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Toxicological analysis of drugs and poisons in formalin-fixed organ tissues

2. Volatile substances

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Abstract Diethylether, chloroform and toluene were administered by inhalation and ethanol intravenously to rabbits. As soon as possible after death, tissue specimens were collected from the brain, lung, liver, kidney and skeletal muscle and fixed in non-buffered 10% formalin at room temperature (10–20° C) for 4 different periods (1, 2, 5 and 14 days). The volatile substances were analyzed and identified by gas chromatography/mass spectrometry (GC-MS). The measured concentrations of ethanol, diethylether, chloroform and toluene in the brain tissue 1 day after fixation decreased to 8, 23, 73 and 84% respectively compared with those in the non-fixed brain tissue (100%). The rank order of the rate of decrease in the fixed state was: ethanol > diethylether ≫ chloroform > toluene. These volatile substances could be detected clearly in all the tissue specimens, even after a 14-day fixation period. These results provide useful toxicological information that will help to differentiate whether volatile substances have been administered antemortem or postmortem.

Key words Toxicology · Formalin-fixed organ tissue Volatile substance · GC/MS analysis

Zusammenfassung Kaninchen erhielten intravenös oder per inhalationem Ethanol, Diethylether, Chloroform und Toluol. Gewebsproben von Gehirn, Lunge, Leber, Niere und Skelettmuskulatur wurden entnommen und in ungepuffertem 10-prozentigem Formalin bei Raumtemperatur (10–20°) über 4 verschiedene Zeiträume (1, 2, 5 und 14 Tage) fixiert. Die flüchtigen Substanzen wurden mit Hilfe der Gaschromatographie/Massenspektrometrie (GC/MS) analysiert und identifiziert. Die gemessenen Konzentrationen von Ethanol, Diethylether, Chloroform und Toluol im Hirngewebe nahmen einen Tag nach der Fixation auf 8, 23, 73 bzw. 84%, – der Konzentrationen im nicht-fixierten Hirngewebe (100%) ab. Die Reihenfolge der Konzentrationsabnahme in fixiertem Material gegenüber dem nicht-fixierten Material lautet: Ethanol > Diethylether ≫ Chloroform > Toluol. Diese flüchtigen Substanzen konnten auch nach einer 14-tägigen Fixationsperiode eindeutig in allen Gewebsproben nachgewiesen werden. Diese Resultate ergeben eine nützliche toxikologische Information zur Differenzierung, ob flüchtige Substanzen vor dem Tode zugeführt wurden oder nicht.

Schlüsselwörter Toxikologie · Formalinfixiertes Gewebe Flüchtige Substanzen · GC/MS-Analyse

Introduction

Histological investigations are important diagnostic procedures for medico-legal and pathological autopsies. Therefore, organ specimens from autopsied bodies are usually fixed in 10% formalin for storage. However, toxicological analysis of illicit chemical substances, such as methamphetamine and toluene, a component of thinners, is also indispensable for medico-legal examinations.

Several studies of the analysis of embalmed and/or formalin-fixed organ tissues for several drugs and poisons: phenobarbital [12]; ethchlorvynol [13]; paraquat [6]; diazepam, phenytoin, desipramine and phenobarbital [14]; amitriptyline and nortriptyline [4]; malathion [11] and tricyclic antidepressants (amitriptyline, nortriptyline, imipramine and desipramine) [15] have been reported. To the best of our knowledge, however, there are hardly any published reports of analytical studies on illicit methamphetamine, amphetamine, narcotics, cannabinoids, some psychotropic drugs and components of thinners in formalin-fixed organ tissues. We have carried out series of experimental studies to detect drugs and poisons in formalin-fixed organ tissues and demonstrated that methamphet-

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amine could be detected qualitatively in formalin-fixed organ tissues at least 28 days after fixation [10].

In this study, the volatile substances toluene, diethylether, chloroform and ethanol in formalin-fixed organ tissues were analyzed by gas chromatography/mass spectrometry (GC-MS).

