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S. D. Ferrara,[1] *M.D., Ph.D.; Luciano Tedeschi;*[1]
Mario Marigo,[1] *M.D., Ph.D.; and Franca Castagna*[1]

Concentrations of Phenobarbital, Flurazepam, and Flurazepam Metabolites in Autopsy Cases

Flurazepam hydrochloride (7-chloro-1-(diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one dihydrochloride) was synthesized in 1965 by Sternbach et al [*1*]. It is used clinically as a hypnotic in the treatment of anxiety and depression syndromes as well as an anticonvulsant in association with other drugs. Its pharmacology and metabolism have been extensively studied by various workers [*2–4*]. The toxicological features of phenobarbital (ethylphenylbarbituric acid) are also almost completely known, and this compound has been used for years as the basic drug in the treatment of various clinical forms of epilepsy.

While numerous cases of fatal intoxication caused by barbiturates have been reported [*5–10*], the same has not been done for flurazepam [*11–13*], nor for flurazepam and phenobarbital. The association of these two drugs, at times clinically indicated in the treatment of epileptic syndromes with depression or anxiety, was responsible for five deaths within a two-year period (1976–1977). These deaths are the basis for this report.

Unlike phenobarbital, flurazepam presents complex problems related to the blood concentration of its main metabolites [*3*]. De Silva et al [*4*] and Schwartz and Postma [*3*] agree that N-1-desalkylflurazepam (III) and N-1-hydroxyethylflurazepam (II) are the major metabolites in human blood and urine, respectively. We report here the levels of both drugs and metabolites III and II in the various organs and body fluids of the five cases considered. There are other known flurazepam metabolites: monodesethylflurazepam (I-A), didesethylflurazepam (I-B), flurazepam-N-1-yl-acetic acid (V), and N-1-desalkyl-3-hydroxyflurazepam (IV); only IV was detected, and its concentrations are reported.

The analysis of therapeutic levels of many benzodiazepine drugs is particularly difficult since only a few nanograms are present. Spectrophotofluorometry [*14–16*], spectrophotometry [*14*], polarography [*4*], and gas chromatography [*4*] have been applied for these determinations. We obtained results employing new gas chromatographic conditions and a selective detector for nitrogen-containing substances. Finally, the results of benzodiazepine quantitation in urine as well as phenobarbital determination in serum employing the EMIT® method [*17–20*] are discussed.

Case Reports

Case 1

A 71-year-old man who lived alone was found submerged in the bathtub. On the kitchen table, two bottles of Dalmadorm® (flurazepam, 30 mg) and two bottles of Sedofen® (phenobarbital, 0.10 g) were found, along with a handwritten suicide note.

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[1]Director, Toxicology Section; laurea in chemistry, forensic toxicologist, assistant of the Toxicology Section; professor of legal medicine; and laurea in biology, forensic toxicologist, assistant of the Toxicology Section, respectively; Istituto Medicina Legale, Padua, Italy.

Autopsy findings excluded the presence of traumatic lesions. Blood was fluid, and a frothy liquid was observed in the oral cavity and upper airways. Pulmonary emphysemic dilatation was also noted.

Toxicological analysis detected phenobarbital and flurazepam (Table 1) and excluded other agents.

Case 2

A 27-year-old woman physician was admitted in deep coma to the Resuscitation Unit. Clinical examination disclosed a state of severe cardiocirculatory collapse, and diffuse cerebral injury was seen on electroencephalographic examination. Three hours after admission, the patient died. At autopsy a marked and diffuse polyvisceral congestion with initial encephalic and pulmonary edema was seen. Postmortem toxicologic analysis disclosed the presence of phenobarbital and flurazepam (Table 1) and excluded other toxic agents.

Case 3

A 5-year-old girl was admitted to the Resuscitation Unit in a state of shock and complete areflexia. The child had been under treatment with phenobarbital and diphenylhydantoin for epilepsy for the past two years. Her mother said that she had found the child lying on the floor with an empty bottle of Luminalette® (phenobarbital, 0.015 g) and a half-empty bottle of Flunox® (flurazepam, 30 mg) nearby. The child died 6 h after admission. Autopsy findings were unremarkable except for a diffuse polyvisceral congestion and edema. Toxicological analysis not only detected phenobarbital and flurazepam (Table 1) but also subtherapeutic levels of diphenylhydantoin (blood, 0.3 mg/100 ml; brain and liver, 0.5 mg/100 mg; kidney, 0.1 mg/100 mg; and gastric content 0.0 mg/100 ml).

