TECHNICAL NOTE

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Stability of Tricyclic Antidepressants in Formalin Solutions

REFERENCE: Takayasu T, Holterman K, Ohshima T, Pounder DJ, Stability of tricyclic antidepressants in formalin solutions. J Forensic Sci 1998;43(6):1213–1219.

ABSTRACT: We investigated the stability of the secondary amines, desipramine (DP) and nortriptyline (NRT), and the tertiary amines, imipramine (IP) and amitriptyline (AT), in formaldehyde (F) and paraformaldehyde (PF) aqueous solutions. NRT showed little instability in 0.37 to 37% F and PF solutions, but AT formation was detected and increased, up to 0.46 to 2.7%, in parallel with rising F and PF concentrations. DP was unstable and levels decreased to 74 to 96% with increasing F concentrations, and fell only to 96% in 10% PF solution. IP formation increased in the same manner as AT to 2.9 to 3.5% of the initial DP. When AT and IP were stored in F and PF solutions, concentrations of AT and IP did not change. DP in F pH 3 to 11 phosphate buffer (PB) solutions showed high recovery in the order: pH 5 > pH 7 > pH 9 > pH 3 and pH 11. DP in PF buffered solutions decreased slightly only at pH 3 (3.5%). By contrast, IP did not change at any pH (pH 3 to 11) of the F or PF solutions. During storage for 21 days at room temperature in 3.7% F and PF solutions, IP and DP degradation was accelerated when compared with the values in pH 3 and 7 PB solutions. However, IP detected in DP F or PF solution was only 0.2% of the initial DP 21 days after storage. Thus, AT, NRT, IP and DP degraded gradually in F and PF solutions during storage at room temperature. TCAs may first react nucleophilically with formaldehyde to form hemiaminals. DP in 3.7% formaldehyde aqueous solution formed little of its methylated product, IP, at room temperature.

KEYWORDS: forensic science, forensic toxicology; desipramine; imipramine; nortriptyline; amitriptyline; formaldehyde, stability

Tricyclic antidepressants (TCAs) are widely used for the treatment of depression (1), and many cases of overdose using TCAs have been reported (2–7). TCA analysis in biological specimens is, therefore, common in forensic toxicology. If tissues suitable for analysis are not retained at autopsy, then tissues in 10% formalin (3.7 to 4% formaldehyde) solution retained for histopathological investigation may be used for analysis. Also, similar material may be obtained from autopsies performed on bodies embalmed using formalin solutions (8,13)

Drug analysis using formalin-fixed tissues is a new challenge for forensic toxicologists (8–18). TCAs (8,13), phenobarbital (9,13), ethchlorvynol (10), paraquat (11,12), diazepam (13), phenytoin (13) and malathion (15) have all been detected in formalin-fixed tissues. However, it is thought that formaldehyde may react with some drugs (8,10,13,16), compromising the analytical result. To further explore this problem, we studied the stability of TCAs when exposed to formalin.

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Received 6 Jan. 1998; and in revised form 18 March 1998; accepted 23 March 1998.

Materials and Methods

Desipramine (DP) hydrochloride and imipramine (IP) hydrochloride, nortriptyline (NRT) hydrochloride and amitriptyline (AT) hydrochloride were obtained from Sigma (St. Louis, MO). Dothiepin hydrochloride was supplied from Boots (Nottingham, U.K.). Standard formaldehyde (37% aqueous solution containing 8 to 10% methanol) abbreviated as formaldehyde (S) and 30% analytical formaldehyde aqueous solution containing less methanol (0.7%) abbreviated as formaldehyde (LM) were obtained from Chemix (Wigan, U.K.) and Synthite (Clwyd, U.K.), respectively. Paraformaldehyde was from Sigma-Aldrich (Dorset, U.K.). All other reagents were of analytical grade.

Formaldehyde (S) and formaldehyde (LM) were diluted to 3.7 and 0.37% with deionized water. Paraformaldehyde was dissolved in hot water to give a 10% solution, and then the solution was diluted to 3.7 and 0.37% with deionized water. The pH of each solution was measured with a model pHASAR-I digital pH meter (Beckman, CA) using a model PHM-110-070N CMAWL electrode (Russell, Fife, U.K.).

Formaldehyde (S), formaldehyde (LM) and paraformaldehyde solutions were finally adjusted to the concentration of 3.7% with deionized water. Before final adjustment of volume, 10 mM phosphate solution was added and then the pH adjusted to 3, 5, 7, 9 or 11 with 0.5 M sodium hydroxide.

DP, IP, NRT or AT hydrochloride was dissolved in 10% ethanol solution at a concentration of 1.0 mg/mL. Dothiepin hydrochloride was also dissolved at 1.0 mg/mL in 10% ethanol solution for the internal standard (IS). An aliquot of 1.0 mg/mL DP, IP, NRT or AT hydrochloride solution was added in each 1 mL of formaldehyde or paraformaldehyde medium, and left for 60 min at room temperature (15 to 25 °C).

