**ABSTRACT** — Due to finalization of the ICH S3A Q&A focusing on microsampling, application of microsampling technique to regular non-clinical animal studies is expected for non-clinical safety assessment of pharmaceuticals. In Europe, microsampling from the tail vein or saphenous vein has often been used, whereas sampling from the jugular vein is thought to be more common for non-clinical studies in Japan. Therefore, we assessed the toxicological effects of serial microsampling from the jugular vein of SD rats in a common 28-day study at 4 independent organizations. Fifty microliter sampling was performed at 6 timepoints on day 1 to 2 and 7 timepoints on days 27 to 28 and its toxicological influences on body weight, food consumption, hematological and clinical chemistry parameters, and organ weights (on day 29 for 3 and day 28 for 1 organizations) were evaluated. The serial microsampling was shown to have no or minimal influences on the assessed parameters. The observed statistical differences for the 18 parameters were sporadic and did not appear to be systemically associated with microsampling. However, the sporadic changes were more often observed in females (14/18 parameters) than in males (6/18), suggesting the possibility that female rats were more susceptible to treatment-based influences. The current results indicate that serial 50 μL sampling from the jugular vein of SD rats had no or very slight toxicological effects, suggesting that this microsampling condition is applicable for toxicokinetic evaluation of non-clinical rat toxicity studies.

**Key words:** Microsampling, Rats, Jugular vein, Toxicological influence

---

**INTRODUCTION**

Non-clinical safety studies using animals are important for the assessment of drug candidates prior to their administration in human subjects/patients. Rodents, especially rats, are commonly used for general toxicology studies. Toxicokinetic (TK) evaluation should be performed to evaluate relationship between toxicological profiles including pathology and clinical chemistry results and systematic exposure to the drug candidate according to the ICH S3A guideline (ICH, 1994). In rodents, subsets of animals, called satellite animals, are specifically utilized for sampling in TK analysis as around 200 μL of blood is taken at each time point, which could affect toxicological assessment in the main study animals. However, increases in the sensitivity of drug measurement apparatuses such as in mass spectrometers have enabled using smaller volume of blood samples for TK evaluation. Considering these circumstances, the ICH S3A Q&A focused on microsampling was finalized and released in Nov. 2017 (ICH, 2017). It stated that the main benefits of microsampling are 1) ability to directly assess the relationship between safety data and drug exposure in the same animal, and 2) contribution to reduce pain and distress in animals (refinement) and elimination or reduction of TK satellite animals (reduction). The Q&A defined micro-
sampling as collection of a very small amount of blood (typically ≤ 50 μL).

For toxicological assessment of microsampled rats, the maximal blood volume that can be withdrawn within 24 hr in multiple sampling was set at 7.5%, 10-15%, and 20% of the circulating blood volume, followed by 1, 2, or 3 weeks of recovery period (Diehl et al., 2001). Since the average blood volume of rats is 64 mL/kg (Diehl et al., 2001), the typical 6-week old rats at 200 g (male) and 150 g (female) are calculated to contain 12.8 and 9.6 mL of blood, respectively, and 7.5% then corresponds to 0.96 and 0.72 mL, respectively. In line with this proposal, Caron et al. showed that sampling of 6 × 200 μL from female rats (~250 g; 7.5% of the blood corresponds to ~1.2 mL) did not influence any toxicological parameters after a week of recovery (Caron et al., 2015). However, as described above, one week of recovery period is needed for such volumes; further, the possibility of transient toxicological effects by hypovolemia due to sampling of conventional volumes of blood from rats exerting an influence on the evaluation of drug candidates cannot be excluded.

So far, several papers have assessed the toxicological influence of microsampling (i.e., ≤ 50 μL). Powles-Glover et al. examined the toxicological effects on the next day of serial blood sampling. They withdrew 6 × 32 μL (microsampling) or 6 × 200 μL (conventional sampling) from the tail vein on day 1 and 14 and then evaluated its effects on day 15 (Powles-Glover et al., 2014). In contrast to the statistically significant effects on many hematological (including hemoglobin, hematocrit, and red blood cell (RBC)) and liver parameters (such as aspartate transaminase (AST) and glutamate dehydrogenase (GLDH)) in conventional sampling (4.9% for male and 8.1% for female of the blood volume taken), the microsampled animals showed statistically significant but minimal effects only on hemoglobin in females and on monocytes in males (0.8% for male and 1.3% for female of the blood volume taken). Caron et al. (2015) reported similar effects with 6 × 200 μL sampled at day 1 and assessment on day 2 or 6 × 200 μL at day 1 and 28 with assessment on day 29 for sampling from the saphenous vein. They showed that the serial sampling volumes of 6 × 50 μL on day 1 showed statistically significant but minimal influences only on fibrinogen and AST (slight increases) in the assessment on day 2. These results suggest that serial microsampling is less influential in rats than conventional sampling even on the next day, and thus appears more suitable for sampling from main study rats of GLP toxicological studies.

