Journal of Chromatography, 87 (1973) 455–472

CHROM. 6993

DETERMINATION OF ETHANOL IN FRESH AND PUTREFIED *POST MORTEM* TISSUES

GEORGE CHRISTOPOULOS*
Cook County Coroner's Laboratory, 1828 W. Polk, Chicago, Ill. 60612 (U.S.A.)
and
E. R. KIRCH and J. E. GEARIEN
University of Illinois Medical Center, Department of Medicinal Chemistry, P.O. Box 6998, Chicago, Ill. 60680 (U.S.A.)
(Received August 6th, 1973)

SUMMARY

Ethanol in various *post mortem* tissues was determined by different methods of analysis. The samples analyzed were collected and stored under various conditions. Decreases as well as increases in the alcohol content were observed depending upon the time and temperature of storage. From the results obtained it is recommended to store the samples under frozen conditions or refrigerated and fluoridated to minimize fluctuations. The variations of ethanol in blood obtained from human cadavers stored up to 90 days are also reported.

INTRODUCTION

In 1971 there were about 2,650 cases requiring toxicological work, while in 1972 there were 3,100 cases. In 1973, it is projected that this number is expected to be still higher. More than 50% of the cases screened have been found to contain ethanol.

In view of the existing conditions mentioned above, it seemed not only desirable, but also imperative to subject various samples of tissue to an analysis more specific than ordinary routine procedure.

In spite of all the investigations and reports concerning the alcohol content of blood and other *post mortem* tissues, there is still a need to investigate further some of the conditions associated with alcohol poisoning. For example, reports in the literature[1–4] do not stress any of the conditions as far as collecting and handling the sample is concerned.

Preliminary work in this laboratory indicated that there is a variance in the alcohol content of identical samples of blood depending on whether they are stored at room temperature or in the refrigerator. Furthermore, the time of collecting the

* To whom inquiries should be directed.

samples of blood to the time of analysis appears to be related to the concentration of the alcohol.

EXPERIMENTAL

Materials and methods

Solvents. The following solvents are all analytical reagent grade unless otherwise specified: methanol, acetaldehyde, isopropyl alcohol, 1-propanol, and 1-butanol.

Reagents. The following reagents were applied: 10% silicotungstic acid; ethanol standard solutions in water containing 25.0 mg%, 50.0 mg%, 100.0 mg%, 150.0 mg%, 200.0 mg% and 300.0 mg%; 1-propanol standard solution in water containing 50.0 mg%, 100.0 mg% and 200.0 mg%.

Equipment

A Barber-Coleman Series 5000 gas–liquid chromatograph, with dual hydrogen flame ionization detector, was employed. The columns were U-shaped borosilicate glass, 4 mm I.D., one of which was 6 ft. long and packed with 60–80 mesh Chromosorb 102. The detector temperature was 280°, the injection temperature 260° and the column temperature 180°. Nitrogen was used as a carrier gas with a flow-rate of 35 ml/min with an inlet pressure of 40 lbs. The hydrogen to air ratio was 4:1.8 and the attenuation used was 1.0.

Procedures

All samples used in this study were submitted by the pathologists of the Cook County Coroner's Laboratories during September 1969 until June 1972.

Alcohol from body fluids. Blood samples were generally collected from the heart; if this was not available the blood was collected from the pleural cavity.

In those cases where the influence of putrefaction was studied, the blood was placed in bottles containing either sodium oxalate or sodium fluoride. The blood sample was divided into three parts and stored as follows prior to the analyses: (1) refrigerated at 3 to 5°, (2) stored at room temperature, and (3) frozen at −1 to −3°.

Blood from twelve cadavers, which had been kept refrigerated at 4° for periods ranging from 2 to 14 weeks, was also examined. The blood from these cadavers was withdrawn and immediately analyzed without subjecting to any preservative treatment.

Blood from heart or pleural cavity. Ethanol was separated quantitatively from blood by steam distillation after precipitation of blood protein with silicotungstic acid. An aliquot of the distillate was oxidized quantitatively to acetic acid by a known excess of dichromate in sulfuric acid solution according to Widmark[5].

Five milliliters of blood (oxalated or fluoridated) were transferred to the proper steam-distilling apparatus containing 35 ml of water. Five milliliters of 10% silicotungstic acid were added and mixed (silicotungstic acid has excellent antifoaming properties), and the steam distillation was carried out with minimum delay in order to avoid loss of any volatile constituents. The distillate was collected in a 25.0-ml volumetric flask which was immersed in an ice water bath. The concentration of the oxidizable substance in the distillate was then determined.

The procedure for other body fluids such as gastric contents, cerebrospinal fluid, bile, and seminal fluid is the same as that for blood.

The procedure for alcohol in tissue, such as (a) heart, (b) brain and spinal cord, (c) spleen, (d) liver, (e) pancreas, (f) adrenals, (g) kidneys, (h) testicles, (i) prostate, (j) lungs, (k) thyroids, (l) muscle, and (m) fat, is the same as that for blood, except that 5.0 g of wet tissue, which had been homogenized or minced, was used in the steam distillation.

As shown in Table I, it is imperative that, to obtain maximum recovery by steam distillation, at least 20.0 to 25.0 ml of the distillate should be collected if the ethanol concentration is 200.0 mg%.

TABLE I

EFFECT OF THE AMOUNT OF DISTILLATE COLLECTED ON ETHANOL DETERMINATION

Mean values of three determinations.

Amount of ethanol standard (ml)	*Amount of distillate collected (ml)*	*Concentration found (mg%)*		*Actual concentration (mg%)*	*% Loss*
		According to Widmark's method	*By GLC*		
5.0	5.0	111.0	109.0	200.0	45.5
5.0	10.0	155.0	156.0	200.0	22.0
5.0	15.0	191.0	192.0	200.0	3.75
5.0	20.0	198.0	196.0	200.0	1.0
5.0	25.0	199.0	198.0	200.0	0.5

Adding enough alcohol to various body fluids and tissues to bring the final concentration to 200 mg% and subjecting these mixtures to the distillation procedure described above, the loss in recoveries was observed to range from 2.0 to 2.5%, as shown in Table II. Furthermore, it should be pointed out that in absence of other volatile reducing substances, excellent correlation exists between the results obtained using dichromate oxidation and gas–liquid chromatography (GLC).