For the extraction of desipramine, imipramine, nortriptyline and amitriptyline from formaldehyde and paraformaldehyde solutions, all procedures were carried out at room temperature (15 to 25°C). An aliquot (10 μL) of IS solution was added to DP, IP, NRT and AT formaldehyde and paraformaldehyde solutions in a 10 mL

screw-capped test tube. To this 2 mL of 0.5 M sodium hydroxide was added and mixed briefly on a vortex mixer (Stuart Scientific, U.K.). To the mixture was added 4 mL of n-heptane/iso-amyl alcohol (98.5:1.5, v/v), and it was again mixed using a Spiramix X10 (Denley, U.K.) for 30 min. The mixture was centrifuged at 3000 rpm for 10 min. The upper organic layer was transferred to a 15 mL screw-capped test tube. To the remaining lower layer, a further 4 mL of n-heptane/iso-amyl alcohol (98.5:1.5,v/v) was added, and Spiramix mixing and centrifugation were carried out in the same manner. The upper organic layer was then transferred to the organic layer from the previous step. To the combined organic layer, 2 mL of 0.05 M sulfuric acid was added, and it was placed on the Spiramix X10 for 30 min. After centrifugation, the upper layer was discarded. To the lower layer, 1 mL of 1.07 M sodium carbonate buffer, pH 9.45 was added, and then mixed briefly on the vortex mixer. To the mixture was added 3 mL of toluene/iso-amyl alcohol (85:15, v/v), and it was then placed on the Spiramix X10 for 30 min. After centrifugation, the upper layer was transferred to a one-dram vial, and evaporated under an airstream at 50°C. The residue was redissolved in 200 or 400 μL of ethylacetate for DP, IP, NRT and AT analysis. One μL of the sample solution was applied to gas chromatography-mass spectrometry (GC-MS).

A model GC8060-MD800 GC-MS system (Fisons, U.K.) was used. The GC was equipped with an autosampler AS800 (Fisons). The GC column used was a WCOT fused silica column coating with CP-SIL 5CB (DF = 0.25), 25 m × 0.25 mm internal diameter (Chrompack, The Netherlands). Column temperature was programmed 100°C for 1 min, to 300°C at the rate of 20°C/min, and then held at 300°C for 2 min. Carrier gas was helium, linear velocity 37.1 cm/s. Analysis was performed using electron impact and positive ion detection modes. Ionization energy was 70 eV. Ionization chamber temperature was 250°C. Both qualitative and quantitative analyses were carried out in a multiple ion monitoring mode. For quantitative analysis, peak areas of DP (44 and 195 μ), NRT (44 μ), IP (58 μ), AT (58 μ) and dothiepin (IS, 58 μ) were used. Calibration curves for NRT, AT, DP and IP were made at concentrations of 1, 10, 30 and 50 μg/mL for each batch of analyses.

Results

Under these analytical conditions, when NRT, AT, DP and IP were measured at concentrations of 30 μg/mL, recoveries (μg/mL), SD values and CV values (%) were 30.78, 1.28, and 4.2% for NRT; 29.41, 0.27, and 0.92% for AT; 29.79, 1.16 and 3.9% for DP; 30.54, 0.23 and 0.75% for IP.

NRT showed almost no change in all the 0.37 to 37% formaldehyde and paraformaldehyde solutions at room temperature, but some AT was detected (Table 1). The amount of AT detected increased, up to 0.46 to 2.7% of the initial NRT, in parallel with increasing formaldehyde and paraformaldehyde concentrations in each solution. DP concentrations in the formaldehyde and paraformaldehyde solutions decreased to 74 to 96%, with increasing formaldehyde and paraformaldehyde concentrations. At the same time as there was a decrease in DP concentration, a small amount of IP was detected. IP concentrations increased in parallel with raising the concentration of formaldehyde and paraformaldehyde (Table 1). AT and IP concentrations in the 0.37 to 37% formaldehyde and paraformaldehyde solutions showed no change when compared with 0.15 M sodium chloride solution and methanol (Table 2).

DP in 3.7% formaldehyde with pH 3 to 11 phosphate buffer (PB) solutions showed a high recovery in the order: pH 5 > pH 7 > pH 9 > pH 3 and pH 11 (Table 3). DP concentrations in formaldehyde (S) buffered solutions decreased only at pH 3, although those in formaldehyde (LM) decreased at pH 9 and 11. A small amount of IP was detected in the same DP formaldehyde and paraformaldehyde solutions. The IP concentrations showed a tendency to increase in parallel with increasing pH (Table 3). In contrast to DP, recoveries of IP in buffered (pH 3 to 11) formaldehyde and paraformaldehyde solutions showed no change (Table 4). In addition, no DP was detected in these solutions.

