24 h and then analyzed by ^{31}P-NMR. The degradation kinetics at 25 °C of another aliquot of all solutions, apart from those at 5.0×10^{-4} *M* and pH 7.0, were continuously monitored in the NMR probe using ^{31}P–NMR.

For the study of stability as a function of storage temperature, 200 ml of human urine (pH 5.8) was divided into four 50-ml fractions. CXCP was dissolved in two of these fractions and the pH of one fraction was adjusted to 5.5 with 1 *M* HCl and that of the other, to 7.0 with 1 *M* NaOH. The same procedure was followed for the CXIF cyclohexylamine salt. The concentration of all the solutions was 6.7×10^{-3} *M*. Each of these solutions was divided into 3-ml fractions, which were stored at 8°, –20°, or –80 °C for various periods and then analyzed by ^{31}P-NMR (at 25° or 4 °C) after being thawed if necessary. A new fraction was used for each time point. Some measurements were duplicated, but using different fractions. Moreover, two studies of the kinetics of CXIF degradation at pH 5.5 and 8 °C were carried out. The pH of each sample was measured before and after the recording of each NMR spectrum. Table 1 lists the number of time points, the duration of storage of the samples, and the change in pH determined over these periods.

^{31}P-NMR analysis

^1H-decoupled NMR spectra were recorded at 121.5 MHz with a Bruker WB-AM 300 spectrometer using the inverse-gated decoupling technique. The magnetic field was shimmed on the free induction decay (FID) from H_2O of the sample. Spectra were acquired using 10-mm-diameter NMR tubes under the following instrumental conditions: probe temperature, 25° or 4 °C; sweep width, 15, 151 Hz; 32K data points zero-filled to 64K; pulse width (PW) 5 μs (i.e., flip angle $\simeq 35°$); repetition time (TR), 2.08 s for the studies on stability as a function of storage temperature or 6.08 s in the experiments on stability at 25 °C; FID spectra processed by exponential multiplication with a line broadening of 2 Hz; and number of transients, 500 for samples with CXCP or CXIF at 6.7×10^{-3} *M* (data sets acquired in 17 or 51 min, depending on the TR used) and 3,000 (3 blocks of 1,000) for samples with CXCP or CXIF at 5.0×10^{-4} *M* (data sets acquired in 3×102 min since only a TR of 6.08 s was used). The concentrations of CXCP and CXIF were measured by comparison of the expanded areas of their ^{31}P-NMR signals with that of MPA, the standard for quantification placed in a sealed coaxial capillary. The areas were determined after the different signals had been cut out and weighed. The external standard [MPA in deuterated water doped at saturation (about 3 m*M*) with the paramagnetic agent $Cr(acac)_3$ to shorten the MPA T_1 relaxation time, the deuterated solvent providing the field frequency lock for the spectrometer] was calibrated against ifosfamide solutions of known concentrations with recording conditions (PW 5 μs, TR 6.08 s) set to produce fully relaxed spectra.

The spectra of samples containing CXCP and CXIF after storage for 24 h at 25 °C were acquired under conditions of full relaxation (PW 5 μs, TR 6.08 s) of the ^{31}P-NMR signals of CXCP, CXIF, and MPA since using a TR of 10.08 s did not modify the signal intensities. The samples were maintained at 4 °C to avoid degradation during the NMR recording. To ensure that degradation of CXCP or CXIF was negligible at this temperature over the time required to record the spectra (about 5 h) of the least concentrated solutions (5.0×10^{-4} *M*) at pH 5.5 (the pH value at which degradation is fastest), fully relaxed ^{31}P-NMR spectra of these solutions were acquired at 4 °C over 10 blocks of 102 min. Quantitative ^{31}P-NMR analysis of these spectra showed that CXCP was not significantly degraded (degradation $< 10\%$) over the 17-h recording period and that degradation of CXIF was significant ($> 10\%$) only after 7 h (i.e., after recording of the 4th block) and was around 30% at the end of the 17-h recording period.

