Model-Independent Procedure for Area Estimation and Intergroup Comparisons

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Abstract □ A method is provided for estimating the area under a response–time curve for an experimental situation where independent observations are made at each time point. The procedure utilizes least-squares spline functions and the jackknife technique to provide area estimates, standard errors, and a statistical test for intergroup comparisons. The method is based on an empirical model and provides a means of analysis when no prior model can be specified or if the incorrect specification of a model will produce invalid results. The method is illustrated using data from a study comparing the pharmacokinetic behavior of a fasciolicide in rats having either a young or old parasitic infestation. The results obtained are comparable with those generated by a model-dependent approach.

Keyphrases □ Pharmacokinetics—area under response–time curve, model-independent estimation, intergroup comparisons □ Spline functions—use with jackknife technique to provide area under response–time curve estimations, model independent

In pharmacokinetics it is often necessary to calculate the area under the response–time curve (AUC) for a subject and then compare the AUC among subjects allocated to several experimental groups. For one type of experimental design, a drug is given to several groups of subjects and a response variable for each subject is then observed at fixed time points (Scheme I, type B). Each subject has its own response–time curve, and an estimate of a subject's AUC could be obtained by (*a*) fitting and then numerically integrating a pharmacokinetic model (1), or (*b*) utilizing a model-independent interpolation procedure such as the trapezoidal rule (2). However, in some situations a time–response curve is not available for each subject; instead independent observations are made at each time point (Scheme I, type A). For example, a drug may be given to several groups of animals with different experimental conditions, and the drug concentration in an organ of the animal is required at several points in time. Since the animals must be serially sacrificed to obtain this information, the measurements are made using different animals at each time point. A model-independent procedure for this experimental design (Scheme I, type A) is introduced which will provide area estimates, standard errors, and a statistical test for intergroup comparisons.

TYPE A STUDIES	TYPE B STUDIES
P experimental groups or treatments	P experimental groups or treatments
↓	↓
N_i subjects in each group	N_i subjects in each group
↓	↓
Drug is given to all the subjects at time t_0	Drug is given to all the subjects at time t_0
↓	↓
A random sample of subjects is selected from each group and the response variable is measured at time t_i, $i = 1, 2, \ldots, k$. Once selected, the subject is removed from the study	Response variable is measured for each subject at all subsequent times $t_1, t_2, \ldots, t_k$
↓	↓
Response–time curve for each group (not individual)	Each subject has a response–time curve
↓	↓
Area calculated for each group's response–time curve	Area calculated for each subject's response–time curve

Scheme I—Two Types of Experimental Designs

In these situations (Scheme I, type A) a model-dependent AUC estimate can be obtained by specifying a pharmacokinetic model. However, this model dependent approach could result in biased and unreliable area estimates in situations where the specific model used is not the correct functional form. In general, it is difficult to discriminate among competing models on the basis of prior biological information and/or statistical goodness-of-fit tests, and this is especially so with the addition of between-subject variability at each time point. Another alternative would be to apply an interpolation procedure to each group's mean response–time curve. However, no estimate of precision would be available and thus, no intergroup comparisons could be made.

This paper presents a model-independent method for estimation of an experimental group's AUC in type A studies, which also allows for intergroup hypothesis testing. The method utilizes least-squares spline functions (3) and the jackknife technique (4), and would appear to be the procedure of choice in obtaining AUC estimates for type A studies unless there is strong evidence for a specific pharmacokinetic model. The method is illustrated on data from a study (5) comparing the pharmacokinetic behavior of a fasciolicide in rats having either young or old parasitic infestations.

THEORETICAL

Estimation and Intergroup Comparison Procedure—The method presented in this paper can be outlined in three steps:

1. Fit an empirical function (spline, Eq. 2) using ordinary least squares to the group response–time curve.
2. Obtain an estimate of the AUC (the integral of the fitted function over time, Eq. 4) and its standard error by least squares or the jackknife procedure (4).
3. Use of the pseudovalues (Eq. 6) generated by the jackknife procedure to evaluate group differences *via* a t test or an ANOVA.

