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Non-linear Regression and Variance Ratio Analysis of Time Based NMR Data

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Biomedical NMR experiments rely frequently on data obtained sequentially over time. A method is presented for analysis of time based NMR data, which allows modelling of continuous and discontinuous functions to observed intensity changes by non-linear regression and which uses variance ratio analysis to compare these models statistically. The method eliminates many of the usual problems in the parametric analysis of experimental values obtained at discrete time points and of comparison of the coefficients of model functions which require unsubstantiated assumptions about the distribution of parameters and ignore internal correlations which may exist between such parameters. The variance ratio method is illustrated for multiple time courses obtained with ^{23}Na NMR of perfused rat kidney undergoing hypoxic perturbation in the presence of different treatments.

INTRODUCTION

Biomedical NMR experiments performed on cells, intact organs or whole animals rely frequently on data obtained sequentially over time. Perturbations such as the induction of hypoxia, ischaemia or the addition of pharmaceutical substances may be made during such experiments. Absolute and relative quantitation of peak intensities within a given experiment represent the first hurdles to be overcome in the biological interpretation of such data. Experimental reproducibility represents an additional hurdle. Finally, the significance of changes induced by a given perturbation must be assessed against the expected biological and spectrometer-induced variability inherent in individual intensity measurements.

Inevitably, the assessment of repeatability of biological experiments requires statistical analysis. Although limited comparisons of the extent of a given perturbation at a given time point by Student's *t*- or other parametric tests are usually valid, such comparisons cannot be meaningfully extended to all time points in a series. Such an extension ignores the contribution of preceding data points to all subsequent intensity measurements. This contribution is important in understanding the effect of a perturbation in a system monitored over time, since the rate of change, its time of onset and the nature of the baseline before the change are important in determining the extent of the final change induced by the perturbation.

One approach is to model the process under observation, to fit a mathematical model to the experimental data sets by regression analysis and to compare data sets by comparing coefficients, such as rate constants. Limitations to this approach include an often arbitrary approach to the selection of a suitable mathematical model, which is usually decided by inspection alone, and assumptions regarding the distribution of the coefficients. Interestingly, such common biological problems have received little attention in standard statistical texts.

A method is presented here for analysis of time based NMR data, which allows modelling of both continuous and discontinuous functions to observed NMR intensity changes and includes a statistical method for comparing models. The same approach is then extended to compare multiple time courses obtained with different treatments in a system undergoing an hypoxic perturbation. The method is based on an approach developed recently for enzyme kinetic analysis.[1] The application to NMR-derived data is illustrated by analysis of changes in total renal ^{23}Na induced by hypoxia in the isolated perfused rat kidney in the course of studies of the mechanisms of hypoxic injury. (M. Cross *et al.*, submitted).

EXPERIMENTAL

Right kidneys from male Wistar rats were perfused at constant pressure at 37°C with recirculating Krebs–Henseleit bicarbonate buffer (KHB) supplemented with 5 mM glucose, 20 natural amino acids and 6.7 g/dL bovine serum albumin and gassed with 95% O_2/5% CO_2 as described previously.[2–4] Perfusion pressure was measured directly within the renal artery using a Statham-type pressure gauge through a polyethylene line contained within the glass cannula used to perfuse the artery. Pressure was held constant by a process controller (model 2703, West Division, Gulton Industries, IL, USA) which regulated the speed of the peristaltic pump (model 501/U, Smith and Nephew, Watson-Marlow, UK). Kidneys were perfused at 90–110 mmHg. Perfusate oxygen tension was monitored in

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Abbreviations used: C_{in}, inulin clearance; DMTU, dimethylthiourea; DMSO, dimethylsulfoxide; *F*, variance ratio; FE_{Na}, fractional sodium excretion; KHB, Krebs–Henseleit bicarbonate buffer; RSS, residual sum of squares; RSS_c, residual sum of squares (complete); RSS_r, residual sum of squares (reduced); $U_{Na}V$, absolute sodium excretion; υ, degrees of freedom; σ^2, variance; σ^2_{pe}, variance due to pure error; σ^2_{lf}, variance due to lack of fit.