The degradation kinetics of CXCP and CXIF at 25 °C were continuously monitored in the NMR probe, and fully relaxed ^{31}P-NMR spectra were acquired in blocks of 51 min over 25 h (solutions

Table 1 Stability of CXCP and CXIF as a function of storage temperature: number of time points, duration of storage of the samples, and change in pH over the storage period

		pH = 7				pH = 5.5			
Temperature	Compound	Duration of storage	Number of time points	Extreme times of measure	Δ pH over storage period	Duration of storage	Number of time points	Extreme times of measure	Δ pH over storage period
8 °C	CXCP	4 months	12	1 day–4 months	−0.3	16 days	7	6 h–16 days	+ 0.3
	CXIF	3 months	10	1 day–3 months	−0.25	9 days	7	6 h–19 days	+ 0.2
−20 °C	CXCP	7 months	14	5 days–7 months	+0.15	3 months	9	2 days–3 months	+ 0.3
	CXIF	6 months	12	5 days–6 months	+0.25	2 months	7	2 days–2 months	+ 0.25
−80 °C	CXCP	9 months	9	9 days–9 months	−0.1	9 months	9	5 days–9 months	+ 0.1
	CXIF	9 months	8	9 days–9 months	−0.15	9 months	8	5 days–9 months	+ 0.1

Table 2 Accuracy and precision of the ^{31}P-NMR assay[a] of CXCP and CXIF

Compound	Concentration (M)	pH	Number of experiments	Mean concentration measured (M)	Accuracy	Precision
CXCP	4.95×10^{-4}	7.0	7	5.18×10^{-4}	+4.6%	± 5.9%
	5.05×10^{-4}	5.5	8	4.65×10^{-4}	−7.9%	± 3.7%
CXIF	4.95×10^{-4}	7.0	7	4.63×10^{-4}	−6.5%	± 7.2%
	4.96×10^{-4}	5.5	8	4.35×10^{-4}	−12.3%	± 7.8%

[a]Measurements were carried out at 4 °C under conditions of full relaxation (TR = 6.08 s)

6.7×10^{-3} M) or in 3 blocks of 102 min over 27 h (solutions 5.0×10^{-4} M). Time points for each spectrum were taken at the midpoint of data acquisition.

To save time for the NMR recording (17 min instead of 51 min), spectra of CXCP and CXIF samples stored at 8°, −20°, and −80 °C were acquired with a TR of 2.08 s, i.e., under conditions of incomplete relaxation. For determination of the true concentrations of CXCP and CXIF, the external standard MPA was thus calibrated against urinary solutions of CXCP and CXIF of known concentration under identical conditions. As the spectra were recorded at 25 °C, we checked that the degradation of CXCP or CXIF was negligible during the NMR recording period (17 min). Indeed, the same degradation kinetics were observed for a solution of CXIF at pH 5.5 (the least stable of the solutions studied) stored at 8 °C whose spectra were recorded at 25° or 4 °C.

Linear least-square fits of pseudo-first-order plots of $ln[CXCP]_t/[CXCP]_{t0}(ln\ C/C_0)$ versus time gave the values for the rate constants. This approximation was also used to calculate the degradation rate constants for CXIF, even if there was a concentration effect on the rates of CXIF decomposition. Indeed, the reaction order was very close to 1 and the rate-constant values were not different from those calculated with the pseudo-first-order approximation. When two measurements were carried out for the same time point, the mean concentration was considered.

The accuracy and precision of the ^{31}P-NMR assay of $\approx 5 \times 10^{-4}$ M solutions (the lower of the two concentrations studied) of CXCP at pH 7.0 and 5.5 or of CXIF at pH 7.0 were $<8\%$ (Table 2). The accuracy was lower for the solution of CXIF at pH 5.5 since this compound is unstable at this pH and was probably slightly degraded during the preparation of the samples. In contrast, the deviation was $<8\%$ for precision as the recording was performed at 4 °C (Table 2).

Stastistical analysis

All results were expressed as mean values ± SD. Statistical significance was determined using Student's t-test. A P value of <0.05 was considered statistically significant.

Results

Stability of CXCP and CXIF at 25 °C in urine

For the reason of accuracy explained below, two sets of experiments were carried out. First, the degradation kinetics of CXCP and CXIF in human urine with pH adjusted to 5.5 or 7.0 to reflect the urinary conditions seen in the patients treated with CP or IF during our previous studies [9, 18]) were continuously monitored in the NMR probe at 25 °C. Second, the percentages of degradation of CXCP and CXIF were evaluated after 24 h of storage at 25 °C and recording of the spectra at 4 °C, the temperature at which degradation is negligible during the NMR recording period.

