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Histogram Analysis of Pharmacokinetic Parameters by Bootstrap Resampling from One-point Sampling Data in Animal Experiments

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Summary: A bootstrap method is proposed for assessing statistical histograms of pharmacokinetic parameters (AUC, MRT, CL and V_{ss}) from one-point sampling data in animal experiments. A computer program, MOMENT(BS), written in Visual Basic on Microsoft Excel, was developed for the bootstrap calculation and the construction of histograms. MOMENT(BS) was applied to one-point sampling data of the blood concentration of three physiologically active proteins (^{111}In labeled Hsp70, Suc_{20}-BSA and Suc_{40}-BSA) administered in different doses to mice. The histograms of AUC, MRT, CL and V_{ss} were close to a normal (Gaussian) distribution with the bootstrap resampling number (200), or more, considering the skewness and kurtosis of the histograms. A good agreement of means and SD was obtained between the bootstrap and Bailer's approaches. The hypothesis test based on the normal distribution clearly demonstrated that the disposition of ^{111}In-Hsp70 and Suc_{20}-BSA was almost independent of dose, whereas that of ^{111}In-Suc_{40}-BSA was definitely dose-dependent. In conclusion, the bootstrap method was found to be an efficient method for assessing the histogram of pharmacokinetic parameters of blood or tissue disposition data by one-point sampling.

Key words: bootstrap method; pharmacokinetics; histogram analysis; hypothesis testing; sparse sampling; Monte Carlo

Introduction

A certain model is assumed in pharmacokinetic studies (PK) which describe the relationship of drug absorption, distribution and elimination to a drug concentration after administration. An attempt is made in PK to evaluate the relationship between the disposition characteristics of a drug and its parameters calculated, based on a particular model. Much interest in PK involves comparing the parameters between two groups of animals under different experimental conditions. In order to identify any statistically significant differences, one time course must be obtained from one animal in the common method.

However, apart from blood, the repeated sampling of tissues from an individual experimental animal is quite difficult, and groups of animals in small numbers are euthanized at each sampling time. This conventional ''one-point sampling'' method has been widely used in various experimental settings in preclinical studies. In such experiments, pharmacokinetic parameters are calculated using the average values of concentrations in the blood or in organs of three or four experimental animals. Since the variances of the parameters are not assessed in this conventional analysis, it is impossible to statistically compare the pharmacokinetic parameters in two animal groups. When the statistical histogram of AUC is approximated by the normal (Gaussian) distribution, Bailer's approach can be adopted as a ''gold standard'' to estimate the standard error of AUC. The linearity and the normality of AUC distribution are implicitly assumed in Bailer's approach.[1] If the parameters are given by a nonlinear function of concentrations, or the statistical distributions of parameters

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Abbreviations used are: Hsp70, heat shock protein 70; Suc-BSA, succinylated bovine serum albumin; AUC, the area under the concentration curve; MRT, mean residence time; CL, total clearance; V_{ss}, volume at steady-state

are unknown, Bailer's method has a limitation.

In the paper (1979) entitled ''BOOTSTRAP METHODS: ANOTHER LOOK AT THE JACKKNIFE'', Efron proposed a 'bootstrap method', a statistical interval analysis, by means of Monte Carlo simulation.[2,3] The bootstrap method has been widely adopted in many areas with the rapid development of computer power and its simple algorithm compared with that of the jackknife. The basis of the bootstrap method involves resampling from the observed data and construction of histogram on computer. The bootstrap method has been applied to estimating the confidence intervals for population pharmacokinetic parameters by resampling n time courses from n population time courses permitting replacement.[4–8] The bootstrap method was also proposed for assessing the bioequivalence between two drug formulations in a draft guidance from the United States Food and Drug Administration (FDA).[9–12] This approach was also implemented in a computer program for linkage analysis, MENDEL4, which was developed for analyzing genetically discrete traits in pedigree and population data sets.[13]

