Table I—TLC Examination of Basic Residue from Urine of Volunteers who had Ingested Cathinone[a]

Urine Sample	Collection Time, hr[b]	Substance Detected Cathinone	Substance Detected *d*-norpseudoephedrine
1	0 (control)	none	none
2	0–4	++[c]	++
3	4–8	++	++
4	8–12	+[d]	++
5	12–15	0[e]	+
6	15–24	0	+

[a] Two unidentified substances in trace amount, associated with cathinone were observed in samples 2, 3, 4 and 5. The R_f values of unidentified substances in solvent system *A* were 0.71 and 0.85. [b] After ingestion of cathinone. [c] ++ = Easily detected. [d] + = Trace amount. [e] 0 = not detected.

mental and control urine was approximately the same. Control experiments had shown recovery of >90% under the standardized conditions.

RESULTS

Both cathinone and *d*-norpseudoephedrine separated well in solvent systems *A* and *B*, the R_f values being as follows: *A*-cathinone 0.55, *d*-norpseudoephedrine 0.42; *B*-cathinone 0.38, *d*-norpseudoephedrine 0.47. Results of TLC are summarized in Table I. Results of GLC were in agreement with those obtained with TLC. With GLC only one *d*-norpseudoephedrine peak was observed at 165° (retention time 7.5 min) while the major cathinone peak was observed at 151° (retention time 5.1 min). Another peak associated with cathinone appeared at 145° (retention time 3.7 min). The results were fairly reproducible, and it was possible to calculate the amount of cathinone and *d*-norpseudoephedrine excreted in the urine. There was no significant variation in the excretion pattern of either cathinone or *d*-norpseudoephedrine in the four volunteers even when the experiment was repeated after 4 weeks. The amount of cathinone and *d*-norpseudoephedrine recovered from the urine of each of the volunteers agreed to within 20% and the results are summarized in Table II.

DISCUSSION

Results obtained in the present study show that some of the cathinone ingested by humans is metabolized to *d*-norpseudoephedrine and possibly two other unidentified metabolites. Metabolism of cathinone to *d*-norpseudoephedrine involves reduction of a ketone group to alcohol, a fairly common metabolic pathway in humans, catalyzed by liver microsomal enzymes. In several drugs (*e.g.*, cortisone and warfarin) reduction of a ketone or aldehyde to an alcohol is often associated with significant change in potency. Usually both the parent drug and the metabolite will contribute to the biological activity, the net result often being determined by such pharmacokinetic parameters as the rate of elimination. The finding that little, if any cathinone is excreted in human urine after 15 hr, may be important in forensic toxicology since *d*-norpseudoephedrine, also present in *C. edulis*, is known to be excreted over a much longer period.

Table II—Amount of Cathinone and *d*-Norpseudoephedrine Excreted in Human Urine (Result of GLC)

Urine Sample(s)	Cathinone Recovered, mg	*d*-norpseudoephedrine Recovered, mg
2 (0–4 hr after ingestion of cathinone)	0.72 ± 0.12 (4.4% of amount ingested)	3.2 ± 0.3 (equivalent to 20% ingested cathinone)
3, 4, 5, 6 (4–24 hr after ingestion of cathinone)	1.04 ± 0.08 (6.5% of amount ingested)	9.9 ± 0.6 (equivalent to 61.9% ingested cathinone)
2, 3, 4, 5, 6 (0–24 hr)	1.765 (11% of amount ingested)	13.12 (equivalent to 81.9% ingested cathinone)

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Nonparametric Pharmacokinetic Calculations: One-Compartment Open Model

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Abstract □ A nonparametric method suitable for estimation of parameters in nonlinear problems was developed for one-compartment pharmacokinetic data. The method was tested by running 500 simulations with various types of error and comparing the results with a standard nonlinear regression computation. The nonparametric method was superior to nonlinear regression techniques if the assumptions for the error structure of the regression were not true.

