We were surprised by the recent article of Highley et al. (1996) on the chemical instability of ifosfamide (IF) in glass vials, and solutions are prepared freshly before use. Alkylating and DNA cross-linking metabolite. IF is supplied as a water-in-oil microemulsion. It becomes cytotoxic, because ifosfamide mustard is the ultimate alkylating agent with significant activity against numerous solid tumors. In the present study, we show that this instability is not intrinsic to the IF powder or aqueous solutions of IF stemmed from degradation compounds of IF, among them chloroethylamine. In contrast, when the trifluoroacetylation reaction is conducted in ethyl acetate, there is high yield of trifluoroacetylated IF, and degradation compounds are minor. In conclusion, we believe that the large amounts of chloroethylamine reported by the authors in both powder and aqueous solutions of IF stemmed from degradation linked to the method of derivatization. Because IF is not readily derivatized by trifluoroacetylation in dichloromethane, the combination of heating with possible uncontrolled evaporation of solvent and the presence of trifluoroacetic acid in the medium leads to degradation of IF and formation of chloroethylamine.

**ABSTRACT:**

This study is a reply to a paper in this journal reporting on the chemical instability of ifosfamide (IF) (Drug Metab. Dispos. 23, 433–437, 1995). The authors describe chloroethylamine as a major degradation product of IF in both the powder and aqueous solutions. In the present study, we show that: i) IF powder remains pure up to 3–5 years after its expiration date; ii) solutions of IF at pH 7 are stable for at least 12 hr at 40°C; and iii) solutions of IF at pH 4 or pH 10 are only slightly degraded (~1%) after standing for 6 hr at 37°C. We also demonstrate that the reported IF instability depends on the analytical procedure used. The trifluoroacetylation procedure used by the authors, which is conducted in dichloromethane, led to low derivatization yields and to the formation of several degradation compounds of IF, among them chloroethylamine. In contrast, when the trifluoroacetylation reaction is conducted in ethyl acetate, there is high yield of trifluoroacetylated IF, and degradation compounds are minor. In conclusion, we believe that the large amounts of chloroethylamine reported by the authors in both powder and aqueous solutions of IF stemmed from degradation linked to the method of derivatization. Because IF is not readily derivatized by trifluoroacetylation in dichloromethane, the combination of heating with possible uncontrolled evaporation of solvent and the presence of trifluoroacetic acid in the medium leads to degradation of IF and formation of chloroethylamine.

**Materials and Methods**

**Chemicals.** IF (Holoxan) was supplied by Sargent Laboratories (Mérignac, France). The expiration dates of the two old batches of IF analyzed were April 1991 (batch number unknown) and November 1992 (batch 104). The NMR analyses were performed between October 1995 and March 1996. TFAA was purchased from Sigma (St. Quentin Fallavier, France). Chloroethylamine and MPA were purchased from Aldrich (St. Quentin Fallavier, France). FBEN was prepared by titrating 4-fluorobenzoic acid (Aldrich) with a solution of NaOH. Cr(acac)₃ was obtained from Spectrométrie Spin Techniques (Paris, France).

**Derivatizations.** The N-trifluoroacetylation reactions were conducted under the following conditions. IF (1 or 2 mg) was dissolved in ethyl acetate (1.2 ml) or dichloromethane (1.2 ml), and 1.2 ml of TFAA was then added. Three reaction conditions were examined: i) in ethyl acetate in tubes stoppered with a glass marble; ii) in dichloromethane in tubes stoppered with a glass marble; and iii) in dichloromethane in a round-bottomed flask fitted with a cooling system. The samples were then heated at 65°C in a sand bath for 1 hr. In experiment (ii), there was complete evaporation of CH₂Cl₂ before heating for 1 hr. The samples were then dried under a stream of nitrogen in experiments (i) and (iii), and the residue was taken up in ethyl acetate for NMR analysis of all the experiments.

- N-Trifluoroacetylation of chloroethylamine (20 mg) was conducted in ethyl acetate (2 ml) with 1 ml of TFAA. The tube stoppered with a glass marble was then heated at 65°C–70°C in a sand bath for 1 hr. The sample was then dried under a stream of nitrogen and the residue taken up in ethyl acetate for NMR analysis.

**NMR Analysis.** The ¹³C NMR spectra were run on a Bruker ARX 400 spectrometer and chemical shifts (δ) related to external trimethylsilyl propane sulfonic acid.