The evaluation method presented here is based on the bootstrap approach for assessing the histograms of pharmacokinetic parameters for data collected by a one-point sampling method in animal experiments. We have developed a computer program, MOMENT(BS), that provides the histograms of the parameters by the bootstrap method, including area under the concentration curve (AUC), mean residence time (MRT), total clearance (CL), and volume of distribution at steady-state (V_{ss}). MOMENT(BS) provides the mean, standard deviation (SD), skewness (SK) and kurtosis (KT) of the parameters by means of bootstrap resampling, in addition to the mean and SD and standard error (SE) by expanding Bailer's method. We selected, as model drugs, three physiologically active proteins: mouse recombinant heat shock protein 70 (Hsp70)[14–18] and two succinylated bovine serum albumins (Suc_{20}-BSA and Suc_{40}-BSA).[19,20] This aim of this study was to carry out a statistical comparison of the pharmacokinetic parameters between two animal groups under different conditions by means of a hypothesis test.

Materials and Methods

Proteins and tissue distribution experiments

Mouse recombinant heat shock protein 70 (Hsp70), succinylated bovine serum albumin (the number of succinic anhydride units conjugated to BSA was 20 or 40; Suc_{20}-BSA and Suc_{40}-BSA) were radiolabeled with ^{111}In using the bifunctional chelating agent DTPA anhydrate. ^{111}In-Hsp70, Suc_{20}-BSA and Suc_{40}-BSA were injected into the tail vein of mice at different doses. The radioactivity in plasma was measured at the indicated times.[21–25]

Data analysis

Bootstrap algorithm: The procedures based on the bootstrap method for one-point sampling are as follows.

Step1. Construction of time course of blood level or tissue concentrations, which consist of three or four points at each time, from data obtained by one-point sampling in animal experiments (one point at each time is collected from one animal).

Step2. Selection of one point from three or four points at each time, permitting replacement using random number, and construction of pseudo-profile.[26]

Step3. Calculation of pharmacokinetic parameters (statistics; Φ) from time course obtained in Step2.

Step4. Construction of the histograms and calculation of moment characteristics (mean, SD, SK and KT) of Φ with resampling of bootstrap number (B) times.

Step5. Assessment of histograms, including normal distribution and log-normal distribution.

Step6. Comparison of Φ between two animal groups depending on type of statistical distribution.

To perform the above procedures, a computer program, MOMENT(BS), was developed, programmed in Visual Basic on Microsoft Excel. When concentrations in an organ are measured, AUC and MRT can be replaced by AUC_i and MRT_i in organ i, respectively.

The means ($\bar{\Phi}$) and the standard deviation (SD) of parameters were calculated by

$$\bar{\Phi} = \frac{1}{B}\sum_{i=1}^{B}\Phi_i$$

$$SD(\Phi) = \sqrt{\frac{1}{B-1}\sum_{i=1}^{B}(\Phi_i - \bar{\Phi})^2}$$

where B is bootstrap resampling number.

The skewness (SK) and kurtosis (KT) are given by

$$SK(\Phi) = \frac{1/B\cdot\sum_{i=1}^{B}(\Phi_i - \bar{\Phi})^3}{\left(1/B\cdot\sum_{i=1}^{B}(\Phi_i - \bar{\Phi})^2\right)^{3/2}}$$

$$KT(\Phi) = \frac{1/B\cdot\sum_{i=1}^{B}(\Phi_i - \bar{\Phi})^4}{\left(1/B\cdot\sum_{i=1}^{B}(\Phi_i - \bar{\Phi})^2\right)^{2}}$$

When the histogram of a pharmacokinetic parameter s close to the normal distribution ($SK \approx 0$ and $KT \approx 3$), the normal distribution test was performed. When the histogram of a parameter is close to a log-normal distribution ($SK \gg 0$ and $KT \gg 3$), the logarithm of the parameter is used for the normal hypothesis test.