Since the dichromate method does not differentiate between ethanol and other

TABLE II

PER CENT RECOVERIES OF ETHANOL IN SEVERAL TISSUES

Case No.	*Sample used*	*Amount added (mg%)*	*Amount found (mg%)*		*Recovery (%)*
			According to Widmark's method	*By GLC*	
72-145	Blood	200.0	196.0*	195.5*	98.0
72-145	Spinal fluid	200.0	198.0*	196.5*	98.6
72-145	Liver	200.0	195.0	196.0	97.5
72-145	Brain	200.0	194.5	195.0	97.3
72-145	Kidney	200.0	195.0	195.0	97.5
72-145	Muscle	200.0	196.0	194.0	97.5
72-145	Fat	200.0	195.0	195.5	97.6

* Mean values of three determinations. Standard deviation ± 0.80 for GLC and ± 0.88 for Widmark's method.

oxidizable material, it is not only desirable, but essential, to subject another aliquot of the same distillate to GLC analysis. This procedure would eliminate the possibility that volatile reducing substances other than ethanol could be present, and, furthermore, the use of GLC would aid in identifying the other volatile reducing substances.

Procedure using GLC for ethanol analysis from body fluids and tissues. Two milliliters of the distillate and 2.0 ml of 50 mg% 1-propanol (used as an internal standard) were transferred to a 5.0-ml vial equipped with a tight-fitting septa. After thorough mixing 6.0 μl of the mixture were injected into the chromatograph. Each determination was carried out in triplicate. Fig. 1 shows a triplicate injection; Fig. 2 shows a triplicate injection of a lower concentration of ethanol; Fig. 3 shows that the concentrations were linear over the range of 0.00 to 350.0 mg% (w/v) of blood ethanol.

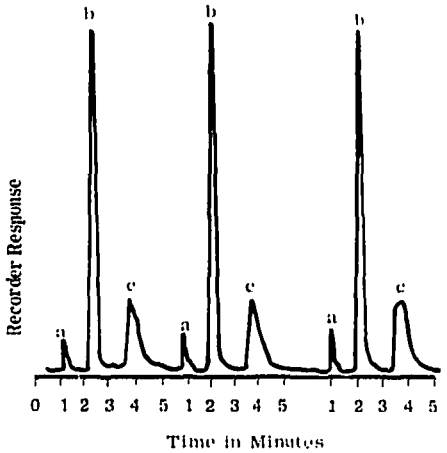

Fig. 1. Demonstration of the internal standard technique used in GLC. Column, Chromosorb 102; column temperature, 180°; flow-rate, 35 ml/min. (a) Water; (b) ethanol; (c) 1-propanol.

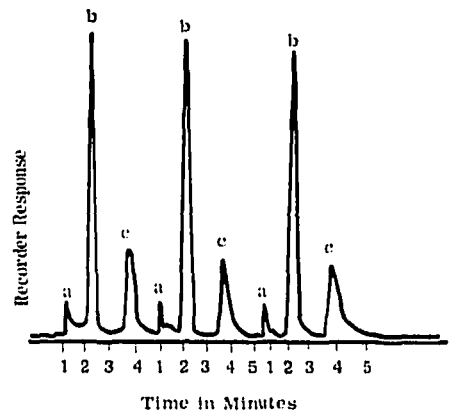

Fig. 2. Demonstration of the internal standard technique used in GLC. Column, Chromosorb 102; column temperature, 180°; flow-rate, 35 ml/min. (a) Water; (b) ethanol; (c) 1-propanol.

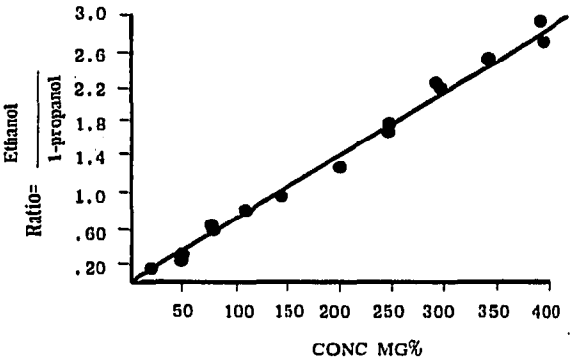

Fig. 3. Graph of the ratio ethanol:1-propanol *vs.* the concentration of blood ethanol.

RESULTS AND DISCUSSION

Determination of Ethanol in Body fluids and tissues

Because of the variance in the different reports in the literature[6–9], in connection with the determination of ethanol in various body fluids and tissues, it seemed essential to investigate several of the procedures used and modify them where necessary in order to obtain more meaningful and reproducible results.

The method used in this laboratory for the determination of ethanol in non-putrefied blood and tissues, is that of Widmark[5], which is based on the oxidation by dichromate of the distillate obtained from steam distillation.

Widmark's method is reliable for the determination of ethanol only in the absence of distillable volatiles such as methanol, acetaldehyde, isopropyl alcohol, 1-propanol and 1-butanol. As seen in Fig. 4, these interfering substances can be separat-

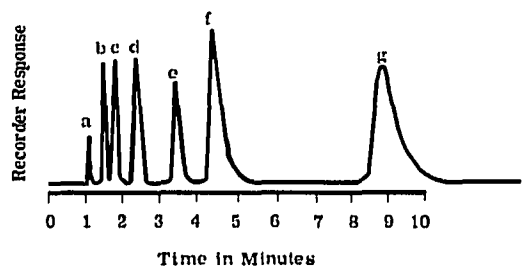

Fig. 4. Mixture of common volatiles. (a) Water; (b) methanol; (c) acetaldehyde; (d) ethanol; (e) isopropanol; (f) 1-propanol; (g) 1-butanol. Column, Chromosorb 102; column temperature, 180°; flow-rate, 30 ml/min.

ed and identified by the GLC method using the conditions stated in the legend to Fig. 4. In the presence of these additional volatiles this alternative method (GLC) should be used[10]. In our laboratory GLC is used as a second method to correlate results for forensic purposes and to prove the presence or absence of volatile reducing substances other than ethanol. The GLC analysis is especially necessary in the analysis

of the putrefied specimen where, as will be shown, the dichromate is of little significance.

