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Analytical Problems with Putrefaction in a Fatal Case Involving Ergotamine and Pentazocine

A drug addict was found dead under suspicious circumstances, but an investigation immediately afterwards did not reveal any drugs in the blood or any pathological indication to explain the cause of death. Six weeks after death a toxicological reinvestigation was requested because of new evidence. By that time, blood decomposition had already started and interfered heavily with normal extraction procedures. This necessitated the use of an acid hydrolysis procedure which revealed the presence of pentazocine and ergotamine, the latter in the form of specific degradation products. This case is of interest as it emphasizes the problems related to the binding of basic drugs to blood proteins or cells, thus rendering them unavailable for solvent extraction procedures. In addition, a search of the literature failed to reveal any other fatal case in which ergotamine was reported to be detectable in postmortem blood.

Case History

According to testimony, a 29-year-old male known to be a drug addict had been using alcohol and marihuana at a bar before going home at 3 a.m. At that time he seemed to be in good control of himself and used a bicycle for transportation. His body was found 35 h afterwards at the bottom of a staircase in his home.

Pathology and Toxicology

Autopsy revealed some minor external bruises and minor internal hemorrhages in various parts of the body including the brain. These injuries could have been the result of a fall down the staircase or other things such as a fight. The possibility that the body had been placed at the bottom of the staircase intentionally so as to simulate a fatal fall also existed. No external blood loss could be seen and there were no needle marks.

A systematic toxicological analysis on the blood and the urine [*1*] did not reveal the presence of any drug. The blood alcohol concentration was 19 mg/100 ml. Six weeks after death new evidence became available indicating that the deceased might have been under the influence of lysergic acid diethylamide (LSD) of questionable quality. This prompted a toxicological reinvestigation. At this time the blood was in a state of decomposition and had a jelly-like appearance. Plasma could not be separated and usual extraction procedures on the whole blood did not provide suitable extracts. It was decided

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to subject the blood to acid hydrolysis [*1*], although this could lead to the destruction of labile drugs, if they were present. To 20 g of the decomposed blood were added 20 ml 6*N* hydrogen chloride and 40 ml of distilled water and the mixture was digested in a boiling water bath for 1 h. After cooling, the slurry was centrifuged for 20 min at 4000 rpm and the clear supernatant was decanted, adjusted to pH 1.5 with 4*N* ammonia, and extracted twice with equal volumes of ether. The aqueous phase was then adjusted to pH 9.5 with 15*N* ammonia and extracted twice with equal volumes of chloroform. An additional extraction with chloroform was carried out after adjusting the pH of the aqueous phase to pH 12.0 with 4*N* sodium hydroxide solution. The respective organic extracts were evaporated to dryness under nitrogen and the residues were each dissolved in 250 μl of ethanol. Aliquots were tested by gas chromatography (GC) and thin-layer chromatography (TLC). Ultraviolet (UV) spectra of the acidic fraction were recorded in ethanol, whereas UV spectra of the alkaline fractions were recorded in 0.1*N* sulfuric acid solution.

Gas chromatography was carried out on glass columns, 1.80 m by 2 mm inside diameter, filled with 3% SE-30 or 3% SP-2250 on Chromosorb G-HP 80-100 mesh. The injection ports were fitted with glass liners and kept at 300°C. Column temperature was 225°C and flame ionization detectors were kept at 300°C. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. The acid etheral extract did not show any drugs under these conditions, nor could drugs be detected in the UV spectra or in thin-layer chromatograms (silica gel with chloroform-acetone 90 + 10 as solvent). The alkaline (pH 9.5) chloroform extract showed a large peak after 6.6 min on SE-30, with a relative retention time to codeine of 0.78. The large peak had a shoulder at 6.1 min. A smaller peak appeared at 4.5 min, relative retention to codeine 0.54. These peaks were thought to be exogenous, whereas a number of peaks with shorter retention times were considered to be endogenous, based on observations on simultaneously analyzed blood samples which were drug-free.

