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# Toxicological analysis of the psychotropic drugs chlorpromazine and diazepam using chemically fixed organ tissues

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**Abstract** Toxicological analysis for chlorpromazine and diazepam was performed using chemically fixed organ tissue specimens. After chlorpromazine and diazepam had been injected into rabbits, organ tissues (brain, lung, liver, kidney and skeletal muscle) were collected and fixed in 3 fixative solutions: buffered 10% formalin solution (pH 7.4, 10% BF), non-buffered 10% formalin solution (pH 5.1, 10% non-BF), buffered 4% paraformaldehyde solution (pH 7.4, 4% BPA). Chlorpromazine and diazepam were determined by GC-MS (gas chromatography-mass spectrometry) after 5 different fixation periods, and were detected even after 28 days of fixation. Recoveries of chlorpromazine and diazepam in 10% BF were within the range 48–86% and 68–171%, respectively after 28-day fixation, those in 10% non-BF were 22–54% and 48–78%, respectively, and those in 4% BPA solution were 13–59% and 14–50%, respectively. Thus, 10% BF was found to be the most suitable fixation medium for analysis of chlorpromazine and diazepam.

**Key words** Toxicology · Fixed organ tissue · GC-MS · Chlorpromazine · Diazepam

**Zusammenfassung** Chlorpromazin- und Diazepamgehalte aus fixiertem Organmaterial wurden bestimmt. Nach intravenöser Gabe von Chlorpromazin und Diazepam an neun Kaninchen, wurde Organmaterial (Gehirn, Lunge, Leber, Niere und Skelettmuskulatur) entnommen und nach drei unterschiedlichen Verfahren fixiert: 1. gepufferte (pH=7.4), 10%ige Formalin-Lsg., (10% BF); 2. ungepufferte (pH=5.1), 10%ige Formalin-Lsg., (10% non-BF) und 3. gepufferte (pH=7.4), 4%ige Paraformaldehyd-Lsg. (4% BPA). Nach 28 Tagen Fixierung wurden die Chlorpromazin- und Diazepamgehalte gaschromatographisch mit massenspezifischer Detektion (GC-MS) bestimmt. Die Wiederfindungsraten für Chlorpromazin und Diazepam lagen für die erste Variante (10% BF) zwischen 48–86% bzw. 68–171%, für die zweite Variante (10% non-BF) zwischen 22–54% bzw. 48–78% und für die dritte Fixierungsart (4% BPA) zwischen 13–59% bzw. 14–50%. Die Fixierung mit gepuffertem, 10%igem Formalin war die geeigneteste Fixierungsvariante zur anschließenden Bestimmung von Chlorpromazin und Diazepam aus fixiertem Organmaterial.

**Schlüsselwörter** Toxikologie · fixiertes Organmaterial · GC-MS · Chlorpromazin · Diazepam

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## Introduction

Forensic toxicological analysis is generally performed using fresh and/or frozen biological specimens collected at autopsy. When no specific biological specimens are preserved for toxicological analysis, for example, in cases when death due to intoxication is suspected after burial, chemically fixed organs prepared for histopathological examination may often be the only specimens available. Recently, there have been some reports on the detection of drugs and poisons in fixed organ tissues [1, 2]. Our laboratory has also reported that methamphetamine can be detected in formalin-fixed organs [3].

The present report describes forensic toxicological analysis for chlorpromazine and diazepam in organ tissue specimens after fixation.

## Materials and methods

### Psychotropic drugs and fixative solutions

Chlorpromazine hydrochloride (5 mg/mL) and diazepam (5 mg/mL) solutions were purchased from Shionogi and Yamanouchi Pharmaceutical Corporations, respectively. Chlorpromazine hydrochloride, clomipramine hydrochloride, diazepam and prazepam were obtained from Yoshitomi, Nippon Ciba-Geigy, Yamanouchi and Sumitomo, respectively. Stock solutions (4 mg/mL) of chlorpromazine hydrochloride and clomipramine hydrochloride were

prepared as the free base with ethanol, and diazepam and prazepam were dissolved in ethanol and acetone, respectively. Working solutions of chlorpromazine (range 1.0–200 μg/mL) and diazepam (range 0.5–100 μg/mL) were prepared, and clomipramine and prazepam (as internal standards) were made up at a concentration of 40 μg/mL.