Materials and methods

Rabbits (ca. 2 kg body weight) were subjected to the vapor of a mixture of diethylether, chloroform and toluene for 40–50 min at room temperature, and 6.7 ml 15% (w/v) ethanol aqueous solution per kg body weight was administered intravenously and they continued to inhale the vapor mixture for 10–15 min until they died. Brain, lung, liver and kidney tissue and skeletal muscle specimens were collected as soon as possible after death.

Each organ collected was cut into blocks (about 1 cm × 2 cm × 2 cm) and fixed in 80 ml non-buffered, 10% formalin (3.7% formaldehyde, pH 5.1) at room temperature (10–20° C) for 1, 2, 5 and 14 days, without renewing the fixative solution.

The animal experiments in the present study were conducted in accordance with the ethical guidelines on animal experiments of the Kanazawa University School of Medicine.

The volatile substances concerned were analyzed by GC-MS using the head space method [1, 3, 7], as follows: the formalin-fixed organ specimens were washed with redistilled water and excess water was removed. A slice weighing approximately 0.2–0.5 g was placed in a vial and an aliquot (0.5 ml) of the internal standard (IS) solution, 0.5 mg/ml ethanol-d6 (d5) and 20 μg/ml toluene-d8 in 10% (v/v) aqueous Tween 20 solution, was added. The vial was incubated at 55° C for 15 min, after which 1 ml head space gas was collected and injected into the GC-MS apparatus.

For the preparation of calibration curves, working solutions were prepared in 10% (v/v) aqueous Tween 20 solution as follows: ethanol (5, 50, 250, 500, 1,000 μg/ml), diethylether and chloroform (1, 10, 50, 100, 200 μg/ml) and toluene (0.75, 7.5, 37.5, 75, 150 μg/ml) and analyzed by GC-MS.

The JEOL JMS DX303 & DA5100 system (Tokyo) was used for GC-MS analysis. The GC column, DB-1 (0.53 mm × 30 m, 5 μm film thickness), was used with the column temperature set at 80° C throughout, the injection, separator and inlet temperatures set at 120° C and the carrier gas (He) flow rate was 15 ml/min. The mass spectrometry conditions were as follows: in addition to the electron impact (EI) and positive ion detection modes, scan (multiple ion monitoring) and selected ion monitoring (SIM) modes were used for qualitative and quantitative analysis respectively. The following ions (M/Z) were recorded for the quantitative analysis of ethanol (45 and 46), ethanol-d6(d5) (IS) (49 and 51), diethylether (74), chloroform (83), toluene (91 and 92) and toluene-d8(IS) (98 and 100). The acceleration voltage was 3 kV, ionization voltage, 70 eV, ionization current, 0.3 mA and conversion dynode voltage, −10 kV.

Results

Qualitative analysis

Figures 1a and 1b show the mass chromatograms and mass spectra of the volatile substances in formalin-fixed brain tissue specimens after fixation for 14 days. The volatile substances administered antemortem could be identified from their mass chromatograms and mass spectra by comparing them with those of the standard substances. Clear mass spectra of the volatile substances were obtained for all the tissue specimens.