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the arterial line by a Clark-type oxygen electrode, attached to a blood gas analyser (PHM 72, Radiometer, Copenhagen, Denmark). After 60 min normoxic perfusion, hypoxia was induced by switching the perfusate gas mixture to 95% N_2/5% CO_2. Perfusion was continued for a further 30 min. In all kidneys, urine samples were collected under oil in tared containers at 5 min intervals and used to measure inulin clearance (C_{in}) and sodium excretion (absolute, $U_{Na}V$, and fractional, FE_{Na}). Only kidneys with an initial (30–45 min) C_{in} of >0.5 mL/min and with sufficient ^{23}Na spectra recorded during this period for baseline evaluation were accepted for analysis.

Data from two separate sets of experiments have been used to illustrate the time series analysis of the effects of hypoxia under different experimental conditions. In the first set, the kidney perfusion medium was supplemented by increasing the concentration of two amino acids such that in group 1, the glycine and alanine concentrations were 5 mM each (increased from 1.18 and 2.30 mM, respectively), whereas in group 2, the serine and glutamine concentrations were 5 mM each (increased from 1.07 and 3.26 mM, respectively). The perfusion conditions and media, including measured osmolality, were otherwise identical. In the second set, five groups of kidneys perfused with normal concentrations of amino acids were compared: (a) hypoxia, but with a free radical scavenger, 15 mM dimethylthiourea (DMTU), added to the recirculating perfusate 15 min before inducing hypoxia; (b) hypoxia as in (a) but with 1.5 mM DMTU; (c) as for (a) but with a different free radical scavenger, 15 mM dimethylsulfoxide (DMSO), added to the perfusate; (d) control hypoxia; and (e) control normoxia. Normal saline was added to the control kidneys in the same volume and at the same time as either DMTU or DMSO was added to groups (a), (b) or (c), at 45 min.

Kidneys were perfused within a CXP 300 vertical bore magnet in a custom-built NMR probe. The kidneys were freely suspended completely within a 22 mm diameter Helmholtz coil tuned to ^{23}Na (79.36 MHz). This allowed gravity drainage of both the venous effluent and urine. ^{23}Na spectra were averaged over 2 min using a pulse length of ~ 80 μs, a sweep width of 15 000 Hz and a repetition interval of 0.56 or 1.00 s. A reference capillary, containing saturated Na-Dy(PPP), was located within one loop of the Helmholtz pair on the outside of the plastic shell housing the suspended kidney, over which the coil was wound.

The intensity of each renal ^{23}Na peak was measured by integration and calibrated against the intensity of the reference ^{23}Na peak at high field, which did not change following the induction of hypoxia. To facilitate comparisons between experiments, the total renal ^{23}Na intensity was normalized to the average intensity of the initial five ^{23}Na spectra collected during normoxic perfusion. The normalized total renal ^{23}Na intensity was averaged at each time point within each group. The data from each group were then fitted to a discontinuous function by non-linear regression (see below). This analysis gave estimates and standard errors for each fitted parameter plus a residual sum of squares, representing the weighted square of the difference between the fitted and the experimental values, summed over all data points. The significance of differences between the parameters for each group was analysed from the variance ratio (F test) calculated from the residual sum of squares, as described in detail below.

Variance ratio (F) test

When a mathematical model is fitted to an experimental data set by regression analysis, a residual sum of squares (RSS_c) is calculated with v_c degrees of freedom given by the number of data points minus the number of fitted parameters. There may exist a second (or 'reduced') model, a subset of the first (or 'complete') model, with one or more parameters treated as fixed constants or omitted entirely. If this second model is fitted to the same data set, a new and greater value of the sum of squares, RSS_r, with a larger number of degrees of freedom, v_r, will be calculated. The question is whether the increase in the sum of squares associated with the reduced model can be ascribed purely to statistical fluctuation, given that the complete model fits the data well.