A DP-storage experiment was carried out in non-buffered and pH 7 buffered 3.7% formaldehyde and paraformaldehyde solutions at room temperature for 21 days. DP concentration in pH 7 PB fell to 91% after 21 days storage while that in pH 3 PB showed almost no change. DP concentrations in the non-buffered formaldehyde and paraformaldehyde solutions were slightly decreased (88 to 94%), although DP concentrations in the formaldehyde and paraformaldehyde pH 7 buffered solutions gradually decreased to 74 to 84% during storage (Tables 5 and 6). A very small amount of IP was detected in the 3.7% formaldehyde and paraformaldehyde DP solutions. IP concentrations in the non-buffered formaldehyde and paraformaldehyde solutions were less than 0.018 μg/mL, while IP concentrations (0.043 to 0.59 μg/mL) in the formaldehyde and paraformaldehyde pH 7 PB solutions after 21-day storage were slightly higher than those of the non-buffered formaldehyde and paraformaldehyde solutions (Table 5).

In the IP storage experiment, IP concentrations in the pH 3 and 7 PB solutions were reduced to 93 and 84% after storage for 21 days, respectively. IP concentrations in the non-buffered 3.7% formaldehyde and paraformaldehyde solutions were decreased to 84 to 87% after storage for 21 days. Also, IP concentrations in the 3.7% formaldehyde and paraformaldehyde pH 7 PB solutions were decreased to 78 to 81% in the same period (Tables 5 and 6). DP was, however, not detected at all in the IP storage solutions.

Discussion

The chemistry of the interaction between TCAs and formaldehyde is that of amines with an aldehyde. Addition of primary amines to an aldehyde give N-substituted hemiaminals which lose water to give the stable Schiff bases (19)

$$R_1CHO + R_2NH_2 \rightarrow R_1CHOH\text{-}NHR_2 \rightarrow R_1CH{=}NR_2 + H_2O$$

When secondary amines are added to aldehydes, initially there are formed N,N-disubstituted hemiaminals: $R_1CHO + R_2R_3NH \rightarrow R_1\text{-}CHOH\text{-}NR_2R_3$. These hemiaminals cannot lose water in the same way as the primary amines (19). Tertiary amines can only give salts: $R_1CHO + R_2R_3R_4N \rightarrow R_1\text{-}CHOH\text{-}NR_2R_3R_4$. When an aldehyde is treated with a primary or secondary amine in the presence of hydrogen and a hydrogenation catalyst, reductive alkylation takes place. Reaction of secondary amines is possible only by a hydrogenolysis pathway. Other reducing agents may be used instead of hydrogen as a catalyst, among them, zinc and hydrochloride, sodium borohydride and formic acid (19). If formic acid is contained in formaldehyde solution, then by the Eschweiler-Clarke reaction, primary and secondary amines are reductively alkylated with formaldehyde and formic acid to form their N-methyl and N,N-dimethyl products, respectively (19,20)

TABLE 1—*Recoveries of nortriptyline and desipramine in formaldehyde and paraformaldehyde aqueous solutions, and amitriptyline and imipramine detected.*

Nortriptyline Incubation* Medium	pH	Nortriptyline† Target‡ concentration (μg/mL)	Nortriptyline† Measured concentration (μg/mL)	Nortriptyline† Recovery (%)	Amitriptyline† Measured concentration (μg/mL)	Amitriptyline† Recovery§ (%)
37% Formaldehyde (S)‖	2.7	32.94	34.27 ± 1.40¶	104	0.93 ± 0.16	2.7
3.7% Formaldehyde (S)	3.3	32.94	32.70 ± 1.49	99.6	0.16 ± 0.03	0.46
0.37% Formaldehyde (S)	4.0	32.94	32.58 ± 2.62	98.9	0.06 ± 0.03	0.17
30% Formaldehyde (LM)**	3.1	32.94	35.14 ± 4.75	107	0.70 ± 0.26	2.0
3.7% Formaldehyde (LM)	3.7	32.94	36.49 ± 1.38	111	0.34 ± 0.07	1.0
0.37% Formaldehyde (LM)	4.5	32.94	33.21 ± 1.68	101	0.20 ± 0.10	0.58
10% Paraformaldehyde	4.6	32.94	36.01 ± 5.12	109	0.52 ± 0.26	1.5
3.7% Paraformaldehyde	4.7	32.94	34.56 ± 8.96	105	0.20 ± 0.06	0.58
0.37% Paraformaldehyde	5.2	32.94	37.81 ± 2.90	115	0.37 ± 0.12	1.1
0.15 M Sodium chloride	5.7	32.94	37.31 ± 3.94	113	0	0
Methanol (>99.8%)		32.94	33.01 ± 1.24	100	0	0
Desipramine Incubation*		Desipramine†			Imipramine†	
37% Formaldehyde (S)‖	2.7	32.99	24.53 ± 1.42**	74.4	0.95 ± 0.27	2.7
3.7% Formaldehyde (S)	3.3	32.99	30.39 ± 1.00	92.1	0.61 ± 0.06	1.8
0.37% Formaldehyde (S)	4.0	32.99	33.94 ± 1.90	103	0.40 ± 0.03	1.2
30% Formaldehyde (LM)**	3.1	32.99	29.77 ± 1.57	90.2	1.15 ± 0.10	3.3
3.7% Formaldehyde (LM)	3.7	32.99	32.34 ± 1.60	98.0	0.75 ± 0.09	2.2
0.37% Formaldehyde (LM)	4.5	32.99	32.10 ± 0.88	97.3	0.40 ± 0.05	1.2
10% Formaldehyde (LM)	4.6	32.99	31.77 ± 2.86	96.3	0.98 ± 0.13	2.8
3.7% Paraformaldehyde	4.7	32.99	34.38 ± 2.81	104	0.55 ± 0.10	1.6
0.37% Paraformaldehyde	5.2	32.99	34.69 ± 1.60	105	0.47 ± 0.10	1.4
0.15 M Sodium chloride	5.7	32.99	33.05 ± 1.14	100	0	0
Methanol (>99.8%)		32.99	35.37 ± 1.67	107	0	0