Table III shows a comparison of Widmark's method with GLC, using putrefied blood samples. These cases were selected at random, where putrefaction was detected. Seventeen blood samples were analyzed, and out of the 17, 6 were negative for ethanol by the GLC method of analysis. With Widmark's method, all were positive and the negative samples by GLC showed as high as 105.0 mg% oxidizable material

TABLE III

COMPARISON OF WIDMARK'S METHOD WITH GLC USING PUTREFIED BLOOD

Mean values of three determinations. Neg. = Negative.

Case No.	*Ethanol concentration (mg%)*	
	According to Widmark's method	*By GLC*
71-1500	105.0	Neg.
71-1485	219.0	20.0
71-1476	251.0	51.0
71-1459	20.0	Neg.
71-1418	190.0	37.0
71-1395	228.0	70.0
71-1384	423.0	138.0
71-1313	102.0	76.0
71-1205	229.0	180.0
71-1129	357.0	51.0
71-1092	280.0	179.0
71-1572	20.0	Neg.
71-1586	26.0	Neg.
71-1603	265.0	49.0
71-1609	25.0	Neg.
71-1619	20.0	Neg.
71-1628	235.0	130.0

calculated as ethanol. It was also observed, that all blood samples analyzed by Widmark's method showed higher ethanol concentration compared to the GLC method of analysis. Table III demonstrates the difference in the two methods, and indicates that if putrefied blood samples are analyzed, only the GLC method should be used, and even then, the ethanol concentration should not be taken as true value in the sample.

From fresh samples of body fluids or tissues, the two methods are usually in agreement and the results can be averaged. In the absence of interfering volatile substances, the two methods show a remarkable correlation, as illustrated in Table II.

Determination of ethanol in oxalated blood

Room temperature storage of blood samples. Samples of blood which had been stored under different temperatures for up to 50 days were analyzed for ethanol at several time intervals by both analytical procedures and the results are shown in Tables

IV and V. In Table IV, some of the problems associated with the determination of blood alcohol in samples undergoing putrefaction are shown. These include:

(a) Initially negative cases which show a rapid increase of oxidizable material in the first few days reaching a maximum in about 15 days followed by a leveling off, or a decrease that sometimes goes to zero. The results from Widmark's method, which measures all volatile oxidizable material, frequently are results (calculated as ethanol) in excess of 100.0 mg% (cases 71-1158, 71-1168, 71-1172, 71-1197 and 71-1198).

TABLE IV

EFFECT OF STORAGE OF OXALATED BLOOD AT ROOM TEMPERATURE ON THE ETHANOL CONTENT OF BLOOD

Mean values of three determinations. I = By GLC; II = according to Widmark's method.

Case No.	*Ethanol concentration (mg%) as received*		*Ethanol concentration (mg%) at room temperature*					
			After 7 days		*After 15 days*		*After 40–50 days*	
	I	*II*	*I*	*II*	*I*	*II*	*I*	*II*
71-1157	196.0	195.0	220.0	289.0	140.0	301.0	115.0	180.0
71-1158	Neg.	Neg.	12.0	22.0	40.0	102.0	31.0	127.0
71-1166	134.0	136.0	144.0	143.0	127.0	163.0	121.0	286.0
71-1167	22.0	51.0	32.0	69.0	63.0	89.0	66.0	86.0
71-1168	Neg.	Neg.	82.0	98.0	89.0	118.0	81.0	127.0
71-1169	76.0	84.0	116.0	122.0	86.0	122.0	Neg.	55.0
71-1170	580.0	582.0	844.0	714.0	758.0	836.0	674.0	1095.0
71-1171	310.0	308.0	348.0	373.0	242.0	582.0	268.0	599.0
71-1172	Neg.	Neg.	10.0	132.0	25.0	155.0	Neg.	173.0
71-1173	356.0	425.0	396.0	375.0	320.0	392.0	311.0	321.0
71-1174	212.0	217.0	230.0	214.0	186.0	220.0	125.0	241.0
71-1176	338.0	349.0	306.0	306.0	285.0	326.0	568.0	637.0
71-1178	414.0	419.0	226.0	395.0	330.0	349.0	68.0	87.0
71-1181	204.0	237.0	127.0	354.0	239.0	510.0	154.0	612.0
71-1182	Neg.	Neg.	14.0	61.0	18.0	55.0		
71-1189	148.0	147.0	86.0	178.0	188.0	408.0	135.0	214.0
71-1194	164.0	166.0	111.0	177.0	164.0	281.0	129.0	270.0
71-1195	106.0	113.0	85.0	152.0	134.0	298.0	146.0	246.0
71-1197	Neg.	Neg.	108.0	196.0	102.0	209.0	203.0	304.0
71-1198	Neg.	Neg.	13.0	109.0	32.0	142.0	37.0	112.0

The actual production of ethanol in these samples is given by the data from the GLC method, which are specific for ethanol, and indicate a rapid rise in ethanol concentration during the first 15 days; however, the actual ethanol concentrations for the 6 initially negative cases listed above averaged less than 60.0 mg%.

(b) Blood samples containing ethanol, which gave initial agreement between the two methods of analysis when the samples were fresh, showed on subsequent analysis, extreme and apparently random fluctuations in ethanol concentration as determined by either procedure. This indicates that the initial blood ethanol concentration cannot be ascertained from samples stored at room temperature.

From the GLC analytical data the variation in actual ethanol concentration

during storage is evident and indicates that samples showing a rising ethanol concentration during the first week will exhibit a subsequent decrease in blood ethanol (cases 71-1171, 71-1174, 71-1157 and 71-1166). On the other hand, the samples which show decreasing blood ethanol during the first week exhibit subsequent increases in ethanol concentration (cases 71-1181, 71-1194, 71-1189 and 71-1195). The significance of these patterns in the concentration changes of ethanol is not apparent.

It is clear, therefore, that alcohol is produced during putrefaction and that partially decomposed blood samples cannot be analyzed for initial ethanol content. If the sample is already decomposed, GLC analysis will yield the ethanol concentration. However, the results should be interpreted with caution, since the ethanol concentration has been shown to be subject to extreme fluctuations.