Bailer's algorithm: In Bailer's original paper,[1] the mean and SE are discussed only for AUC. Therefore, the present discussion is the expansion of Bailer's

algorithm. The mean $E(\Phi)=\bar{\Phi}$ and variance ($V(\Phi)$) of MRT, CL and V_{ss} in addition to those of AUC were calculated by the following equations which were derived according to the formula of four arithmetic operations of probability variable.

$$E(AUC)=\sum_{i=2}^{m}(E(C_i)+E(C_{i-1}))\cdot(t_i-t_{i-1})/2$$

$$V(AUC)=\sum_{i=2}^{m}(V(C_i)+V(C_{i-1}))\cdot((t_i-t_{i-1})/2)^2$$

$$E(AUMC)=\sum_{i=2}^{m}(t_i\cdot E(C_i)+t_{i-1}\cdot E(C_{i-1}))\cdot(t_i-t_{i-1})/2$$

$$V(AUMC)=\sum_{i=2}^{m}(t_i^2\cdot V(C_i)+t_{i-1}^2\cdot V(C_{i-1}))\cdot(t_i-t_{i-1})/2)^2$$

$$E(MRT)=E(AUMC)/E(AUC)$$

$$V(MRT)=\frac{V(AUMC)}{E(AUC)^2}+\frac{V(AUC)\cdot E(AUMC)^2}{E(AUC)^4}$$

$$E(CL)=D/E(AUC)$$

$$V(CL)=D^2\cdot V(AUC)/E(AUC)^4$$

$$E(V_{ss})=E(CL)\cdot E(MRT)$$

$$V(V_{ss})=E(MRT)^2\cdot V(CL)+E(CL)^2\cdot V(MRT)$$

where m is point number of time course (pseudo-profile), t_i and C_i are time and concentration, respectively, D is dose, and AUMC is area under moment curve. The time course is integrated by the trapezoidal formula without extrapolation. The standard deviation (SD) of each parameter is given as the root of the variance $V(\Phi)$.

$E(C_i)$ and $V(C_i)$ are the mean and variance of the concentrations at time t_i, respectively, and defined by

$$E(C_i)=\frac{1}{n_i}\sum_{j=1}^{n_i}C_{ij}$$

$$V(C_i)=\frac{1}{n_i-1}\sum_{j=1}^{n_i}(C_{ij}-E(C_i))^2$$

where n_i is point number of concentrations at time t_i.

Now, we define the variance of mean (Φ) by the following equations.

$$V(\overline{AUC})=\sum_{i=2}^{m}(V(C_i)/n_i+V(C_{i-1})/n_{i-1})\times((t_i-t_{i-1})/2)^2$$

$$V(\overline{AUMC})=\sum_{i=2}^{m}(t_i^2\cdot V(C_i)/n_i+t_{i-1}^2\cdot V(C_{i-1})/n_{i-1})\times((t_i-t_{i-1})/2)^2$$

$$V(\overline{MRT})=\frac{V(\overline{AUMC})}{E(AUC)^2}+\frac{V(\overline{AUC})\cdot E(AUMC)^2}{E(AUC)^4}$$

$$V(\overline{CL})=D^2\cdot V(\overline{AUC})/E(AUC)^4$$

$$V(\bar{V}_{ss})=E(MRT)^2\cdot V(\overline{CL})+E(\overline{CL})^2\cdot V(\overline{MRT})$$

where we note the relationship: $E(\Phi)=E(\bar{\Phi})$.

When n_i are the same (n), we find the following simple relationships:

$$V(\overline{AUC})=\frac{1}{n}V(AUC)$$

$$V(\overline{AUMC})=\frac{1}{n}V(AUMC)$$

$$V(\overline{CL})=\frac{1}{n}V(CL)$$

$$V(\bar{V}_{ss})=\frac{1}{n}V(V_{ss})$$

The standard error (SE) of each pharmacokinetic parameter is given as the root of the variance $V(\bar{\Phi})$.

The normal hypothesis test is carried out using the following equation.

$$Z_0=\frac{|\bar{\Phi}_1-\bar{\Phi}_2|}{\sqrt{SE_1^2+SE_2^2}}$$

where $\bar{\Phi}_1$ and $\bar{\Phi}_2$ are means of a pharmacokinetic parameter, and SE_1 and SE_2 are standard errors in groups 1 and 2, respectively. If $Z_0>1.96$ (confidence interval $p<0.05$), the difference is assumed to be significant between groups 1 and 2.