Although the ICH S3A Q&A has been finalized, microsampling has been applied limitedly to non-clinical toxicokinetic studies, especially in GLP studies in Japan. Additionally, in microsampling studies from Europe, researchers often use sampling from the tail vein or saphenous vein (Jonsson et al., 2012; Powles-Glover et al., 2014; Caron et al., 2015; Coleman et al., 2017). However, sampling from the jugular vein is thought to be more commonly used for non-clinical rodent studies in Japan. Therefore, we decided to assess the toxicological effects of serial microsampling from the jugular vein of rats in a common 28-day study. Furthermore, we examined whether minor differences in apparatus and procedures, for examples, type of feed and sampling needles’ thickness among 4 independent organizations would not influence the toxicological effects of serial microsampling. We chose the sampling volume of 50 μL at a time-point because the ICH S3A Q&A document defines microsampling as ≤ 50 μL typically, and this relatively large volume of blood is useful for incurred sample reanalysis as well as for other purposes such as biomarker assessment.

**MATERIALS AND METHODS**

**Organizations and animals**

Four organizations (A-D) except National Institute of Health Sciences participated in the animal experiments. Sprague-Dawley (Crl:CD(SD)) rats (5-week age) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Rats took food and water ad libitum. Organization B used CRF-1 (Oriental Yeast Co., Tokyo, Japan) and the others used CR-LPF (Oriental Yeast Co.,) as the feed. After 5-8 days of habituation, the study was initiated at each organization. This study was approved by the Animal Experiment Committee of each organization.

**Study protocol**

Two animal groups were set per organization: 0 (group I) and 50 μL (group II) sampling groups. Each group comprised 6 males and 6 females. Fifty microliters of blood were taken from the jugular vein of group II rats at each of 0.5, 1, 2, 4, and 8 hr on day 1 and at 24 hr on day 2. The same volume of blood sampling was performed at 0, 0.5, 1, 2, 4, and 8 hr on day 27 and at 24 hr on day 28. Time 0 was set in the morning. Blood sampling was conducted using 1-mL low-dose syringes with 29-30G needles (Organization A and C; Becton Dickinson Co., Franklin Lakes, NJ, USA) or using 1-mL syringes with 27G needles (Organization B and D; Terumo Corp., Tokyo Japan), with heparin. All animals were inspected daily for clini-
Lack of toxicological effects by microsampling in rats

Clinical signs, and body weights and food consumption were measured at least once a week.

On day 29 (Organization A-C) and 28 (Organization D), rats were anaesthetized with isoflurane (Mylan V.V., Tokyo, Japan, or Pfizer Inc., New York, NY, USA) and 3.5 to 6 mL of blood was withdrawn from the abdominal aorta (Organization A) or abdominal vena cava (Organization B, C and D) for hematological and clinical chemistry assessments. The rats were then sacrificed by cut the blood vessels and release the blood, and the organs were collected with sufficient blood loss from the body, and anatomized to measure the organ weights of the heart, lung, liver, pancreas, kidneys, thymus, spleen, adrenal glands, and submandibular gland (at maximum).

The hematological parameters assessed included RBC counts, hematocrit, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte, mean corpuscular volume (MCV), and the neutrophil, lymphocyte, monocyte, basophil, eosinophil, and platelet counts (at maximum). Clinical chemistry parameters measured included total protein, albumin, glucose, total cholesterol, triacylglycerol, alanine transaminase (ALT), AST, GLDH, alkaline phosphatase (ALP), total bilirubin, lactate dehydrogenase (LDH), creatine kinase (CK), fibrinogen, prothrombin time (PT), activated partial thromboplastin time (APTT), blood urea nitrogen (BUN), creatinine (Cre), Na, K, Ca, inorganic P, and Cl (at maximum).

Statistical analysis
Statistical comparisons were performed using Student’s t-test between 0 (group I) and 50 μL (group II) sampling groups at each organization. To find the minor influences of microsampling, correction for multiple comparison was not applied.