Refrigerated storage of blood samples. In Table V the effect of refrigeration on oxalated blood samples was studied. Nineteen blood samples were selected at random

TABLE V

EFFECT OF STORAGE OF OXALATED BLOOD UNDER REFRIGERATED CONDITIONS ON THE ETHANOL CONTENT OF BLOOD

Mean values of three determinations. I = By GLC; II = according to Widmark's method.

Case No.	*Ethanol concentration (mg%) as received*		*Ethanol concentration (mg%) after refrigeration*					
			For 7 days		*For 15 days*		*For 40–50 days*	
	I	*II*	*I*	*II*	*I*	*II*	*I*	*II*
71-1157	196.0	195.0	205.0	214.0	192.0	190.0	218.0	234.0
71-1158	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	5.0	20.0
71-1166	134.0	136.0	144.0	132.0	111.0	153.0	107.0	107.0
71-1167	22.0	51.0	12.0	31.0	12.0	30.0	35.0	55.0
71-1168	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	10.0	15.0
71-1169	76.0	84.0	76.0	79.0	80.0	82.0	89.0	102.0
71-1170	580.0	582.0	278.0	271.0	260.0	320.0	268.0	282.0
71-1171	310.0	308.0	285.0	290.0	272.0	295.0	269.0	301.0
71-1172	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	10.0	15.0
71-1173	356.0	425.0	346.0	414.0	370.0	389.0	363.0	358.0
71-1174	212.0	217.0	215.0	204.0	200.0	214.0	179.0	187.0
71-1176	338.0	349.0	308.0	350.0	280.0	354.0	272.0	355.0
71-1178	414.0	419.0	368.0	344.0	408.0	377.0	382.0	390.0
71-1181	204.0	237.0	115.0	263.0	256.0	286.0	164.0	242.0
71-1182	Neg.	Neg.	Neg.	Neg.	88.0	122.0	—	—
71-1189	148.0	147.0	67.0	142.0	30.0	143.0	162.0	179.0
71-1194	164.0	166.0	182.0	172.0	134.0	148.0	151.0	158.0
71-1195	106.0	113.0	122.0	138.0	86.0	107.0	83.0	93.0
71-1197	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	2.0	Neg.
71-1198	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	3.0	Neg.

and tested for ethanol using both Widmark's method and GLC over a period of time. These 19 samples are the same samples as used in the room temperature storage study summarized in Table IV.

Six blood samples which were originally negative after a period of 20 days of refrigeration showed production of oxidizable material calculated as ethanol (using the Widmark method). GLC showed the actual production of ethanol during refriger-

ation and the increase was not as much as indicated using the Widmark method (10 mg%).

Blood samples which contained ethanol originally were not refrigerated and were analyzed by both methods at several time intervals. Fluctuations in ethanol concentration during storage was again observed as determined by either method of analysis. However, the variations in concentration were not as pronounced as those associated with room temperature storage. In 16 of the samples the variation amounted to less than $\pm$ 25.0 mg% for the duration of the study and appeared to be independent of the level of blood ethanol. It is clear that even refrigerated oxalated blood shows a putrefaction effect. For medicolegal purposes, subsequent analysis of such samples cannot yield the same ethanol content which was originally present.

Frozen blood and spinal fluid storage. Curry[3] maintains that it is not likely that endogenous chemical reaction results in a loss of ethanol in samples of blood while stored at room temperature or refrigerated. He suggests that if any decrease is observed in the concentration of ethanol, this may be due to the presence of certain types of bacteria.

Since bacterial action should be negligible in frozen samples, GLC analysis of blood ethanol was studied as a function of time with the samples stored in a frozen condition (Table VI). In addition, spinal fluid, which shows ethanol levels in the body comparable to that in blood, was included in the study in the hope that if any changes in blood ethanol were observed, the changes in spinal fluid samples would correspond.

TABLE VI

EFFECT OF FREEZING OF SAMPLES ON DETERMINATION OF ETHANOL CONCENTRATION

Mean values of three determinations. Analysis done by GLC only.

Case No.	*Specimen*	*Ethanol concentration (mg%) as received*	*Ethanol concentration (mg%) after freezing*			
			For 1 month	*For 2 months*	*For 6 months*	*For 1 year*
70-1152	Blood	111.0	112.0	115.0	116.0	101.0
	Spinal fluid	115.0	109.0	111.0	110.0	98.0
70-1215	Blood	156.0	150.0	153.0	145.0	140.0
	Spinal fluid	179.0	170.0	171.0	160.0	159.0
70-1311	Blood	215.0	201.0	211.0	198.0	190.0
	Spinal fluid	195.0	190.0	185.0	188.0	173.0
70-1480	Blood	Neg.	Neg.	Neg.	Neg.	Neg.
	Spinal fluid	Neg.	Neg.	Neg.	Neg.	Neg.
70-1515	Blood	335.0	330.0	331.0	315.0	310.0
	Spinal fluid	369.0	360.0	355.0	350.0	341.0
70-1645	Blood	Neg.	Neg.	Neg.	Neg.	Neg.
	Spinal fluid	Neg.	Neg.	Neg.	Neg.	Neg.
70-1990	Blood	401.0	395.0	380.0	375.0	383.0
	Spinal fluid	429.0	415.0	411.0	395.0	398.0
71-15	Blood	Neg.	Neg.	Neg.	Neg.	Neg.
	Spinal fluid	Neg.	Neg.	Neg.	Neg.	Neg.
71-23	Blood	Neg.	Neg.	Neg.	Neg.	Neg.
	Spinal fluid	Neg.	Neg.	Neg.	Neg.	Neg.
71-35	Blood	79.0	71.0	70.0	69.0	75.0
	Spinal fluid	83.0	80.0	88.0	90.0	71.0

In ten cases, samples of blood and of spinal fluid were analyzed under frozen storage conditions at several time intervals. Four samples of blood and four samples of spinal fluid were originally negative for ethanol, and, after being frozen for a year, there was no ethanol production observed.

In Table VI, using GLC for ethanol analysis, only a very slight fluctuation was noticed in the concentration of ethanol. Most samples showed a small decrease of ethanol after a period of one year. From these results, it is clear that frozen forensic samples should give reliable values of ethanol concentration even after a long period of storage.