This is addressed[5,6] by comparing the variance due to lack of fit (σ^2_{lf}) in the reduced model with the variance due to pure error (σ^2_{pe}), the latter being assumed to be approximated by the variance of the fit by the complete model. The value of F is then calculated from the ratio of these variances:

$$\sigma^2_{lf} = (RSS_r - RSS_c)/(v_r - v_c)$$
$$\sigma^2_{pe} = RSS_c/v_c$$
$$F = \sigma^2_{lf}/\sigma^2_{pe} \quad (1)$$

If the calculated value of F is larger than that found for probability p with $(v_r - v_c, v_c)$ degrees of freedom in the F statistical tables, then there is less than p chance that the increased sum of squares is due to random fluctuations. That is, there is a significant lack of fit in the reduced model, which is therefore rejected as providing an unsatisfactory fit.

An extension of this procedure is to test whether two or more data sets fitted by the same model can be satisfied when keeping one or more parameter values in common.[1] Suppose the model is given by $y = f(a, b, c, x)$, where a, b and c are three fitted parameters and x is an independent variable. If there are two data sets (with N_1 and N_2 data points), each is first fitted to this model to give RSS_1 and RSS_2, which are then added to give RSS_c. The two data sets are then merged into a single larger data set, with the source of each data point identified by an indicator variable (i). We may then fit with a common value, for example, for parameter (b) using the modified five-parameter model $y = f(a_i, b, c_i, x, i)$ to give a value for RSS_r. Using $v_c = (N_1 - 3) + (N_2 - 3)$ and $v_r = (N_1 + N_2)$ – five degrees of freedom, the variance ratio is calculated and interpreted as before. The procedure may be extended to combine any number of data sets and to specify any combination of common and independent parameters.

RESULTS AND DISCUSSION

Mathematical model

When the rat kidney is subjected to hypoxic perfusion, there is an immediate increase in the total renal ^{23}Na intensity (Fig. 1). This increase has been attributed

solely to the intracellular compartment.[7] We wished to describe this process in terms of a mathematical model in order to use this model to investigate the effects of various pretreatments of the kidneys.

Since control experiments indicate that the preparation is stable over 90 min, we concluded that prior to inducing hypoxia at 60 min, the ^{23}Na intensity is constant. Starting from 60 min there is an exponential increase in ^{23}Na intensity. Mathematically this can be described as:

$$y = \begin{cases} y_0 & t \leq 60 \\ y_0 + A[1 - e^{-k(t-60)}] & t > 60 \end{cases} \quad (2)$$

where y_0 is the baseline intensity, A the asymptotic intensity change following hypoxia and k is the first-order rate constant for the increase in intensity.

Regression analysis

DNRP53[8] is a general non-linear regression computer program written in BASIC. It will readily fit equations containing a discontinuity such as Eqn (2) to experimental data. Using this program, the data in Fig. 1 were fitted to Eqn (2) and yielded the following values for the parameters: $y_0 = 1.0016 \pm 0.0017$ ($\pm$SE), $A = 0.130 \pm 0.006$ and $k = 0.161 \pm 0.025$/min. This fitted line can be seen to provide an excellent description of the experimental data (Fig. 1). We also fitted a double exponential function (with four parameters) to the data and obtained a fit which was both visually and statistically indistinguishable from the fit to a single exponential. A similar result was obtained by fitting the function in which the rate of change in intensity is a saturable process (the integrated Michaelis–Menten equation—with five parameters). Thus, a single exponential increase in intensity following anoxia (with three parameters) was taken to be a sufficient description of the data.