The GC on SE-30 is shown in Fig. 1. On SP-2250 the major peak shifted to a retention time of 19.5 min. Ultraviolet spectra and TLC (silica gel with methanol-concentrated ammonia 100 + 1.5 as solvent) on the basic extracts were inconclusive and as the GC information did not match any drug in our reference system, aliquots of the extracts were analyzed on a computerized gas chromatography/mass spectrometry (GC/MS) system (Finnigan Model 3200). The chemical ionization (CI) spectrum (methane) of the smaller peak at 4.5 min showed this component to have a molecular weight of 228 (MH^+ at *m/e* 229, M + 29^+ at *m/e* 257) and a base peak at *m/e* 181. This component could not be identified. The CI spectrum of the major peak at 6.6 min is given in Fig. 2, indicating a molecular weight of 244 (MH^+ at *m/e* 245, M + 29^+ at *m/e* 273). Exact mass determinations by means of high-resolution MS revealed a molecular weight of 244.1213. This is consistent with $C_{14}H_{16}N_2O_2$ and the fragments in the CI spectrum can be attributed to $(C_7H_9N_2O_2)^+$ at *m/e* 153, $(C_6H_9N_2O)^+$ at *m/e* 125, $(C_7H_7)^+$ at *m/e* 91, and $(C_4H_8N)^+$ at *m/e* 70 [*2*]. This indicated that the major peak was due to a cyclic dipeptide, phenylalanine-proline lactam (Phe-Pro lactam). The shoulder peak at 6.1 min, in addition to giving some mass fragments that were thought to be impurities, also showed a molecular weight of 244 and the same fragmentation as above, which may indicate an isomer of the major peak.

After some deliberation, it was suggested that Phe-Pro lactam could emanate from ergotamine. Acid hydrolysis of the parent compound in water or in spiked blood indeed gave peaks with the same retention and MS behavior as the major and shoulder peaks in the blood extracts but, in addition to that, the ergotamine extract showed a third peak with an even longer retention time (Fig. 3). The latter had a molecular weight of 314 and was tentatively identified as a pyruvoyl precursor of Phe-Pro lactam [*2*]. A careful GC/MS study with MS in the CI-single ion detection mode (*m/e* 315) at highest sensitivity then revealed that the postmortem blood extract also contained a very small

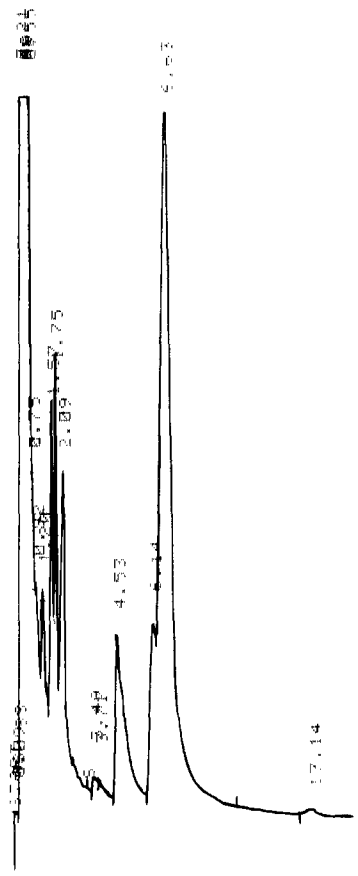

FIG. 1—*Gas chromatogram on SE-30, obtained with the alkaline extract (pH 9.5) of the acid-hydrolyzed postmortem blood.*

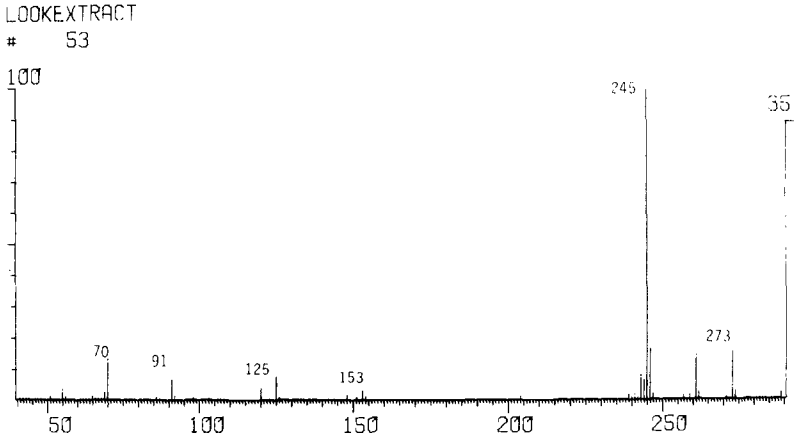

FIG. 2—*Chemical ionization mass spectrum (methane) of the major peak at 6.6 min in Fig. 1.*