Results and Discussion

Figure 1 shows the time courses of the plasma concentration of ^{111}In-Hsp70, Suc_{20}-BSA, and Suc_{40}-BSA after intravenous injection into mice, where each plot is expressed as the average (± standard deviation) of three mice.[14,19] The resampling number in the bootstrap calculation was considered for evaluating the optimum value. As a representative example, **Fig. 2** shows the effect of the bootstrap resampling number (B) on the variance of parameters calculated by MOMENT(BS). **Figures 2a** and **b** show the averages and standard deviations of the mean(AUC) and mean(CL) versus B for three trial bootstrap calculations, whereas **Figs. 2c** and **d** show the averages and standard deviations of SD(AUC) and SD(CL) versus B. The means were almost constant and the standard deviations were close to zero with B of 200 or more, whereas the means and standard deviations exhibited marked fluctuations in B from 5- to 100-fold. **Figure 3** shows statistical histograms of AUC (a, b, c) and CL (d, e, f) and the theoretical curves of the normal distribution of AUC with B values of 10, 200 and 1000, and **Figs. 3d**, **e** and **f** show the histograms and the theoretical curves of CL. The histograms at B (10) deviate from the theoretical curves of a normal distribution, whereas they are close to the theoretical curves at B values of 200 or 1000. Therefore, bootstrap resampling of 200 or more is enough to calculate the mean and SD, which is in good agreement with the conclusion by Efron *et al.*[27,28] Considering these findings, B(1000) was

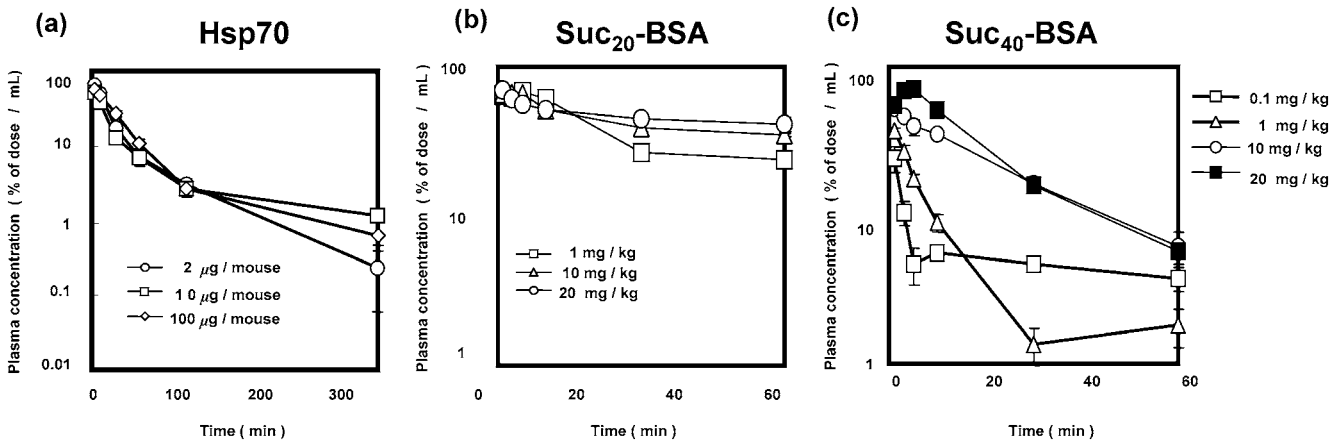

Fig. 1. Plasma concentration and liver accumulation of ^{111}In-Hsp70, Suc_{20}-BSA, Suc_{40}-BSA after intravenous injection into mice at several doses. These results are expressed mean ± standard deviation of three mice.