The reason suggested for any observed increase of blood ethanol during storage is that it may be due to glycogenolysis, which was first observed by Bernard[11], and later proven by Hamilton-Paterson[12]. Bacteria and yeasts could then produce ethanol from the glycolysis products[13–15]. Sturner *et al.*[16], suggested that glycolysis should be complete between 6 to 8 h after death.

Determination of ethanol in blood with preservative

Room temperature storage of blood samples. The addition of 1% sodium fluoride to a blood sample inhibits glycolysis and prevents bacteria or yeasts from producing ethanol[3]. The variation with time in ethanol concentration of blood samples containing 1% sodium fluoride stored at room temperature was studied and the results are presented in Table VII. A comparison should also be made between the results of

TABLE VII

EFFECT OF STORAGE AT ROOM TEMPERATURE ON ETHANOL CONCENTRATION IN BLOOD WITH 1% NaF

Mean values of three determinations. Analysis done by GLC only.

Case No.	*Ethanol concentration (mg%) as received*	*Glucose concentration (mg%) as received*	*Ethanol concentration (mg%) at room temperature*		
			After 5 days	*After 15 days*	*After 40–50 days*
71-4	259.0	600.0	265.0	260.0	255.0
71-5	78.0	30.0	75.0	90.0	15.0
71-6	298.0	Neg.	301.0	335.0	360.0
71-10	246.0	Neg.	260.0	273.0	291.0
71-11	135.0	830.0	130.0	139.0	126.0
71-12	233.0	200.0	235.0	240.0	260.0
71-14	187.0	0.0	185.0	198.0	219.0
71-15	52.0	15.0	75.0	91.0	115.0
71-16	350.0	420.0	365.0	381.0	395.0
71-18	31.0	0.0	33.0	38.0	40.0
71-25	187.0	70.0	201.0	230.0	231.0
71-30	207.0	45.0	215.0	222.0	226.0
71-43	199.0	1,200.0	201.0	215.0	225.0
71-50	100.0	15.0	95.0	103.0	98.0
71-58	157.0	90.0	168.0	190.0	195.0
71-63	Neg.	900.0	Neg.	Neg.	15.0
71-78	Neg.	0.0	Neg.	Neg.	Neg.
71-82	Neg.	0.0	5.0	22.0	28.0
71-92	Neg.	15.0	11.0	33.0	48.0
71-105	Neg.	750.0	Neg.	Neg.	Neg.

this study and those reported in Table IV, in which no inhibitor was added.

Of the five blood samples which had no blood ethanol initially, three samples produced ethanol with time, even in the presence of sodium fluoride (up to 48.0 mg%).

Two samples showed no ethanol content even after 45 days at room temperature. A comparison with the fluctuations reported for the similar experiment as shown in Table IV reveals that the presence of sodium fluoride greatly diminished the variation in ethanol concentration during storage with most samples remaining in a ± 25.0 mg% range.

Refrigerated storage of blood samples. In Table VIII, the effect of refrigerated storage on ethanol was studied. Twenty blood samples were analyzed for ethanol

TABLE VIII

EFFECT OF REFRIGERATED STORAGE ON ETHANOL CONCENTRATION IN BLOOD WITH 1% NaF

Mean values of three determinations. Analysis done by GLC only.

Case No.	*Ethanol concentration (mg%) as received*	*Glucose concentration (mg%) as received*	*Ethanol concentration (mg%) after refrigeration*		
			For 5 days	*For 15 days*	*For 45–50 days*
71-4	259.0	600.0	261.0	268.0	270.0
71-5	78.0	30.0	80.0	81.0	73.0
71-6	298.0	Neg.	286.0	275.0	291.0
71-10	246.0	Neg.	250.0	240.0	236.0
71-11	135.0	830.0	139.0	140.0	128.0
71-12	233.0	200.0	239.0	226.0	229.0
71-14	187.0	0.0	185.0	179.0	189.0
71-15	52.0	15.0	55.0	53.0	46.0
71-16	350.0	420.0	342.0	358.0	336.0
71-18	31.0	0.0	33.0	29.0	26.0
71-25	187.0	70.0	181.0	173.0	177.0
71-30	207.0	45.0	215.0	230.0	201.0
71-43	199.0	1,200.0	190.0	183.0	195.0
71-50	100.0	15.0	101.0	96.0	98.0
71-58	157.0	90.0	150.0	151.0	145.0
71-63	Neg.	900.0	Neg.	Neg.	Neg.
71-78	Neg.	0.0	Neg.	Neg.	Neg.
71-82	Neg.	0.0	Neg.	Neg.	Neg.
71-92	Neg.	15.0	Neg.	Neg.	Neg.
71-105	Neg.	750.0	Neg.	Neg.	Neg.

after refrigeration for different time intervals. Samples of blood which were fluoridated and which were originally negative, produced no ethanol with refrigeration up to 45 days. Samples of blood which were positive originally, showed only a slight variation (± 10.0 mg%) with most samples showing a small decrease in ethanol concentration. These data show that very little fluctuation in ethanol concentration occurs when fluoridated samples are refrigerated. From the results of this study, it can be recommended that samples of blood for forensic purposes be fluoridated and refrigerated unless fluoride poisoning is suspected.

TABLE IX

EFFECT OF REFRIGERATION OF CADAVERS ON ETHANOL CONCENTRATION (BLOOD USED ONLY)

Mean values of three determinations. I = By GLC; II = according to Widmark's method.