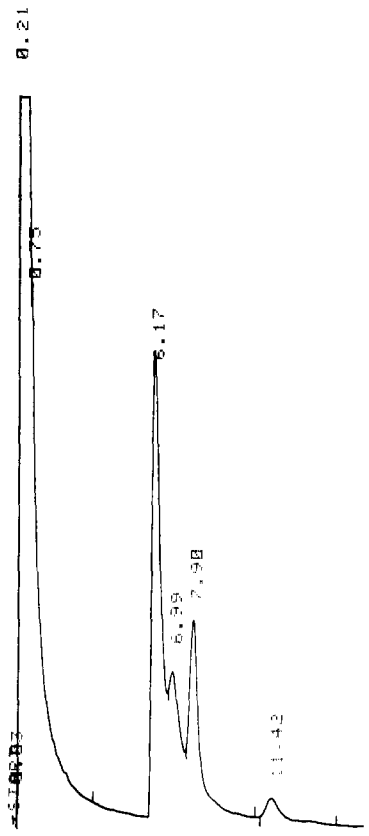

FIG. 3—*Gas chromatogram on SE-30 obtained with the alkaline extract (pH 9.5) of an acid-hydrolized blood sample that had been spiked with ergotamine. The peak at 7.9 min had a molecular weight of 314, whereas those at 6.2 and 6.9 min both had molecular weight 244.*

amount of this pyruvoyl precursor of Phe-Pro lactam, riding on the tail of the major peak.

The occurrence of two peaks with molecular weight 244 and identical fragmentation patterns could be explained by assuming a partial conversion of L-proline into D-proline in the acid hydrolysis procedure. This characteristic conversion has been observed earlier by Hofmann in his studies on the structure of ergotamine [*3*]. Authentic samples of L-Phe-L-Pro lactam and L-Phe-D-Pro lactam confirmed these suggestions and showed the first peak to be the L-D isomer and the second peak to be the L-L isomer. The component with molecular weight 314 is either pyruvoyl-L-Phe-D-Pro lactam or L-D pyroergotamine, but as a reference sample of the former was not available, we were unable to distinguish between the two, if this is at all possible. We therefore wish to refer to the molecular weight 314-component as the pyruvoyl precursor of L-Phe-D-Pro lactam, which is applicable to both structures. Figure 4 depicts the various suggested conversions of ergotamine under acid hydrolysis conditions. Question marks indicate conversions that were not observed by Hofmann [*3*], but which may be typical under our conditions.

During the part of the work that concentrated on establishing proof of the presence of the isomeric lactams, another interesting phenomenon was observed when scanning the up- and down-slopes of the two lactams and the valley in between. The "impurities" found in the shoulder peak could be attributed to a component that was completely

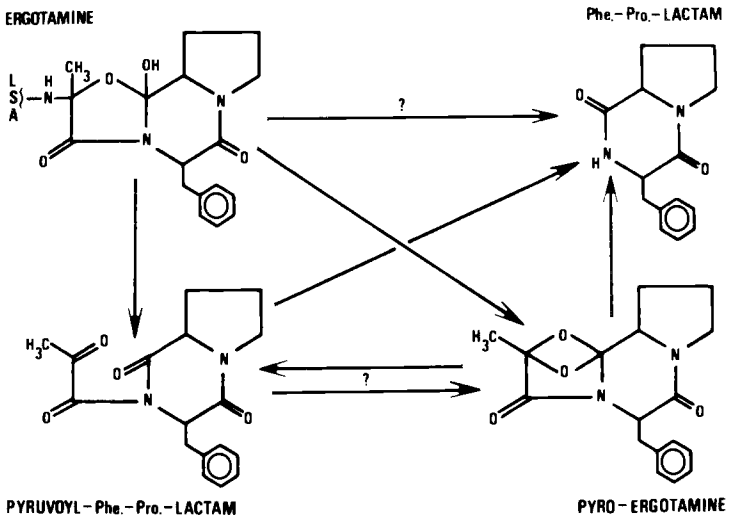

FIG. 4—*Suggested decomposition pathways of the peptide part of ergotamine under acid hydrolysis conditions.* LSA *denotes the lysergic acid moiety of ergotamine.* Question marks *indicate conversions that have not yet been proven; all other conversions have been shown to be possible by Hofmann* [3]. *It should be noted that it cannot be excluded that some of the above reactions may also occur in the injection port of the gas chromatogram.*

overlapped by the two lactams in the total ion current chromatogram. This component had its maximum intensity at the bottom of the valley at 6.2 min. Its CI spectrum is shown in Fig. 5. Subtraction of the lactam peaks at *m/e* 245 and *m/e* 273 leaves a spectrum consisting of MH^+ at *m/e* 286, M + 29^+ at *m/e* 314, and prominent fragments at *m/e* 284, 230, and 217, which can be attributed to pentazocine [*4*]. This was confirmed by GC on SP 2250. On this column, both lactams had a retention time of 19.55 min, whereas pentazocine showed up as a small peak after 10.22 min. It has been observed [*5*] that under acidic conditions pentazocine may take up one molecule of water at the double bond in the side chain to form 2′-hydroxy-2-(3-methyl-3-hydroxy-butyl)-5,9-dimethyl-6,7-benzomorphan. We did not detect this decomposition product in our extracts.