Although Eqn (2) fits the data well, it should be noted that the values of the fitted parameters may not be particularly reliable, despite their relatively small SEs. This is because A and k are correlated with one another. This means that within limits, a very similar exponential curve can be obtained for any rate constant provided an appropriate amplitude is selected. For example, decreasing k by one-third to 0.107/min will give nearly as good a fit if A is increased from 0.123 to 0.147 (Fig. 1, dotted line). This emphasizes the difficulty of comparing several curves and suggests that comparisons based on the values and SEs of fitted parameters could be quite misleading.

The error quoted for any parameter derived by regression analysis here is given as the SE. This represents a measure of the uncertainty of the derived parameter and is the only relevant error associated with parameters derived by a regression analysis of a single data set. This should be contrasted with the SD, which describes the dispersion of a series of measurements.

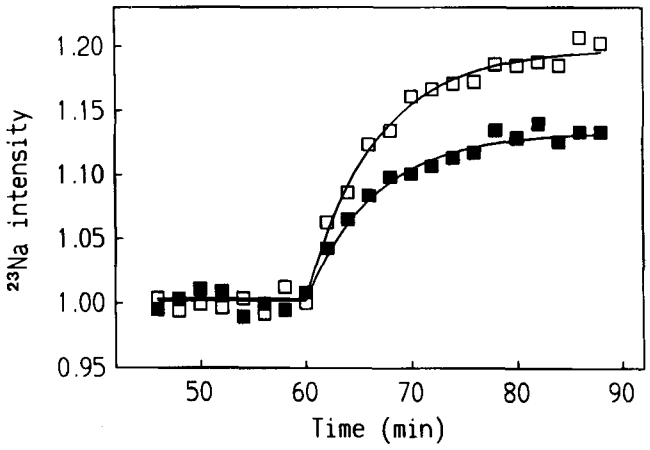

Figure 2. Averaged normalized total renal ^{23}Na intensity measurements from perfused rat kidney. The closed squares show the same data as Fig. 1. The open squares illustrate perfusate supplemented with serine and glutamine to 5 mM each (also $n = 4$). Other conditions were identical for both sets of kidneys. The fitted lines illustrate non-linear regression fits of Eqn (2) to the data with final parameters as shown in the bottom section of Table 1.

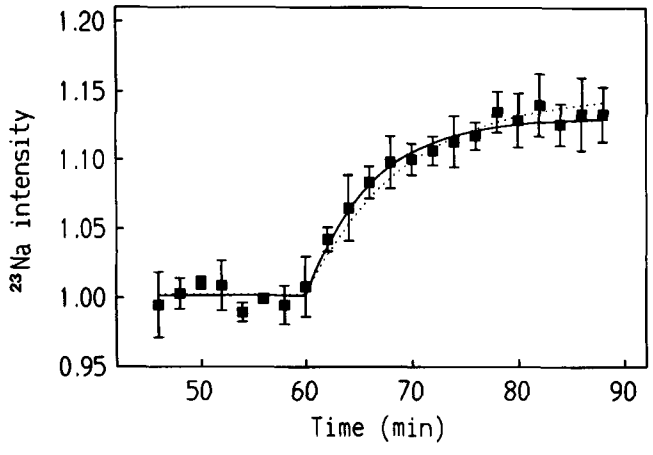

Figure 1. Averaged normalized total renal ^{23}Na intensity measurements ($n = 4$) from perfused rat kidney obtained at 7.2 T for perfusate supplemented with glycine and alanine to 5 mM each. Hypoxia was induced at 60 min while perfusion continued. The solid line shows the fit of Eqn (2) obtained using the DNRP53 non-linear regression program. The dotted line illustrates the effect of arbitrarily decreasing k by one-third with a compensating increase in A. Error bars represent $\pm$1SD.