Keyphrases □ One-compartment pharmacokinetic model—nonparametric method, comparison with standard nonlinear regression procedure, estimation of parameter

Calculating the parameters of a pharmacokinetic model containing more than one exponential term requires the use of either the method of residuals (sometimes referred to as stripping or feathering) or one of various nonlinear regression techniques. As they have been applied classically, both general techniques have proven somewhat

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Table I—Mean and Coefficient of Variation of Parameter Estimates from 500 Simulations Using a One-Compartment Pharmacokinetic Model with Data of Constant Variance and Containing One Randomly Selected Outlier of Increasing Magnitude [a]

Outlier Magnitude	Marquardt Nonlinear Regression [b]			Nonparametric [b]		
	k_a	K	FD/V	k_a	K	FD/V
1	2.00	0.200	3.00	2.00	0.200	3.00
	(2.67)	(2.04)	(0.99)	(3.04)	(2.55)	(1.11)
2	2.00	0.200	3.00	2.00	0.200	3.00
	(3.13)	(2.39)	(1.17)	(3.43)	(2.82)	(1.24)
4	2.00	0.200	3.00	1.99	0.200	3.01
	(4.57)	(3.58)	(1.74)	(4.17)	(3.00)	(1.37)
6	2.00	0.200	3.00	1.99	0.201	3.01
	(6.33)	(4.79)	(2.40)	(4.66)	(3.02)	(1.46)
8	2.00	0.201	3.01	1.98	0.201	3.01
	(8.26)	(6.21)	(3.11)	(5.31)	(3.05)	(1.48)

[a] Variance = 0.2, k_a = 2, K = 0.2, FD/V = 3.0, the outlier randomly selected from one of the seven points with a variance from 1 × 0.2 (no outlier) to 8 × 0.2. The concentrations were computed at t = 0.5, 1.0, 1.5, 2.0, 4.0, 8.0, and 12 hr. [b] Numbers in parentheses are coefficients of variation.

unsatisfactory. The method of residuals lacks objectivity and may be theoretically biased, and the nonlinear regression techniques may fail to converge under certain circumstances (1).

Recently, several investigators have examined the use of nonparametric methods and have found them to be superior to parametric regression techniques in the analysis of enzyme kinetic (2) and receptor-binding data (3). Indeed, some statisticians are beginning to question the general application of conventional regression procedures. Endrenyi and Tang (4) found that a nonparametric method was superior to conventional linear regression techniques when applied to the analysis of a single exponential kinetic model. Koup (5) used a similar technique, in combination with the method of residuals, to solve for the parameters in a biexponential model using a nonparametric method to determine the best fit for the terminal portion of the curve. The second linear component was determined by the method of residuals and the appropriate parameters ascertained. It was found that, for his system, the nonparametric results were markedly superior for two constants and slightly inferior for the remaining two constants.

The purpose of this paper is to formulate a nonparametric method for the solution of nonlinear equations. The method is tested by its performance with a conventional nonlinear regression technique, using data with different error structures and in the presence of outliers of increasing importance.

BACKGROUND

In 1974, Eisenthal and Cornish-Bowden (6) developed a nonparametric method for processing enzymatic data. The method, frequently referred to as a "direct linear plot," was originally devised as a graphical procedure for estimating enzyme kinetic parameters. Basically it involves plotting all possible sets of data in parameter space. This plot leads to a series of line intersections, where each intersecting point represents a different estimate of the desired parameters. The median value of this series of parameters is then selected as the best estimate. Since medians rather than means are used, the method is extremely resistant to the effects of outliers, providing at least the majority of points are good. In searching the literature, no reference applying this nonparametric technique to determine the parameters of nonlinear equations was found.

One approach is to divide the experimental data into sets, which allows the determination of the desired parameters and the estimation of the parameter values for each set. The selected nonparametric technique is then applied to the resultant list of estimates. The most complex problem is determining the parameter estimates for each set. This can be accomplished by using numerical solutions to solve the nonlinear equations. Application of this technique to the one-compartment, first-order absorption pharmacokinetic model follows.

THEORETICAL

The one-compartment, first-order absorption model in pharmacokinetics is described by the general equation:

$$C = \frac{FD}{V}\frac{k_a}{k_a - K}(e^{-Kt} - e^{-k_a t}) \qquad \text{(Eq. 1)}$$

where C corresponds to the plasma drug concentration at time t, F is the fraction of dose absorbed, D is the dose, V is the volume of distribution, and k_a and K are the absorption and elimination rate constants, respectively. If FD/V is treated as a single value, then the equation can be solved once we know the three parameters FD/V, K and k_a. The determination of three parameters requires the solution of three simultaneous equations of the form:

$$C_1 = \frac{FD}{V}\frac{k_a}{k_a - K}(e^{-Kt_1} - e^{-k_a t_1}) \qquad \text{(Eq. 2)}$$

$$C_2 = \frac{FD}{V}\frac{k_a}{k_a - K}(e^{-Kt_2} - e^{-k_a t_2}) \qquad \text{(Eq. 3)}$$

$$C_3 = \frac{FD}{V}\frac{k_a}{k_a - K}(e^{-Kt_3} - e^{-k_a t_3}) \qquad \text{(Eq. 4)}$$