* Nortriptyline or desipramine was incubated with each medium for 60 min at room temperature and then extracted by the procedure described in the text.

† Concentrations of nortriptyline, amitriptyline, desipramine and imipramine are shown as fee base.

‡ Target concentrations (μg/mL) of nortriptyline and desipramine are calculated from 6.588 μg/200 μL and 6.597 μg/200 μL, respectively.

§ Recovery of amitriptyline and imipramine calculated as follows; measured concentration of amitriptyline (or imipramine) × 100/34.69 (or 34.72).

‖ Formaldehyde (S) indicates standard formaldehyde.

¶ Mean value ± SD ($n = 5$). Underlined values are significant ($p < 0.05$) by the Student t-test, when compared with the control values (0.15 M sodium chloride and methanol).

** Formaldehyde (LM) indicates formaldehyde containing less methanol.

TABLE 2—*Recovery of amitriptyline and imipramine in formaldehyde and paraformaldehyde aqueous solutions.*

Amitriptyline Incubation* Medium	Amitriptyline† Target‡ concentration (μg/mL)	Amitriptyline† Measured concentration (μg/mL)	Amitriptyline† Recovery (%)	Nortriptyline† Measured concentration (μg/mL)
37% Formaldehyde (S)§	16.57	18.07 ± 0.85‖	109	0
30% Formaldehyde (LM)	16.57	17.69 ± 0.55	107	0
10% Paraformaldehyde	16.57	16.87 ± 0.36	102	0
0.15 M Sodium chloride	16.57	16.56 ± 0.64	99.9	0
Methanol (>99.8%)	16.57	17.17 ± 0.75	104	0
Imipramine Incubation*	Imipramine†			Desipramine†
37% Formaldehyde (S)§	16.59	15.32 ± 1.64‖	92.3	0
30% Formaldehyde (LM)¶	16.59	15.44 ± 1.56	93.1	0
10% Paraformaldehyde	16.59	15.53 ± 2.19	93.6	0
0.15 M Sodium chloride	16.59	15.17 ± 2.66	91.4	0
Methanol (>99.8%)	16.59	16.04 ± 1.71	96.7	0

* Amitriptyline or imipramine was first incubated with each medium for 60 min at room temperature and then extracted by the procedure described in the text.

† Concentrations of nortriptyline, amitriptyline, desipramine and imipramine are shown as free base.

‡ Target concentrations (μg/mL) of amitriptyline and imipramine are calculated from 6.628 μg/400 μL, and 6.637 μg/400 μL, respectively.

§ Formaldehyde (S) indicates standard formaldehyde.

‖ Mean value ± SD ($n = 5$).

¶ Formaldehyde (LM) indicates formaldehyde containing less methanol.

TABLE 3—*Recovery of desipramine in phosphate buffered formaldehyde and paraformaldehyde aqueous solutions, and imipramine detected.*