RESULTS AND DISCUSSION

Body weights and food consumption were evaluated every week. As shown in Table 1, body weights increased from 0 to 4 weeks in both sexes of microsampling group II, in a similar manner as the no treatment group I at all 4 organizations. Body weights of the female group at organization D were higher in group II than in group I at 0 week (starting point). This statistical significance disappeared from 1 to 4 weeks. Regarding food consumption, females of group II at organization D showed more consumption (~12% more) at 1 and 3 weeks than those of group I, but no statistical significance was observed for all weeks as well as in both sexes at other organizations. These results suggested that the microsampling treatment had no or minimal influence, which was not observed in

Table 1. Body weights and food consumption in rats treated with 50 μL microsampling and corresponding unsampled controls in 4 organizations.

|                | A¹ | B² | C³ | D⁴ |
|----------------|----|----|----|----|
| **Body weight (g)** |    |    |    |    |
| No treatment (Group I) |    |    |    |    |
| 0 wk           | 180 ± 7 | 143 ± 7 | 204 ± 8 | 162 ± 4 | 204 ± 6 | 155 ± 8 | 229 ± 6 | 145 ± 8 |
| 1 wk           | 227 ± 10 | 158 ± 13 | 262 ± 9 | 187 ± 8 | 260 ± 8 | 175 ± 9 | 291 ± 8 | 170 ± 8 |
| 2 wks          | 275 ± 14 | 173 ± 18 | 320 ± 14 | 214 ± 8 | 315 ± 10 | 194 ± 12 | 350 ± 12 | 189 ± 14 |
| 3 wks          | 317 ± 20 | 191 ± 20 | 371 ± 23 | 234 ± 9 | 354 ± 12 | 208 ± 14 | 396 ± 12 | 206 ± 12 |
| 4 wks          | 354 ± 25 | 201 ± 21 | 401 ± 30 | 246 ± 8 | 380 ± 16 | 217 ± 17 | 425 ± 15 | 219 ± 17 |
| 50 μL microsampling (Group II) |    |    |    |    |
| 0 wk           | 181 ± 9 | 142 ± 6 | 204 ± 8 | 162 ± 4 | 202 ± 6 | 155 ± 3 | 224 ± 9 | 155 ± 5* |
| 1 wk           | 226 ± 13 | 162 ± 11 | 255 ± 15 | 184 ± 5 | 257 ± 12 | 179 ± 8 | 287 ± 12 | 178 ± 9 |
| 2 wks          | 276 ± 20 | 180 ± 13 | 312 ± 22 | 210 ± 8 | 309 ± 17 | 201 ± 4 | 342 ± 19 | 202 ± 10 |
| 3 wks          | 321 ± 28 | 201 ± 18 | 361 ± 33 | 231 ± 7 | 347 ± 29 | 219 ± 4 | 386 ± 23 | 216 ± 18 |
| 4 wks          | 363 ± 33 | 215 ± 23 | 396 ± 42 | 245 ± 16 | 372 ± 37 | 228 ± 4 | 412 ± 31 | 234 ± 15 |
| **Food consumption (g/day)** |    |    |    |    |
| No treatment (Group I) |    |    |    |    |
| 1 wk           | 25 ± 1 | 18 ± 2 | 28 ± 2 | 20 ± 2 | 180 ± 10 | 122 ± 6 | 29 ± 1 | 17 ± 1 |
| 2 wks          | 27 ± 2 | 17 ± 2 | 30 ± 3 | 21 ± 1 | 192 ± 9 | 125 ± 10 | 29 ± 1 | 17 ± 1 |
| 3 wks          | 28 ± 2 | 18 ± 2 | 29 ± 3 | 20 ± 2 | 195 ± 7 | 128 ± 11 | 29 ± 1 | 17 ± 1 |
| 4 wks          | 28 ± 2 | 18 ± 3 | 29 ± 3 | 20 ± 2 | 191 ± 8 | 130 ± 9 | 30 ± 1 | 18 ± 0* |
| 50 μL microsampling (Group II) |    |    |    |    |
| 1 wk           | 25 ± 2 | 18 ± 2 | 27 ± 3 | 20 ± 2 | 175 ± 15 | 123 ± 10 | 28 ± 1 | 18 ± 0* |
| 2 wks          | 27 ± 3 | 18 ± 2 | 28 ± 3 | 19 ± 2 | 186 ± 11 | 128 ± 5* | 28 ± 2 | 18 ± 1 |
| 3 wks          | 29 ± 4 | 19 ± 3 | 28 ± 4 | 20 ± 3 | 185 ± 16 | 129 ± 5* | 28 ± 3 | 19 ± 2* |
| 4 wks          | 30 ± 4 | 19 ± 3 | 28 ± 4 | 20 ± 5 | 182 ± 16 | 128 ± 3* | 28 ± 3 | 19 ± 2 |

Significantly different from the corresponding "no treatment" group by Student t-test. *p < 0.05.