Case No.	*Ethanol concentration (mg%) as received*		*Ethanol concentration (mg%) after*											
			10 days		*20 days*		*30 days*		*50 days*		*70 days*		*90 days*	
	II	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*
205	Neg.	Neg.	Neg.	Neg.	15.0	38.0	26.0	89.0	40.0	90.0	41.0	110.0	32.0	89.0
5	Neg.	Neg.	Neg.	Neg.	5.0	25.0	24.0	111.0	32.0	155.0	60.0	195.0	55.0	186.0
89	Neg.	Neg.	5.0	80.0	20.0	112.0	30.0	168.0	39.0	187.0	40.0	230.0	49.0	246.0
160	415.0	400.0	395.0	370.0	391.0	393.0	380.0	390.0	370.0	404.0	360.0	490.0	340.0	500.0
214	Neg.	Neg.	Neg.	Neg.	9.0	34.0	9.0	68.0	—	—	—	—	—	—
166	9.0	11.0	10.0	15.0	16.0	32.0	18.0	40.0	23.0	84.0	11.0	118.0	0	145.0
15	230.0	241.0	229.0	252.0	230.0	280.0	236.0	298.0	218.0	301.0	201.0	312.0	190.0	360.0
25	356.0	359.0	345.0	360.0	340.0	368.0	315.0	390.0	308.0	401.0	—	—	—	—
24	793.0	791.0	785.0	785.0	601.0	790.0	635.0	801.0	645.0	820.0	131.0	180.0	Neg.	Neg.
10	284.0	288.0	289.0	295.0	290.0	328.0	327.0	348.0	340.0	345.0	327.0	348.0	301.0	380.0
20	357.0	359.0	360.0	372.0	355.0	388.0	340.0	390.0	306.0	340.0	290.0	310.0	236.0	258.0
8	227.0	229.0	230.0	245.0	233.0	260.0	248.0	290.0	208.0	330.0	108.0	180.0	Neg.	94.0

Determination of ethanol in cadaver blood

Blood containing no oxalate or fluorides was obtained from cadavers that had been kept refrigerated for up to three months; the blood was analyzed for ethanol at various time intervals (Table IX). Four blood samples obtained from the cadavers at the start of the experiment contained no ethanol. After a period of 90 days all showed ethanol to be present. The samples were analyzed by both Widmark's method and GLC. It can be seen in Table IX that the negative samples showed as high as 55.0 mg% ethanol at the end of the experiment, using the GLC method of analysis. On the other hand, using the same samples in Widmark's method of analysis, it is observed that the oxidizable material calculated as ethanol gives a concentration more than three times greater. The other blood samples which were positive as received, showed extreme, and apparently random variation of ethanol concentration with time by both methods of analysis. It can be concluded, again, that using refrigeration alone will not prevent decomposition, and the production of ethanol and other decomposition products is evident.

Therefore, the ethanol content measure should be interpreted with caution for cases which were refrigerated for a period of time prior to autopsy and ethanol analysis.

Distribution of ethanol in other body fluids and tissues

In Table X, the distribution of ethanol in body fluids and tissues was studied. Body fluids and tissues were investigated with special emphasis on different sections of the brain. This study was done to evaluate the distribution of ethanol throughout the body, the relationship of blood ethanol to other body tissues and fluids and to evaluate the best specimen for ethanol analysis.

The six cases used in this study were selected at random and all had different histories. For example, Case No. 72-878 was shot to death, Case No. 72-699 was found dead with history of alcoholism, and gross pathology indicated fatty metamorphosis of the liver. Case No. 72-580 was found dead under a porch with possible low-temperature exposure. Case No. 72-736 was killed in an automobile accident and Case No. 72-535 was found dead in bed with possible overdose. In Case No. 72-878, the blood/right lung ratio was 0.82, while the blood/left lung ratio was 2.05. The deceased was shot through the left lung, the left lung had collapsed and the blood volume had concentrated in the right lung, showing a very high ethanol content. In body fluids, such as spinal fluid, the blood/spinal fluid ratios ranged between 0.6 and 1.1, the bood/bile ratios ranged between 0.7 and 1.04, and the blood/urine ratios ranged between 0.7 to 1.0. In body tissues the blood/liver ratios ranged between 1.70 to 3.0. The blood to different sections of the brain ratios were also determined. Although it is anatomically difficult at times to separate out certain sections of the brain, it was observed that the highest concentration of alcohol was in the cerebellum and pituitary gland. It was also observed that the concentration of ethanol in the right lung is usually higher, as seen in Table X.

In body fluids, the ethanol is evenly distributed in accordance with the law of diffusion provided that the diffusion equilibrium has been attained. Muscle and fat contained the least amount of ethanol. This is in agreement with the amount of water present in these tissues[17].

TABLE X

DISTRIBUTION OF ETHANOL CONTENT IN TISSUES AND FLUIDS—BLOOD/TISSUE OR FLUID RATIOS

Mean values of three determinations. Results of GLC and Widmark's method are averaged.

Source	*Case No./Concentration of ethanol in blood (mg%)*					
	72-878/338.0	*72-699/129.0*	*72-580/316.0*	*72-656/141.0*	*72-736/154.0*	*72-535/320.0*
Bile	0.70	—	0.90	1.04	1.12	0.99
Urine	1.0	—	0.72	0.82	0.93	0.72
Gastric contents	0.39	0.62	—	—	—	1.22
Seminal fluid	3.31	—	1.0	1.08	—	—
Testicular fluid	—	—	1.46	—	—	—
Spinal fluid	1.03	0.62	0.90	0.99	0.87	—
Liver	3.19	1.79	3.63	2.24	2.96	2.05
Kidney	1.85	1.22	2.93	1.72	1.36	1.70
Spleen	1.69	1.40	3.26	1.16	2.48	1.75
Pancreas	1.91	1.34	4.05	0.97	1.79	2.18
Right lung	0.82	1.21	2.25	1.22	1.62	1.57
Left lung	2.05	1.40	2.74	1.43	1.73	1.85
Right ventricle	1.98	1.79	3.09	2.20	2.10	2.90
Left ventricle	—	1.98	3.50	2.35	—	—
Tongue	2.99	—	—	—	—	—
Testis	2.05	—	—	1.70	—	—
Prostate	2.58	—	2.02	—	—	—
Adrenals	2.70	2.39	7.18	1.76	3.20	—
Right ventricle fat	—	3.39	6.58	4.55	8.10	—
Aortic fat	—	—	22.5	5.88	—	—
Adipose	—	4.96	22.5	5.64	6.69	20.0
Mesentary fat	—	1.63	—	—	—	—
Frontal lobe	—	1.84	2.28	2.16	2.61	2.50
Temporal	—	1.37	2.44	2.35	2.08	—
Parietal	—	1.72	4.0	—	3.14	3.37
Occipital	—	1.72	3.59	3.13	2.52	2.62
Cerebellum	2.19	1.19	1.80	1.66	1.52	1.52
Medulla	4.63	2.48	7.80	5.22	2.80	2.34
Pituitary	1.78	—	2.70	—	1.12	—
Pons	2.99	2.08	3.23	4.07	4.40	5.08
Thalamus	2.99	1.74	2.63	—	3.70	—
Corpus striatum	2.48	—	2.12	4.14	—	—
Cerebral pendicles	2.99	—	4.15	3.20	—	—

Ethanol levels in fatalities

Estimation of acute alcoholic intoxication. Several investigators[3,17] have concluded that the fatal concentration of ethanol in blood is between 400.0 mg% to 600.0 mg%.