Case 4

A man of 54 years was found dead in his home. Some years previously, myasthenia gravis had been diagnosed. At the time of his death, he was convalescing from an episode of polytraumatic fracture and consequent posttraumatic epilepsy. At autopsy bronchial pneumonia with confluent foci was seen but this was not considered to be the sole cause of death. Toxicological analysis of body fluids and viscera disclosed the presence of phenobarbital and flurazepam (Table 1) and excluded other toxic agents. Subsequently it was discovered that the patient had attempted suicide in the past by ingesting drugs and was under treatment with Gardenal® (phenobarbital, 0.10 g) and Dalmadorm (30 mg).

Case 5

A 54-year-old man was found dead in his home with several empty bottles of Sedofen (0.10 g) and Flunox (30 mg) nearby. He had been under treatment for a depressive syndrome with flurazepam and other similar drugs and had been admitted various times to neurology clinics. At autopsy hepatic cirrhosis and marked polyvisceral congestion were observed. Toxicological analysis confirmed the presence of phenobarbital and flurazepam as well as a blood ethanol level of 2.98 g/litre.

Materials and Methods

Reagents

All reagents employed were analytical-reagent grade. Peroxide-free diethyl ether, distilled

TABLE 1—*Assay of phenobarbital, flurazepam, and flurazepam metabolites in organic fluids and viscera.*[a]

Case	Drugs	Concentration, μg/ml or μg/g					
		Plasma	Urine	Brain	Lung	Liver	Kidney
1	phenobarbital	80.2	6.4	20.5	...	60.3	32.2
	flurazepam	0.5	nd[b]	1.1	0.8	1.7	0.6
	metabolite II	0.8	2.7[c]	0.5	0.4	0.8	nd
	metabolite III	0.6	...	0.9	0.5	2.8	0.4
	metabolite IV	...	0.4[c]	nd	nd	0.08	nd
2	phenobarbital	25.2	3.8	45.4	...	89.2	61.3
	flurazepam	nd	nd	0.3	0.4	0.8	0.2
	metabolite II	0.65	2.8[c]	0.3	nd	0.3	0.5
	metabolite III	1.1	...	0.4	0.3	1.7	0.3
	metabolite IV	...	nd[c]	nd	nd	nd	nd
3	phenobarbital	7.3	...	6.2	...	25.8	20.4
	flurazepam	3.2	...	0.8	1.9	2.7	0.9
	metabolite II	2.5	...	0.7	0.8	3.5	1.1
	metabolite III	1.8	...	0.65	0.6	3.1	0.6
	metabolite IV	...	...	nd	nd	nd	nd
4	phenobarbital	50.7	4.2	27.4	...	56.5	42.3
	flurazepam	nd	nd	nd	nd	nd	nd
	metabolite II	nd	2.4[c]	0.2	nd	nd	nd
	metabolite III	0.4	...	nd	nd	0.4	nd
	metabolite IV	...	0.6[c]	nd	nd	0.3	nd
5	phenobarbital	15.5	1.6	26.1	...	24.5	16.2
	flurazepam	1.7	0.3	1.9	1.8	7.3	2.4
	metabolite II	1.5	5.4[c]	1.2	1.0	2.1	0.9
	metabolite III	1.9	...	2.1	0.9	3.7	1.5
	metabolite IV	...	0.3[c]	nd	nd	0.4	nd

[a]Assays were not always carried out at the limits of sensitivity of the gas chromatographic method employed.
[b]nd = concentration less than 0.2 μg/ml or μg/g in every case.
[c]Concentration of conjugated metabolite.

daily, was used throughout. Flurazepam metabolites were obtained from Roche Co. and stored in the dark at 4°C.

Extraction Procedure

Phenobarbital—Blood levels of phenobarbital were determined according to Barret's method [*21*] for the determination of diphenylhydantoin. We had already used this procedure for the simultaneous quantitation of diphenylhydantoin and phenobarbital [*22*].

Urine and tissue phenobarbital levels were assayed by using this same method except that urine and homogenized tissue were first diluted 1:1 with distilled water. The method employs 5-(*p*-methylphenyl)-5-ethyl barbituric acid as the internal standard and 0.2*M* trimethylanilinium hydroxide in methanol (Methelute®, Pierce Chemical Co.) as the methylating agent.