To study the stability of IF in aqueous solutions, ¹H-decoupled ³¹P NMR spectra were recorded at 161.98 MHz on a Bruker ARX 400 spectrometer with a sweep width of 18,518 Hz, a pulse width of 8 μsec (i.e. flip angle ~ 35°), a repetition time of 1.88 sec, and a number of transients of 300–1,000. The relative concentrations of the phosphorylated compounds observed in the ³¹P NMR spectra were determined from the peak intensities. Chemical shifts were related to external 85% H₃PO₄.

Solutions of IF in ethyl acetate after derivatization with TFAA were doped with Cr(acac)₃ (2.5 mg in 2.5 ml) before ³¹P and ¹⁹F NMR analysis. ³¹P NMR was used to assess underivatized IF, whereas ¹⁹F NMR was used to assess trifluoroacetylated IF and all other derivatized products formed by degradation of IF (assuming that they were all monotrifluoroacetylated). This led to a complete quantitative analysis. ¹H-decoupled ³¹P NMR and ¹⁹F NMR spectra were recorded with a Bruker WB-AM 300 spectrometer in the following instrumental conditions: probe temperature, 25°C; sweep width, 15,151 Hz for ³¹P spectra and 20,833 Hz for ¹⁹F spectra; 32 K data points zero-filled to 64 K; pulse width, 5 μsec (i.e. flip angle ~ 45°) for ³¹P spectra and 6 μsec (i.e. flip angle ~ 45°) for ¹⁹F spectra.
Stability of IF Powder. We recorded the $^{31}$P and $^{13}$C NMR spectra of two old batches of commercial IF. One batch had expired 4.5 years and the other 3 years before the analysis. Spectra of the aqueous solutions of IF prepared from these two batches at a concentration of 3.8 M displayed the sole presence of IF whose spectral characteristics are given in table 1.

To check that the NMR method was sensitive enough to detect the amounts of chloroethylamine reported by Highley et al. (1), we recorded the $^{13}$C NMR spectrum of a 3.8 M solution of IF enriched with 18.7% (w/w) of chloroethylamine, because the amounts of chloroethylamine found by the authors in solutions of IF analyzed immediately after being made up were in the range of 13.6–21.3% of chloroethylamine found by the authors in solutions of IF enriched with 18.7% (w/w) of chloroethylamine, because the amounts of chloroethylamine reported by Highley et al. (1)

Stability of IF in Aqueous Solutions. Using $^{31}$P NMR, we investigated the degradation of buffered aqueous solutions of IF (3.8 × 10$^{-3}$ M and 3.8 × 10$^{-2}$ M) at pH 4 (acetate 0.2 M), pH 7 (cacodylate 0.1 M), and pH 10 (carbonate 0.1 M) warmed to 37°C or 40°C [i.e. in the conditions of pH, temperature, and concentration (3.8 × 10$^{-3}$ M only) reported by Highley et al. (1)].

In contrast to these authors, we did not observe any degradation of IF (3.8 × 10$^{-3}$ M) for 5 hr at 40°C at any of the values of pH tested (4, 7, or 10). Similar results were obtained for solutions of IF (3.8 × 10$^{-2}$ M) at pH 7 for 12 hr at 40°C, or for 6 hr at 37°C. A degradation of 1.1 mol% was observed in solutions of IF (3.8 × 10$^{-2}$ M) left for 6 hr at 37°C and pH 4 leading to two phosphorylated products with $^{31}$P NMR $\delta = 1.1$ ppm and 1.0 ppm, corresponding to 0.7% and 0.4% of the degradation, respectively. After 24 hr, the degradation reached 3.9 mol%, with 3.2% of the compound resonating at 1.1 ppm and 0.7% of that resonating at 1.0 ppm. The degradation product with a $^{31}$P NMR signal at $\delta = 1.1$ ppm was identified as compound III (fig. 1) by spiking with standard whose spectral characteristics are given in table 1. The compound with a $^{31}$P NMR signal at $\delta = 1.0$ ppm was not identified, but it did not correspond to any of the phosphorylated standards I, II (fig. 1), or V ([Cl(CH$_2$)$_2$NH(CH$_2$)$_3$OP(O)(OH)]$_2$O nor to phosphate ion. The solutions of IF (3.8 × 10$^{-2}$ M) at pH 10 gave rise to 0.7% of a single $^{31}$P NMR signal at $\delta = 20.5$ ppm. By spiking with the authentic standard, we showed that, at pH 10, the product formed was the aziridine of IF (table 1), which is in acid-base equilibrium with IF (KOH 2.5 M/HCl 2.5 M) and had been observed but not identified by Kämpfer et al. (2). In view of the slight degradation of 3.8 × 10$^{-2}$ M solutions of IF at pH 4 and pH 10 (≈1%), $^{31}$P NMR spectroscopy is not sensitive enough to assess degradation of the same order of magnitude in 10-fold less concentrated solutions of IF.