Medium	Desipramine*			Imipramine*	
	Target concentration (μg/mL)	Measured concentration (μg/mL)	Recovery (%)	Measured concentration (μg/mL)	Recovery‡ (%)
pH3PB§	32.99	33.42 ± 1.34‖	101	0	0
pH5PB	32.99	34.73 ± 0.86	105	0	0
pH7PB	32.99	34.30 ± 0.07	104	0	0
pH9PB	32.99	33.36 ± 0.83	101	0	0
pH11PB	32.99	35.65 ± 0.93	108	0	0
3.7% Formaldehyde (S)¶—pH3PB	32.99	31.70 ± 0.79††	96.1	0.10 ± 0.09	0.3
3.7% Formaldehyde (S)—pH5PB	32.99	36.52 ± 0.93	111	0.32 ± 0.11	1.0
3.7% Formaldehyde (S)—pH7PB	32.99	35.08 ± 2.14	106	0.21 ± 0.06	0.6
3.7% Formaldehyde (S)—pH9PB	32.99	34.30 ± 2.68	104	0.35 ± 0.13	1.0
3.7% Formaldehyde (S)—pH11PB	32.99	32.38 ± 0.93	98.2	0.43 ± 0.06	1.2
3.7% Formaldehyde (LM)**—pH3PB	32.99	30.80 ± 2.47	93.4	0.15 ± 0.07	0.4
3.7% Formaldehyde (LM)—pH5PB	32.99	33.07 ± 0.65	100	0	0
3.7% Formaldehyde (LM)—pH7PB	32.99	32.16 ± 0.77	97.5	0.21 ± 0.09	0.6
3.7% Formaldehyde (LM)—pH9PB	32.99	31.21 ± 1.49	94.6	0.18 ± 0.11	0.5
3.7% Formaldehyde (LM)—pH11PB	32.99	28.77 ± 2.89	87.2	0.21 ± 0.18	0.6
3.7% Paraformaldehyde—pH3PB	32.99	31.85 ± 1.04	96.5	0.18 ± 0.13	0.5
3.7% Paraformaldehyde—pH5PB	32.99	33.26 ± 1.15	101	0.31 ± 0.11	0.9
3.7% Paraformaldehyde—pH7PB	32.99	33.84 ± 0.97	103	0.39 ± 0.11	1.1
3.7% Paraformaldehyde—pH9PB	32.99	35.01 ± 0.71	106	0.43 ± 0.25	1.2
3.7% Paraformaldehyde—pH11PB	32.99	34.62 ± 1.79	105	0.48 ± 0.06	1.4

* Concentrations of desipramine and imipramine are shown as free base. Desipramine was first incubated with each medium for 60 min at room temperature and then extracted by the procedure described in the text.

† Target concentration (μg/mL) is calculated from 6.597 μg/200 μL.

‡ Recovery of imipramine was calculated as follows: measured concentration of imipramine × 100/34.72.

§ pH3PB indicates 0.01 M phosphate sodium buffer, pH3.

‖ Mean value ± SD ($n = 4$).

¶ Formaldehyde (S) indicates standard formaldehyde.

** Formaldehyde (LM) indicates formaldehyde containing less methanol.

†† Dotted underline or solid underline indicates a value which is respectively close to significant or significant ($p < 0.05$), by the Student t-test, when compared with each control value (pH 3–11 PB).

$$R_1NH_2 + HCHO \leftrightarrow R_1N{=}CH_2 + H_2O$$

$$R_1N = CH_2 + HCOOH \leftrightarrow R_1NHCH_3 + CO_2 \xrightarrow{\text{Repeat sequence}} R_1N(CH_3)_2$$

For example, AT and IP are produced from NRT and DP, respectively.

Dettling et al. (14) reported stability of NRT under various conditions (formaldehyde concentration, pH and incubation time) in formaldehyde solution. For example, at pH 4.5 in 40% formaldehyde solution, NRT concentration decreased 68% along with a concomitant formation of AT after 24 hours. The formation of the N-methylated product, AT, is favored at an elevated pH. In pH 9.5, 20% formaldehyde solution, loss (%) of NRT (35%, 53%, 69% and 89%) and production of AT (35%, 54%, 71% and 89%) was found after incubation for 1, 2, 4 and 7 days, respectively. Production of AT was almost 100% of the loss of NRT at pH 9.5 in 20% formaldehyde solution for 1 to 7 days. Production of AT, however, ranged from 13 to 37% of the loss of NRT at pH 4 in 20% formaldehyde solution. These results might be explained by the Eschweiler-Clarke reaction, if formic acid was present in the formaldehyde solution, although Dettling et al. did not raise this issue (14).

Winek et al. (8) reported that TCAs (NRT, AT, DP and IP) could be detected in formalin-fixed human liver tissue and formalin solutions stored for 7 to 22 months (except for NRT in formalin-fixed liver tissue). Concentrations of TCAs in formalin-fixed liver tissues did not correlate with those in frozen liver tissues. Some methylation of the secondary amine, NRT, to the corresponding tertiary amine, AT, and of DP to IP took place in the formalin-fixed liver tissues and in formalin solutions. NRT was not detected in most cases, suggesting that it may degrade more rapidly than DP (8). Winek et al. (8) postulated that secondary amines such as NRT and DP react with formaldehyde to form Schiff bases, which can undergo reduction to form the methylated analogs AT and IP. However, in general, secondary amines do not form Schiff bases (19). Therefore we speculate that AT or IP detected in the NRT or DP formalin solution probably formed by the Eschweiler-Clarke reaction (19,20).

Winek et al. (13) reported an experimental study using blood spiked with drugs and liver tissue collected at autopsy. DP in the liver tissues 21 day after storage in 5 and 8% formalin (1.9 and 3% formaldehyde) solutions decreased to 34 and 41%, respectively, of DP in the frozen liver tissues. DP was detected in the formalin fixed liver tissue for at least 28 days.

