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Note

Investigation of the isolation of a mixed disulphide, diethylthiocarbamoyl 2-pyridyl disulphide by analytical and preparative high-performance liquid chromatography

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Recent studies have demonstrated that the use of C_{18}-microparticulate silica, which is packed in flexible-walled cartridges that can be subjected to radial compression, allows the rapid and effective separation of peptide and protein mixtures[1–5]. In addition to these reversed-phase studies, we and other workers[6,7] have discovered that preparative normal phase chromatography can also be successfully carried out with the corresponding silica cartridges. This technique clearly has potential for the rapid isolation of materials of limited stability, such as the isolation of the unsymmetrical disulphide which is described in this report.

In a previous communication[8], it has been reported that certain symmetrical disulphide compounds have quite different effects on hepatic aldehyde dehydrogenase. On the one hand, disulfiram (tetraethylthiuram disulphide) is a potent inactivator of the enzyme. Small concentrations of this compound react rapidly with aldehyde dehydrogenase by a disulphide-exchange mechanism with consequent loss of activity[9]. This phenomenon has been widely utilised in therapy against alcoholism — a patient taking disulfiram experiences a very unpleasant reaction if he drinks ethanol, largely due to the build-up of acetaldehyde which normally would be metabolised by aldehyde dehydrogenase[10]. On the other hand, 2,2′-dithiodipyridine is an activator of the enzyme, again reacting probably by a disulphide-exchange process. Aldehyde dehydrogenase pre-modified by 2,2′-dithiodipyridine is largely protected against the inactivating effect of disulfiram. However, when the enzyme is presented with an equimolar mixture of the two modifiers, an extensive loss of activity results, showing that the enzyme has a greater affinity for disulfiram than for 2,2′-dithiodipyridine. We thought it would be interesting to investigate the effect on aldehyde dehydrogenase of the mixed disulphide analogue of disulfiram and 2,2′-dithiodipyridine, *i.e.*, diethylthiocarbamoyl 2-pyridyl disulphide. The present paper describes the rapid isolation of this disulphide by preparative normal-phase high-performance liquid chromatography.

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EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for the analytical separations. This consisted of two M6000A solvent delivery units, a M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to a M450 variable-wavelength UV spectrophotometer (Waters Assoc.) and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). A μPorasil column (10 μm, 30 cm × 4 mm I.D.) purchased from Waters Assoc. was used for all analyses. Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.).

The preparative separations were carried out on a Waters Assoc. Prep LC/System 500 instrument with a built in refractive index detector and recorder. A M450 variable-wavelength detector (Waters Assoc.) was connected in series with the refractive index detector, and coupled to an Omniscribe two-channel recorder (Houston Instruments). A Waters Assoc. Prep-Pak-500 silica cartridge (mean particle size 75 μm; 30 × 5.7 cm) was used for the purification. Sample injections were made using a Gastight 1010W syringe (Hamilton).

For both analytical and preparative work solvents were filtered using a Pyrex filter holder (Millipore, Bedford, MA, U.S.A.) while samples were filtered using a Swinney Filter (Millipore). Millipore HA grade, 0.45-μm filters were used at all times for solvent and sample preparation, except for filtration of the methanol when a Millipore FH grade filter was used.

Materials

The following compounds were obtained from the sources indicated: disulfiram and 2,2′-dithiodipyridine, Sigma (St. Louis, MO, U.S.A.); 2-pyridinethiol, Aldrich (Milwaukee, WI, U.S.A.); sodium diethyldithiocarbamate, BDH (Poole, Great Britain). Dichloromethane was obtained from BDH and distilled before use.

Methods

The equilibrium between disulfiram, 2,2′-dithiodipyridine, 2-pyridinethiol and diethyldithiocarbamate. The equilibration between these species was investigated as follows. Sodium diethyldithiocarbamate (2 m*M* in water, 0.1 ml) and 2,2′-dithiodipyridine (1 m*M* in ethanol, 0.1 ml) were mixed in 2.8 ml phosphate buffer (pH 7.3, 0.01 *M*). The increase in absorbance at 342 nm (due to the liberation of the 2-pyridinethiolate ion) was followed at room temperature using a Perkin-Elmer 124 spectrophotometer. Alternatively, disulfiram (1 m*M* in ethanol, 0.1 ml) and 2-pyridinethiol (2 m*M* in water, 0.1 ml) were mixed in the same buffer and the decrease in absorbance at 342 nm was followed.