The chemical fixative solutions used to preserve the organ tissue specimens were non-buffered 10% formalin solution (10% non-BF, containing 3.7% formaldehyde, pH 5.1), 10% formalin solution buffered with 0.1 M Na phosphate (10% BF, containing 3.7% formaldehyde, pH 7.4), and 4% paraformaldehyde solution buffered with 0.1 M Na phosphate (4% BPA, pH 7.4).

### Animal experiments and tissue fixation

Nine rabbits (weighing ca. 2 kg) were divided into 3 groups; group A was used for clarifying the fixative effect of 10% buffered formalin (10% BF), group B for 10% non-buffered formalin (10% non-BF), and group C for 4% buffered paraformaldehyde (4% BPA). The experimental procedure for each group is shown in Fig. 1.

3 rabbits

↓ 20 mg/kg chlorpromazine hydrochloride and 20 mg/kg diazepam were injected subcutaneously into the dorsum and thigh of each rabbit

↓ Sacrificed by carbon monoxide inhalation (2–3 h after the injection)

Organ tissues (brain, lung, liver, kidney and skeletal muscle) were collected as soon as possible after death of each rabbit

↓ Cut into 6 parts (measuring about 1 cm × 1 cm × 2 cm)

5 of these were immersed in 80 ml fixative (group A; 10% BF, group B; 10% non-BF, group C; 4% BPA) for 5 different fixation periods (i.e. 1, 3, 7, 14 and 28 days)

Remaining 1 part was used as a control for analysis without fixation

**Fig. 1** Flow chart of animal experiments and tissue fixation. The preparations fixed in 10% BF (group A) and 10% non-BF (group B) were stored at room temperature (10–20°C), and that in 4% BPA (group C) was stored at 4°C

**Fig. 2** Mass chromatograms and mass spectra of diazepam (1) and chlorpromazine (2) in the eluate from brain tissue fixed in 10% non-BF for 28 days; IS 1, clomipramine; IS 2, prazepam

### Extraction method and analytical conditions

The fixed organs were rinsed once in water, wiped, minced and weighed to prepare extraction samples as follows: 3 mL of 0.1 M borate buffer (pH 11) was added to all samples (1.0 g) except for skeletal muscle (6 mL of 0.1 M borate buffer). Clomipramine (20 μg) and prazepam (10 μg) were added as internal standards, except for lung tissue where 40 μg clomipramine was added, the samples were homogenized, and centrifuged at 10,000 rpm. The supernatant was transferred to an Extrelute column, allowed to soak for 15 min, and eluted with 20 mL n-heptane. Fixatives used for storage of the organ tissues during the 28-day fixation period were adjusted to pH 11 with 1 N NaOH solution, and eluted by the same method. The eluates were evaporated to dryness under nitrogen gas at 50°C, and then dissolved in ethanol for gas chromatography - mass spectrometry (GC-MS) analysis.