From our studies the fatal concentration for blood ethanol is frequently in excess of 400.0 mg%, as shown in Table XI. In Table XI, the distribution of ethanol in known alcoholic intoxication was studied. Ten cases were selected where the cause of death was given as "acute alcoholic intoxication." In cases No. 453, 1155, and 1299, the bile ethanol content is the highest and greater than the blood ethanol level. The history in all three cases is that they all won drinking contests. Case No. 453 won a bet for drinking a quart of whiskey in 10–15 min. He collected the bet and as he

TABLE XI

DISTRIBUTION OF ETHANOL IN KNOWN ACUTE ALCOHOLIC INTOXICATIONS

Mean value of three determinations. Analysis done by GLC only.

Case No.	*Ethanol concentration (mg%) in*								
	Blood	*Bile*	*CSF*	*Urine*	*Liver*	*Spleen*	*Kidney*	*Brain*	*Gastric contents*
453	662.0	1100.0	611.0	540.0	360.0	450.0	450.0	340.0	200 g
490	796.0	594.0	690.0	630.0	440.0	465.0	455.0	490.0	—
461	495.0	480.0	418.0	530.0	290.0	315.0	360.0	340.0	1000 mg
590	915.0	125.0	817.0	940.0	540.0	595.0	640.0	580.0	—
715	615.0	580.0	590.0	680.0	365.0	389.0	401.0	395.0	500 mg
945	423.0	390.0	401.0	485.0	245.0	—	285.0	320.0	600 mg
1053	515.0	485.0	520.0	560.0	310.0	—	345.0	310.0	700 mg
1155	680.0	900.0	—	580.0	370.0	—	440.0	360.0	—
1215	545.0	410.0	—	630.0	390.0	395.0	415.0	380.0	—
1299	1766.0	2795.0	—	—	1161.0	—	1043.0	912.0	320 g*

* Highest seen in our laboratories.

walked out of the bar collapsed on the sidewalk. The high bile ethanol level shows that equilibrium has not yet been attained and the blood ethanol would have increased as time went on. In other words, reabsorption and redistribution of ethanol from bile and gastric contents would have occurred.

From the clinical histories of the above cases and other cases seen by this investigator, it can be concluded that when bile ethanol content is higher than blood or other body fluids, death occurred rapidly. For the rest of the cases shown in Table XI, the diffusion equilibrium is believed to have been attained[17].

Ethanol levels in violent deaths. In Table XII, the ethanol concentration of selected tissues and fluids for 15 cases where death was not due to "acute alcoholic intoxication," but to various violent means, was determined by the GLC method of analysis. Blood, bile, urine and liver fluids and tissues were used for the analysis. Blood to other body fluids and tissue ratios were determined. The range of these ratios is very much the same range noticed in Table X. These cases were included in this study to demonstrate some of the medicolegal problems the toxicologist is faced with.

In the cases shown in Table XII, most likely murder has been committed and the alcohol results have to be defended and perhaps interpreted in courts. If a comparison of the ethanol content found in cases listed in Table XI with the ethanol content found in cases in Table XII is made, the ethanol concentration and distribution is occasionally similar, and yet the immediate cause of death in the cases presented in Table XII was by gunshot or other violent means.

The problem of interpreting the level of alcohol in the blood of a living subject is very easy in comparison with the blood level in a dead person. In the latter area considerable attention has been paid in the last decade or so, ever since the discovery that yeasts and some bacteria could produce alcohol in *post mortem* blood[13–15].

The responsibility for correct interpretation of alcohol concentration in body fluids and tissues can only be assumed if one is familiar with storage and handling of the samples.

TABLE XII

TYPICAL CASES OF HIGH ETHANOL CONTENT ON VIOLENT DEATHS

Mean values of three determinations. Analysis done by GLC only.

Case No.	*Ethanol concentration (mg%) in*						
	Blood	*Bile*	*Urine*	*Liver*	*Blood/bile ratio*	*Blood/urine ratio*	*Blood/liver ratio*
72-1232	334.0	240.0	361.0	169.0	1.39	0.93	1.97
72-1252	363.0	376.0	494.0	282.0	0.96	0.73	1.28
72-1013	475.0	462.0	505.0	271.0	1.03	0.94	1.79
72-1083	302.0	303.0	375.0	159.0	0.99	0.81	1.90
72-1006	392.0	444.0	534.0	219.0	0.88	0.73	1.78
72-898	300.0	277.0	361.0	—	1.08	0.83	—
72-948	375.0	303.0	447.0	230.0	1.23	0.83	1.63
72-951	312.0	328.0	418.0	259.0	0.95	0.75	1.20
72-878	338.0	485.0	338.0	206.0	0.70	1.0	1.64
72-863	320.0	438.0	409.0	241.0	0.73	0.78	1.33
72-785	316.0	293.0	363.0	197.0	1.08	0.87	1.60
72-773	451.0	461.0	530.0	246.0	0.97	0.85	1.83
72-823	419.0	395.0	494.0	274.0	1.06	0.85	1.53
72-712	349.0	321.0	335.0	—	1.08	1.04	—
72-723	391.0	419.0	479.0	—	0.93	0.81	—
72-493	422.0	498.0	603.0	—	0.84	0.70	—
72-171	288.0	—	346.0	187.0	—	0.83	1.54

TABLE XIII

EFFECT OF REFRIGERATION ON ETHANOL CONCENTRATION IN URINE

Mean values of three determinations. Calculation done by GLC only.