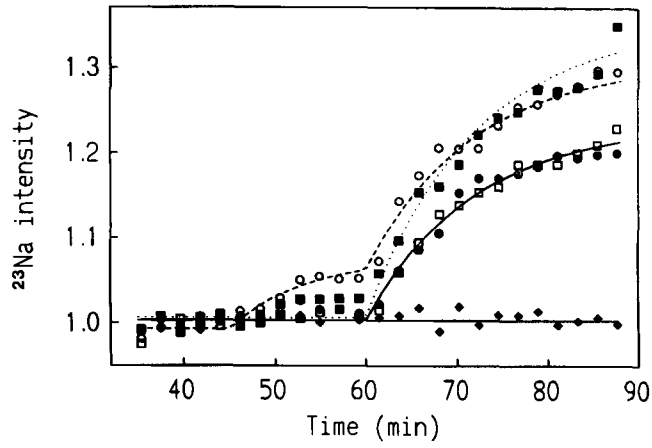

Figure 3. Effect of adding free radical scavengers to perfusion medium prior to the induction of hypoxia. The fitted lines represent Eqn (2) except for curve (a), where Eqn (3) has been used. The lines have been fitted using the parameters in row 11 in Table 2. Treatment key: (a) ----○, 15 mM DMTU; (b) ——●, 1.5 mM DMTU; (c) ——□, 15 mM DMSO; (d) · · ·■, control with hypoxia; (e) ——◆, normoxic control.

Thus, a set of replicate measurements such as the mean ^{23}Na intensity data illustrated in Fig. 1 will have an SD associated with each mean and the error bars shown represent ±1 SD. For simplicity, the error bars (SD) have not been shown at all in Figs 2 and 3. In contrast, parameters, such as k in Eqn (2), are quoted with an SE. The only situation where an SD of a fitted parameter could be obtained is if there were several identical experiments which were each analysed separately. From the several values of each fitted parameter, either or both an SD and an SE could be calcualted, depending on the purpose. Even in this situation, only the SE is informative, if the concern is knowing the deviation of the parameter from the 'true' value. In this latter case, an SD will still indicate the dispersion of the parameter values within the data set.

Pretreatment with supplementary amino acids

When the kidney perfusion medium is supplemented with additional glycine and alanine, there is an apparent reduction in the extent of increase in ^{23}Na intensity following hypoxia compared with kidneys perfused with supplementary serine and glutamine (Fig. 2). We wished to assess what the nature of this difference was (rate or amplitude) and whether the apparent differences were statistically significant. Individual fits of Eqn (2) to each data set using DNRP53 yielded the parameters listed in the upper part of Table 1. These demonstrate that the two curves have similar rate constants, but differnt amplitudes.

The significance of these parameter differences was assessed in the following way. A combined data set was created wherein the source of each data point remained specified by the use of an indicator variable. Equation (2) was then fitted to the combined data set with a single common rate constant but allowing each curve to have an individual baseline and amplitude. For comparison both curves were also fitted to the combined data with a common amplitude but individual baselines and rate constants. Parameters obtained by both fits to the combined data are shown in the remainder of Table 1, which also shows the residual sum of squares (RSS) obtained with each fitting procedure.

Table 1. Parameter estimates and statistical analysis following fitting Eqn (2) to the data illustrated in Fig. 2 (22 points per curve). Errors represent SEs of the fitted parameters

	Baseline	Amplitude	Rate constant	Sum of squares
Individual fits				
Gly + Ala	1.0016	0.1296	0.1608	10.012
	±0.0017	±0.0064	±0.0247	
Ser + Gln	1.0031	0.1976	0.1442	2.770
	±0.0008	±0.0046	±0.0097	
			Total sum of squares = 12.782	
Combined fits: common amplitude				
Gly + Ala	1.0022		0.0689	
	±0.0019		±0.0667	
		0.1842		26.623
		±0.0076		
Ser + Gln	1.0036		0.1774	
	±0.0017		±0.0272	
$F = (26.623 - 12.782)/(12.782/38) = 41.15$ ($p < 0.001$)				
Combined fits: common rate constant				
Gly + Ala	1.0017	0.1318		
	±0.0013	±0.0043		
			0.1505	12.923
			±0.0119	
Ser + Gln	1.0031	0.1950		
	±0.0012	±0.0053		
$F = (12.923 - 12.782)/(12.782/38) = 0.42$ ($p > 0.2$)				