¹A, B, C and D indicate organizations.
The results of blood parameters at 4 weeks are shown in Table 2. Among the parameters, RBC counts were significantly lower in female rats of group II at organization D. Further, the hematocrit and hemoglobin values were significantly lower in both male and female rats of group II than those of group I at organization D. However, the differences between groups I and II were less than 8% and there were no statistically significant differences in other organizations for these 3 red blood cell-related parameters. Neutrophil counts of group II at organization C were incidentally higher in male rats and the value was outside the range of background values, but no statistically significant difference was found between group I and II. These results suggest that the microsampling treatment might influence the red blood cell-related parameters, but the influences were minimal and only sporadically observed (not in more than 1 organization per condition).

The blood clinical chemistry was analyzed next (Table 3). Again, statistically significant differences between groups I and II were detected for 10 parameters sporadically (observed only at 1 organization per parameter). Among the 10 parameters, six were for females and four were for males. Over 30% differences between the two groups were found in 1) total cholesterols in the male rats at organization C, 2) GLDH in female rats at organization A, 3) LDH in female rats at organization A, and 4) Cre in female rats at organization B. However, for these 10 parameters, there were no significant differences between the animal groups in the other sex group at the same organization or in both sex groups at the other 3 organizations. Thus, the results suggested that the detected significant differences for the 10 parameters were observed by chance, and these appeared to be independent of the serial microsampling.

The final assessment was performed for organ weights more than 1 organization per condition.

The results of blood parameters at 4 weeks are shown in Table 2. Among the parameters, RBC counts were significantly lower in female rats of group II at organization D. Further, the hematocrit and hemoglobin values were significantly lower in both male and female rats of group II than those of group I at organization D. However, the differences between groups I and II were less than 8% and there were no statistically significant differences in other organizations for these 3 red blood cell-related parameters. Neutrophil counts of group II at organization C were incidentally higher in male rats and the value was outside the range of background values, but no statistically significant difference was found between group I and II. These results suggest that the microsampling treatment might influence the red blood cell-related parameters, but the influences were minimal and only sporadically observed (not in more than 1 organization per condition).

Table 2. Blood parameters in rats treated with 50 μL microsampling and corresponding unsampled controls in 4 organizations.

| Parameter       | No treatment: Group I | A | B | C | D |
|-----------------|-----------------------|---|---|---|---|
| 50 μL microsampling: Group II | Male | Female | Male | Female | Male | Female |
| RBC (×10^6/μL)  | 7.72 ± 0.36 | 8.00 ± 0.22 | 7.54 ± 0.37 | 7.58 ± 0.29 | 7.79 ± 0.28 | 8.02 ± 0.40 | 7.83 ± 0.32 | 7.43 ± 0.32 |
| 50 μL microsampling  | 7.71 ± 0.24 | 7.68 ± 0.38 | 7.57 ± 0.21 | 7.52 ± 0.50 | 7.99 ± 0.48 | 7.81 ± 0.22 | 7.50 ± 0.45 | 6.85 ± 0.33 * |
| Hematocrit (%)   | 46.9 ± 2.22 | 46.1 ± 1.3 | 45.6 ± 1.9 | 42.8 ± 1.7 | 46.3 ± 1.2 | 45.7 ± 1.9 | 42.7 ± 0.6 | 38.9 ± 1.0 |
| Hemoglobin (g/dL) | 15.6 ± 1.0 | 15.4 ± 0.4 | 15.1 ± 0.5 | 14.8 ± 0.5 | 15.0 ± 0.4 | 15.2 ± 0.8 | 15.8 ± 0.3 | 14.6 ± 0.5 |
| MCH (pg)        | 20.3 ± 1.5 | 19.2 ± 0.3 | 20.0 ± 0.7 | 19.6 ± 0.6 | 19.3 ± 0.4 | 19.0 ± 0.7 | 20.2 ± 0.8 | 19.6 ± 0.8 |
| MCHC (%)        | 33.4 ± 1.8 | 33.3 ± 0.4 | 33.0 ± 0.4 | 34.7 ± 0.5 | 32.5 ± 0.5 | 33.4 ± 0.5 | 36.9 ± 0.4 | 37.4 ± 0.8 |
| Eosinophil (%)  | 2.73 ± 0.49 | 1.90 ± 0.52 | 2.90 ± 0.77 | 2.39 ± 0.45 | 2.53 ± 0.3 | 1.84 ± 0.38 | 3.43 ± 0.43 | 3.01 ± 0.48 |
| Basophil (%)     | 60.8 ± 2.2 | 57.7 ± 1.0 | 60.5 ± 2.5 | 56.5 ± 1.2 | - | - | 54.7 ± 2.5 | 52.4 ± 2.8 |
| Lymphocyte (%)  | 5.17 ± 1.1 | 58.0 ± 1.6 | 60.9 ± 1.5 | 56.3 ± 1.0 | - | - | 55.1 ± 3.0 | 54.0 ± 1.6 |
| Monocyte (%)     | 0.15 ± 0.08 | 0.09 ± 0.04 | 0.15 ± 0.05 | 0.10 ± 0.04 | 0.27 ± 0.07 | 0.20 ± 0.09 | 0.35 ± 0.09 | 0.24 ± 0.07 |
| Eosinophil (%)  | 0.020 ± 0.006 | 0.005 ± 0.005 | 0.01 ± 0.01 | 0.01 ± 0.01 | - | - | 0.007 ± 0.005 | 0.002 ± 0.004 |
| Neutrophil (%)  | 0.013 ± 0.005 | 0.007 ± 0.005 | 0.02 ± 0.01 | 0.01 ± 0.01 | - | - | 0.003 ± 0.005 | 0.003 ± 0.005 |
| Platelet (×10^4/μL) | 1141 ± 81 | 1294 ± 83 | 1161 ± 66 | 1140 ± 88 | 1147 ± 120 | 1183 ± 100 | 1115 ± 135 | 1195 ± 90 |