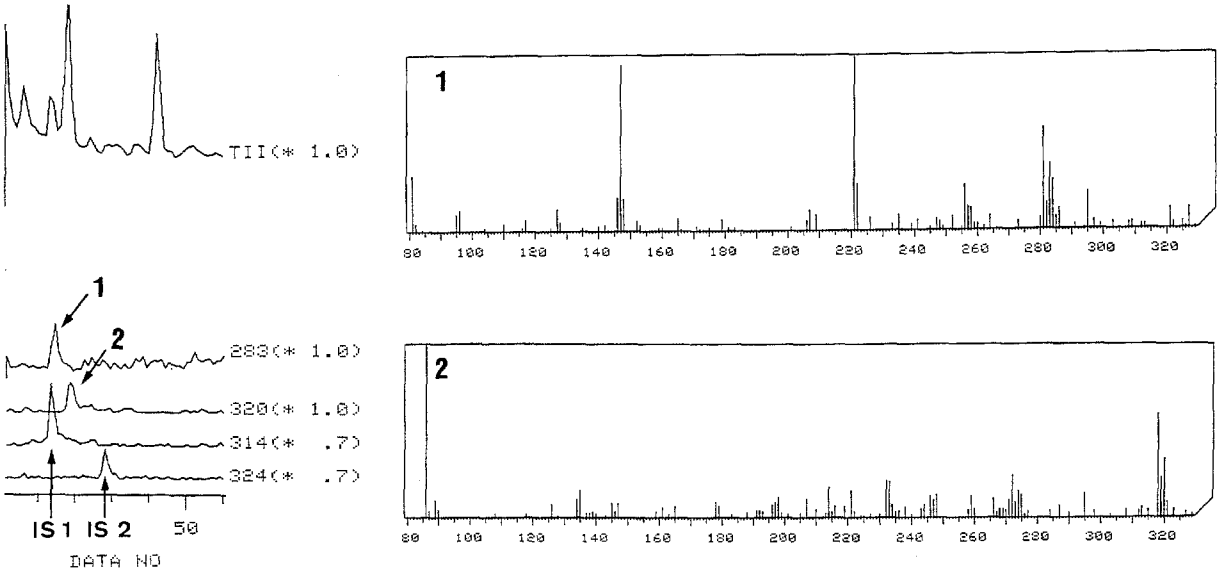
For GC-MS, a Shimadzu QP-1000 apparatus, equipped with a DB-1 column (0.53 mm × 15 m, J & W Scientific) was used. The column was programmed at 250°C for 1 min, followed by an increase of 8°C/min with a hold at 280°C for 5 min. The GC injection port was set at 250°C and the carrier gas was helium at a flow rate of 15 mL/min.

The mass spectrometer was operated in the multiple ion monitoring and selected ion monitoring modes in the positive electron impact mode. Quantitative analysis was performed using the peak height ratios of chlorpromazine (m/z 320) to clomipramine (m/z 314), and diazepam (m/z 283) to prazepam (m/z 324). The detection limits of chlorpromazine and diazepam were approximately 0.5 and 0.1 μg/mL, respectively.

## Results

### Qualitative analysis

Figure 2 shows the mass chromatograms and mass spectra of the eluate of brain after 28-day fixation with 10% non-BF formalin solution, which were consistent with those of the authentic substances. Diazepam was detected in the eluate from fixatives, but chlorpromazine was not.



Quantitative analysis

The standard curves for chlorpromazine and diazepam revealed a linear relationship between the concentration and the peak height ratio. Recoveries of chlorpromazine and diazepam from non-fixed frozen liver tissue were 73–98%, and 57–63%, respectively. The recoveries of these drugs from liver tissue fixed with 10% non-BF were 68–97% and 58–66%, respectively.

Extraction samples from experimental organ tissue specimens of rabbits were measured, and a comparison was made of the chlorpromazine and diazepam levels after each fixation period with those before fixation. Three rabbits were used for each experiment with each fixative solution.

Mean chlorpromazine concentrations in non-fixed organ tissues in 10% BF, 10% non-BF and 4% BPA are shown in Table 1. The chlorpromazine concentrations were

**Table 1** Mean chlorpromazine concentrations in non-fixed organ tissues before fixation in 10% BF, 10% non-BF and 4% BPA

| Organ | Buffered 10% formalin | Non-buffered 10% formalin | 4% Paraformaldehyde |
|---|---|---|---|
| Brain | 63.50 ± 3.10* | 74.78 ± 12.20 | 91.18 ± 18.97 |
| Lung | 405.7 ± 117.2 | 601.7 ± 128.6 | 600.4 ± 147.2 |
| Liver | 33.09 ± 6.59 | 56.56 ± 22.64 | 65.87 ± 25.52 |
| Kidney | 91.78 ± 16.81 | 158.9 ± 54.93 | 153.9 ± 10.64 |
| Skeletal muscle | 26.90 ± 2.31 | 48.33 ± 9.52 | 83.83 ± 11.67 |

* Mean values (μg/g) ± SD. ($n$=3).