These experiments show that: i) IF powder remains pure up to 3–5
years after its expiration date; ii) solutions of IF at pH 7 are stable for at least 12 hr at 40°C; and iii) solutions of IF at pH 4 or pH 10 are only slightly degraded (~1%) after standing for 6 hr at 37°C. These observations are in complete disagreement with those of Highley et al. (1), who reported the presence of large amounts of chloroethylamine in solutions of IF immediately after preparation (13.6–21.3% w/w) or after standing for 6 hr at 37°C (26.0–45.6% w/w). These authors also observed the same degradation products at both acidic and basic pH. On the other hand, our results are in agreement with those of Radford et al. (3), who only observed 7% degradation after leaving a 0.31 M solution of IF for 9 days at 37°C, those of Zon et al. (4) who measured ~50% degradation in 0.1 M solution of IF over a period of 119 days at 37°C, and those of Muñoz et al. (5), who failed to observe any degradation of IF in 0.9% sodium chloride (10–80 mg/ml) over 8 days at either 25°C or 35°C. For aqueous solutions of IF in the pH range of 5–9, Kaijser et al. (6) reported a half-life of 20 hr at 70°C, 254 days at room temperature, and almost 6 years at 4°C.

Explanation of the Results Reported by Highley et al. (1). In a previous study (7), we showed that degradation of IF in aqueous acid medium at 20°C gave rise to four degradation products (fig. 1). After derivatization with TFAA, Highley et al. (1) demonstrated the presence of IF and some of its products of acid degradation, such as chloroethylamine, which they quantified with respect to IF, and 2-chloroethyl-3-hydroxypropylamine arising from cleavage of the PO bond of product III.

The following trifluoroacetylation procedure was used by Highley et al. (1) for the analysis of IF solutions. The first step consisted of extracting an aliquot (volume not specified) of aqueous solutions of IF (5.2–2,000 μg/ml), either previously heated or not, with dichloromethane. After drying over sodium sulfate, filtration and evaporation to a residual volume of 300 ml, an equal volume of TFAA was then added and the mixture heated at 65°C for 1 hr. After evaporation to dryness, the residue was taken up in ethyl acetate for injection into the GC/MS apparatus.

To check that the acid degradation products of IF were extracted into dichloromethane [i.e. the first stage in the process used by Highley et al. (1)], we performed a liquid/liquid extraction of an aqueous solution adjusted to pH 7 containing IF and all its degradation products (I–IV) with dichloromethane (1 volume/3 volumes). The 31P NMR analysis showed that this extracted IF into the organic phase, leaving the four degradation products (I–IV) in the aqueous phase. On the basis of these results, we concluded that the compounds of degradation of IF observed by Highley et al. (1) stemmed from degradation related to the derivatization procedure.

The choice of dichloromethane as solvent for derivatization by TFAA is somewhat surprising, because previous studies on derivatization of IF by TFAA have used ethyl acetate (8, 9). We found that derivatization yields are much lower in dichloromethane than in ethyl acetate (table 2). We tested three conditions for the derivatizations of IF with TFAA by heating at 65°C for 1 hr: i) in ethyl acetate in a tube stoppered with a glass marble (table 2, A and B); ii) in dichloromethane under the same conditions, which led to complete solvent evaporation (table 2, C and D); and iii) in dichloromethane using a cooling system to prevent evaporation of solvent (table 2, E and F). The table 2

<table>
<thead>
<tr>
<th>Reactions in Ethyl Acetate</th>
<th>Reactions in Dichloromethane with Complete Evaporation</th>
<th>Reactions in Dichloromethane with Cooling</th>
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<tr>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Initial IF</td>
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<td>% Recovery</td>
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<tr>
<td>% IF</td>
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<td>95.2</td>
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<tr>
<td>CF2CONH(CH3)2Cl (compound VII)</td>
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<td>0</td>
</tr>
<tr>
<td>% Other trifluoroacetylated compounds (unidentified)</td>
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<td>5.5</td>
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</table>