Fitting an Empirical Function (Spline) Using Ordinary Least Squares—In this method it is assumed that for each group and response variable there is a continuous (smooth) function, $g(t)$, which represents the average response *versus* time relationship, *i.e.*:

$$Y_{ism} = g(t_{is}) + e_{ism} \quad m = 1, 2, \ldots M_{is}, \quad s = 0, \ldots S_i, \quad i = 1, 2, \ldots P, \quad \sum_s M_{is} = N_i \qquad \text{(Eq. 1)}$$

where Y_{ism} is the response of the mth subject at the sth time of the ith group; $g(t)$ is a continuous function whose exact form is not known; e_{ism} are independent identically distributed random errors with mean 0 and variance σ^2; and N_i is the number of subjects in the ith group.

To obtain model-independent AUC estimates for each group, an em-

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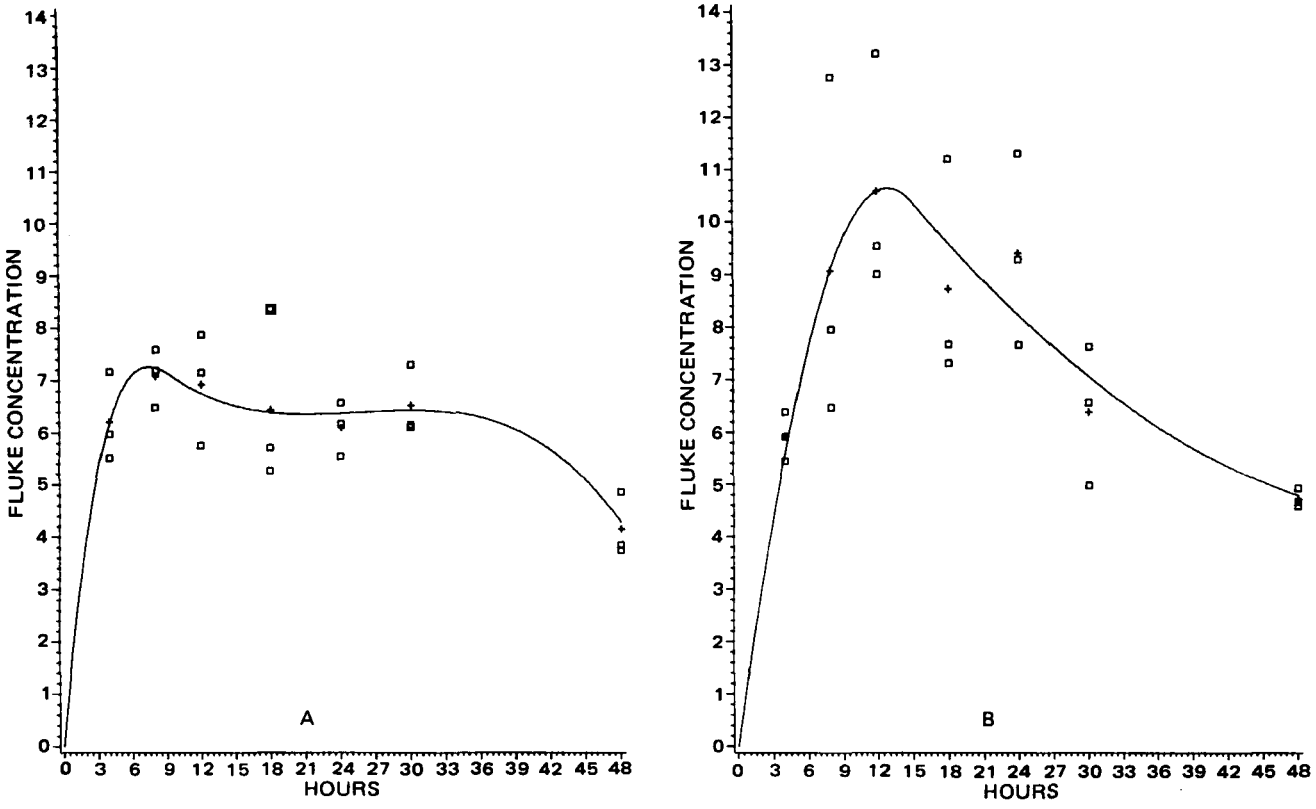