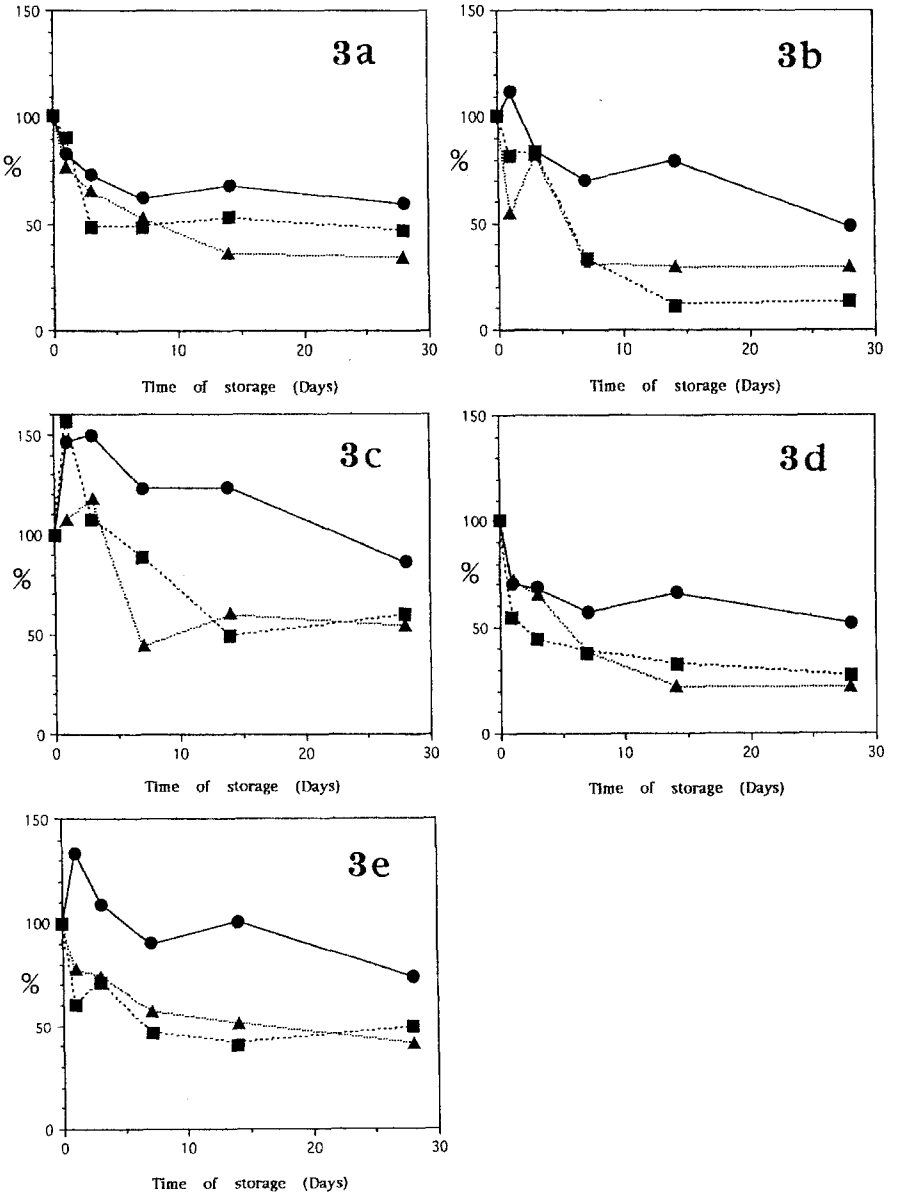



**Fig. 3** Concentrations of chlorpromazine in fixed organ tissues calculated by comparing the measured values in Table 1 with those of non-fixed organ tissues. In each panel, the solid circles (●) represent 10% BF, the solid triangles (▲) represent 10% non-BF, and the solid squares (■) represent 4% BPA solution. Fig. 3a, brain; 3b, lung; 3c, liver; 3d, kidney; 3e, skeletal muscle

highest in the lungs for each fixative solution. Taking the values for chlorpromazine in non-fixed organ tissues before each chemical fixation procedures as 100%, percentages of chlorpromazine remaining in the organ tissue fixed with each fixative solution are shown in Fig. 3. The percentages of chlorpromazine decreased gradually as the fixation period became longer.