Synthesis of diethylthiocarbamoyl 2-pyridyl disulphide. 2-Pyridinethiol (2.22 g, 0.02 mol), sodium hydroxide (0.8 g, 0.02 mol) and sodium diethyldithiocarbamate (4.5 g, 0.02 mol) were dissolved in water (100 ml). Potassium iodide solution containing iodine (5.08 g, 0.02 mol) was added. The resulting insoluble material was extracted into chloroform, the chloroform solution was dried over magnesium sulphate and evaporated under reduced pressure to give a yellow oil.

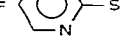
HPLC conditions. Analytical HPLC was carried out at a flow-rate of 1.5

ml/min using dichloromethane as the mobile phase. All chromatography was carried out at room temperature (*ca.* 22°C). Samples were dissolved in dichloromethane at a concentration of 5 mg/ml and 10–25 μl volumes injected on to the columns. Similarly 25-μl aliquots from the preparative HPLC runs were analysed. For the preparative separations, a flow-rate of 150 ml/min was maintained (back pressure 100 p.s.i.). The dichloromethane was degassed by vacuum aspiration before use. The crude sample was loaded in amounts of 5 g in 10 ml of eluent.

RESULTS AND DISCUSSION

It was intended to synthesize diethylthiocarbamoyl 2-pyridyl disulphide by the iodine oxidation of a mixture of sodium diethyldithiocarbamate and sodium 2-pyridinethiolate:

$$A^- + B^- \xrightarrow{KI_3} A\text{–}A + A\text{–}B + B\text{–}B$$

where $A = (C_2H_5)_2N \cdot CS \cdot S$ and $B =$ 

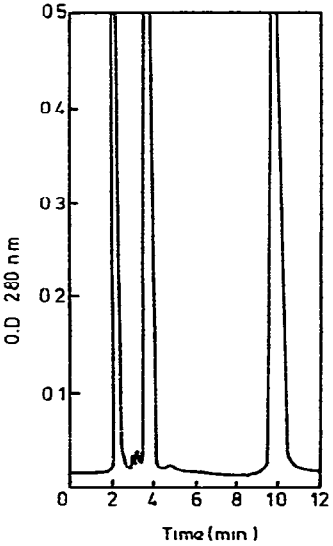
If A^- and B^- are oxidised equally readily then a statistical distribution of the products would be expected (25% A–A, 50% A–B, 25% B–B). On the other hand if A^- (say) was oxidised very much more readily than B^- we would expect, in the reaction above, rapid production of A–A, followed subsequently by B–B, with a very low yield of the mixed disulphide. (Likewise of course, B–B might be preferentially formed first.) To help clarify whether we could expect an appreciable amount of A–B we performed the equilibration experiments described in *Methods*.

It was found that diethyldithiocarbamate reacted with 2,2′-dithiodipyridine to give 39% of the 2-pyridinethiol which would be released if the reaction went to completion. Equilibrium was reached in approximately 12 min. In the other direction, it was found that 2-pyridinethiol reacted with disulfiram to reach a similar equilibrium position (a limiting 2-pyridinethiol concentration of 45% of the initial value) after approximately 25 min. In other words, disulfiram and 2,2′-dithiodipyridine are evidently of very similar oxidation potential. (If they were identical in this regard a figure of 50% would be obtained in the equilibration experiments.) Therefore it was concluded that in oxidising an equimolar mixture of diethyldithiocarbamate and 2-pyridinethiol we could indeed expect to produce a substantial amount of the required mixed disulphide (close to the statistically calculated amount).