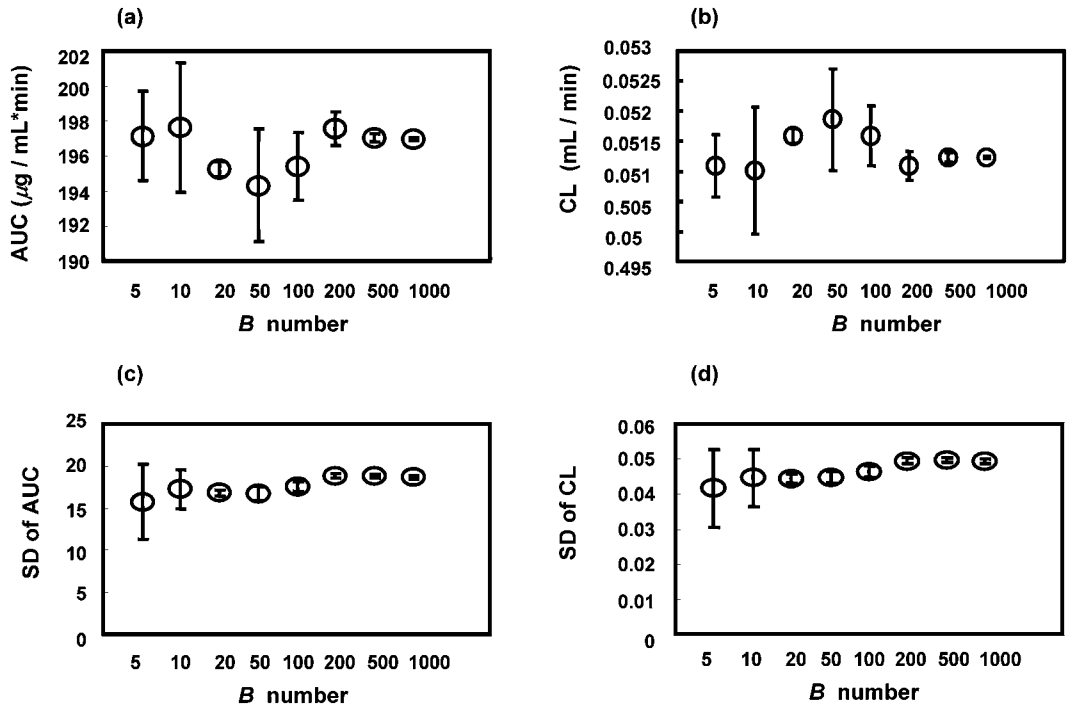

Fig. 2. Effect of bootstrap number on the estimated pharmacokinetic parameters. (a) and (b) represent the average of AUC and CL of ^{111}In-Hsp70 with standard deviation after intravenous injection at a dose of 10 μg/mouse. (c) and (d) represent the average of SD with standard deviation, calculated at three trials by MOMENT(BS).

selected in the following calculations.

Table 1 shows the means of AUC, MRT, CL and V_{ss} and the standard deviations (SD) calculated by MOMENT(BS) after intravenous injection of ^{111}In-Hsp70, Suc_{20}-BSA and Suc_{40}-BSA into mice. The mean values and SD by bootstrap method were in good agreement with those calculated by Bailer's method. **Table 2** shows the skewness (SK) and kurtosis (KT) of the pharmacokinetic parameters of the three physiologically active proteins. All SK fluctuate around zero, whereas all KT were about two to three. Based on the agreement between the bootstrap and Bailer's approaches and on the fact that SK were close to 0 and that KT were close to 3, all histograms of the parameters calculated by MOMENT(BS) were assumed to be normal (Gaussian) distributions, not log-normal distributions. These discussions are ignored in Bailer's approach. Thus, an attempt was made to compare the parameters between two groups under different conditions based on normal distribution, where it is noted that *SE* of parameters are