Figure 1—*Plot of observed individual drug concentration in the liver fluke of rats (□) and mean drug concentration (+)* versus *time (hr) for young (A) and old (B) infestation groups. Curves represent least-squares spline fit to the individual drug concentration* versus *time data (Table I).*

pirical function must be chosen that adequately describes the variation of response over time and gives a close approximation to $g(t)$. Spline functions, which are defined as piecewise continuous polynomials of degree n, are considered to be excellent empirical functions (3, 6, 7) for this purpose. The pieces of the spline are joined together at points called knots (abscissa values) in such a fashion that the function and the first $n-1$ derivatives agree at the join points. The degree of the polynomial pieces and the number and position of the knots may be varied in different situations to provide a good fit to the data. Quadratic and cubic splines (polynomial pieces of degree at most 2 or 3) are the most popular among practitioners because of their computational simplicity, smoothness, and flexibility, and have been used in a variety of biological and chemical applications (3, 7, 8–12).

There are several numerical methods for fitting splines to data (3). The method employed here is that of ordinary least squares (OLS). This method requires that the number of knots, as well as their location, be specified. The fitting of the spline to data is then accomplished by multiple linear regression techniques. The choice of the number and position of the knots is an important and difficult problem that has to be addressed. Knots should be chosen so as to correspond to the overall behavior of the data (number of time points, position of maxima and minima, *etc.*). Some rules of thumb for knot positioning have been presented by Wold (3). However, in most applications judgment and experience together with simulation results seem to be the only criteria for making these decisions.

Spline functions can be considered as a multiple linear regression model by employing the "+" function representation of a spline (7). For example, a cubic spline is given as follows: Let $S(t)$ for $t_0 \le t \le t_L$ be a cubic spline with knots at $k_2, \ldots k_{r-1}$. Then:

$$S(t) = b_0 + b_1 t + b_2 t^2 + b_3 t^3 + \sum_{j=2}^{r-1} b_{j+2}\,(t-k_j)_+^3 \qquad \text{(Eq. 2)}$$

where:

$$(t-k_j)_+ = \begin{cases} t-k_j, \text{ if } t > k_j \\ 0, \text{ otherwise} \end{cases} \qquad \text{(Eq. 3)}$$

and b_j are the parameters to be estimated. Notice that the parameters b_j appear linearly in Eq. 1 and thus can be estimated by OLS. A quadratic spline can be defined in an analogous manner (7).

Area Estimation Using Least-Squares Theory—Once the appropriate spline has been fitted to the time–response data for each experimental group, an estimate of the AUC can be obtained. Let the fitted spline function for a group be denoted by $S_i(\hat{t})$. An estimate of the area under the fitted function ($\hat{\text{AUC}}_i$) is given by the integral of $S_i(\hat{t})$ evaluated from t_0 to t_L, *i.e.*:

$$\hat{\text{AUC}}_i = \int_{t_0}^{t_L} S_i(\hat{t})\,dt \qquad \text{(Eq. 4)}$$

If $S_i(\hat{t})$ is a cubic spline, then:

$$\hat{\text{AUC}}_i = \hat{b}_{0i}t + \frac{\hat{b}_{1i}t^2}{2} + \frac{\hat{b}_{2i}t^3}{3} + \frac{\hat{b}_{3i}t^4}{4} + \sum_{j=2}^{r-1} \frac{\hat{b}_{ji+2}}{4}(t-k_j)_+^4 \Bigg|_{t_0}^{t_L} \qquad \text{(Eq. 5)}$$

Estimates of the AUC are linear combinations of the spline parameter estimates, $\hat{b}_{ji}$, so that an estimate of their standard error can be obtained using linear regression methods (13). Note that different spline functions ($\hat{b}_{ji}$) and area estimates ($\hat{\text{AUC}}_i$) are obtained for the different experimental groups.