As described above, Winek et al. (8) reported that methylated products of NRT and DP could be detected in some NRT and DP cases in accord with the experimental results of Dettling et al. Therefore, in this study we re-examined in a simple experimental system whether DP or NRT reacted with formaldehyde at room temperature or not, and whether TCAs were stable in various formaldehyde solution at room temperature or not.

TABLE 4—*Recovery of imipramine in phosphate buffered formaldehyde and paraformaldehyde aqueous solutions.*

Medium	Imipramine*			Desipramine*
	Target† concentration (μg/mL)	Measured concentration (μg/mL)	Recovery (%)	Measured concentration (μg/mL)
pH3PB‡	16.59	17.85 ± 2.38§	108	0
pH5PB	16.59	17.73 ± 0.53	107	0
pH7PB	16.59	17.58 ± 0.35	106	0
pH9PB	16.59	17.41 ± 0.58	105	0
pH11PB	16.59	18.00 ± 0.65	108	0
3.7% Formaldehyde (S)‖—pH3PB	16.59	17.39 ± 0.57	105	0
3.7% Formaldehyde (S)—pH5PB	16.59	17.65 ± 1.13	106	0
3.7% Formaldehyde (S)—pH7PB	16.59	18.29 ± 0.43	110	0
3.7% Formaldehyde (S)—pH9PB	16.59	18.21 ± 0.65	110	0
3.7% Formaldehyde (S)—pH11PB	16.59	18.29 ± 0.56	110	0
3.7% Formaldehyde (LM)¶—pH3PB	16.59	16.09 ± 0.17	97.0	0
3.7% Formaldehyde (LM)—pH5PB	16.59	17.19 ± 0.29	104	0
3.7% Formaldehyde (LM)—pH7PB	16.59	16.46 ± 0.70	99.2	0
3.7% Formaldehyde (LM)—pH9PB	16.59	16.27 ± 0.73	98.1	0
3.7% Formaldehyde (LM)—pH11PB	16.59	17.51 ± 0.62	106	0
3.7% Paraformaldehyde—pH3PB	16.59	17.83 ± 0.85	107	0
3.7% Paraformaldehyde—pH5PB	16.59	17.99 ± 0.58	108	0
3.7% Paraformaldehyde—pH7PB	16.59	16.73 ± 1.15	101	0
3.7% Paraformaldehyde—pH9PB	16.59	18.11 ± 0.37	109	0
3.7% Paraformaldehyde—pH11PB	16.59	16.88 ± 1.38	102	0

* Concentrations of desipramine and imipramine are shown as free base. Imipramine was first incubated with each medium for 60 min at room temperature and then extracted by the procedure described in the text.

† Target concentration (μg/mL) is calculated from 6.637 μg/400 μL.

‡ pH3PB indicates 0.01 M phosphate sodium buffer, pH3.

§ Mean value ± SD (n = 4).

‖ Formaldehyde (S) indicates standard formaldehyde.

¶ Formaldehyde (LM) indicates formaldehyde containing less methanol.

TABLE 5—*Desipramine and imipramine concentration changes in formaldehyde and paraformaldehyde solutions during storage at room temperature*