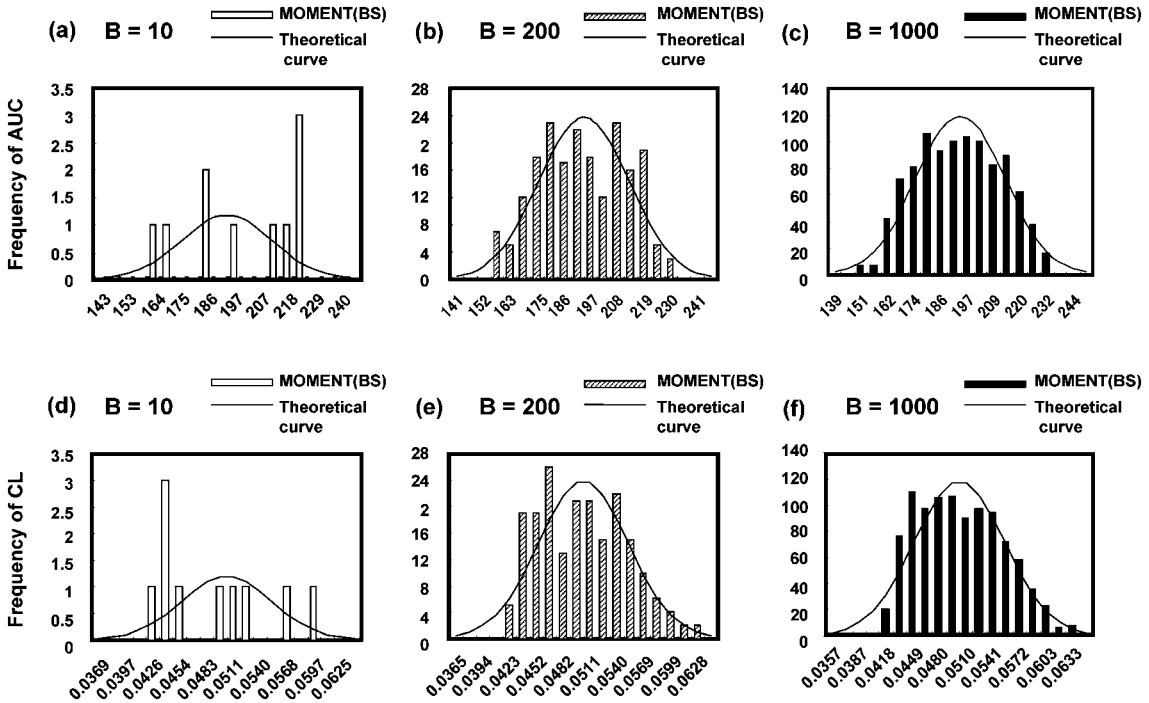

Fig. 3. (a), (b) and (c) represent the frequency histograms of AUC of Hsp70 after intravenous injection at a dose of 10 μg/mouse into mice, whereas (d), (e) and (f) show the frequency of the CL at B numbers (10, 200 and 1000).

Table 1. Mean and Standard Deviation (SD) of AUC, MRT, CL and V_{ss} calculated by MOMENT(BS)

	Dose	AUC (μg/mL·min)				MRT (min)				CL (mL/min)				V_{ss} (mL)			
		Bootstrap		Bailer's		Bootstrap		Bailer's		Bootstrap		Bailer's		Bootstrap		Bailer's	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hsp70	2 μg/mouse	45.4	2.8	45.4	2.6	49.0	3.1	49.1	6.3	0.044	0.003	0.044	0.003	2.16	0.12	2.16	0.30
	10 μg/mouse	195	19	196	18	68.0	6.8	67.6	9.8	0.052	0.005	0.051	0.005	3.53	0.61	3.44	0.59
	100 μg/mouse	2640	165	2640	157	53.4	3.1	53.4	6.4	0.040	0.002	0.038	0.002	2.03	0.13	2.02	0.27
Suc_{20}-BSA	0.1 mg/kg	102	3	101	3	27.0	0.6	27.0	1.3	0.030	0.001	0.030	0.001	0.800	0.038	0.799	0.05
	1 mg/kg	673	8	673	8	22.7	0.3	22.7	0.7	0.046	0.001	0.045	0.001	1.01	0.02	1.01	0.03
	10 mg/kg	7850	261	7850	237	26.2	0.3	26.2	1.2	0.038	0.001	0.038	0.001	1.00	0.03	1.00	0.05
	20 mg/kg	16380	638	16400	686	28.2	0.9	28.1	2.4	0.037	0.001	0.037	0.002	1.03	0.03	1.03	0.10
Suc_{40}-BSA	0.05 mg/kg	4.41	0.29	4.41	0.31	29.7	1.5	29.7	3.8	0.342	0.023	0.340	0.002	10.2	0.7	10.1	1.5
	0.1 mg/kg	9.56	0.62	9.57	0.62	24.7	1.4	24.7	3.1	0.315	0.021	0.314	0.002	7.76	0.60	7.73	1.1
	1 mg/kg	106	7	106	7	12.5	1.4	12.5	2.4	0.283	0.020	0.282	0.020	3.52	0.38	3.52	0.73
	10 mg/kg	3890	175	3900	173	18.8	1.0	18.8	1.9	0.077	0.004	0.077	0.003	1.45	0.06	1.45	0.16
	20 mg/kg	9760	364	9770	367	16.0	0.4	16.0	0.9	0.062	0.002	0.061	0.002	1.00	0.05	0.982	0.069

given by $SD/\sqrt{n}$, respectively, in bootstrap and Bailer's approaches.