Area Estimation and Measurements of Precision Using the Jackknife Procedure—If the residuals (observed values minus fitted values) obtained from the spline regression were normally distributed, then the estimated standard errors for AUC estimates computed by least squares could be used to calculate confidence intervals and test for group differences. However, in addition to the random variation, the residuals contain a degree of unknown bias. The bias occurs from fitting an empirical model [$S(t)$] and not the true functional form of the underlying model [$g(t)$]. Also, the random component of the residuals may not be normally distributed. Thus, the standard least-squares method when

Table I—Least-Squares Parameter Estimates for Spline Functions Fitted to Drug Uptake Data in the Liver Fluke in Rats

Parameter	Infestation Group	
	Young	Old
t	2.4767	1.6480
t^2	−0.2714	−0.06386
t^3	0.0093730	—
$(t-10)_+^3$	−0.0095835	—
$(t-15)_+^2$	—	0.06676
S^a	0.8624	1.8322

[a] Denotes root mean square error.

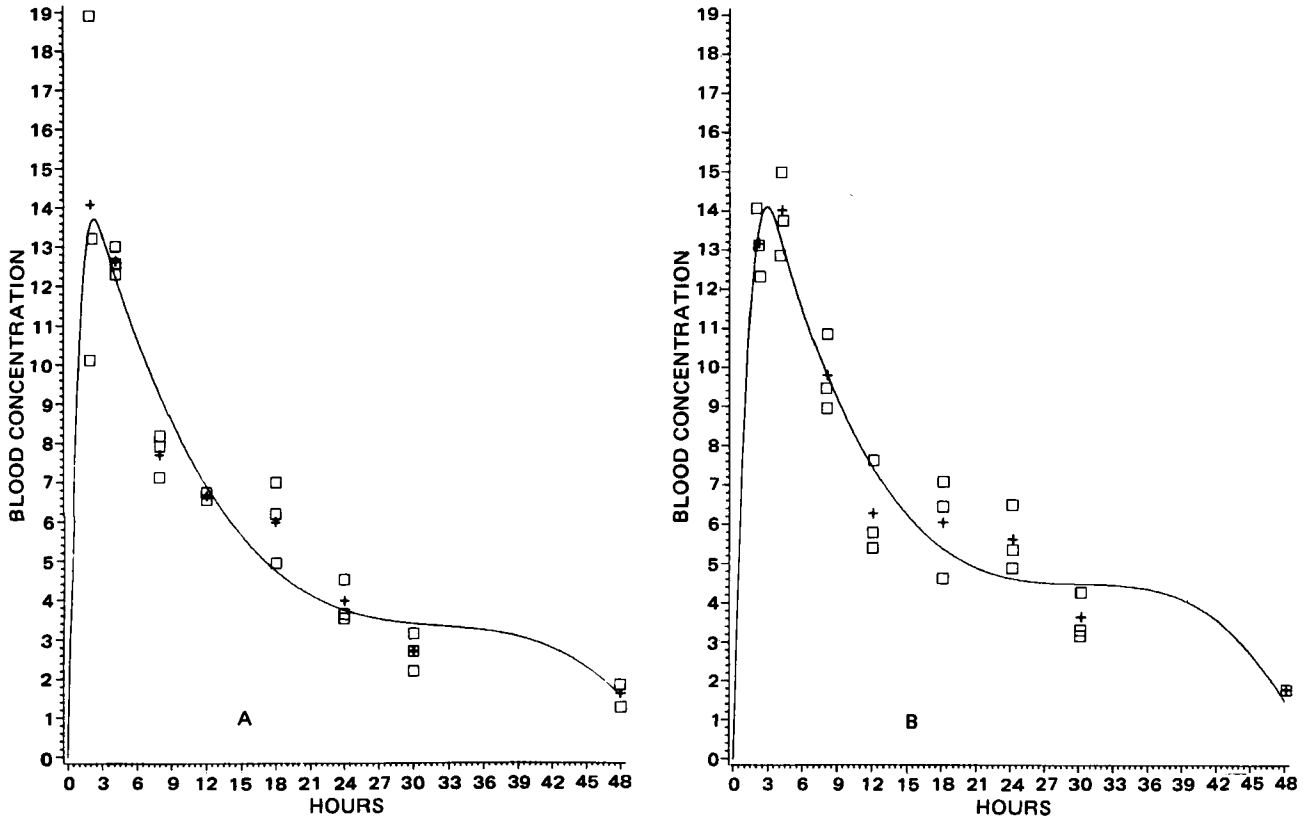

Figure 2—*Plot of observed individual drug concentration in blood of rats (□) and mean drug concentration (+) versus time (hr) for the young (A) and old (B) infestation groups. Curves represent least-squares spline fit to the individual drug concentration versus time data (Table III).*

used to test group differences could yield misleading results. In complex situations where standard methods might not be applicable, a useful statistical technique has been the jackknife procedure (4). The jackknife procedure was introduced by Quenouille (14) as a method for eliminating bias due to finite sample sizes in estimating parameters. Tukey (15) proposed that the jackknife be used for robust interval estimation. A clear nonmathematical exposition of the jackknife with examples is provided by Mosteller and Tukey (4), while an excellent review of the statistical literature dealing with the jackknife is presented by Miller (16).