After 24 h in the NMR probe at 25 °C (Table 3), both CXCP and CXIF at pH 7.0 were relatively stable, showing a statistically nonsignificant difference ($P > 0.05$) in percentage of degradation (about 10%). On the other hand, at pH 5.5 their stability was markedly reduced and CXIF was more unstable than CXCP; nearly 80% of the CXIF and around 50% of the CXCP was degraded ($t_{1/2}$ = 11.6 h for CXIF and 23.9 h for CXCP). These results were obtained for a CXCP and CXIF concentration around 6.5×10^{-3} M. In comparison, the mean concentration of CXIF in the urine of patients treated with a dose of 3 g/m^2 IF injected i.v. over 3 h is around 5×10^{-4} M [9]. To find out whether the rates of degradation of CXCP and CXIF depended on concentration, we followed the degradation kinetics of these two compounds at a concentration of 5×10^{-4} M in urine at pH 5.5 (degradation is faster at this pH than at pH 7) in the NMR probe at 25 °C. Although the rate of degradation of CXCP did not alter over the concentration range of 6.5×10^{-3} M to 5×10^{-4} M ($t_{1/2} \approx 24$ h), that of CXIF was faster at the lowest concentration ($t_{1/2}$ = 9.1 h versus $t_{1/2}$ = 11.6 h at the highest concentration; Table 3). The same was found for the residual percentages of CXCP and CXIF after 24 h in the NMR probe. Almost identical percentages (≈50%) were found for CXCP at both concentrations, whereas for CXIF, less degradation was found (16%) at 5.0×10^{-4} M than at 6.6×10^{-3} M (23%). The rate of degradation of CXCP at pH 5.5 was thus not a function of concentration over the range studied. On the other

Table 3 Stability of CXCP and CXIF in human urine as monitored in the NMR probe at 25 °C

Compound	pH	Concentration (M)	Number of experiments	residual percentage after 24 h (range)	$t½$ in h (range)
CXCP	7.0	6.6×10^{-3}	3	89 (86–91)	
CXIF	7.0	6.6×10^{-3}	3	86 (83–90)	
CXCP	5.5	6.6×10^{-3}	3	48 (46–50)	23.9 (21.3–25.8)
CXIF	5.5	6.7×10^{-3}	4	23 (16–28)	11.6 (9.6–13.5)
CXCP	5.5	5.1×10^{-4}	3	48 (45–51)	24.3 (23.1–25.7)
CXIF	5.5	5.0×10^{-4}	3	16 (10–23)	9.1 (7.1–11.8)

Table 4 Stability of CXCP and CXIF in human urine at 25 °C

Compound	pH	Concentration (M)	Number of experiments	Residual percentage after 24 h of storage at 25 °C (± SD)[a]
CXCP	7.0	6.6×10^{-3}	3	91 ± 2
		5.1×10^{-4}	4	93 ± 4
	5.5	6.6×10^{-3}	3	45 ± 1
		5.1×10^{-4}	7	45 ± 2
CXIF	7.0	6.6×10^{-3}	6	88 ± 3
		1.1×10^{-3}	12	84 ± 4
		5.0×10^{-4}	6	81 ± 4
		2.5×10^{-4}	12	76 ± 5
	5.5	1.0×10^{-2}	12	25 ± 2
		6.7×10^{-3}	6	21 ± 2
		1.0×10^{-3}	10	15 ± 1
		5.0×10^{-4}	7	13 ± 2

[a] ^{31}P-NMR spectra were acquired at 4 °C so as to avoid any degradation during the recording period

hand, we could not conclude that the rate of degradation of CXIF was higher at the lower concentration, as the differences between the $t_{1/2}$ values and the percentages of residual CXIF observed after 24 h at the two different concentrations were not statistically significant ($P > 0.05$) due to uncertainties in the determinations of the half-lives and the residual percentages of CXIF (Table 3).