Equations 2–4 are generated by substituting the values for three data points (time and concentation) into the general equation (Eq. 1). Unfortunately, the nonlinear nature of these equations makes them difficult to solve algebraically. To simplify the problem, ratios of the above equations were taken to give Eqs. 5 and 6:

$$\frac{C_1}{C_2} = \frac{e^{-Kt_1} - e^{-k_a t_1}}{e^{-Kt_2} - e^{-k_a t_2}} = R_1 \qquad \text{(Eq. 5)}$$

$$\frac{C_2}{C_3} = \frac{e^{-Kt_2} - e^{-k_a t_2}}{e^{-Kt_3} - e^{-k_a t_3}} = R_2 \qquad \text{(Eq. 6)}$$

This step eliminates FD/V from the equations, and yields two equations and two unknowns which can be solved using the Newton-Raphson method (7) for approximating the roots of simultaneous equations. This technique uses expressions in the form of those shown in Eqs. 7 and 8:

$$X = X_0 - \frac{\frac{F\partial G}{\partial Y} - \frac{G\partial F}{\partial Y}}{\frac{\partial G}{\partial Y}\frac{\partial F}{\partial X} - \frac{\partial F}{\partial Y}\frac{\partial G}{\partial X}} \qquad \text{(Eq. 7)}$$

$$Y = Y_0 + \frac{\frac{F\partial G}{\partial X} - \frac{G\partial F}{\partial Y}}{\frac{\partial G}{\partial Y}\frac{\partial F}{\partial X} - \frac{\partial F}{\partial Y}\frac{\partial G}{\partial X}} \qquad \text{(Eq. 8)}$$

where X is unknown 1, *i.e.*, K_1; Y is unknown 2, *i.e.*, k_{a_1}, and F and G are Eqs. 5 and 6. Once the appropriate partial derivatives are taken then the parameters K and k_a are estimated by iteration until they do not change significantly. The third parameter, FD/V, is then determined by substituting the estimated values of K and k_a into Eq. 9.

$$FD/V = \frac{C(k_a - K)}{k_a(e^{-Kt} - e^{-k_a t})} \qquad \text{(Eq. 9)}$$

The above process gives one set of parameter estimates corresponding to the use of one set of three data points. Another combination of three

Table II—Mean and Coefficient of Variation of Parameter Estimates from 500 Simulations Using a One-Compartment Pharmacokinetic Model with Data of Constant Coefficient of Variation and Containing One Randomly Selected Outlier of Increasing Magnitude[a]

Outlier Magnitude	Marquardt Nonlinear Regression[b]			Nonparametric[b]		
	k_a	K	FD/V	k_a	K	FD/V
1	2.03	0.201	3.01	1.918	0.204	3.06
	(12.30)	(5.74)	(4.51)	(11.76)	(3.77)	(4.42)
2	2.03	0.201	3.02	1.90	0.205	3.08
	(14.59)	(6.87)	(5.41)	(13.23)	(4.49)	(5.31)
4	2.06	0.203	3.03	1.86	0.206	3.11
	(26.49)	(10.32)	(7.90)	(15.74)	(5.68)	(5.74)
6	2.52	0.206	3.05	1.85	0.208	3.12
	(191.78)	(17.33)	(10.86)	(17.77)	(6.61)	(6.09)
8	81.67	0.206	3.06	1.84	0.208	3.14
	(2116.61)	(17.06)	(12.38)	(17.36)	(6.81)	(7.35)

[a] Coefficient of variation = 5%, $k_a = 2$, $K = 0.2$, $FD/V = 3.0$, the outlier randomly selected from one of the seven points with a coefficient of variation from $1 \times 0.05 \times C$ (no outlier) to $8 \times 0.05 \times C$. The concentrations were computed at $t = 0.5$, 1.0, 1.5, 2.0, 4.0, 8.0, and 12 hr. [b] Numbers in parentheses are coefficients of variation.

data points is selected and the procedure repeated to give a new set of parameter estimates. The process is again repeated until all possible combinations of data have been used. In the present paper, seven data points (seven time and concentration combinations) were used. This gave a list of 35 estimates (all combinations of seven items taken three at a time). The median value from each list was then selected as the best estimate for that particular parameter. If desired, confidence limits can be obtained by the method of Cornish-Bowden *et al.* (8).