In situations where linear least-squares methods are employed, there are two methods of jackknifing: the regular jackknife and the weighted jackknife (17). The weighted version provides improved bias reduction and variance estimation for nonlinear functions of linear regression parameters. However, in contrast to the regular jackknife, the weighted jackknife does not address the issue of bias for linear functions of the regression parameters. Since the AUC estimate for each group is a linear function of the fitted spline parameters, the regular jackknife is the procedure of choice for this situation.

To obtain the regular jackknife estimate of the AUC for each group, the function given by Eq. 4 is first calculated with all of the group's observations included. Let $A_{\text{all},i}$, $i = 1, 2, \ldots P$ denote this estimate of area. Next, let A_{ism} denote the estimate of area with the mth subject at the sth time deleted (*i.e.*, the $_{ism}$th subject is deleted, the spline function for the ith group is fitted, and the AUC is computed). Then, pseudovalues are defined as:

$$P_{ism} = N_i A_{\text{all},i} - (N_i - 1) A_{ism}, \quad \begin{matrix} i = 1, 2, \ldots, p \\ s = 1, 2, \ldots, S_i \\ m = 1, 2, \ldots, M_{is} \end{matrix} \qquad \text{(Eq. 6)}$$

The jackknife estimate of AUC for the ith group is then the mean of the pseudovalues:

$$J_i(\hat{\text{AUC}}) = \frac{1}{N_i} \sum_{sm} P_{ism} \qquad \text{(Eq. 7)}$$

with estimated variance:

$$s^2(\hat{J}_i) = \frac{1}{N_i(N_i - 1)} \sum_{sm} [P_{ism} - J_i(\hat{\text{AUC}})]^2 \qquad \text{(Eq. 8)}$$

For each group the pseudovalues can be treated as if they follow a Student's t distribution, and a $(1 - \alpha)$% confidence interval is given by:

$$J_i(\hat{\text{AUC}}) \pm t_{(1-\alpha/2),Ni-1} s(\hat{J}_i) \qquad \text{(Eq. 9)}$$

where $t_{(1-\alpha/2)}, N_i - 1$ is the $(1 - \alpha/2)$ percentage point of the Student's t distribution with $N_i - 1$ degrees of freedom.

Intergroup Comparisons—The pseudovalues for each group can be used as if they were identically distributed estimates of the AUC, and hence, standard statistical procedures can be applied to make intergroup comparisons (18). Various statistical analyses can be used depending on the experimental design, *e.g.*, a one-way or factorial ANOVA procedure. In addition, trend tests for factors with quantitative levels or comparisons of various linear functions of treatment groups using multiple-comparison procedures can be obtained easily.