Small amounts of both drugs could be detected in liver extracts after acid digestion, but in view of the low recoveries no attempts were made to correlate these amounts with those found in the blood. No drugs could be detected in the brain.

Discussion

A comparison between Fig. 1, the chromatogram of the hydrolyzed blood, and Fig. 4, the chromatogram of hydrolyzed ergotamine, shows a large difference in peak sizes: In the blood extract, L-Phe-L-Pro lactam is the major component and the pyruvoyl precursor is present only in minute quantities. In the hydrolysate of ergotamine in water and in spiked, nondecomposed blood, L-Phe-D-Pro lactam is the major component, followed by the pyruvoyl precursor and L-Phe-L-Pro lactam, respectively. This may be because in decomposed blood the formation of the L-L lactam out of ergotamine prevailed over that of the L-D lactam; however, it is possible that L-Phe-L-Pro lactam is an endogenous hydrolysis product of blood proteins and that its concentration increases because of the putrefaction of the blood. In experiments on the blank blood samples which were stored for various periods of time at room temperature we observed a small L-Phe-L-Pro lactam

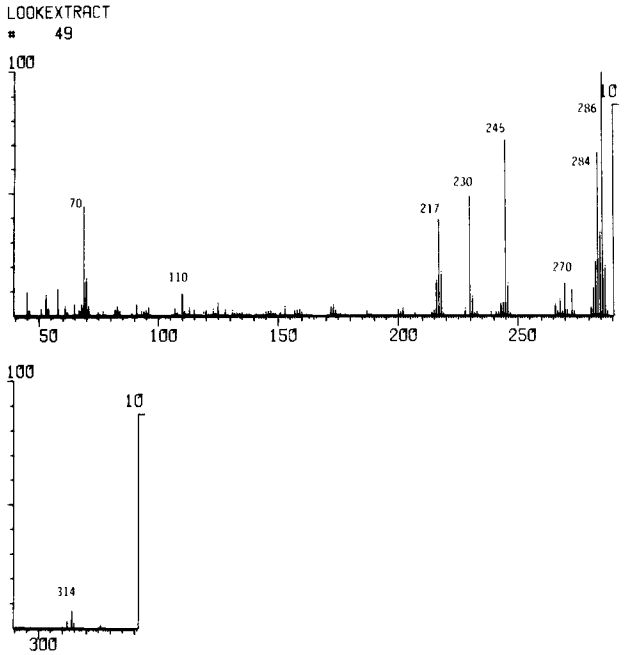

FIG. 5—*Chemical ionization mass spectrum (methane) of the valley between the major peak (6.6 min) and its shoulder (6.1 min) in Fig. 1. The fragments at* m/e *245 and* m/e *273 are from Phe-Pro lactam, but the remaining ones at 286 (MH^+),* m/e *314 ($M + 29^+$),* m/e *284,* m/e *230, and* m/e *217 can be attributed to pentazocine.*

peak in most of the samples, but it may be that in postmortem decomposed blood the formation rate of this component is much greater. Phenylalanine-proline lactam and other cyclodipeptides have also been found in *Candida* cultures [*6*]. Although it was not mentioned explicitly, it seems likely that in the latter case the L-L lactams were involved.

Yet, the presence in the blood of L-Phe-D-Pro lactam, together with its pyruvoyl precursor with molecular weight 314 seems to be specific for ergotamine, its synthetic derivative dihydroergotamine, and their respective -inine stereoisomers.