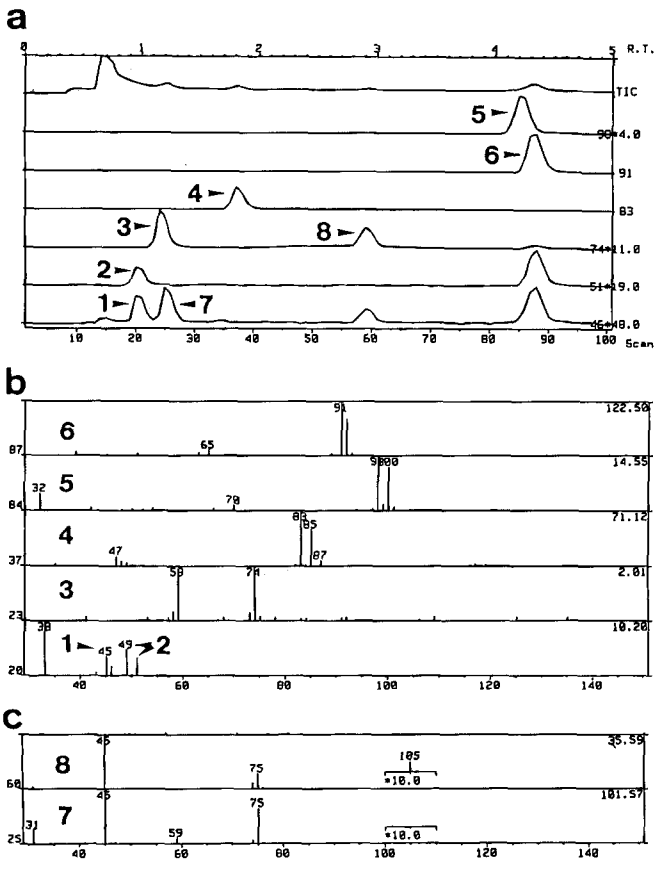

Fig. 1 Mass chromatograms and mass spectra, obtained using the head space method, of volatile substances in the brain after 14-day formalin fixation. Mass chromatograms (a) and mass spectra (b and c) are as follows: 1) ethanol, 2) ethanol-d6 (d5) (IS), 3) diethylether, 4) chloroform, 5) toluene-d8 (IS), 6) toluene. The volatile substances 7) and 8) were assumed to be CH_3-CH_2-O-CH_2-OH and CH_3-CH_2-O-CH_2-O-CH_2-OH respectively (see text). The analytical conditions are described in the text

The mass chromatograms and mass spectra patterns of the volatile substances in the formalin solution after the 14-day fixation period were the same as those of the fixed tissue specimens.

Figure 1c shows that additional products, which had probably formed as a result of a reaction between ethanol and formaldehyde (formaldehyde dimer), were detected in the organ tissues and formalin solution. Their structures were assumed to be CH_3-CH_2-O-CH_2-OH (mass spectrum of volatile substance 7 in Fig. 1 c) and CH_3-CH_2-O-CH_2-O-CH_2-OH (mass spectrum of volatile substance 8 in Fig. 1 c).

Quantitative analysis

The concentrations of ethanol, diethylether, chloroform and toluene were measured by GC-MS using the SIM mode for quantitative analysis. The relative peak areas (ethanol/ethanol-d6(d5); chloroform/toluene-d8, diethylether/toluene-d8; toluene/toluene-d8) were used to measure the concentrations. There was a linear relationship

Table 1 Measured concentrations of ethanol, diethylether, chloroform and toluene in formalin-fixed organ tissues