Significantly different from the corresponding "no treatment" group by Student t-test. *p < 0.05, **p < 0.01. Grey background represents the data are outside the range of background values.

A, B, C and D indicate organizations.

*: not evaluated.
### Table 3. Blood clinical chemistries in rats treated with 50 μL microsampling and corresponding unsampled controls in 4 organizations.

|                  | 50 μL microsampling: Group II | 50 μL microsampling | 50 μL microsampling | 50 μL microsampling |
|------------------|-------------------------------|---------------------|---------------------|---------------------|
|                  | Male                          | Female              | Male                | Female              |
| Total protein    | 5.7 ± 0.1                     | 5.7 ± 0.2           | 5.7 ± 0.2           | 5.7 ± 0.1           |
| (g/dL)           | 5.6 ± 0.2                     | 5.8 ± 0.4           | 5.7 ± 0.3           | 5.5 ± 0.2           |
| Albumin          | 3.8 ± 0.1                     | 4.0 ± 0.2           | 2.6 ± 0.2           | 2.8 ± 0.2           |
| (g/dL)           | 3.8 ± 0.1                     | 4.1 ± 0.3           | 2.5 ± 0.2           | 2.7 ± 0.2           |
| Glucose          | 158 ± 17                      | 127 ± 13            | 118 ± 11            | 114 ± 8             |
| (mg/dL)          | 156 ± 12                      | 120 ± 12            | 122 ± 5             | 124 ± 14            |
| Total Cholesterol| 62 ± 6                        | 58 ± 15             | 48 ± 11             | 55 ± 7              |
| (mg/dL)          | 54 ± 8                        | 68 ± 14             | 57 ± 11             | 52 ± 11             |
| Triglyceride     | 42 ± 9                        | 13 ± 4              | 40 ± 18             | 15 ± 7              |
| (mg/dL)          | 54 ± 18                       | 18 ± 5              | 39 ± 18             | 13 ± 3              |
| ALT              | 32 ± 2                        | 25 ± 5              | 27 ± 3              | 23 ± 2              |
| (UL)             | 33 ± 3                        | 26 ± 5              | 28 ± 3              | 21 ± 2              |
| AST              | 72 ± 2                        | 66 ± 5              | 81 ± 4              | 73 ± 8              |
| (UL)             | 75 ± 11                       | 66 ± 6              | 77 ± 5              | 72 ± 6              |
| GLDH             | 18.2 ± 4.2                    | 14 ± 4.5            | 15 ± 4              | 16 ± 4              |
| (UL)             | 27.7 ± 11.2                   | 19.0 ± 4.0*         | -                   | -                   |
| ALP              | 674 ± 55                      | 354 ± 71            | 651 ± 133           | 364 ± 99            |
| (UL)             | 606 ± 66                      | 400 ± 65            | 633 ± 134           | 382 ± 80            |
| Total bilirubin  | 0.05 ± 0.01                   | 0.06 ± 0.01         | 0.01 ± 0.02         | 0.02 ± 0.01         |
| (mg/dL)          | 0.05 ± 0.01                   | 0.05 ± 0.01         | 0.02 ± 0.01         | 0.03 ± 0.01         |
| LDH              | 182 ± 106                     | 86 ± 24             | 252 ± 49            | 222 ± 44            |
| (UL)             | 183 ± 139                     | 171 ± 73*           | 261 ± 50            | 185 ± 34            |
| CK               | 143 ± 13                      | 94 ± 21             | 203 ± 23            | 142 ± 13            |
| (UL)             | 139 ± 10                      | 104 ± 26            | 200 ± 42            | 141 ± 14            |
| Fibrinogen       | 365 ± 28                      | 235 ± 38            | 200 ± 15            | 155 ± 9             |
| (mg/dL)          | 377 ± 24                      | 284 ± 53            | 225 ± 24            | 167 ± 10            |
| PT               | 15.6 ± 1.7                    | 13.5 ± 1.0          | 17.1 ± 3.0          | 10.4 ± 0.3          |
| (sec)            | 17.0 ± 2.5                    | 14.0 ± 0.7          | 16.5 ± 1.8          | 10.3 ± 0.3          |
| APTT             | 18.2 ± 8.9                    | 13.8 ± 2.5          | 21.1 ± 2.3          | 16.3 ± 1.1          |
| (sec)            | 16.0 ± 1.9                    | 13.1 ± 1.5          | 20.5 ± 2.2          | 15.6 ± 1.3          |
| BUN              | 11 ± 2                        | 14 ± 2              | 12 ± 2              | 15 ± 3              |