Case No.	*Ethanol concentration in urine (mg%) as received*	*Ethanol concentration in urine (mg%) on refrigeration*					
		With preservative			*Without preservative*		
		10 days	*20 days*	*30 days*	*10 days*	*20 days*	*30 days*
72-1013	505.0	513.0	501.0	498.0	488.0	491.0	495.0
72-1083	375.0	375.0	368.0	369.0	380.0	379.0	368.0
72-1123	342.0	345.0	338.0	330.0	335.0	340.0	342.0
72-1126	360.0	365.0	358.0	353.0	350.0	358.0	350.0
72-848	361.0	361.0	352.0	355.0	360.0	368.0	370.0
72-951	418.0	401.0	415.0	410.0	415.0	408.0	401.0
72-948	447.0	440.0	442.0	447.0	456.0	450.0	459.0
72-1006	534.0	530.0	495.0	496.0	485.0	493.0	490.0
72-878	338.0	340.0	350.0	351.0	345.0	330.0	335.0
72-833	159.0	150.0	152.0	155.0	150.0	160.0	166.0
72-336	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-340	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-342	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-347	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-348	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-349	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-353	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-354	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-355	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-356	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Determination of ethanol in urine samples

Refrigerated storage of urine samples. Trojanowska[18] has suggested that if alcohol was found in *post mortem* urine, then prior ingestion of alcohol could be inferred, which means, in the absence of glucose and proteins in urine, there should be little variance in the alcohol concentration in urine if handled properly. Especially, one should not expect an increase of alcohol under these conditions.

In Table XIII, the effect of refrigeration on ethanol concentration in urine with and without phenylmercuric nitrate as a preservative was studied.

Twenty urine samples were used in this experiment. Of these 10 were positive as received and the other 10 were negative for ethanol. The 10 urine samples which were negative after refrigeration up to 30 days with preservative and without preservative showed no ethanol. Moreover, the urine samples containing ethanol showed little fluctuation in concentration regardless of the presence or absence of preservative.

Room temperature storage of urine samples. In Table XIV, the effect of room temperature storage on ethanol concentration in urine with and without phenylmercuric nitrate as preservative was studied. Out of the ten negative urine samples for ethanol, two showed some ethanol production. The rest of the samples showed some fluctuation with all showing lower than initial concentration after 30 days. The fluctuations observed are small in comparison with those observed in blood stored at room temperature.

TABLE XIV

EFFECT OF ROOM TEMPERATURE STORAGE ON ETHANOL CONCENTRATION IN URINE

Mean values of three determinations. Analysis done by GLC only.

Case No.	*Ethanol concentration in urine (mg%) as received*	*Ethanol concentration in urine (mg%) at room temperature*					
		With preservative			*Without preservative*		
		10 days	*20 days*	*30 days*	*10 days*	*20 days*	*30 days*
72-1013	505.0	508.0	507.0	495.0	501.0	480.0	393.0
72-1083	375.0	378.0	360.0	363.0	351.0	340.0	301.0
72-1123	342.0	348.0	336.0	333.0	315.0	290.0	245.0
72-1126	360.0	360.0	354.0	348.0	301.0	215.0	180.0
72-898	361.0	350.0	356.0	345.0	310.0	290.0	260.0
72-951	418.0	401.0	407.0	395.0	390.0	370.0	315.0
72-948	447.0	440.0	390.0	398.0	407.0	336.0	215.0
72-1006	534.0	501.0	515.0	495.0	480.0	415.0	345.0
72-878	338.0	335.0	330.0	326.0	310.0	280.0	201.0
72-833	159.0	150.0	153.0	145.0	85.0	63.0	Neg.
72-336	Neg.	Neg.	Neg.	Neg.	5.0	Neg.	Neg.
72-340	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-342	Neg.	Neg.	3.0	6.0	3.0	5.0	4.0
72-347	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-348	Neg.	Neg.	10.0	7.0	15.0	7.0	3.0
72-349	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-353	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-354	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-355	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
72-356	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

In both Tables XIII and XIV, it was observed that no increase of alcohol occurred in samples of urine whether they were preserved with phenylmercuric nitrate or kept without a preservative for the same time, under the same conditions. While no bacteriological counts were carried out in samples of urine, it should be further pointed out that any reduction of alcohol might be due to bacteriological action, especially, since some reduction of ethanol was observed in urine samples without preservative.

REFERENCES

1 C. P. Stewart and A. Stolman, *Toxicology*, Vols. I and II, Academic Press, New York, 1960–1961.
2 A. Stolman, *Progress in Chemical Toxicology*, Vols. I–IV, Academic Press, New York, London, 1963–1969.
3 A. S. Curry, *Advances in Forensic and Clinical Toxicology*, Chemical Rubber Co., Cleveland, Ohio, 1972, pp. 17–28, 93–106.
4 F. Lundquist, *Methods of Forensic Science*, Vol. II, Interscience, London, New York, 1963.
5 E. M. P. Widmark, *Biochem. Z.*, 131 (1922) 473.
6 L. M. Shupe and K. M. Dubowski, *Amer. J. Clin. Pathol.*, 22 (1952) 901.
7 A. S. Curry, G. W. Walker and G. S. Simpson, *Analyst (London)*, 91 (1966) 742.
8 M. Wolthers, *Acta Med. Leg. Soc.*, 9 (1956) 325.
9 L. Karmitis and L. J. Porter, *J. Forensic Sci.*, 17 (1972) 318.
10 M. K. Roach and P. J. Creaven, *Clin. Chim. Acta*, 21 (1968) 275.
11 C. Bernard, *C.R. Acad. Sci., Ser. D*, 83 (1876) 369.
12 J. L. Hamilton-Paterson, *J. Pathol. Bacteriol.*, 50 (1940) 473.
13 V. D. Pleuckhahn, *J. Forensic Med.*, 15 (1968) 12.
14 D. J. Blackmore, *J. Forensic Sci. Soc.*, 8 (1968) 73.
15 V. D. Pleuckhahn, *J. Forensic Med.*, 15 (1968) 12.
16 W. A. Sturner and G. E. Gantner, Jr., *J. Forensic Sci.*, 9 (1964) 485.
17 S. Felby and J. Olsen, *J. Forensic Sci.*, 14 (1969) 93.
18 M. Trojanowska, *Acta Pol. Pharm.*, 24 (1967) 331.