To find out whether the rate of degradation of CXIF was or was not a function of concentration, we required a more accurate method of evaluating the percentage of degradation. The percentages of residual CXIF and CXCP after 24 h of storage at 25 °C were thus measured at 4 °C, the temperature at which degradation is negligible during the NMR recording period. The results obtained (Table 4) were not significantly different (for those in which comparison was possible) from those observed after 24 h degradation at 25 °C in the NMR probe (Table 3). They indicated that over the concentration range of 6.5×10^{-3} M to 5×10^{-4} M the rate of degradation of CXCP was independent of concentration ($P > 0.05$ between the percentages of residual CXCP) at both pH 7.0 and pH 5.5. However, being more accurate, they showed that the degradation of CXIF was significantly faster at the 5×10^{-4} M concentration ($P < 0.01$ between the percentages of residual CXIF at pH 7.0 and $P < 0.005$ at pH 5.5). This statement was supported by experiments conducted at two additional concentrations (Table 4); indeed, the lower the CXIF concentration, the higher the degradation of CXIF. The percentages of residual CXIF were significantly different at both pH 7.0 [$P < 0.05$ except when concentrations of 1.1 10^{-3} M and 5.0×10^{-4} M were compared ($P < 0.1$)] and pH 5.5 ($P < 0.005$).

In conclusion, at 25 °C in urine, these results showed that (1) the rates of degradation of CXCP and CXIF were higher in urine at acid than at neutral pH: (2) at pH 5.5, CXIF was degraded much faster than CXCP; (3) after 24 h at 25 °C at pH 7.0, CXIF was less stable than CXCP ($P < 0.005$) at $\approx 5 \times 10^{-4}$ M, whereas at $\approx 6.5 \times 10^{-3}$ M there was no difference in stability ($P > 0.05$); and (4) there was a concentration effect on the rate of decomposition of CXIF.

Figure 3 depicts characteristic ^{31}P-NMR spectra of urine samples containing CXCP or CXIF after 24 h of evolution at 25 °C and pH 5.5. CXCP ($\delta = 20.48$ ppm) led to two phosphorated degradation compounds resonating at 1.88 and 1.69 ppm, respectively (Fig. 3A). They were identified by spiking with authentic standards and had the following respective structures: H_2NOC

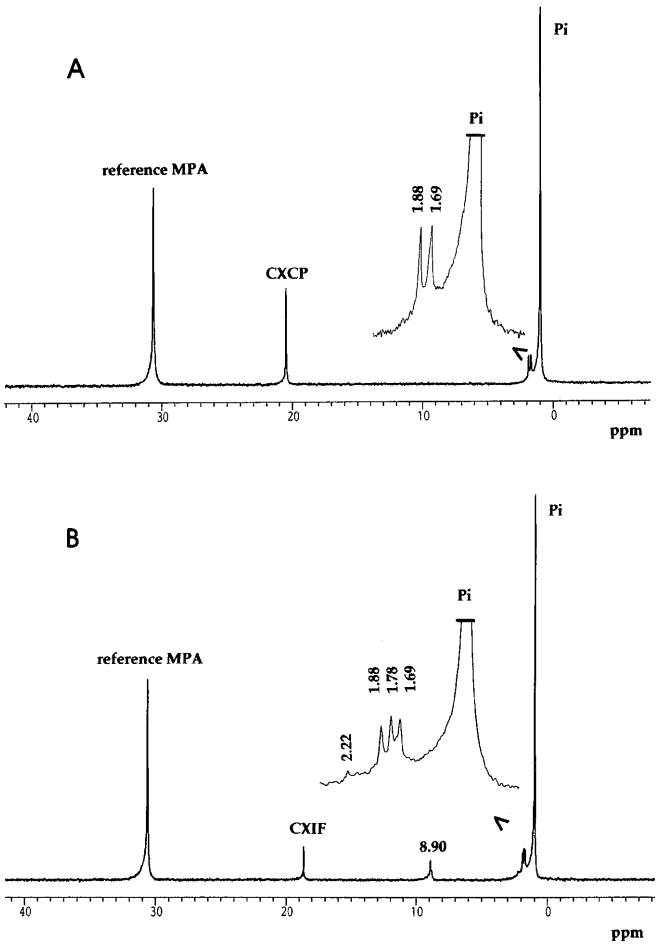

Fig. 3 ^{31}P-NMR spectra of **A** CXCP and **B** CXIF in human urine at pH 5.5 after 24 h evolution at 25 °C. The spectra were recorded at 4 °C and pH 5.7