Organ tissue	Time after formalin fixation (days)	Measured concentration (μg/ml)			
		Ethanol	Diethylether	Chloroform	Toluene
Brain	0	1,809 ± 353[a]	67.05 ± 13.47	118.0 ± 12.92	95.78 ± 3.48
	1	140 ± 44	14.49 ± 2.48	87.05 ± 15.62	78.89 ± 21.33
	2	99 ± 48	10.47 ± 0.73	64.63 ± 20.32	48.04 ± 6.16
	5	138 ± 16	20.20 ± 2.31	72.08 ± 9.98	104.64 ± 0.68
	14	90 ± 7	16.32 ± 3.32	25.97 ± 3.70	62.24 ± 18.08
Lung	0	1,610 ± 382	36.70 ± 15.21	67.16 ± 20.88	14.72 ± 1.27
	1	61 ± 9	5.64 ± 1.09	30.06 ± 6.87	15.02 ± 4.38
	2	72 ± 20	6.51 ± 1.20	42.44 ± 17.49	7.22 ± 0.88
	5	100 ± 14	11.53 ± 3.23	39.37 ± 11.25	10.48 ± 2.00
	14	90 ± 8	5.90 ± 2.68	9.78 ± 1.47	5.86 ± 2.03
Liver	0	2,105 ± 512	99.96 ± 5.66	197.7 ± 19.23	94.56 ± 5.29
	1	182 ± 51	13.37 ± 1.44	130.9 ± 23.92	100.67 ± 11.62
	2	139 ± 55	9.28 ± 2.65	144.3 ± 40.59	94.04 ± 22.58
	5	122 ± 25	10.89 ± 0.47	84.98 ± 27.57	103.87 ± 13.09
	14	92 ± 9	12.04 ± 3.47	34.01 ± 2.97	83.46 ± 11.87
Kidney	0	2,145 ± 494	87.03 ± 6.42	145.9 ± 14.88	76.14 ± 2.55
	1	214 ± 103	13.14 ± 5.18	115.5 ± 70.12	59.32 ± 18.86
	2	161 ± 24	8.49 ± 1.42	112.7 ± 45.23	55.78 ± 2.53
	5	133 ± 17	10.01 ± 2.35	65.03 ± 2.34	58.04 ± 11.63
	14	93 ± 19	16.04 ± 5.28	34.91 ± 8.13	58.27 ± 6.58
Skeletal muscle	0	715 ± 352	28.54 ± 8.47	49.54 ± 11.38	15.74 ± 5.45
	1	122 ± 21	12.10 ± 2.76	38.26 ± 13.23	17.20 ± 5.67
	2	122 ± 34	7.19 ± 1.29	36.80 ± 11.62	13.39 ± 3.42
	5	119 ± 21	6.60 ± 1.01	26.18 ± 6.50	17.32 ± 1.86
	14	93 ± 14	12.55 ± 4.72	21.44 ± 10.41	17.09 ± 3.21

[a] Mean value ± SD ($n = 3$)

between the relative peak area and substance concentration over the range tested for each substance.

After formalin-fixation for one day (24 h), the ethanol concentration ratios relative to the levels before fixation (100%) were reduced to 8% (brain), 4% (lung), 9% (liver), 10% (kidney) and 17% (skeletal muscle) (Tables 1 and 2). These levels remained almost constant after a 14-day fixation period.

The diethylether concentrations of each organ were reduced after formalin fixation for one day compared with those before fixation (100%) and remained relatively constant thereafter up to 14 days fixation (Tables 1 and 2).

The chloroform concentration ratio of each organ decreased after formalin fixation for one day compared with before fixation (100%) and declined further thereafter up to 14 days fixation (Tables 1 and 2).

The toluene concentration ratios were 83% (brain), 102% (lung), 106% (liver), 78% (kidney) and 109% (skeletal muscle) after formalin fixation for one day compared with those of each organ before fixation (100%) (Tables 1 and 2). They decreased gradually thereafter, except for that in skeletal muscle, to 65% (brain), 40% (lung), 88% (liver) and 76% (kidney). The skeletal muscle ratio was 109% after a 14-day fixation period.

Figure 2 shows the time courses of the concentration ratios of the 4 volatile substances in formalin-fixed to non-fixed kidney tissue. The same pattern was obtained for the other organs, except skeletal muscle. The rank order of the rate of decrease of the concentration ratios was: ethanol > diethylether ≫ chloroform > toluene (Fig. 2).

Discussion

Despite many studies on volatile substances, few investigations on their analysis in formalin-fixed organ tissues have been reported. Generally, volatile substances have been considered to evaporate easily from tissue specimens. However, they could be detected in organ tissues from severely burned bodies [8], indicating that "volatiles" do not necessarily evaporate so easily under certain conditions.

In order to examine the rate of evaporation in formalin-fixed organ tissues, the following typical volatile substances were utilized: hydrophilic ethanol, low boiling point diethylether, hydrophobic chloroform and aromatic toluene (a component of thinners, an illicit substance in Japan).