First consider the fit to a common amplitude (Table 1). The RSS is 26.623, somewhat larger than the total sum of squares, 12.782, obtained by fitting each curve separately. However, some increase in the sum of squares is to be expected, as the combined fit uses five parameters while the individual fits use six, resulting in some additional flexibility. The question is whether this increase in the sum of squares is statistically significant. The variance ratio gave an F value of 41.15 for (1, 38) degrees of freedom, corresponding to $p < 0.001$. In other words there is <0.1% chance that this inflated sum of squares resulted from statistical fluctuation and we can safely conclude that the fit to a common amplitude is not a satisfactory description of the data.

The fit with a common rate constant is shown in the lower section of Table 1 from which $p > 0.2$. There is thus a >20% probability that the small elevation in the sum of squares has arisen by chance, so that both curves seem to be described by the same rate constant. The curves shown in Fig. 2 illustrate the fit with identical rate constants. The good agreement between the curves and the data provides further evidence that this fit represents a good model.

Effect of free radical scavengers

The effect of adding either the free radical scavenger DMTU or DMSO to the perfusion medium prior to the induction of hypoxia is shown in Fig. 3. This illustrates a further complication, since the experimental conditions change twice during the experiment for curves (a)–(c): at 45 min when the scavenger is added and again at 60 min when hypoxia is superimposed. In analysing these data the first question is whether the scavenger itself alters ^{23}Na intensity. This was determined by fitting an expanded form of Eqn (2) to the data, namely:

$$y = \begin{cases} y_0 & t \leq 45 \\ y_0 + A_1[1 - e^{-k_1(t-45)}] & 45 < t \leq 60 \\ y_0 + A_1[1 - e^{-k_1(t-45)}] + A_2[1 - e^{-k_2(t-60)}] & t > 60 \end{cases} \quad (3)$$

A fit of Eqns (2) and (3) to the data for 15 mM DMTU gave RSS of 38.534 and 6.580, respectively. Comparison using the F test gave $F = 48.56$ for (2, 20) degrees of freedom corresponding to $p < 0.001$, indicating that inclusion of the additional relaxation event at 45 min is necessary to provide an adequate description of the data. For 1.5 mM DMTU, the values were $F = 3.43$ and $p > 0.05$, not significant. Similarly for 15 mM DMSO, $F = 0.22$ and $p > 0.2$. Thus, the introduction of the scavengers 1.5 mM DMTU and 15 mM DMSO at 45 min do not perturb the system whereas 15 mM DMTU does.

^{23}Na intensity curves from the three treatment groups

Table 2. Statistical analysis of the data illustrated in Fig. 3 (25 points per curve). The letters (a)–(e) represent the groups described in the Experimental section (see also legend to Fig. 3). Equation (3) was fitted to curve (a), while Eqn (2) was fitted to curves (b)–(d). An entry such as 'b, a+c+d' means that the corresponding parameter was forced to have a common value for groups (a), (c) and (d) while the fitted curve for group (b) was not constrained in this manner. The *F* values were calculated for each of the reduced models (rows 2–11) taking row 1 as the complete model. See text for a detailed description.

	Baseline	Amplitude	Rate constant	Sum of squares	*v*	*F*	*p*
1	a, b, c, d	a, b, c, d	a, b, c, d	37.372	86	–	–
2	a+b+c+d	a+b+c+d	a+b+c+d	53.091	95	4.02	<0.001
3	a, b, c, d	a+b+c+d	a+b+c+d	45.836	92	3.25	<0.01
4	a, b, c, d	a, b, c, d	a+b+c+d	37.748	89	0.29	>0.2
5	a, b, c, d	a+b+c+d	a, b, c, d	38.014	89	0.49	>0.2
6	a, b, c, d	a, b+c+d	a, b+c+d	45.781	90	4.83	<0.01
7	a, b, c, d	b, a+c+d	b, a+c+d	45.185	90	4.49	<0.01
8	a, b, c, d	c, a+b+s	c, a+b+d	44.225	90	3.94	<0.01
9	a, b, c, d	d, a+b+c	d, a+b+c	37.573	90	0.12	>0.2
10	a, b, c, d	a+b+c+d	d, a+b+c	38.137	91	0.35	>0.2
11	a, b, c, d	d, a+b+c	a+b+c+d	37.843	91	0.22	>0.2