Mean diazepam concentrations in non-fixed organ tissues for 10% BF, 10% non-BF and 4% BPA are shown in Table 2. The values for non-fixed organ tissues (100%) before each chemical fixation procedures compared to percentages of diazepam remaining in the organ tissues fixed with each fixative solution are shown in Fig. 4. The percentages of diazepam in the lung and skeletal muscle in the case of 10% BF were notably increased after the fixation.

**Table 2** Mean diazepam concentrations in non-fixed organ tissues before fixation in 10% BF, 10% non-BF and 4% BPA

| Organ | Buffered 10% formalin | Non-buffered 10% formalin | 4% Paraformaldehyde |
|---|---|---|---|
| Brain | 16.68 ± 6.45* | 47.47 ± 9.09 | 20.94 ± 12.60 |
| Lung | 16.78 ± 5.42 | 42.23 ± 5.12 | 38.73 ± 6.65 |
| Liver | 14.71 ± 4.62 | 43.44 ± 5.96 | 24.42 ± 9.00 |
| Kidney | 9.65 ± 4.30 | 26.01 ± 5.91 | 15.34 ± 1.91 |
| Skeletal muscle | 9.20 ± 4.27 | 20.86 ± 5.36 | 22.56 ± 7.55 |

* Mean values (μg/g) ± SD. ($n$=3).

## Discussion

Chlorpromazine and diazepam are the most commonly available psychotropic drugs in Japan, and are often the cause of acute poisoning, either accidentally or intention-