| (mg/dL)          | 11 ± 1                        | 16 ± 2              | 12 ± 1              | 12 ± 2              |
| Cre              | 0.3 ± 0.1*                    | 0.3 ± 0.0*          | 0.2 ± 0.0           | 0.3 ± 0.0           |
| (mg/dL)          | 0.3 ± 0.0*                    | 0.4 ± 0.0*          | 0.2 ± 0.0           | 0.3 ± 0.0           |
| Na               | 141 ± 2                       | 140 ± 2             | 145 ± 1             | 143 ± 1             |
| (mmol/L)         | 141 ± 1                       | 141 ± 2             | 145 ± 1             | 143 ± 1             |
| K                 | 3.9 ± 0.2                     | 4.0 ± 0.2           | 3.4 ± 0.2           | 3.3 ± 0.3           |
| (mmol/L)         | 4.0 ± 0.2                     | 3.9 ± 0.2           | 3.6 ± 0.2           | 3.4 ± 0.3           |
| Ca                | 9.7 ± 0.2                     | 9.4 ± 0.1           | 9.4 ± 0.3           | 9.2 ± 0.3           |
| (mg/dL)          | 9.7 ± 0.2                     | 9.5 ± 0.3           | 9.4 ± 0.1           | 9.2 ± 0.1           |
| Inorganic P      | 6.47 ± 0.29                   | 5.13 ± 0.23         | 7.2 ± 0.8           | 5.5 ± 1.0           |
| (mg/dL)          | 6.25 ± 0.08                   | 5.62 ± 0.63         | 7.1 ± 0.6           | 5.8 ± 0.8           |
| CI                | 106 ± 2                       | 107 ± 2             | 103 ± 2             | 106 ± 2             |
| (mmol/L)         | 107 ± 1                       | 104 ± 2             | 105 ± 2             | 108 ± 2             |

Significantly different from the corresponding "no treatment" group by Student t-test. *p < 0.05.

Grey background represents the data are outside the range of background values.

*Values in plasma (Otherwise in serum)

A, B, C and D indicate organizations.

*: not evaluated.
As shown in Table 4. The weights of the liver from the female rats at organization D, and those of the thymus and spleen from female rats at organization C were significantly higher (9.4% for liver, 37% for thymus and 31% for spleen) in group II than those in group I. Supplementary Table 1 shows the results of organ weights per 100 g body weight. Again, statistically significant differences were sporadically detected in 4 parameters, in which the weights of the thymus and spleen from the females of organization C are common with Table 4. However, there were no significant differences between the animal groups either in the other sex group at the same organization or in both sex groups at the other 3 organizations.

Based on the current results, serial 50 μL × 6 (day 1 to 2) + 7 (day 27 to 28) point-microsampling from the jugular vein of SD rats was indicated to have no or only minimal influences on body weight gain, food consumption, hematological and blood clinical chemistry parameters, and organ weights at all the organizations in the 28-day study. Although the results from organization D showed slightly more number of the statistically different parameters (8 parameters other than body weight and food consumption) than the other 3 organizations (1-5 parameters), probably due to their assessments on day 28 (day 29 in the other 3 organizations) and/or difference in body weights of female rats between groups I and II from 0 week, there were no meaningful inter-organization variations on the microsampling effects that could interfere with the toxicological evaluation of pharmaceuticals. Thus, the present results could be applied to other pharmaceutical companies/contract research organizations (CROs). The observed statistical differences were sporadic and did not appear to be systemically associated with microsampling. However, sporadic changes in the assessed toxicological parameters were observed more often in females (14/18 parameters) than in males (6/18). At 0 week, the sampled blood volumes corresponded to 2.1-2.6% in males and 2.9-3.3% in females, and at 4 weeks, 1.3-1.5% in males and 2.2-2.5% in females: i.e., the percentages of blood volume sampled were larger in females. We could not fully exclude the possibility that microsampling treatment affected the toxicological parameters through leakage of blood from the sampling points (i.e., jugular vein) as we detected dark red nests or clotted blood at these points in some treated rats. Training for the microsampling technique could thus be important to obtain constant and invariable results.