and the control with hypoxia alone [curve (d) in Fig. 3] were then combined and analysed systematically (Table 2). An exponential component commencing at 60 min was included in the analysis of all four curves. An additional exponential component at 45 min was included for the 15 mM DMTU curve alone [curve (a) in Fig. 3]. The first row of Table 2 corresponds to analysing each curve separately so that each experimental group has a separate baseline, amplitude and rate constant. The second row represents the opposite extreme with each curve having the same baseline, amplitude and rate constant; clearly this does not give a satisfactory fit ($p<0.001$).

As noted above (Experimental), the mean ^{23}Na intensity data for each group was separately normalized to a baseline value of unity using spectra collected during the 35–45 min period. While it would be expected that each treatment would have a similar baseline after 45 min, we did not wish to force all curves to adhere to the same baseline. For this reason, all the remaining analyses in Table 2 allow each curve to follow an individual baseline. Even with this added flexibility, forcing each curve to have the same amplitude and rate constant (row 3) is not satisfactory ($p<0.01$).

In rows 4 and 5, the curves are forced to have either a common rate constant or amplitude, respectively; in each case the *F* value is low and $p>0.2$. Thus, although it was not possible to describe all four curves by the same rate constant and amplitude, a satisfactory fit can be obtained by selecting either one of these as a common parameter. This result reflects the correlation between the fitted values of the rate constant and amplitude as discussed earlier.

We then went on to pool the curves in four different ways, each time using a separate rate constant and amplitude for one curve and a common rate constant and amplitude for the remaining three; these are shown in rows 6–9 of Table 2. Only row 9 was satisfactory, where curve (d), the hypoxic control, was analysed separately. Thus it appears that each of the scavengers has a similar effect, which is significantly different from that of the control curve. While the hypoxic control may differ in both the amplitude and the rate constant, we tested the possibility that the difference could be ascribed either to a difference in rate constant (row 10) or amplitude (row 11). Each fit was satisfactory and since the latter gave a slightly smaller RSS value, this was accepted as giving the best description of the data. The adequacy of fit using the parameters from row 11 is shown by the fitted lines in Fig. 3.

Summarizing, the effect of pretreatment with any of the three radical scavengers tested was to alter the maximum amplitude of the change in ^{23}Na intensity following the induction of hypoxia without affecting the rate constant of the change. Additionally, pretreatment with 15 mM DMTU induced an independent increase in ^{23}Na intensity prior to the induction of hypoxia.

CONCLUSION

The methods illustrated allow a useful approach to modelling and statistical analysis of time series data typically acquired in *in vivo* and *in vitro* biomedical NMR studies, namely, multiple treatments prior to or after an applied system perturbation, resulting in discontinuous functions. An alternative approach could have been to fit the same model to each experimental data set of sodium intensities and then average the parameter values within each group of experiments to obtain a parameter mean and SD. Comparing groups with different treatments by comparing coefficients from parametric analysis could have then been performed. This requires assumptions regarding the distribution of the coefficients when usually only a small number of experiments are available. Apart from the above-mentioned risks of relying on correlated parameters, this would have made the analysis, required to compare the multiple subsets shown in Fig. 3 and Table 2, very complex. By contrast, the analysis presented here was relatively simple and intuitive using the method outlined. Furthermore the variance ratio method provides an objective means of assessing the suitability of the model itself.

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