EXPERIMENTAL

Data Description—A study (5) investigating the pharmacokinetic basis for increased efficacy of a fasciolicide against older liver flukes (*Fasciola hepatica*), *i.e.*, 39–44 weeks old, in rats led to the development of the model-independent estimation and hypothesis-testing procedure described above. The fasciolicide was administered as a single oral dose when the fluke infestation in the rats were either young (9–16 weeks) or

Table II—Area Under the Drug Uptake in Liver Fluke of Rats–Time Curve

		Model Independent[a]		Model Dependent	
Group	N^b	Least Squares[c]	Jackknife[c]	Least Squares[d]	Jackknife[c]
Young	21	289.31 (11.35)	289.83 (9.66)	278.89	277.66 (10.02)
Old	21	344.16 (19.96)	344.23 (18.94)	347.80	348.67 (17.32)
t statistic		—	-2.56^f (30)[e]	—	-3.55^g (32)[e]

[a] Results taken from Ref. 21. [b] Number of observations. [c] Number in parentheses are standard errors. [d] Standard errors were not computed. [e] Since the within-group variances were statistically significantly different ($p < 0.05$), a t test for unequal variances was applied to the pseudovalues and adjusted degrees of freedom are reported (in parentheses). [f] Statistically significant difference, $p < 0.05$ between young and old groups. [g] Statistically significant difference ($p < 0.001$) between young and old groups.

Table III—Least-Squares Parameter Estimates for Spline Functions Fitted to Blood Level Data of Drug in Rats

Parameter	Infestation Group	
	Young	Old
t	15.2758	12.1734
t^2	−5.4484	−3.3347
t^3	0.6090	0.2811
$(t-3)_+^3$	−0.6094	—
$(t-4)_+^3$	—	−0.2816
S^a	1.6970	1.1907

[a] Denotes root mean square error.

old (39–44 weeks). Twenty-one rats were started in each group, with three rats being sacrificed at each time point. The drug uptake in the liver fluke was one of the variables measured (Fig. 1). Blood levels of the rats were also taken at these time points (Fig. 2). It was hypothesized that the drug uptake in the liver fluke was greater in rats having the older infestation, while the drug concentrations in the blood profile were similar for both groups. The areas under the respective time–response curves were considered to be one of the parameters of interest.

Data Treatment—To apply the estimation procedure to these data, the degree of the spline and the number and position of the knots had to be determined. Based on the work of Wold (3) and previous studies[1], it was thought that a spline with a single knot situated near the peak of the response–time curve would provide an adequate fit in this kind of situation (where the response variable rises to a peak and slowly decreases toward zero). Thus, for each group and response variable, either a quadratic or cubic spline with one knot and no intercept term was fitted by OLS. The specific form of the spline was determined by varying the location of the knot and degree of the spline and choosing the result that gave the smallest sum of squared errors. The OLS AUC estimates were obtained by computing the definite integral of the fitted spline from 0 to 48 hr. The jackknife was applied to the AUC estimates. The old *versus* young infestation group comparison was accomplished by performing a two-sample Student's t test on the pseudovalues obtained with the jackknife.

For purposes of comparison, a model-dependent approach was also utilized. An open one-compartment model that is often used for these kind of data (19) was fitted using nonlinear regression. The least-squares and jackknifed AUC estimates were obtained in an analogous manner as described in the model-independent approach.

Computation—All necessary computations were made by utilizing the Statistical Analysis System (SAS) computer package (20). The spline functions were fitted with the SYSREG procedure and the no-intercept option. A SAS PROC MATRIX macro (21) was used to obtain the area estimates and to apply the jackknife procedure. The between-infestation group comparison was made with the TTEST procedure. The results for the model-dependent case were obtained by using the SAS NLIN (nonlinear least-squares) procedure. The plots (Figs. 1 and 2) were computer-generated using a ZETA 1536 plotter which was driven by the SAS/GRAPH package.