The preparation was then carried out using the procedure described in *Methods*. At the same time an analytical normal-phase HPLC separation was developed using authentic samples of the disulphides used in the preparation. Maximal resolution of the two disulphide standards, namely disulfiram and 2,2′-dithiodipyridine, was important since it was anticipated that the required mixed disulphide would elute from the column with a retention time intermediate between that of the standards. This assumption was based on a consideration of the polarity of the sample. A variety of mobile phase conditions were tested by varying the ratios of the two most suitable solvents dichloromethane and chloroform. For maximum resolution of the standards dichloromethane was chosen as eluent. Since the ultimate purpose of the analytical

HPLC was to develop a scale-up to preparative HPLC, a single phase solvent had a number of advantages. These advantages included ease of concentration of the purified product by rotary evaporation and ease of recovery of the solvent. Fig. 1 shows the analysis of a sample of the bright yellow crude product from the potassium iodide oxidation of 2-pyridinethiol and sodium diethyldithiocarbamate. The first peak, eluting at 2.1 min corresponds to the 2,2′-dithiodipyridine, the peak at 3.9 min to the mixed disulphide diethylthiocarbamoyl 2-pyridyl disulphide and the peak at 10 min to the disulfiram. Such a separation, achieving baseline resolution of all components lends itself well to chromatography of very large amounts.

Using the procedure described in *Methods*, approximately 10 g of diethylthiocarbamoyl 2-pyridyl disulphide were formed. The preparative separation of the crude mixture was then carried out using the Prep LC system 500 and a single silica cartridge. Equilibration of the cartridge was rapid and economical in terms of solvent used, due to the ease of recycling the equilibration solvent. Fig. 2 shows the preparative separation of 5 g of the crude mixture. The numbers on the chromatogram represent collected fractions. As can be seen in Fig. 2B, the compounds being chromatographed were ideally suited to detection by refractive index changes. The use of an UV detector allowed a ready comparison of the preparative elution profile with the chromatogram obtained on the analytical system, which used only UV detection. The run



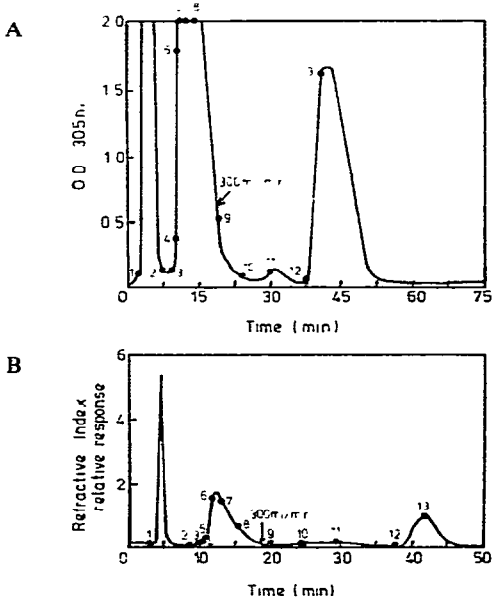


Fig. 1. The analysis of the crude product formed by oxidation of a mixture of 2-pyridinethiol and sodium diethyldithiocarbamate. The mixture was analysed on a μPorasil column with dichloromethane as the mobile phase, at a flow-rate of 1.5 ml/min.

Fig. 2. The preparative separation of 5 g of the crude disulphide mixture. The sample was loaded in 10 ml of dichloromethane. The mobile phase was dichloromethane, and the flow-rate was maintained at 150 ml/min until after the second peak had eluted, when it was increased to 300 ml/min. A, The trace obtained on the UV detector; B, that obtained from the refractive index detector. The detectors were coupled in series. The numbers adjacent to the dots refer to the fraction which was collected until the next dot.

was complete in 50 min, with the mixed disulphide product being completely eluted after 20 min.

Immediately after collection, aliquots of each fraction were subjected to analytical HPLC. Fig. 3 shows a composite of chromatograms obtained from each analysis. Chromatogram 1 is obviously due to 2,2′-dithiodipyridine which is absent in fractions 2 and 3. The impurities eluting before the required product peak are evident in chromatograms 3–5, while the product is beginning to appear in chromatogram 5. Fractions 6–8 show the diethylthiocarbamoyl 2-pyridyl disulphide with the most pure portion being fraction 8. The disulfuram appears as expected in the colourless frac-