Medium	Substance detected	Storage time (days)					
		0 (30–60 min)	1	3	7	14	21
Desipramine storage*							
pH 3 PB	DP†	33.62 ± 1.96‡	33.85 ± 0.91	33.31 ± 1.11	33.34 ± 1.48	34.22 ± 0.77	33.17 ± 0.78
	IP†	0	0	0	0	0	0
pH 7 PB	DP	34.49 ± 2.82	34.00 ± 1.81	33.43 ± 1.05	33.78 ± 1.10	34.16 ± 1.16	31.31 ± 0.41
	IP	0	0	0	0	0	0
3.7% formalin (S)	DP	32.53 ± 0.48	32.66 ± 0.93	31.93 ± 0.69	29.59 ± 0.38	30.87 ± 1.50	28.72 ± 1.24
	IP	0.010 ± 0.003	0.010 ± 0.002	0	0.007 ± 0.006	0.003 ± 0.001	0.001 ± 0.001
3.7% formalin (S)—pH 7 PB	DP	32.11 ± 0.62	31.17 ± 0.07	31.62 ± 2.34	30.17 ± 0.87	29.33 ± 0.26	23.74 ± 0.38
	IP	0.007 ± 0.006	0.012 ± 0.003	0.023 ± 0.004	0.050 ± 0.009	0.039 ± 0.002	0.059 ± 0.004
3.7% formalin (LM)	DP	32.41 ± 1.20	32.70 ± 0.44	32.55 ± 0.68	32.36 ± 0.61	30.24 ± 0.69	28.64 ± 0.88
	IP	0.009 ± 0.007	0.008 ± 0.002	0	0.016 ± 0.004	0.004 ± 0.000	0.001 ± 0.000
3.7% formalin (LM)—pH7PB	DP	32.63 ± 0.92	33.02 ± 1.08	32.57 ± 0.66	30.33 ± 5.74	26.44 ± 0.69	24.79 ± 1.87
	IP	0.007 ± 0.007	0.011 ± 0.001	0.020 ± 0.004	0.057 ± 0.025	0.037 ± 0.004	0.055 ± 0.001
3.7% Paraformaldehyde	DP	34.16 ± 1.95	34.02 ± 1.03	33.53 ± 0.48	32.32 ± 1.01	29.25 ± 1.57	32.24 ± 0.44
	IP	0.004 ± 0.003	0.008 ± 0.003	0.004 ± 0.003	0.008 ± 0.002	0.008 ± 0.002	0.018 ± 0.001
3.7% Paraformaldehyde—pH 7 PB	DP	34.16 ± 1.03	32.80 ± 1.57	31.65 ± 0.27	30.80 ± 0.88	28.00 ± 0.66	28.93 ± 0.68
	IP	0.012 ± 0.009	0.010 ± 0.002	0.014 ± 0.002	0.035 ± 0.003	0.028 ± 0.001	0.043 ± 0.002
Imipramine storage*							
pH 3 PB	IP	17.03 ± 1.30	17.71 ± 0.58	16.34 ± 0.81	17.08 ± 1.37	16.34 ± 0.81	15.81 ± 0.52
pH 7 PB	IP	18.16 ± 1.20	17.38 ± 0.69	16.09 ± 0.45	17.01 ± 2.07	14.32 ± 0.45	15.30 ± 0.74
3.7% formalin (S)	IP	17.08 ± 1.42	17.38 ± 0.63	15.65 ± 2.01	16.17 ± 1.15	16.63 ± 1.24	14.80 ± 0.29
3.7% formalin (S)—pH 7 PB	IP	17.65 ± 0.83	16.96 ± 0.71	16.48 ± 1.58	16.96 ± 1.68	14.84 ± 0.49	14.26 ± 0.18
3.7% formalin (LM)	IP	17.35 ± 0.42	17.76 ± 1.21	16.77 ± 1.88	16.84 ± 1.71	15.51 ± 0.80	14.72 ± 0.61
3.7% formalin (LM)—pH 7PB	IP	17.48 ± 0.86	17.63 ± 1.22	15.90 ± 0.83	16.45 ± 1.26	14.69 ± 0.21	13.97 ± 0.62
3.7% Paraformaldehyde	IP	17.84 ± 0.77	17.14 ± 0.71	14.90 ± 0.63	15.47 ± 1.35	15.53 ± 0.69	14.91 ± 0.63
3.7% Paraformaldehyde—pH 7 PB	IP	18.50 ± 0.58	16.24 ± 0.13	15.57 ± 1.44	15.68 ± 1.91	14.81 ± 0.63	14.50 ± 0.31

* Desipramine (32.99 μg/mL) or imipramine (16.59 μg/mL) was stored in each medium at room temperature. After storage desipramine and imipramine were extracted by the procedure described in the text.

† DP and IP show desipramine and imipramine, respectively, with their concentrations in μg/mL.

‡ Mean value ± SD (n = 4)

TABLE 6—*Ratio of recoveries of desipramine and imipramine in formaldehyde and paraformaldehyde solutions during storage at room temperature.*

Medium	Substance detected	Storage time (days) 0 (30–60 min)	1	3	7	14	21
Desipramine storage*							
pH 3 PB	DP†	100‡	101	99.1	99.2	102	98.7
	IP†	0.0	0.0	0.0	0.0	0.0	0.0
pH 7 PB	DP	100	98.6	96.9	97.9	99.0	90.8
	IP	0.0	0.0	0.0	0.0	0.0	0.0
3.7% formalin (S)	DP	100	100	98.2	91.0§	94.9	88.3
	IP	0.0	0.0	0.0	0.0	0.0	0.0
3.7% formalin (S)—pH 7 PB	DP	100	97.1	98.5	94.0	91.3	73.9
	IP	0.0	0.0	0.1	0.1	0.1	0.2
3.7% formalin (LM)	DP	100	101	100	99.8	93.3	88.4
	IP	0.0	0.0	0.0	0.0	0.0	0.0
3.7% formalin (LM)—pH 7 PB	DP	100	101	99.8	93.0	81.0	76.0
	IP	0.0	0.0	0.1	0.2	0.1	0.2
3.7% Paraformaldehyde	DP	100	99.6	98.2	94.6	85.6	94.4
	IP	0.0	0.0	0.0	0.0	0.0	0.1
3.7% Paraformaldehyde—pH 7 PB	DP	100	96.0	92.7	90.2	82.0	84.7
	IP	0.0	0.0	0.0	0.1	0.1	0.1
Imipramine storage*							
pH 3 PB	IP	100	104	95.9	100	95.9	92.8
pH 7 PB	IP	100	95.7	88.6	93.7	78.9	84.3
3.7% formalin (S)	IP	100	102	91.6	94.7	97.4	86.7
3.7% formalin (S)—pH 7 PB	IP	100	96.1	93.4	96.1	84.1	80.8
3.7% formalin (LM)	IP	100	102	96.7	97.1	89.4	84.8
3.7% formalin (LM)—pH 7 PB	IP	100	101	91.0	94.1	84.0	79.9
3.7% Paraformaldehyde	IP	100	96.1	83.5	86.7	87.1	83.6
3.7% Paraformaldehyde—pH 7 PB	IP	100	87.8	84.2	84.8	80.1	78.4

* Concentrations of desipramine and imipramine stored were respectively 32.99 and 16.59 μg/mL as free base.