Flurazepam—Flurazepam and metabolites II and III were determined in blood according to the method of de Silva et al [*4*]. A 4-ml blood sample, previously diluted with borate buffer, pH 9, was extracted with diethyl ether. The final residue after back extraction was dissolved with at least 100 μl ethyl acetate and aliquots up to 10 μl were injected into the gas chromatograph.

For the urine assay, the same extraction with diethyl ether was carried out but the samples (1 ml) were buffered at pH 7 instead of pH 9. Urinary assay of metabolites II and IV was limited to their conjugated forms; after elimination of the nonconjugated basic metabolites by extraction at pH 11, the urines were hydrolyzed enzymatically (Glusulase® enzyme preparation) at pH 5.5 and successively extracted with diethyl ether at pH 9 according to the method of de Silva [*4*]. For the tissue assay of the benzodiazepine compounds and metabolite IV, extraction was carried out as for blood, with diethyl ether at pH 9 following 2:1 dilution of the homogenates with distilled water.

Gas Chromatographic Conditions

Phenobarbital—A Hewlett-Packard gas chromatograph Series 5700 equipped with dual silyl-treated glass columns [*23*] and double flame ionization detector was employed. The columns (1.5 m by 3 mm) were packed with 2% OV-17 (Carlo Erba) on Chromosorb W 80–100 mesh (Analabs). The column was programmed to start at 170°C and rise at 8°C/min to 240°C, where it was held for 10 min; the injector and detector temperature were 250 and 300°C, respectively. The carrier gas was nitrogen at 30 ml/min. Under these conditions phenobarbital was identified at a relative retention time R_R of 0.75, relative to the internal standard.

Flurazepam—A Hewlett-Packard Series 5700 gas chromatograph equipped with dual glass silyl-treated columns and double organic nitrogen selective detector was employed. The columns (1.2 m by 2.5 mm) were packed with 1% SP-1000 (Supelco, Inc.) on 100–120 Supelcoport. Conditioning was carried out at 270°C for 16 h with flowing helium (15 ml/min). Gas chromatographic conditions were as follows: column temperature, 260°C; injector and detector temperature, 300°C; and carrier gas, helium at 45 ml/min. The response of the detector was optimized from time to time by adjusting the flow of hydrogen (3.0 to 3.2 ml/min) and air (50 to 60 ml/min) as necessary for plasma at low temperature. The thermoionic emission current was kept adjusted to 20% full scale with an attenuation of 1×32.

Under these conditions, flurazepam (I) and its metabolites (II, III, IV) were identified at R_R 1.4, 3.57, 2.56, and 0.63, respectively, relative to the reference standard (diazepam, $R_T \simeq 4.2$ min).

A Hewlett-Packard integrator, Model 3380, was employed for the recording and integration of the chromatograms.

Calibrations

Phenobarbital—Details of this method have been previously reported [*22*].

Flurazepam—The method proposed by de Silva et al [*4*] was employed except that the reference solutions were prepared to obtain higher concentrations. All standard solutions contained 1 μg/ml of diazepam as reference compound.

When therapeutic levels are to be determined it is possible to assay all the benzodiazepine compounds considered with the gas chromatographic conditions adopted, if diazepam is used as internal standard. In this case, the calibration curves obtained with the ratio benzodiazepine area/diazepam area are straight lines. In cases of toxic levels, however, precise determination is not possible with this procedure despite the wide range of linearity for each single compound; the concentration of the unchanged drug and the metabolites may vary widely and, therefore, their simultaneous assay using the internal standard technique is not possible. Figure 1 illustrates a gas chromatogram of a plasma assay with diazepam as the internal standard (Case 5).

Immunoenzymatic Assays

EMIT assays of phenobarbital in plasma and benzodiazepine compounds in urine were carried out by using the kits furnished by Syva (Palo Alto, Calif.) and following the enclosed instructions.

A Beckman spectrophotometer, Model 24, equipped with a sipper system, thermal control, and a timer-printer (Model 701), was used.

Results and Discussion

The gas chromatographic conditions employed resolved satisfactorily the problems related to the assay of flurazepam and its metabolites. The gas chromatographic peaks of flurazepam and its metabolites II, III, and IV, which were identified on the basis of the reference or internal standard, all show a perfectly symmetrical tendency, which renders derivatization unnecessary.