IF (1 or 2 mg), dissolved in 1,200 μl of ethyl acetate or dichloromethane, was derivatized with 1,200 μl TFAA. Indicated values are the means of two separate experiments, except for the percentage of IF in experiments C and D. 31P NMR was used solely to assay underivatized IF. 19F NMR was used to assay the fluorinated compounds VI, VII, and unidentified products (assuming they were all monotrifluoroacetylated).
reactions were conducted in duplicate with 1 mg (table 2, A, C, and E) or 2 mg (table 2, B, D, and F) of IF. The residues were taken up in ethyl acetate and analyzed by $^{19}$F and $^{31}$P NMR. For derivatization in ethyl acetate, the trifluoroacetyl derivative of IF (compound VI, δ = 5.9 ppm; spectral characteristics in table 1) was the predominant constituent in the $^{19}$F NMR spectrum, along with TFA, and several other unidentified minor derivatization products producing signals in the 0.4/0.2 ppm and −10.8/−11.8 ppm ranges (fig. 2A). The trifluoroacetyl derivative of chloroethylamine (compound VII) resonating at −0.35 ppm was not observed as demonstrated by spiking with the authentic standard. The $^{31}$P NMR spectrum showed complete disappearance of the signal of IF and the sole signal of trifluoroacetylated IF at 0.7 ppm. In a derivatization reaction in dichloromethane without controlling solvent evaporation, the $^{19}$F NMR spectrum (fig. 2B) showed a small amount of compound VI, much TFA along with several other unidentified derivatization products giving signals in the 7.1–7.0 ppm, 6.5–6.3 ppm, and 0.4–0.2 ppm ranges, and a relatively large amount of compound VII (identified by spiking with the authentic standard) with respect to compound VI. The $^{31}$P NMR spectrum exhibited a weak signal of IF at 13.2 ppm in 2 of 4 experiments conducted, and a broad weak unidentified signal at 1.2 ppm in the other two experiments. For a derivatization reaction in dichloromethane with control of solvent evaporation, the $^{19}$F NMR spectrum (fig. 2C) exhibited signals of compound VI, TFA, compound VII, and the same unidentified derivatization products as observed in the previous experiments. The $^{31}$P NMR spectrum exhibited a strong signal of IF and a weak signal of trifluoroacetylated IF in 2 of 4 experiments, or a weak unidentified signal at 0.2 ppm in the other two experiments.

Table 2 lists the proportions of the various products obtained under the three different reaction conditions. In ethyl acetate (table 2, A and B), the total recovery was quantitative. Trifluoroacetylated IF represented >95 mol% of the initial IF and no trifluoroacetylated chloroethylamine was detected. When dichloromethane was allowed to evaporate during the derivatization reaction (table 2, C and D), there was a maximum total recovery of 35 mol% of the initial IF, probably due to the evaporation of the trifluoroacetylated compounds. Trifluoroacetylated IF represented <1 mol% of the initial IF, whereas trifluoroacetylated chloroethylamine was observed in ~10-fold higher amounts (~10 mol% of the initial IF). There was little residual IF, although large amounts of its unidentified trifluoroacetylated derivatization products were observed. If the trifluoroacetylation reaction was conducted in dichloromethane under conditions in which evaporation of solvent was prevented by cooling (table 2, E and F), the overall recovery was nearly quantitative (~80 mol%). Nearly all of the recovery was represented by IF and its unidentified trifluoroacetylated degradation products. Small amounts of trifluoroacetylated IF (2.4–2.9 mol% of the initial IF) and trifluoroacetylated chloroethylamine (0.3–0.4 mol%) were detected, although the molar ratio—derivatized chloroethylamine/derivatized IF—was nonnegligible (13.8–12.5% for experiments E and F, respectively).

In conclusion, we believe that the large amounts of chloroethylamine reported by Highley et al. (1) in both powder and aqueous solutions of IF stemmed from degradation linked to the method of derivatization. Because IF is not readily derivatized by TFAA in dichloromethane, the combination of heating, inadequate control of solvent evaporation, and the presence of TFA in the medium induces degradation of IF with formation of chloroethylamine.

References


2. A. Küpfer, T. Cerny, and J. R. Idle: Intramolecular rearrangement of