† DP and IP show desipramine and imipramine, respectively.

‡ Ratio of recovery (percent value) of desipramine (or imipramine) just after storage (0) to that after storage (1 to 21 days). Original data are shown in Table 5.

§ Underlined values are significant ($p < 0.05$) by the Student t-test, when compared with corresponding control values (pH 3 PB and pH 7 PB) at the same storage time.

In this study, NRT at pH 2.7 in 37% formaldehyde solution containing 8 to 10% methanol (abbreviated as S) as well as formaldehyde solution with lower methanol content (0.7%) (LM) could be recovered almost 100%. DP in the same solution could be recovered at 74% (formaldehyde, S) and 90% (formaldehyde, LM). The methylated products AT from NRT and IP from DP were detected in the NRT and DP formaldehyde and paraformaldehyde solutions, respectively. The concentrations of AT and IP recovered were each 2.7% of the drug spiked, 32.94 and 32.99 μg/mL, respectively. The recoveries of AT and IP were the same without regard to the methanol content in the formaldehyde solutions. Therefore the methanol in the formaldehyde solution was not obviously implicated in methylation of NRT or DP. These results qualitatively correspond with those of Dettling et al. (14) and Winek et al. (8), in that the methylated products, AT and IP, were detected in the NRT and DP formaldehyde solutions, respectively. However, recoveries of AT (2.7%) and IP (2.7%) were much lower than the recovery of AT (13 to 37%) found by Dettling et al. (14). The possible reasons may be: the content of formic acid as impurity in the formaldehyde was different; contamination by reducing agents in the water and formaldehyde solutions used; and the analytical methodology, especially the extraction procedure for NRT or DP, was different.

With regard to the methylation of the secondary amines, NRT and DP, one of the obvious interpretations is that secondary amines reductively react with formaldehyde and formic acid to form their methylated products (19,20).

From our results tertiary amines, AT and IP, were detected only at about 3% of the concentration of secondary amines (NRT and DP). The results may be interpreted as formation of the tertiary amines (AT and IP) as a result of the analytical methodology if a small amount of formaldehyde and formic acid as impurity were co-extracted with NRT or DP and then heated at 50°C for dry-up. Also, it may be that a small amount of AT or IP is produced by the Eschweiler-Clarke reaction from NRT or DP with formaldehyde and formic acid (an impurity of 37% formaldehyde aqueous solution) at room temperature within one hour.

Recoveries of tertiary amines (AT and IP) were almost unchanged in some formaldehyde and paraformaldehyde solutions when compared with the secondary amines. In general the N atom of tertiary amines is less reactive than primary and secondary amines because of steric hindrance. Therefore we believe that AT and IP do not react with formaldehyde at room temperature.

DP concentrations at pH 3, 9 or 11 in 3.7% formaldehyde PB solutions decreased compared with those at pH 5 and 7 solutions. The reason may be that reactivity of these secondary amines and formaldehyde is accelerated by both acid and alkaline conditions.

Since 10% formalin (3.7 to 4.0% formaldehyde) aqueous solution is commonly used for tissue-fixation, we selected this concentration of formaldehyde solution for studies of storage at room temperature for 21 days. DP concentration at pH 7 PB fell to 91% in contrast to that in pH 3 PB (99%). This suggests that DP may be unstable in a neutral PB solution when contrasted with an acidified PB solution. In a non-buffered 3.7% formaldehyde solution,

DP concentrations fell to about 88% after 21 days storage, while DP in a pH 7 formaldehyde solution fell to 74 to 76%. However, IP detected was only 0.2% of the initial concentration of DP (32.99 μg/mL) 21 days after storage. So it seems that DP degradation was accelerated by formaldehyde and paraformaldehyde. It may be that DP reacted nucleophilically with formaldehyde and formed an additional product (a hemiaminal). Also, it demonstrates that DP in 3.7% formaldehyde aqueous solution hardly forms its methylated product, IP, at room temperature after 21 days.

We conclude that the reactions between some drugs and formaldehyde solutions are likely to prove chemically complex and will need to be explored further, if analysis of formaldehyde-fixed tissues is to be used other than qualitatively. In the meantime it would be of value to record the pH and the formaldehyde type and concentration used in both experimental studies and casework.

Acknowledgment

The first author (T.T.), is grateful for financial support from the Pfizer Health Research Foundation (Tokyo).

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