RESULTS

Liver Fluke Uptake—The spline function used for area estimation for the young infestation group was a cubic spline with a knot at 10 hr; the spline function for the old infestation group was a quadratic spline with a knot at 15 hr. The spline parameter estimates are given in Table I.

The OLS and jackknife estimates of the AUC are contained in Table II. The AUC estimates and standard errors are similar for the least-squares and jackknife procedures. The two-sample t test for unequal variances performed on the AUC pseudovalues showed that old infestation groups had a statistically significantly greater AUC ($p < 0.05$) after 48 hr than the young group. The AUC estimates obtained with the model-dependent approach gave results that were comparable to the model-independent approach.

Blood Level of Drug—The following splines were fitted: (*a*) for the young infestation group, a cubic spline with a knot at 3 hr and (*b*) for the old infestation group, a cubic spline with a knot at 4 hr. The spline estimates are given in Table III. Results of the AUC estimation procedure are presented in Table IV.

The least-squares and jackknife AUC estimates yield similar estimates and standard errors. Although the jackknife AUC estimates for the old infestation group of rats were higher than those for the young group, the two-sample t test using the pooled within-group variance showed that this result was not statistically significant ($p > 0.05$). The AUC estimates for the model-dependent approach are comparable (although smaller) to corresponding estimates obtained with the model-independent procedures.

[1] Unpublished data, Merck Sharp & Dohme.

Table IV—Area Under the Blood Level of Drug in Rats–Time Curves

Group	N^b	Model Independent[a]		Model Dependent	
		Least Squares[c]	Jackknife[c]	Least Squares[d]	Jackknife[c]
Young	24	251.20 (20.53)	250.01 (17.25)	239.94	242.68 (13.67)
Old	22	285.79 (15.18)	281.67 (15.41)	262.12	260.83 (15.61)
t statistic		—	−1.35[e]	—	−0.88[e]

[a] Results taken from Ref. 21. [b] Number of observations. [c] Number in parentheses are standard errors. [d] Standard errors were not computed. [e] No statistically significant difference, $p > 0.05$, between young and old groups based on a two-sample t test.

DISCUSSION

AUC estimation for type A studies is different from what is usually encountered, in that independent observations are made on different subjects at each point in time rather than each subject having its own time–response curve (type B studies). The model-independent approach developed for this situation can be considered as a viable option when there is no strong evidence for choosing a particular model. The flexibility in fitting least-squares spline functions could provide protection against the possibility of systematic bias resulting from the choice of an improper functional form. Simulation results (22) have shown that splines are competitive in situations where they are compared with the correct functional form and yield much better results when compared with an incorrect model specification. This flexibility of spline functions was also evident in the analysis of the illustrative data. For the liver fluke uptake data, the spline approach gave similar results to the more classical one-compartment open model. However, for the blood level of drug data, the paucity of data in the absorption phase of response–time curve would seem to make the spline approach more applicable than that of the one-compartment open model. This property of least-squares splines could prove extremely useful in situations where very complicated nonlinear models seem warranted, but are difficult to fit using the usual nonlinear regression techniques.

Fitting the spline function is somewhat subjective in that the number and position of the knots must be specified. However, this subjective process is also encountered in some pharmacokinetic situations where the number of terms of a multiexponential model must be chosen. With some experience, one should be able to select knots so that the resulting spline will give a good fit to the data. Also, it should be noted that because of the excellent local properties of spline functions, the effects of incorrect knot positioning should not cause difficulties.

Although the AUC estimates and standard errors are similar for the least-squares and jackknife procedures, the jackknife also gives protection against potentially unsatisfied assumptions. The advertised advantages of the jackknife in situations such as this are (*a*) bias reduction, (*b*) validity robustness, and (*c*) robustness against violation in homogeneity of variance. The jackknife is quite versatile and flexible and has been applied to problems in several biological settings, *e.g.*, toxicology (23) and enzyme kinetics (18). For this problem, standard errors, which were not readily obtainable for the model-dependent approach using least-squares theory, were relatively easy to compute using the jackknife.