Table 5 Stability of CXCP and CXIF in urine samples at pH 7.0: residual percentages at various storage times as a function of storage temperature

Storage time	Storage temperature					
	8 °C		−20 °C		−80 °C	
	Residual percentage					
	CXCP	CXIF	CXCP	CXIF	CXCP	CXIF
1 day	93	97				
3 days	97	95				
5 days	92	91	89	94		
9 days	93	89	96	91	95	100
16 days	82	82	92	85		
1 month	80	70	82	74	100	99
2 months	60	37	68	55	91	85
3 months	45	26	58	44	81	80
4 months	36		52	34	79	79
6 months			39	18	73	60
9 months					65	48
Rate constants[a] (day^{-1})	8.5×10^{-3}	16.3×10^{-3}	5.2×10^{-3}	9.4×10^{-3}	1.8×10^{-3}	2.9×10^{-3}
Half-life (days)	81	43	132	74	391	242

[a]All the time points analyzed were not reported in the table, but all were used to calculate the rate constants

$(CH_2)_2OP(O)(OH)_2$ and $HOOC(CH_2)_2OP(O)(OH)_2$. CXIF (δ = 18.66 ppm) gave five phosphorated decomposition compounds whose ^{31}P-NMR signals were located at δ = 8.90, 2.22 (very low intensity), 1.88, 1.78, and 1.69 ppm, respectively (Fig. 3B). The compound resonating at δ = 8.90 ppm remains unidentified. The four other compounds were identified by spiking with authentic standards. They had the following respective structures: $HO(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$, H_2N-$OC(CH_2)_2OP(O)(OH)_2$, $Cl(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$, and $HOOC(CH_2)_2OP(O)(OH)_2$. Endogenous inorganic phosphate (P_i) resonated at 1.01 ppm in both spectra and could obscure P_i coming from the degradation of CXCP or CXIF.

Stability of CXCP and CXIF as a function of the storage temperature of urine samples

It can be seen from the results listed in Tables 5 and 6 that the rate of degradation of both CXCP and CXIF fell with storage temperature at both pH 7.0 and pH 5.5. Figure 4 illustrates this behavior for CXCP. It can be seen that degradation remained quite fast at −20 °C. After storage for 1 month at this temperature the percentage of degradation of CXCP and CXIF was around 20% and 30%, respectively, at pH 7.0 and around 40% and 70%, respectively, at pH 5.5. Even on storage at −80 °C the two compounds were not stable in urine, as after 3 months, 20–25% degradation was observed,

Table 6 Stability of CXCP and CXIF in urine samples at pH 5.5: residual percentages at various storage times as a function of storage temperature

Storage time	Storage temperature					
	8 °C		−20 °C		−80 °C	
	Residual percentage					
	CXCP	CXIF	CXCP	CXIF	CXCP	CXIF
6 hours	93	79				
1 day	86	65				
2 days	84	55	93	82		
3 days	71	45				
5 days	61	29	88	76	91	94
9 days	48	15	79	69		
16 days	29		73	54		
1 month			60	33	94	86
2 months			44	11	87	80
3 months			18		77	76
4 months					78	68
6 months					72	53
9 months					59	39
Rate Constants[a] (day^{-1})	7.3×10^{-2}	20.1×10^{-2}	1.9×10^{-2}	3.5×10^{-2}	1.9×10^{-3}	3.3×10^{-3}
Half-life (days)	9.5	3.5	37	20	364	207

[a]All the time points analyzed were not reported in the table, but all were used to calculate the rate constants

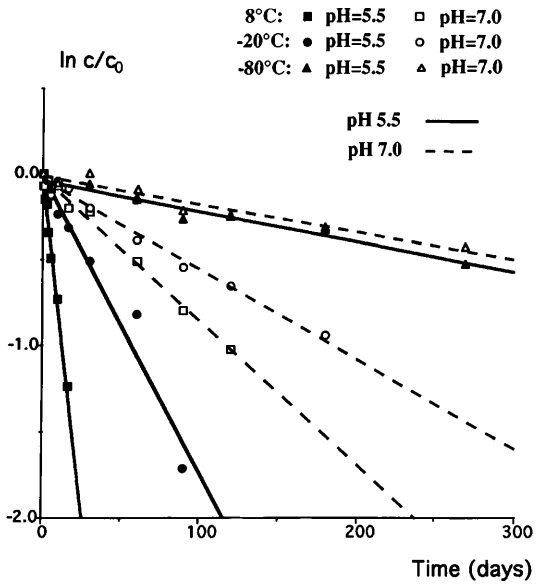

Fig. 4 Degradation of CXCP at pH 5.5 and pH 7.0 as a function of storage temperature

which rose to 30–50% after 6 months, CXCP being more stable than CXIF.