The difference of MRT of Hsp70 was insignificant between 2 and 100 μg. The difference of SUC_{20}-BSA was insignificant between 0.1 and 10 mg, between 0.1 and 20 mg, and between 10 and 20 mg. The difference of Suc_{40}-BSA was insifnificant between 0.05 and 0.1 mg. The difference of MRT was significant among others.

The difference of CL of Suc_{20}-BSA was insignificant between 10 and 20 mg. The difference of Suc_{40}-BSA was insignificant between 0.05 and 0.1 mg, and between 0.1 and 1 mg. The difference of CL was significant among others.

The difference of V_{ss} of Hsp-70 was insignificant between 2 and 100 μg. The difference of Suc_{40}-BSA was insignificant between 1 and 10 mg, between 1 and 20 mg, and between 1 and 20 mg. The difference of Vss was significant among others.

Generally, it was concluded that the pharmacokinetic parameters of Hsp70 and Suc_{20}-BSA are almost

Table 2. Skewness (SK) and Kurtosis (KS) of AUC, MRT, CL and V_{ss} calculated by MOMENT(BS)

Dose		AUC		MRT		CL		V_{ss}	
		SK	KT	SK	KT	SK	KT	SK	KT
Hsp70	2 μg/mouse	0.217	2.48	0.178	1.89	0.075	2.39	0.112	2.62
	10 μg/mouse	0.155	2.14	0.265	2.28	0.190	2.15	0.200	2.01
	100 μg/mouse	0.163	2.49	0.547	1.98	0.094	2.48	0.207	2.62
Suc_{20}-BSA	0.1 mg/kg	0.298	2.35	−0.099	2.30	−0.186	2.28	−0.355	2.13
	1 mg/kg	−0.215	2.51	−0.255	1.99	0.686	2.67	0.180	2.74
	10 mg/kg	0.301	2.00	0.164	2.23	−0.232	1.98	0.063	2.39
	20 mg/kg	−0.033	2.37	−0.327	1.83	0.179	2.42	−0.089	2.28
Suc_{40}-BSA	0.05 mg/kg	−0.335	2.72	−0.623	2.23	0.643	3.09	0.072	2.50
	0.1 mg/kg	0.097	2.66	−0.107	2.17	0.221	2.62	0.401	2.70
	1 mg/kg	0.244	2.48	−0.111	1.94	0.040	2.41	0.007	2.24
	10 mg/kg	−0.253	2.33	0.007	2.20	0.441	2.49	0.133	2.70
	20 mg/kg	0.356	2.12	0.069	2.26	−0.225	2.06	−0.326	1.91

independent of dose, whereas those of Suc_{40}-BSA are strictly dose-dependent.

The bootstrap method has been applied exclusively to estimating the confidence intervals of statistics (Φ).[29–31] The interval analysis is equivalent to the test of statistical hypothesis. The test of hypothesis in addition to the histogram analysis by the bootstrap method is the first trial for the analysis of one-sampling data. The means and SD of the pharmacokinetic parameters estimated by the bootstrap method agreed with those obtained by Bailer's method, using MOMENT(BS). Consequently, SE by the bootstrap method were in good agreement with those by Bailer's method. The histograms of the parameters showed a normal distribution, which was close to the theoretical curve, and SK and KT were close to zero and three, respectively, for the present data using three physiologically active proteins. The proposed method is expected to be useful for assessing the histogram of a pharmacokinetic parameter. After assessment of the statistical distribution, it was shown that the hypothesis test can be applied to statistically comparing the disposition of two groups under different conditions, using one-point sampling data from animal experiments.

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