Because of the decomposed character of the blood the quantitative aspects of this case could not be fully assessed. Studies on spiked fresh blood, spiked putrefied blood, and the decomposed blood, to which an extra amount of ergotamine was added, revealed that in all cases considerable losses occurred, yielding recoveries on the order of 10% and less. For pentazocine, similar recovery results were obtained. This, together with the fact that postmortem degradation of ergotamine and pentazocine may have occurred in the six weeks before analysis, made it impossible to come up with exact data on the blood concentration of these drugs at the time of death. Blood levels of ergotamine and pentazocine after therapeutic doses have been reported to be between 1 and 10 ng/ml for ergotamine [*7*] and between 10 and 80 ng/ml for pentazocine [*8*]. However, a rough estimate, based on the response factors obtained in the GC run and assuming 10% recovery, indicated that the ergotamine concentration in the blood at the time of analysis must have been at least 1 μg/ml, which is some 100 to 1000 times higher than the therapeutic blood levels. For pentazocine we arrived at figures 10 to 100 times the therapeutic blood levels.

Although these figures would suggest a massive overdose of both drugs, it should be noted that the normal extraction procedures used in obtaining the therapeutic blood

levels may not have been able to liberate irreversibly bound drugs or drugs present in blood cells [*9*]. It can be assumed that after the acid hydrolysis procedures the sum of free and bound drug was measured, together with possible metabolites of ergotamine.

Pentazocine can be obtained in The Netherlands by prescription only. However, it is not surrounded by the strict protection measures that are in effect for other drugs of abuse such as opiates and, consequently, it can be obtained fairly easily. More and more well-informed addicts and dealers turn to it as a relatively cheap and "safe" substitute for heroin and morphine.

Apart from the above findings on the two drugs as such, this case also offers some aspects of a more general nature. The initial systematic analysis was carried out within 48 h after the body was found and within 83 h after victim was last seen alive. Common extraction and screening techniques were applied, but they did not reveal the presence of any drug. Only after we used acid hydrolysis did we become aware of the presence of ergotamine and pentazocine in what seemed to be rather large quantities. Apparently, these drugs can be tightly bound by blood proteins or stored in blood cells. Ehrnebo et al [*9*] have studied the binding of radioactive pentazocine to blood cells and plasma proteins in volunteers. They found 48% of the total amount of pentazocine present in blood cells, 33% reversibly bound to plasma proteins, and only 19% free in plasma water. For ergotamine we have no binding data available. In postmortem or decomposed blood, the amounts of free and reversibly bound drugs may even be lower, so that they become no longer detectable after normal extraction procedures. If this phenomenon applies to other basic drugs as well, it seems necessary to adopt acid hydrolysis as a standard technique in systematic toxicology in addition to the normal extraction procedures. The presently used hydrochloric acid system has good liberation properties but may be too drastic for general use. Further research needs to be done to establish which system combines adequate liberation with minimal degradation of the drugs involved.

The complexity of multiple drug analysis is amply demonstrated. It requires not only the availability of sophisticated instrumentation such as a computerized, rapid-scanning GC/MS system but also a continuous alertness of the analyst in order not to miss any clues. As Sunshine has pointed out [*10*], one is tempted to stop if one drug is found, and after having found the Phe-Pro lactam, we almost overlooked the pentazocine. An important factor in this respect is that the analyst usually adjusts his analytical system to those components that are present in higher concentrations. For example, if he runs a GC, he may want to record a nice chromatogram with the highest peak giving some 80 to 90% full-scale deflection. As a result, minor peaks may easily disappear in the baseline noise and remain undetected. Therefore, with multiple drug use being very common today, it is imperative that systematic toxicological analyses are carried out under conditions of highest sensitivity.

Finally, this case illustrates the formidable problems in systematic toxicology, when the analytical data cannot be matched to any of the drugs or metabolites present in the reference systems on hand. Even the best selection of sophisticated instrumentation may not be able to help the toxicologist because of the very nature of his samples: highly complex mixtures in which the components to be analyzed are present in very low quantities, limited sample size, and no opportunities to check blank values. With ergotamine, we slowly proceeded from molecular weight to exact molecular weight to general formula to the structure of the cyclic lactam and, finally, the link to ergotamine. With the component with molecular weight 228 we were less successful. Determination of the exact molecular weight failed because of interfering substances and the limited amount of sample available, which did not permit adequate purification or the application of other techniques. Therefore, its identity still remains undetermined, and the question remains whether other drugs may have been involved in this case but gone unnoticed.

Summary

A 29-year-old male drug addict was found dead at the bottom of a staircase. Analysis of the acid-hydrolized blood showed the presence of pentazocine and two characteristic compounds that contained L-phenylalanine and D-proline, linked together by peptide bounds. It was shown that the latter two components could emanate from the peptide part of ergotamine under the conditions used. It seemed likely that, at the time of analysis, pentazocine and ergotamine were present at concentrations far above therapeutic values. A third component in the blood could not be identified.

Acknowledgments

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