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Safe Disposal of Diisopropyl Fluorophosphate (DFP)

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ABSTRACT

Diisopropyl fluorophosphate (DFP), a volatile highly toxic enzyme inhibitor, in buffer (pH 3, pH 5, pH 7, pH 9, pH 11, Hank's, Dulbecco's, PBS, TBE, and HEPES) or water (10 m*M*), in DMF solution (200 m*M*), and bulk quantities can be degraded by adding 1*M* NaOH. The DFP was completely degraded, as determined by enzymatic assay, and the final reaction mixtures were not mutagenic.

Index Entries: Safety; degradation; stability; enzyme inhibitor; diisopropyl fluorophosphate; dyflos; Isoflurophate.

INTRODUCTION

Diisopropyl fluorophosphate (*1*) (DFP, DIFP, diisopropyl phosphorofluoridate, Dyflos, Isoflurophate) is a practically odorless (*2*), colorless, volatile (bp 73°C 16 mmHg [*2*]) liquid that is widely used as an enzyme inhibitor (*3–8*). It is also used to treat glaucoma (*9*). DFP is highly toxic (LC_{50} 360 μg/L for rats, LD_{50} 500 μg/kg for rabbits) with a toxicity comparable to hydrogen cyanide (*2*). Because of the volatile nature of this compound and because DFP is a cholinesterase inhibitor (*9,10*) and a

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neurotoxin (*11*), it is important to have validated procedures for the decontamination of equipment and the safe disposal of this compound in reaction mixtures, reagent preparations, and bulk quantities.

By analogy with the nerve gases Sarin (GB) (*12–15*) and Soman (GD) (*15*), which also possess a P—F bond, DFP should be readily hydrolyzed by strong base, presumably to diisopropylphosphate (*16*). Strong base has been recommended for degrading DFP (*1,2,17*), but validation procedures are not given so the efficacy of these procedures is unknown. We decided to investigate the use of 1*M* NaOH solution for the destruction of DFP. Residual DFP was determined by its inhibitory effect on chymotrypsin.

MATERIALS AND METHODS

Warning! DFP is a volatile, highly toxic liquid, and it should be handled only in a properly functioning chemical fume hood. Appropriate protective clothing should be worn at all times.

Materials

BTEE, α-chymotrypsin (Type II from bovine pancreas, cat C 4129), MOPS, Tris base, HEPES, Triton X-100, and some buffers (*see below*) were obtained from Sigma Chemical Co., St. Louis, MO. DFP and all other reagents were obtained from Aldrich Chemical Co., Milwaukee, WI. The DFP was stored in the refrigerator. It was supplied in a vial with a septum, and portions were removed as required using a Hamilton 100-μL syringe.

Solutions of DFP in buffer and water (10 m*M*) were prepared by dilution of a 200 m*M* stock solution of DFP in dimethylformamide (DMF). Solutions of DFP in DMF were prepared fresh each week, and solutions of DFP in MOPS for the analytical procedure were prepared immediately before use. The BTEE solution was prepared by adding 1 mL of a 100-m*M* solution of BTEE in methanol to 200 mL of a 100 m*M* pH 7.8 Tris buffer containing 100 m*M* calcium and 0.2% of Triton X-100. The solution should be prepared at room temperature immediately before use, but is useable for several hours. Chymotrypsin solutions were prepared fresh each week and stored in the refrigerator. The MOPS buffer was 100 m*M*, pH 6.5, and the MOPS/Ca buffer contained 100 m*M* MOPS and 100 m*M* $CaCl_2$ adjusted to pH 6.5.

Hank's Balanced Salts (Hank's) (Sigma H 6136, pH 6.4), Dulbecco's phosphate buffered saline (Dulbecco's) (Sigma D 5773, pH 7.2), and phosphate-buffered saline (PBS) (Sigma 1000-3, pH 7.2) were purchased from Sigma. Hank's was 0.78 m*M* in phosphate, and Dulbecco's and PBS were 10 m*M* in phosphate. The 50-m*M*, pH 8.0, Tris-Borate-EDTA (TBE) buffer was prepared by diluting Tris-EDTA-Borate concentrate (Sigma TEB-9) 1 to 27. The pH 3 and 5 buffers were 50 m*M* phthalate buffers, and the pH 7 buffer was a 50 m*M* phosphate buffer. The pH 9 buffer was 50

m*M* borax (actual pH 9.1), and the pH 11 buffer was 50 m*M* sodium carbonate. The pH 7.5 50 m*M* HEPES buffer contained 500 m*M* NaCl.

Destruction Procedures

To each 1 mL of 10 m*M* DFP in buffer or water, add 200 μL of 1*M* sodium hydroxide solution and check that the reaction mixture is strongly basic, pH ≥12. Allow to stand at room temperature for 18 h, analyze for completeness of destruction, neutralize by the addition of 100 μL of glacial acetic acid, and discard it.

To each 1 mL of 200 m*M* DFP in DMF, add 2 mL of 1*M* sodium hydroxide solution and check that the reaction mixture is strongly basic, pH ≥12. Allow to stand at room temperature for 18 h, analyze for completeness of destruction, neutralize by the addition of 500 μL of glacial acetic acid, and discard it.