Using GC-MS analysis, additional products (7 and 8 in Fig. 1 c) were detected in the non-buffered 10% formalin itself before it was used for tissue fixation, but no ethanol was detected. These additional products may have formed

Table 2 Ratios of the concentrations of the four volatile substances in the formalin-fixed to those in the non-fixed organ tissues

Organ tissue	Time after formalin fixation (t_n) (days)	Fixed organ (t_n) / non-fixed organ (t_0) ratio			
		Ethanol	Diethylether	Chloroform	Toluene
Brain	0 (t_0)	1	1	1	1
	1 (t_1)	0.08	0.22	0.74	0.83
	2 (t_2)	0.06	0.16	0.55	0.50
	5 (t_5)	0.08	0.30	0.61	1.09
	14 (t_{14})	0.05	0.24	0.22	0.65
Lung	0	1	1	1	1
	1	0.04	0.15	0.45	1.02
	2	0.05	0.18	0.63	0.49
	5	0.06	0.31	0.59	0.71
	14	0.06	0.16	0.15	0.40
Liver	0	1	1	1	1
	1	0.09	0.13	0.66	1.06
	2	0.07	0.09	0.73	0.99
	5	0.06	0.11	0.43	1.10
	14	0.04	0.12	0.17	0.88
Kidney	0	1	1	1	1
	1	0.10	0.15	0.79	0.78
	2	0.08	0.10	0.77	0.73
	5	0.06	0.12	0.45	0.76
	14	0.04	0.18	0.24	0.76
Skeletal muscle	0	1	1	1	1
	1	0.17	0.42	0.77	1.09
	2	0.17	0.25	0.74	0.85
	5	0.17	0.23	0.53	1.10
	14	0.13	0.44	0.43	1.09

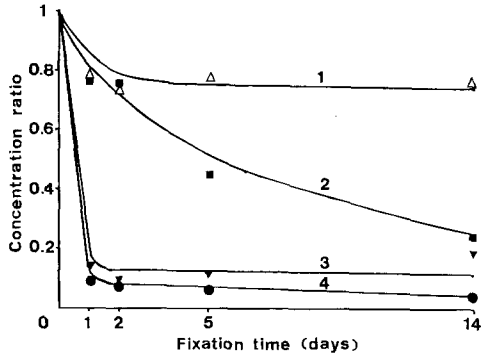

Fig. 2 Ratios of the concentrations of the volatile substances in the kidney after to those before formalin fixation. The measured and calculated values are shown in Tables 1 and 2 respectively. (△) Toluene (1), (■) chloroform (2), (▼) diethylether (3), (●) ethanol (4)

due to an acid-catalyzed nucleophilic reaction between ethanol and formaldehyde [9].

The concentration of water-soluble ethanol in each organ remaind at 4–17%, compared with that of non-fixed organs (100%), after formalin fixation for one day. Subsequently, the concentrations in the fixed organs did not decrease so rapidly after fixation for 2 days or longer. The reason for this phenomenon was thought to be that the ethanol concentration equilibrated only one day later, since ethanol in the fixed organs diffused rapidly into the formalin solution in a closed system, such as a plastic vessel.

Diethylether, chloroform and toluene are only slightly soluble in water: their respective solubilities are 6.0, 0.74 and 0.063% (Table 3) [2, 16]. Approximating curves were constructed to examine the relationships between the water solubilities of the 4 volatile substances and the rate of decrease of their concentrations in the fixed organ tissues using the formula:

$$Y = e^{-bt},$$

where Y represents the ratio of the concentration of each volatile substance in the formalin-fixed to that in the non-fixed tissue (data shown in Table 2), t is the fixation time (days) and b is the coefficient evaluated by fitting the curve in Fig. 2 (Table 3). The coefficient (b) was considered to represent the rate of decrease of the volatile substance concentration in the formalin-fixed organ tissue. Figure 3 shows the relationships between the water solubilities of the 3 hydrophobic volatile substances and the coefficients of their approximating curves in kidney (a in Fig. 3) and brain (b in Fig. 3) tissues. Both factors were closely related, indicating a close relationship between the water solubilities of the 3 hydrophobic volatile substances and their decreasing ratios in the formalin-fixed organ tissues. Further studies with other hydrophobic volatile substances are needed to determine whether such a relationship is generally applicable to such compounds.