Flurazepam, metabolites II and III, and the reference standard are eluted on a 1% SP-1000 column without concomitant significant interferences except those seen when urinary extracts are eluted. In the elution of urinary extracts many unidentified peaks were noted, and some of these had retention times which were very close to those of metabolites II and III. Ether extraction, which was employed for urine, was therefore allowed for the assay of flurazepam only and not metabolites II and III.

In the gas chromatographic elution of the ether extracts of plasma and urine, a peak caused by an endogenous substance always appears and has the same retention time as metabolite IV (see Fig. 1). In these fluids, therefore, assay of metabolite IV was not possible. In urine, metabolites IV and II were assayed only in their conjugated form because it is known that they are eliminated mostly in this form, especially in cases of overdose [*4*].

The recovery carried out in blood samples confirmed the values reported by de Silva [*4*] for the various benzodiazepine compounds with the exception of metabolite IV, where the total recovery (35 ± 8 standard deviation) was lower than that reported [*4*]. However, even if recovery of the interfering endogenous substance on the same blank was constant in our hands, a precise assay of metabolite IV could not be carried out. The limits of detection allowed the quantitation of therapeutic levels of flurazepam (0.01 μg/ml), metabolite III (0.01 μg/ml), and metabolite II (0.02 μg/ml) in the blood; for metabolite IV the limit of detection in viscera was 0.08 μg/ml.

The extraction technique for the five cases reported was the method for therapeutic levels

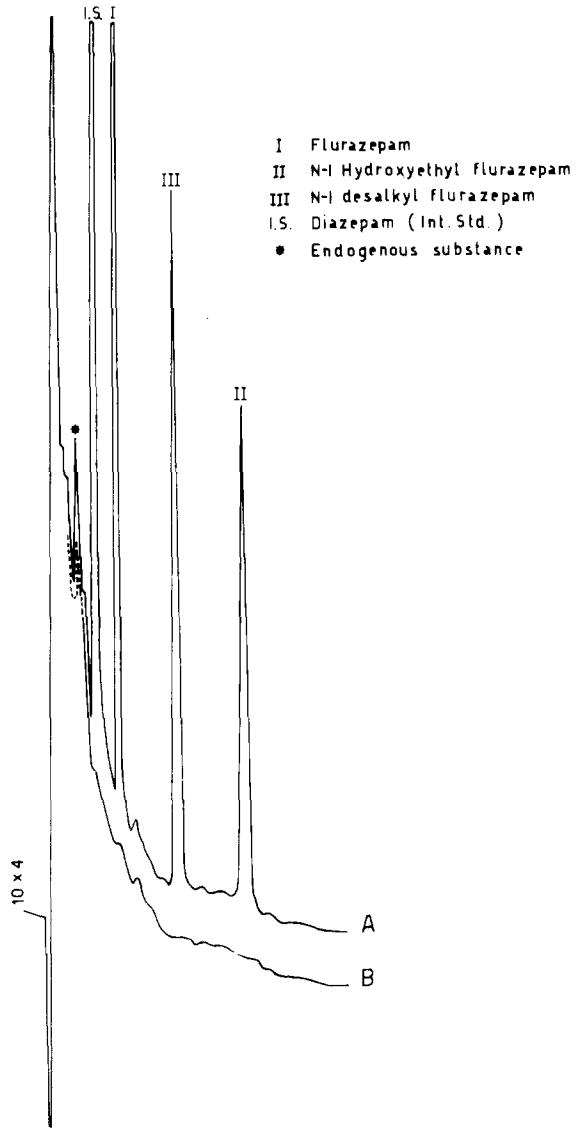

FIG. 1—*Chromatograms of the GLC analyses of blood diethyl ether extracts:* (A) *blood extract from Case 5 and* (B) *control blood extract.*

[*4*] since it leads to cleaner extracts despite the high concentration of benzodiazepine which would be expected on the basis of the first screening.

EMIT assay of phenobarbital in serum was carried out only in Cases 1, 3, and 4. These analyses were in good agreement with the values reported in Table 1 (maximum coefficient of variation, 12.5%) and confirm the merits of this method, which we had already evaluated in the analysis of anticonvulsants and other drugs [*24-27*].

The results of EMIT assay of urinary benzodiazepine compounds in four cases were negative with the exception of Case 4, where a positivite finding corresponding to 0.9 μg/ml of oxazepam was obtained.