One limitation of this study was that we do not plan to evaluate the some hematological parameters (MCV and basophil in organizations C) (Table 2), clinical chemistry parameters (GLDH in organizations B and D, total bilirubin and inorganic P in organizations C) (Table 3),

| Table 4. Organ weights in rats treated with 50 μL microsampling and corresponding unsampled controls in 4 organizations. |
|---------------------------------------------------------------|
| 50 μL microsampling: Group II | Male | Female | Male | Female | Male | Female |
| Heart (g) | No treatment | 1.10 ± 0.14 | 0.72 ± 0.08 | 1.20 ± 0.06 | 0.87 ± 0.06 | 1.26 ± 0.25 | 0.73 ± 0.06 | 1.32 ± 0.13 | 0.74 ± 0.08 |
| | 50 μL microsampling | 1.04 ± 0.09 | 0.72 ± 0.08 | 1.25 ± 0.13 | 0.86 ± 0.06 | 1.24 ± 0.09 | 0.78 ± 0.08 | 1.26 ± 0.09 | 0.80 ± 0.06 |
| Lung (g) | No treatment | 1.24 ± 0.16 | 0.93 ± 0.06 | - | - | 1.18 ± 0.05 | 0.90 ± 0.07 | 1.51 ± 0.07 | 1.06 ± 0.11 |
| | 50 μL microsampling | 1.15 ± 0.09 | 0.97 ± 0.08 | - | - | 1.21 ± 0.09 | 0.91 ± 0.04 | 1.50 ± 0.09 | 1.12 ± 0.04 |
| Liver (g) | No treatment | 8.82 ± 0.86 | 5.03 ± 0.61 | 10.46 ± 1.38 | 6.39 ± 0.62 | 9.80 ± 0.75 | 5.67 ± 0.31 | 11.19 ± 0.91 | 5.63 ± 0.46 |
| | 50 μL microsampling | 8.86 ± 1.11 | 5.45 ± 0.65 | 10.10 ± 1.69 | 6.36 ± 0.39 | 9.64 ± 1.38 | 5.99 ± 0.33 | 10.14 ± 0.82 | 6.16 ± 0.17* |
| Pancreas (g) | No treatment | 1.10 ± 0.20 | 0.83 ± 0.10 | - | - | - | - | - | - |
| | 50 μL microsampling | 1.10 ± 0.09 | 0.83 ± 0.09 | - | - | - | - | - | - |
| Kidneys (g) | No treatment | 2.35 ± 0.23 | 1.45 ± 0.17 | 2.90 ± 0.29 | 1.67 ± 0.08 | 2.69 ± 0.04 | 1.57 ± 0.11 | 3.02 ± 0.14 | 1.51 ± 0.15 |
| | 50 μL microsampling | 2.30 ± 0.14 | 1.47 ± 0.13 | 2.73 ± 0.37 | 1.75 ± 0.07 | 2.67 ± 0.36 | 1.60 ± 0.16 | 2.75 ± 0.20* | 1.68 ± 0.18 |
| Thymus (g) | No treatment | 5.88 ± 0.157 | 0.420 ± 0.148 | 0.512 ± 0.111 | 0.518 ± 0.122 | 0.501 ± 0.106 | 0.346 ± 0.080 | - | - |
| | 50 μL microsampling | 0.474 ± 0.099 | 0.444 ± 0.110 | 0.486 ± 0.064 | 0.453 ± 0.058 | 0.459 ± 0.055 | 0.475 ± 0.083* | - | - |
| Spleen (g) | No treatment | 0.56 ± 0.07 | 0.39 ± 0.09 | 0.67 ± 0.10 | 0.49 ± 0.05 | 0.67 ± 0.10 | 0.35 ± 0.05 | 0.69 ± 0.11 | 0.46 ± 0.09 |
| | 50 μL microsampling | 0.57 ± 0.05 | 0.40 ± 0.04 | 0.66 ± 0.07 | 0.49 ± 0.04 | 0.64 ± 0.10 | 0.46 ± 0.08* | 0.64 ± 0.06 | 0.49 ± 0.07 |
| Adrenal glands (mg) | No treatment | 52 ± 7 | 52 ± 7 | 50 ± 7 | 67 ± 10 | 56 ± 20 | 55 ± 8 | 58 ± 8 | 60 ± 6 |
| | 50 μL microsampling | 50 ± 3 | 56 ± 7 | 53 ± 9 | 67 ± 7 | 51 ± 7 | 61 ± 9 | 59 ± 6 | 60 ± 8 |
| Submandibular gland (g) | No treatment | 0.600 ± 0.061 | 0.378 ± 0.035 | 0.639 ± 0.060 | 0.431 ± 0.053 | - | - | - | - |
| | 50 μL microsampling | 0.572 ± 0.046 | 0.426 ± 0.039 | 0.605 ± 0.056 | 0.423 ± 0.022 | - | - | - | - |