CXCP and CXIF were more stable in urine at pH 7.0 than at pH 5.5, although the difference was less great at the lower temperatures (Tables 5, 6; Fig. 4). The ratio of the degradation rate constants determined at pH 5.5 and 7.0 fell markedly from around 10 at 8 °C to ≈4 at −20 °C and slightly more than 1 at –80 °C. Thus, at –80 °C, CXCP and CXIF were almost equally stable at both values of pH.

CXCP was more stable than CXIF at all three temperatures of storage as illustrated at pH 5.5 in Fig. 5. At

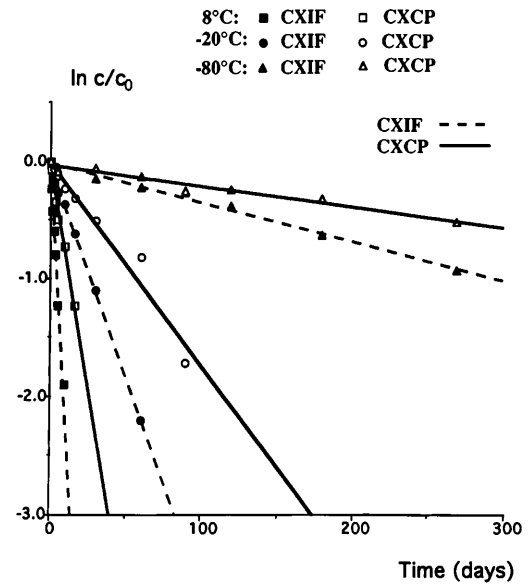

Fig. 5 Degradation of CXCP and CXIF at pH 5.5 as a function of storage temperature

both pH 7.0 and pH 5.5 the ratio of the degradation rate constants, CXIF/CXCP, was around 2, showing a slight fall on decrease in storage temperature.

All these experiments were conducted with concentrations of CXCP and CXIF of 6.7×10^{-3} *M*, i.e., at least 10-fold that encountered in patients' urine. As the rate of degradation of CXIF (as opposed to that of CXCP) is a function of concentration, it should be borne in mind that the stability of CXIF in patients' urine will be lower than that reported herein.

Discussion

It has been known for some time that CXCP is unstable. Jardine et al. [14] first reported in 1978 that CXCP at 0.20 m*M* in plasma or urine decomposed (58% and 13%, respectively) to nornitrogen mustard (NNM) during both storage (overnight after freezing) and the procedures of NNM extraction (pH 8.5 with chloroform) and derivatization (with trifluoroacetic anhydride). Most, if not all, of the decomposition occurred during brief exposure of the sample at pH 8.5, i.e., during extraction of NNM. Jarman et al. [15] reported in 1979 that keeping a 0.68 m*M* solution of CXCP in urine at pH 5.5 for 24 h at room temperature led to 50% decomposition. We obtained a comparable result (50–55% degradation) after maintaining a solution of CXCP in urine at pH 5.5 and 25 °C for 24 h. Ludeman et al. [17] demonstrated in 1992 that the hydrolysis of CXCP (23.3 m*M*) was pH-dependent and was facilitated under acidic conditions. They reported $t_{1/2}$ values of 77 min at 37 °C and pH 4, of 7 days at pH 7.4, and of 23 days at pH 9. More recently, Yule et al. [31] demonstrated the increasing instability of CXCP (0.17 m*M*) with falling pH from 8 to 5.8 after incubation of urine samples for 3 h at 37 °C. The CXCP concentration measured at pH 5.8 was reduced to 14% of that observed at pH 8, the pH of maximal stability of this compound.