Table 3 Physicochemical properties of the volatile substances and coefficients of their approximating curves

Volatile substance	Molecular weight[a]	Boiling point (°C)[a]	Solubility in water[a]	Coefficient of the approximating curve (b)[c]			
				Brain	Lung	Liver	Kidney
Ethanol	46.07	78.5	Freely soluble	0.173	3.00	2.41	2.30
Diethylether	74.12	34.6	8.43% (w/w) (15°C), 6.05% (w/w) (25°C)	0.170	1.05	1.97	1.83
Chloroform	119.39	61–62	1 ml/200 ml water (25°C) (= 0.74% (w/v))	0.098	0.511	0.160	0.163
Toluene	92.13	110.6	0.627 g/l water (25°C)[b] (= 0.0627% (w/v))	0.033	0.065	0.0037	0.073

[a] Data from Merck Index [16]
[b] Data from Bohn and Claussen [2]
[c] Approximating curve, $Y = e^{-bt}$ [Y, ratio of concentration of each substance in the formalin-fixed to that in the non-fixed tissue (Table 2); t, fixation time (days), b, coefficient]

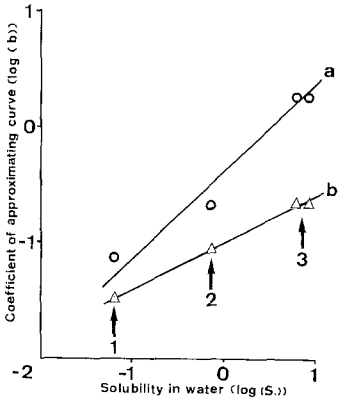

Fig. 3 Relationship between water solubility and the coefficient of an approximating curve. The data are listed in Table 3. (○) Kidney (a); (△) brain (b); 1, toluene; 2, chloroform; 3, diethylether *(double plots)*

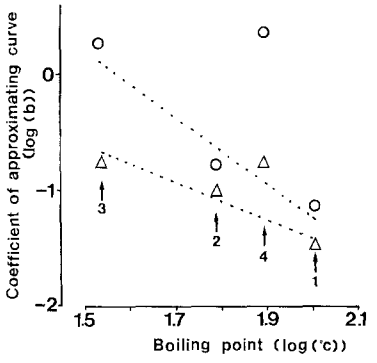

Fig. 4 Relationships between the boiling point and coefficient of the approximating curve. Data are listed in Table 3. (○) Kidney; (△) brain; 1, toluene; 2, chloroform; 3, diethylether; 4, ethanol

The relationship between the coefficients (b) of the 4 volatile substances and their boiling points can be interpretated as follows (Fig. 4). If ethanol is the exception, there is a relationship between the coefficients (b) and boiling points of the other 3 substances. If, however, ethanol is not an exception, no such relationship can be deduced. It would appear therefore that further studies are needed to resolve this problem.

The coefficients (b) values of the 4 volatile substances in the fixed tissues differed among the organs (Table 3). This may be due to different interactions (affinity and/or structural difference) between the organ tissue components and volatile substances [5, 17].

In a preliminary test (data not shown), the volatile substances were detected in the fixed organs and the formalin solution, which had been stored in a closed plastic vessel at room temperature (10–20° C) without being renewed even after 3 months. Therefore it was assumed that volatile substances which underwent no chemical reaction with formaldehyde did not evaporate readily in a closed system.

In the light of these results, it was concluded that volatile substances administered antemortem could be detected in formalin-fixed organ tissues after at least 14 days fixation at room temperature in a closed system. This shows great promise for clarifying whether volatile substances have been administered antemortem or postmortem.

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