Significantly different from the corresponding "no treatment" group by Student t-test. *p < 0.05.

A, B, C and D indicate organizations. -: not evaluated.
and organ weights (lung in organization B, pancreas in organizations B, C and D, thymus in organization D and submandibular gland in organizations C and D) (Table 4).
Evaluation of microsampling effects on these parameters would be insufficient as a multi-organization study.

In 39 companies, including 38 in the US/EU with 90% being pharmaceutical and CRO companies, a recent survey on microsampling for quantitative bioanalysis revealed that more than half these companies have used rodent microsampling data from GLP studies for investigative new drug (IND) applications and that about 1/4 of the companies used rodent and non-rodent microsampling data from GLP studies for new drug application (NDA) (Patel et al., 2019). Our results could accelerate the usage of microsampling techniques in rat toxicity studies in Japanese pharmaceutical and CRO companies.

ACKNOWLEDGMENTS

This work was supported in part by AMED under Grant Numbers JP17ak0101073j0001 and JP18ak0101073j0002.

Conflict of interest–Hideaki Yokoyama, Kyotaka Muta, and Akio Kobayashi are employees of Japan Tobacco Inc. Norimichi Hattori and Asuka Takumi are employees of Ajinomoto Co., Inc. Hirohiko Ohtsuka, Harumi Kitaura, and Fumihiro Jinno are employees of Axcelead Drug Discovery Partners Inc. Eiji Murata, Kanae Mori, Keiko Nakai, and Atsushi Iwai are employees of LSI Medience Co. No other conflicts of interest were declared for this work.

REFERENCES

Caron, A., Lelong, C., Bartels, T., Dorchies, O., Gury, T., Chalier, C. and Benning, V. (2015): Clinical and anatomic pathology effects of serial blood sampling in rat toxicity studies, using conventional or microsampling methods. Regul. Toxicol. Pharmacol., 72, 429-439.

Coleman, D., Smith, G., Lawrence, R., McManus, D., Diaram, S. and Edwards, J. (2017): Capillary microsampling in nonclinical safety assessment: practical sampling and bioanalysis from a CRO perspective. Bioanalysis, 9, 787-798.

Diehl, K.H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.M. and van de Vorstenbosch, C.; European Federation of Pharmaceutical Industries Association and European Centre for the Validation of Alternative Methods. (2001): A good practice guide to the administration of substances and removal of blood, including routes and volumes. J. Appl. Toxicol., 21, 15-23.

ICH. (1994): ICH S3A: Note for guidance on toxicokinetics: The assessment of systematic exposure in toxicity studies. (https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S3A/Step4/S3A_Guideline.pdf)

ICH. (2017): ICH S3A Q&A focus on microsampling. (https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S3A/S3AIWG_Step4_2017_1116.pdf).

Jonsson, O., Palma Villar, R., Nilsson, L.B., Norsten-Höög, C., Brogren, J., Eriksson, M., Königsson, K. and Samuelsson, A. (2012): Capillary microsampling of 25 µl blood for the determination of toxicokinetic parameters in regulatory studies in animals. Bioanalysis, 4, 661-674.

Patel, S.R., Bryan, P., Spooner, N., Timmerman, P. and Wickremesinhe, E. (2019): Microsampling for quantitative bioanalysis, an industry update: output from an AAPS/EBF survey. Bioanalysis, 11, 619-628.

Powles-Glover, N., Kirk, S., Wilkinson, C., Robinson, S. and Stewart, J. (2014): Assessment of toxicological effects of blood microsampling in the vehicle dosed adult rat. Regul. Toxicol. Pharmacol., 68, 325-331.