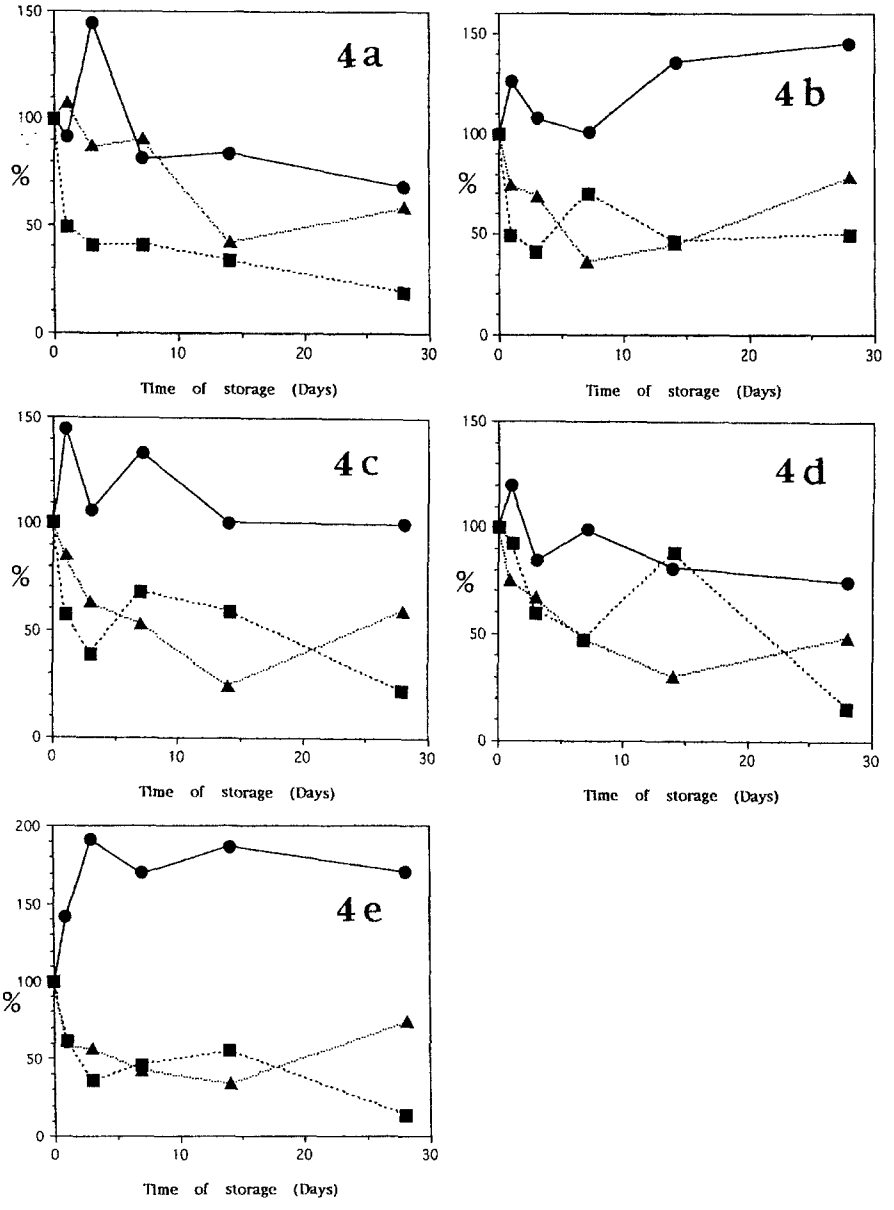



**Fig. 4** Concentrations of diazepam in fixed organ tissues calculated by comparing the measured values in Table 2 with those of non-fixed organ tissues. In each panel, the solid circles (●) represent 10% BF, the solid triangles (▲) represent 10% non-BF, and the solid squares (■) represent 4% BPA solution. Fig. 4a, brain; 4b, lung; 4c, liver; 4d, kidney; 4e, skeletal muscle

ally. The Investigation Committee of the Medico-Legal Society of Japan has already reported that chlorpromazine is responsible for approximately half of all cases of poisoning by psychotropic drugs [4].

After chemical fixation procedures, the total chlorpromazine content in tissue specimens decreased, although chlorpromazine could still be detected in each organ tissue even after 28 days. The organs fixed in 10% BF showed higher amounts of residual chlorpromazine than those treated with other fixatives, and similar results were obtained for diazepam.

The fatal concentration of chlorpromazine in serum is reported to be 3.0–12.0 μg/mL in man [5]. Another study has shown that chlorpromazine concentrations in brain, liver and kidney were 12.0, 84.0 and 34.0 μg/mL, respectively, when the lethal concentration of the drug in blood was 6.6 μg/mL [6]. Cardauns and Iffland [7] reported the drug concentrations in a case of diazepam-alcohol fatality as 30 μg/mL in blood, 16 μg/g in liver and 0.8 μg/g in kidney. The levels of chlorpromazine and diazepam used in our animal experiments were much higher than those found in humans. However, it is thought that chlorpromazine and diazepam would still be detectable by our method, even if the concentrations in human specimens were about 10-fold lower, since the detection limits for chlorpromazine and diazepam in this study were about 0.5 and 0.1 μg/mL, respectively. These are much lower than the concentrations seen in cases of fatal chlorpromazine or diazepam intoxication in humans.

In our study, the percentage recoveries of chlorpromazine and diazepam after fixation in 10% BF were higher than those using other fixatives. Dennis [8] reported that the carbonium ion ($^{+}CH_2OH$) is responsible for the major fixative effects of formaldehyde in aqueous solution. Since the production of carbonium ions decreases as the pH increases, buffered formaldehyde solutions produce rather weaker fixative effects than non-buffered solutions. It is assumed from our findings that percentage recoveries of chlorpromazine and diazepam depend on the pH of the fixative. That is, neutral buffered fixative has no marked influence on the percentage recoveries of these 2 drugs for at least 28 days, whereas acidic fixatives reduce the recoveries. Moreover, although 10% BF contains about 1.0% (w/v) methanol as a stabilizer of formaldehyde, and also formic acid produced by oxidation of formaldehyde, 4% BPA does not contain methanol or formic acid. From this, it is assumed that the effects of 10% BF on chlorpromazine and diazepam concentrations were weaker than those of the other fixative solutions used in this study.