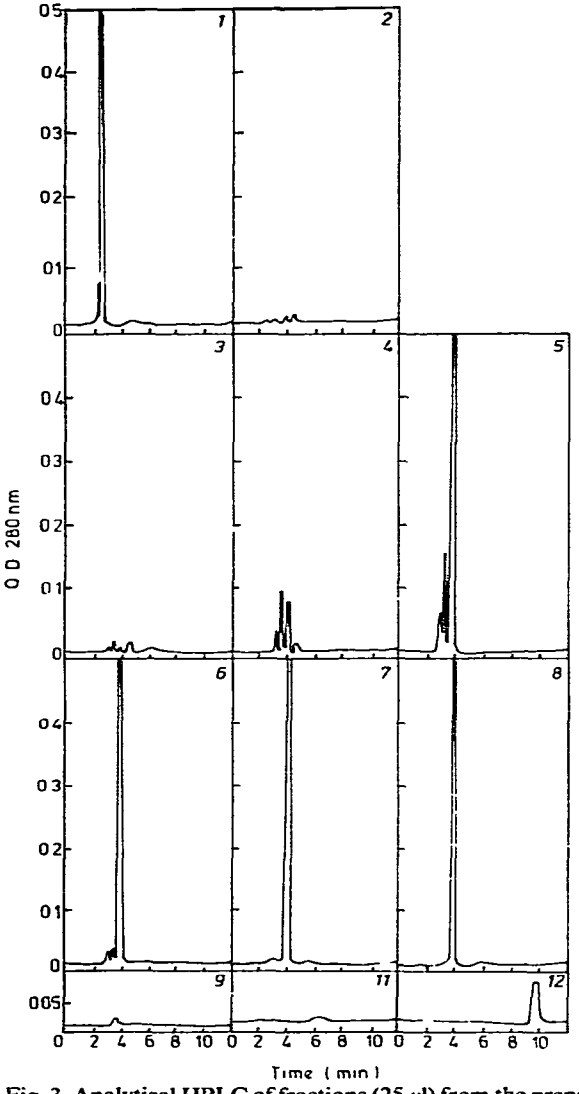


Fig. 3. Analytical HPLC of fractions (25 μl) from the preparative run shown in Fig. 2. The chromatographic conditions used are described in Fig. 1.

tions 12 and 13, the latter of which is not shown. The most significant feature of Fig. 3 is the high degree of purity of the three separate products obtained using preparative HPLC. It is apparent that the disulphide of interest, shown in fractions 7 and 8, has been purified to homogeneity in multigram quantities within 20 min of injection.

The rapid preparative separation described in this study is particularly important for solutes, such as mixed disulphides, which can exhibit limited stability and thus can not be isolated by slower, conventional chromatographic techniques. If the purified mixed disulphide was left in solution at room temperature, the solution gradually changed from colourless to yellow. If a sample of pure mixed disulphide was concentrated by removal of dichloromethane under reduced pressure on a rotary evaporator and then analysed by HPLC, the elution profile shown in Fig. 4 was obtained. Clearly the diethylthiocarbamoyl 2-pyridyl disulphide had disproportionated to reform disulfuram and 2,2′-dithiopyridine, according to the reaction scheme presented earlier. Unfortunately the lack of stability of the mixed disulfide made its handling difficult, and precluded normal concentration of the fractions after the preparative separation. At the same time, it is likely that HPLC with radial compression of flexible-walled cartridges is the only preparative chromatographic technique which is sufficiently rapid to allow isolation of this mixed disulphide without significant disproportionation occurring during the separation.

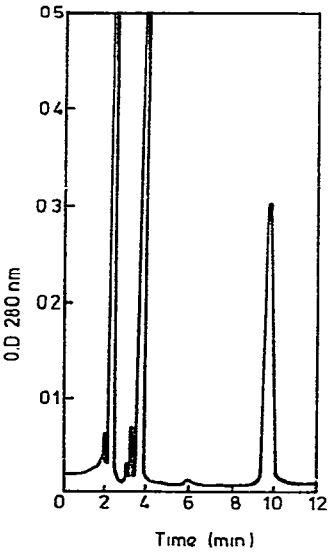


Fig. 4. Analytical HPLC of a sample of the purified diethylthiocarbamoyl 2-pyridyl disulphide after concentration by rotary evaporation under reduced pressure.

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