To each 40 μL of pure DFP, add 1 mL of 1*M* sodium hydroxide solution and check that the reaction mixture is strongly basic, pH ≥ 12. Stir the reaction mixture for 1 h at room temperature, analyze for completeness of destruction, neutralize by the addition of 200 μL of glacial acetic acid, and discard it.

To decontaminate spills or equipment, add at least 1 mL of 1*M* sodium hydroxide solution for each 40 μL of DFP that is estimated to be present. Add more NaOH if required to wet all contaminated surfaces thoroughly. Check that the reaction mixture is strongly basic, pH ≥ 12, and that all the oily DFP has dissolved. Allow to stand at room temperature for at least 2 h and preferably 18 h, analyze the solution for completeness of destruction, neutralize by the addition of 200 μL of glacial acetic acid for each 1 mL of NaOH used, and discard it. Decontamination is faster if it is possible to agitate or stir the reaction mixture, e.g., when the inside of a flask is being decontaminated. Clean the equipment or the spill area in a conventional fashion.

Analytical Procedures

The analytical system measures the rate at which *N*-benzoyl-L-tyrosine ethyl ester (BTEE) is hydrolyzed by chymotrypsin by determining the increase in absorption at 256 nm (*18,19*). DFP inhibits the activity of chymotrypsin, and so the rate of hydrolysis acts as an indicator for the presence or absence of DFP.

The absorbance of the analytical samples was determined at 256 nm using quartz UV cells in a Perkin Elmer Lambda 2 UV/VIS spectrometer fitted with a 13-cell changer. The cells were cleaned with chromic acid, which was discarded appropriately (*20*). If required the spectrometer could be programmed to record the absorbance of each cell periodically, so that the progress of the reaction could be followed graphically. The temperature in the cell compartment was 28°C.

The analytical samples are prepared as follows. Add 100 μL of the reaction mixture to be tested (40 μL for reactions involving neat DFP) to 1 mL of MOPS/Ca buffer. (Buffers that contain phosphate [particularly pH 7 buffer, Dulbecco's buffer, and PBS] may produce a precipitate of calcium phosphate that will interfere with the spectrophotometric determination. If this is the case, add 0.5 mL of the reaction mixture to 5 mL of buffer, allow to stand for several hours, and then centrifuge for 5 min. Use 1.1 mL of the supernatant.) To each buffered analytical sample, add either 100 μL of MOPS buffer (for unspiked samples) or 100 μL of 3.1 μg/mL DFP in MOPS buffer (for spiked samples). Finally, add 100 μL of a 0.1 μg/mL solution of chymotrypsin of 1 m*M* HCl. Allow the mixture to stand at room temperature for 1 h, then add 3 mL of BTEE solution, place this mixture in a quartz UV cell, and immediately determine the absorbance. An air blank is used to avoid problems with spontaneous hydrolysis and bubble formation. Leave the mixture in the cell, and after 16–20 h, again determine the absorbance. Calculate the rate of hydrolysis (R_{rm}) in mAU/h.

The rate of hydrolysis of BTEE when the reaction mixture is present should be compared with the rate of hydrolysis of BTEE when a reaction mixture blank is present (R_{rb}). Add 50 μL of DMF to 950 μL of buffer, and then add 200 μL of 1*M* sodium hydroxide solution. Allow this mixture to stand at room temperature for 18 h, then take 100 μL of this mixture, and analyze it as described above.

The blank for measuring the spontaneous hydrolysis rate (R_{sp}) consists of 100 μL of 0.2*M* sodium hydroxide solution, 1 mL of MOPS/Ca buffer, 100 μL of MOPS buffer, and 100 μl of 1 m*M* hydrochloric acid. After 1 h add 3 mL of BTEE solution and measure the rate of increase of absorbance as described above.

A measure of the activity of the chymotrypsin when the DFP degradation reaction mixture is present is $A_{rm} = R_{rm} - R_{sp}$. Similarly, a measure of the activity of the chymotrypsin when no DFP degradation reaction mixture is present (but a reaction blank is present) is $A_{rb} = R_{rb} - R_{sp}$. Thus, if the DFP has been completely degraded $A_{rm} = A_{rb}$, but if a large amount of DFP is present $A_{rm} = 0$. The limit of detection for DFP was arbitrarily set at the concentration that would the inhibit the activity of the chymotrypsin by 50%, i.e., $A_{rm} = 0.5 \times A_{rb}$. Note that this does not represent a 50% decrease in the initial concentration of DFP, because the initial concentration of DFP was many times that needed to inhibit the chymotrypsin completely. Spiking experiments were used to determine the limit of detection. The rate of hydrolysis when a reaction mixture spiked with 100 μL of 3.1 μg/mL DFP in MOPS buffer was used is R_{rs}, and the activity of chymotrypsin when a reaction mixture spiked with DFP is added is $A_{rs} = R_{rs} - R_{sp}$. If the analytical system is working satisfactorily, A_{rs} should be $< 50\%$ of A_{rm}.