The first and sole study of the stability of CXIF is that reported by Hartley et al. [13] in 1994. Using XAD-2 solid-phase extraction cartridges, these authors determined the recovery of CXIF added to blank urine (0.07 and 0.17 m*M*) at pH 12, 6–7, and 2 and room temperature after storage for 0, 4, and 24 h. The optimal recovery was obtained at neutral pH (45%) when the urine samples were extracted immediately after the addition of CXIF to urine, whereas only 27% of CXIF was assayed at pH 12 and no CXIF was detected at pH 2. Their study of temporal stability showed that at pH 6–7, CXIF was stable at room temperature, at least over 4 h, but that it was degraded by one-third after 24 h (CXIF recovery 30%). At pH 7.0 and 25 °C we found that around 20% of CXIF (0.5 m*M*) was degraded in 24 h, which is in line with these results.

Our results are in agreement with the general conclusion of these previous studies that both CXCP and CXIF are not stable at room temperature and are more unstable at acid pH than at neutral pH. Moreover, they

provided more quantitative information on the rates of degradation of CXCP and CXIF in urine samples at pH 7.0 and 5.5 at 25 °C as well as at much lower temperatures. To our knowledge, there is no report on the long-term stability of these compounds at low temperatures. Also, there is no report on the compounds resulting from the degradation of CXIF. Only NNM [14, 17] and a phosphorated compound tentatively assigned to phosphoramidic acid , $H_2NP(O)(OH)_2$ [17], were found during CXCP degradation.

Our data showed that the hydrolysis of P-N bonds in CXCP and CXIF led to four phosphoric acid esters. Two of them were common to both compounds, $HOOC(CH_2)_2OP(O)(OH)_2$ and $H_2NOC(CH_2)_2OP(O)(OH)_2$, resulting from the attack of ammonia (endogenous and/or coming from the breakdown of the CXCP P-NH_2 bond) on carboxyl groups. Two additional compounds were identified in the hydrolysis of CXIF: $Cl(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$, resulting from the attack of chloroethylamine liberated by the hydrolysis of CXIF P-N bonds on the carboxyl group, and a very low level of $HO(CH_2)_2HNOC(CH_2)_2OP(O)(OH)_2$, arising from the hydrolysis of the chloroethyl group of the previous compound.

Three main features of the stability of CXCP and CXIF in urine emerged from the present study: (1) CXCP and CXIF decompose with time in urine at all storage temperatures tested (25°, 8°, −20°, and −80 °C) – although the rate of degradation was a function of temperature, degradation at −20° and −80 °C was nonetheless significant; (2) CXCP is more stable than CXIF in urine at both values of pH (7.0 or 5.5); and (3) CXCP and CXIF are more stable at pH 7.0 than at pH 5.5, although this difference fell with decreasing temperature to be almost negligible at −80 °C .

On the basis of these results it can be seen that to obtain valid estimates of CXCP and CXIF levels in urine, the samples must be collected on ice in 6 to 8-h periods (or, failing that, at room temperature but with freezing following within a few hours), frozen immediately after collection, and stored at −80 °C until analysis. Moreover, the samples should be analyzed within 2 months for CXCP and within 1 month for CXIF. Under these conditions, breakdown will be minimized and the levels of these compounds determined in urine samples would accurately reflect their systemic production.

In quantitative studies of the urinary excretion of CP or IF, urine samples were collected at room temperature over periods of 6–8 h [4, 9, 13, 18, 27, 31] and, in some cases, over periods of 2 [15], 16, or even 24 h [2, 12, 16]. In one study, urine samples were stored at 5 °C after each micturition until the end of the 6-h collection period [3]. Moreover, in all these studies, urine samples were frozen and stored at −20 °C until analysis, apart from those of Jarman et al. [15] and those used in our previous studies [9, 18], which were stored at −30° and −80 °C, respectively. Neither the duration of storage nor the pH of the urine samples was taken into account in any of these studies. It is thus almost certain that the rates of excretion of CXCP and CXIF were underestimated as pointed out by Jarman et al. [15] and Yule et al. [31] for CXCP or by Hartley et al. [13] in the case of CXIF. Moreover, the wide interpatient variation observed in CXCP and CXIF recovery could stem (at least partly) from various and excessively long storage periods prior to analysis [9, 18] and from various urinary pH values, which led to different degradation rates of these compounds when samples were stored at −20 °C.

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