Chlorpromazine and diazepam are highly lipophilic basic drugs, which accumulate in tissues over a long period in a bound form with plasma components and/or tissue proteins. Yokogawa et al. [9] reported the tissue-to-plasma partition coefficients of these drugs in the organs of rabbits, that of chlorpromazine in the lungs being especially high (64 times that in plasma). In this study also, the chlorpromazine concentrations in the lungs were the highest (range 405.7–601.7 μg/g) among all the organ tissues in each fixative solution. After 28 days of fixation, the decrease of chlorpromazine concentration in lung tissue fixed with each fixative was more conspicuous than that in the other organ tissues; 48% decrease in 10% BF, 13% in 4% BPA, and 29% in 10% non-BF. Chlorpromazine in lung tissue might thus be greatly influenced by the fixative solution as well as the organ tissue, since its concentration in the lungs was high.

Fixed organ tissues provide useful alternative materials for forensic toxicology, and studies on the stability and degradation of drugs in formalin – containing solutions and formalin – fixed tissues have recently been reported. Dettling et al. [10] reported that amitriptyline was produced from nortriptyline by N-methylation in formalin-containing solutions. Winek et al. [11] also reported that the concentration of amitriptyline and imipramine (tertiary amines) were increased by N-methylation of nortriptyline and desipramine (secondary amines), respectively. In our study, N-demethylated chlorpromazine (secondary amine) might have been present in the organ tissues, although there was no significant increase in chlorpromazine.

On the contrary, Winek et al. [12] reported that the concentration of diazepam decreased in formalin-blood solutions, thus suggesting that diazepam was not methylated. Therefore, we consider that methylation of desmethyldiazepam to diazepam has hardly occurred and it has almost no influence on the diazepam concentrations in organ tissues.

However, concentrations of diazepam in lung tissue and skeletal muscle fixed with 10% BF were higher than those in non-fixed organ tissues. In this study, the ion at m/z 283 was selected for quantitative analysis of diazepam. Therefore it might be reasonable to consider that nordiazepam (N-demethylated derivative of diazepam) and/or other substances may have influenced the quantitative analysis of diazepam in organ tissues. However, Kudo et al. [13] reported that nordiazepam itself showed a base ion at m/z 242 and a molecular ion at m/z 270. Since m/z 283 is one of the most suitable ions for quantitative analysis of diazepam, disturbance of the m/z 283 peak by nordiazepam has never been speculated theoretically. Therefore we conclude that our measured values of diazepam in organ tissues are theoretically reliable and practically useful. Moreover, diazepam extraction and analysis from each organ fixed with 10% BF, 10% non-BF and 4% BPA, were performed under the same conditions. Although diazepam concentrations in the lungs and skeletal muscle fixed with 10% BF increased, diazepam concentrations in other organ tissues fixed with 10% BF did not increase. In addition, in the case of fixation using 10% non-BF and 4% BF, diazepam concentrations did not increase in every organ tissue. Although, we cannot fully explain the reason why the diazepam concentration in the 10% BF-fixed lungs and skeletal muscle increased, we consider that the increase in the lungs and skeletal muscle fixed with 10% BF was not due to contamination.

A tendency for increased diazepam concentrations was observed in lung tissue and skeletal muscle. Qualitative

analysis detected diazepam not only in lung tissue and skeletal muscle but also in the eluate from the fixatives. Since the partition coefficients of diazepam in these 2 tissues are higher than in brain tissue [9], it is speculated that the diazepam eluted from these tissues into 10% BF may reaccumulate or be redistributed in the same tissues during fixation. A disproportionate or uneven redistribution of diazepam in non-fixed and fixed organ tissues may be responsible for the increased concentration of the drug in lung tissue and skeletal muscle. The increase of diazepam concentration in fixed lung tissue and skeletal muscle may thus be attributable to these phenomena, although further experiments are needed to confirm this.

In the present study chlorpromazine and diazepam were detected successfully in organ tissues fixed in 10% BF, 10% non-BF and 4% BPA, even after 28 days. Among these fixatives, 10% BF was the most suitable for chlorpromazine and diazepam analysis.

All of the animal experiments in the present study were done under the ethical guidelines for animal experimentation of the Takara-machi area of Kanazawa University.

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