Since diazepam or other benzodiazepines were not detected by gas-liquid chromatography in the urine of Case 4, and bearing in mind the data referring to the cross-reactivity of the kit in regard to flurazepam, the value of 0.9 μg/ml would correspond to more than 65 μg/ml flurazepam, which is much higher than values obtained in gas chromatography. This discordance may be explained if remarkable differences in cross-reactivity for the various metabolites compared to flurazepam are assumed.

The results obtained in our cases (Table 1) agree with the reports of other workers in cases of intoxication with barbiturates [*5,6,8,10,28*].

No comparison with flurazepam is possible since specific indications are not given in the literature [*12,13,29*].

The association of the two drugs, as seen in our cases, and the consequent phenomenon of toxicologic interaction [*30–32*] do not permit the evaluation of the death-producing potential for each drug in question, even though it should be stressed that various studies [*33,34*] demonstrate that the toxicity of the benzodiazepines in general and flurazepam in particular [*30,32*] appears lower than that of phenobarbital.

For these reasons, the values obtained in our cases permit limited considerations and deductions.

A critical analysis of the phenobarbital concentrations found in Cases 3 to 5 excludes the conclusion that the deaths were due to an overdose; in addition, as reported by others [*5,10,35*], a higher concentration of phenobarbital in the liver compared to plasma was confirmed.

The distribution of flurazepam and its metabolites in the various fluids and organs was similar in all the cases examined. Values higher than therapeutic plasma levels of flurazepam and all the metabolites tested were detected in Cases 1, 2, 3, and 5. Flurazepam was always present in higher quantities in the liver than in the brain with a liver/brain ratio varying from a minimum of 1.55 to a maximum of 3.84. In Cases 1 and 3, the plasma level of metabolite II was even higher than that of metabolite III, in contrast with what is observed when therapeutic doses are administered. Furthermore, metabolite III was present in higher concentrations than metabolite II in brain and lung tissue, while in liver this difference was less marked.

In addition, metabolite IV was found in the urine and liver of only those subjects who had been under treatment with flurazepam for some time (Cases 4 and 5); it was absent in Cases 2 and 3, who were not under chronic treatment with drug. The finding of this metabolite in Case 1 suggests that the decedent was also under long-term treatment with flurazepam.

It seems opportune to consider the importance of the interaction between phenobarbital and diazepam, especially in cases of epileptics under chronic treatment with the barbiturate (Cases 3 and 4). Since barbiturate constitutes the typical drug able to provoke enzymatic induction, reduced reactivity towards overdose from barbiturate and benzodiazepine itself appears possible. Moreover, according to Benetello et al [*36*], in epileptics under chronic phenobarbital treatment the association of a benzodiazepine (diazepam, bromazepam, clonazepam) brings about a reduction in plasma barbiturate levels. In the attempt to explain the mechanism of this phenomenon, these workers suggest an interaction at the site of entry into the body (during absorption) or at the site of exit from the body (during excretion), and consider enzyme induction or competition for protein binding sites unlikely. Nevertheless, if a reduction in the blood levels of phenobarbital through an interaction with a benzodiazepine acquires an essential clinical significance in the treatment of epilepsy, it appears irrelevant in cases of overdose from both drugs, regardless of pretreatment with barbiturate, because of the summational effect of these compounds at the receptor level.

Finally, from an analysis of the cases presented, the indispensable role of toxicologic examination clearly emerges. In fact, in the absence of circumstantial evidence (Cases 2 and 4) or significant pathological findings (all cases), the suspicion of intoxication as the sole cause of death (Cases 2, 3, 5) or as concurring cause of death (Cases 1, 2) may be confirmed.

Summary

In five cases of death resulting from acute intoxication with phenobarbital and flurazepam, the blood, urine, brain, lung, liver, and kidney levels of these drugs as well as the levels of N-1 hydroxyethyl, N-1 desalkyl, and N-1-desalkyl-3-hydroxy flurazepam metabolites were determined.

Concentration of flurazepam and its metabolites was determined by using new gas chromatographic conditions employing a selective detector for nitrogen-containing substances and a column of 1% SP-1000. In addition, the EMIT technique was also employed on blood and urine samples and the results compared with GLC data.

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Address requests for reprints or additional information to
S.D. Ferrara
Istituto di Medicina Legale
V. Falloppio 50
35100 Padova, Italy