When carrying out the procedures described above, it was found that the rate of hydrolysis of BTEE (and hence the rate of increase in absorbance)

Table 1
Destruction of Undiluted DFP and DFP Dissolved in Water and Buffers

	A_{rm}/A_{rb}, %		A_{rs}/A_{rm}, %	
Buffer	Mean	SD	Mean	SD
pH 3	104.8	13.6	46.5	15.3
pH 5	97.0	10.6	47.1	8.2
pH 7	126.7	23.7	39.2	11.4
pH 9	96.5	17.7	33.1	10.2
pH 11	99.3	10.7	43.8	12.0
Hank's	98.4	9.2	31.6	12.3
HEPES	106.7	8.2	27.9	7.4
Tris	100.0	11.3	29.8	11.7
PBS	104.6	8.6	37.8	6.5
Dulbecco's	103.7	13.6	28.8	7.1
Water	107.1	9.2	39.9	5.0
Undiluted	97.8	17.9	38.4	3.8
Average of all buffers	103.6	12.8	37.0	9.2

was linear. This was checked periodically by measuring the absorbance every hour and graphing the readings. The result was a straight line. If the concentration of the enzyme was too high, the graph rose sharply and then leveled off as all the BTEE was consumed. If the concentration of enzyme was too low, the graph differed little from that for spontaneous hydrolysis.

Mutagenicity Tests

Reaction mixtures were neutralized by the addition of glacial acetic acid and tested for mutagenicity using the plate incorporation technique of the *Salmonella*/mammalian microsome mutagenicity assay as previously described (*21*). DFP itself was tested for mutagenicity as a solution in DMF.

RESULTS AND DISCUSSION

When solutions of DFP in buffer or water were degraded as described above in the Destruction Procedures section, no DFP remained in the final reaction mixtures (Table 1). Each value in the table is the mean of six runs (after outliners were removed [22]). Table 1 shows that the activity of chymotrypsin when the reaction mixture was added was the same as the activity of chymotrypsin when a blank was added (A_{rm}/A_{rb} = 100%). When a small amount (0.31 μg) of DFP was added to an aliquot of the

reaction mixture, the activity of chymotrypsin was reduced by at least 50% ($A_{rs}/A_{rm} \leq 50\%$). Since 100 μL of reaction mixture were used in the analytical procedure, this means that the concentration of DFP was <3.1 μg/mL. The initial concentration of DFP in buffer was 10 m*M* (1.84 mg/mL). Dilution with NaOH made the starting concentration of DFP 1.53 mg/mL, and so the amount of DFP in the final reaction mixture was <0.2% of the initial amount.

In a similar fashion, stock solutions of DFP in DMF can be degraded by the addition of NaOH. The quantity of DFP remaining was <0.03% of the initial amount.

When neat DFP was allowed to react with 1*M* NaOH, there was no significant inhibitory activity after stirring for 1 h. When the reactants were allowed to stand together with no agitation (as would be the case when spills or equipment was being decontaminated), decontamination was slower because the oily DFP tends to stick to the side of the reaction vessel and is slow to go into solution, but no significant inhibitory activity was seen after 2 h. In these experiments, the entire reaction mixture, not just an aliquot, was taken up in the MOPS/Ca buffer by adding the buffer to the reaction flask and agitating the resulting mixture. Thus, if any DFP remained on the sides of the flask, it would have been detected by the analytical procedure. The quantity of DFP remaining was <0.02% of the initial amount. In practice, however, because of the inherent variability of these systems, it might be prudent to let 18 h elapse before discarding the reaction mixture.

In almost all cases, the reaction mixtures were not mutagenic. In a few instances, slight mutagenic activity toward strains TA1530 and TA1535 of *Salmonella typhimurium* with and without activation was seen when DFP in TBE and Dulbecco's buffers was degraded, but the activity was only slightly greater than the level of significance (twice the mean of the control values). In addition the effect was not reproducible, and mutagenic activity was sometimes detected in control reactions in which DFP was omitted. DFP itself was mutagenic to strains TA100, TA1530, and TA1535 with and without activation. A dose–response effect was seen.

Table 2 shows the time required until DFP in buffer was completely degraded to the limit of detection. This was defined as the time at which the addition of buffer solution containing DFP did not inhibit chymotrypsin activity by >50%. At this point, the amount of DFP was <0.17% of the initial amount. The stability of DFP is pH-dependent and decreases markedly as the pH increases. DFP is somewhat less stable in pH 7 buffer than in PBS or Dulbecco's buffer (both pH 7.2), but this may be because of the higher phosphate concentration in the pH 7 buffer (50 m*M*) than in the other buffers (10 m*M*). The hydrolysis of the related compounds Sarin and Soman is accelerated by the addition of acetate ion (23).

Table 2
Degradation of a 1-m*M* Solution of DFP in Buffer

Buffer	pH	Time required until DFP was completely degraded to the limit of detection, h
pH 3	3.0	>330
pH 5	5.0	>330
Hank's	6.4	>330
pH 7	7.0	168
PBS	7.2	330
Dulbecco's	7.2	330
HEPES	7.5	168
Tris	8.0	168
pH 9	9.1	18
pH 11	11.0	4

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