In this situation, the usual least-squares techniques may be sensitive to the violation of the assumptions of normality and homogeneity of variance required in Eq. 1. The jackknife is an excellent tool for constructing nonparametric confidence intervals, since it is relatively insensitive to the manner in which the random errors are distributed. An added advantage of the jackknife is that one can use the pseudovalues to evaluate group differences, especially in situations involving more complicated experimental designs (18). Additional protection against violations of assumptions can be obtained by employing a transformation in conjunction with the jackknife (4, 16, 18). Since the distribution of the AUC estimates might be adversely affected by asymmetrical residuals (*e.g.*, log normal distribution) resulting from the spline regression, a logarithmic transformation might be required in conjunction with the jackknife. This can be accomplished by using log ($A_{\text{all},i}$) and log (A_{ism}) in Eq. 6 to compute pseudovalues. Such a transformation may also provide protection against variance heterogeneity, although the jackknife is considered robust against such a violation in assumptions (17).

For the above transformation, the spline function would still be fit to the untransformed response data. An alternative that might be necessary if the final concentrations are close to zero is to fit the spline function to a transformed response variable (3). However, the situation is more complicated[2] since the AUC becomes a nonlinear function of the spline parameters. In this case, the weighted jackknife (17) should be employed. Also note that this discussion relates to the within-experimental group analysis. The necessity for transformation for purposes of comparisons between experimental groups is a separate consideration. For instance, in the analysis of the study data, a log transformation was employed in the hope of achieving homogeneity of the within-infestation group variance for the liver fluke data. However, the results were similar to the untransformed case and, thus, were not reported.

The jackknife estimate of variance is known to be slightly inflated in theory (24)[1]. However, this is a minor defect since the standard error estimates from nonlinear regression procedures are often optimistically low (25)[1], and the jackknife precision estimates are closer to reality because they are data based. The results of a study which examined the jackknife estimation of rate constants for multiexponential functions fitted to biochemical data seemed to corroborate this claim (26).

The chief drawback to the widespread use of the jackknife has been concern about computational issues. However, with growing sophistication of computers, software packages, and more efficient jackknife procedures (18, 27), this may no longer be an issue. As noted above, the entire model estimation procedure developed in this paper can be automatically perfomed with a SAS macro (21).

In summary, the model-independent approach gave reasonable AUC estimates and allowed for intergroup comparisons in this study (type A design, two groups studied). Careful application of this procedure should prove to be a valuable technique for type A studies. This method should also be investigated for other parameters that can be computed by model-independent methods.

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[2] As noted by a referee.

Synthesis and Antidiabetic Activity of Some Sulfonylurea Derivatives of 3,5-Disubstituted Pyrazoles

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Abstract □ Two series of 3,5-disubstituted pyrazolesulfonylurea derivatives were prepared and evaluated as hypoglycemic agents. Preliminary biological testing revealed that the new compounds possess potent hypoglycemic activity.

Keyphrases □ 3,5-Disubstituted pyrazolesulfonylurea derivatives—synthesis, potential hypoglycemic agents □ Potential hypoglycemic agents—preparation, antidiabetic activity of 3,5-disubstituted pyrazolesulfonylurea derivatives

Previous work showed that 3,5-dimethylpyrazole and its active metabolite, 5-methylpyrazole-3-carboxylic acid, had potent hypoglycemic activity (1–5). The present study, which is a continuation of previous work (6–11), describes the preparation of derivatives of 3,5-disubstituted pyrazolesulfonylureas and their evaluation as potential hypoglycemic agents.

Derivatives of *p*-[3-ethoxycarbonyl-5-α-phenyl-*p*-chlorostyryl)-1-pyrazolyl]benzenesulfonylurea and *p*-[3 - ethoxycarbonyl-5-(α-phenyl-*p*-methoxystyryl)-1-

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