In their comparison of methods of analyzing enzyme kinetic data, Atkins and Nimmo (2) emphasize the importance of thoroughly testing new methods by simulation before applying them in practice. To test the present method, 500 simulations were performed as described in the *Experimental* section.

EXPERIMENTAL

To provide an adequate test for the method, two computer programs[1] were formulated to generate data and determine parameters. Both programs generated data by taking assumed constants ($k_a = 2.00$, $FD/V = 3.00$ and $K = 0.200$), calculating the concentration seven times ($t = 0.5$, 1.0, 1.5, 2.0, 4.0, 8.0, and 12.0), and adding randomly generated, normally distributed error with a mean of 0 and a variance of one times either a percentage (5%) of the generated concentration (constant coefficient of variation) or a constant (0.02) (constant variance), depending on the error structure desired. Both programs generated the same set of data points. Outliers were produced by randomly selecting one of the times and multiplying the error added to the term by a constant (1, 2, 4, 6, or 8). The outlier concentration thus generated had a larger error than the rest of the points. One program analyzed the data by a standard nonlinear regression procedure (9), and the other program utilized the proposed nonparametric method. Five hundred simulations were carried out and the average of each parameter was computed along with the coefficient of variation and other summary statistics.

RESULTS AND DISCUSSION

The use of nonweighted, nonlinear regression assumes a constant variance and, as expected in the nonlinear regression technique, performed well with this error structure when no outliers or only a small outlier was present (Table I). As the size of the outlier was increased, the nonparametric method demonstrated its superiority by showing a much smaller coefficient of variation. The presence of one outlier with a variance eight times the variance of the other points gave coefficients of variation for the nonlinear regression twice the corresponding nonparametric coefficients of variation. Neither computational procedure demonstrated appreciable bias.

When an error structure with a constant coefficient of variation was analyzed (Table II) (which violates the assumptions of nonweighted, nonlinear regression), the nonparametric method was slightly superior when no outliers were present, and markedly superior when outliers reached moderate size with regard to coefficients of variation. Although a weighted nonlinear regression would have improved the performance of the nonlinear regression technique, the proper use of weighting is nearly impossible in practice because of lack of knowledge of the appropriate weights. Consequently, the superiority of the nonparametric method is clearly demonstrated. The nonparametric method demonstrated a slight bias.

The nonparametric method is not a panacea for all the ills of pharmacokinetic data processing because of two concerns. Since all possible sets of three data points are utilized in the calculations, a problem exists if these data points do not contain the proper information. For example, the later points in a collection do not contain information concerning k_a if the data are collected after the absorption phase is essentially complete. Consequently, k_a values computed from the later data points will be essentially meaningless and will probably be represented at the extremes of the distribution. Should a sufficient number of these occur, the median may be affected. A second deficiency of the nonparametric method arises from data points too close together. This problem is similar to determining the slope of a line using two points close together. The slope in the case of the line, or the parameter estimates in the case of the nonparametric method, will be determined more by error than by the location of the points. For our simulation, we chose a conventional experimental design, with points selected at short intervals at the onset to ensure a reasonable determination of k_a, and the time period extending over sufficient time to ensure adequate determination of K (roughly 3.5 half-lives). While this experimental design is not optimum for either nonlinear regression or nonparametric determinations, it approximates data normally used for the determination of nonparametric parameters, without straining either computational technology.

Occasionally the nonparametric method will not converge because of either excessive error in the points or too little information for calculating one of the parameters. In our computer program, if the estimated parameter is 100 or 0.01 times the original estimate, the iteration is stopped and the values of 0.01 times or 100 times the original estimate are assigned. This places these values at the extremes (where they would normally be), and the median is relatively unaffected. The Newton–Raphson method is unstable if the estimate is not close to the true value, so the above safeguard prevents instability of the computer program during the simulations. In actual practice, although these results would produce outliers and hence not affect the results, a different algorithm might be beneficial.

In conclusion, the utility of a nonparametric technique for the determination of parameters of nonlinear equations is demonstrated. As anticipated, the nonparametric technique outperforms conventional nonlinear regression computations unless the error structure coincides with the weighting used for the nonlinear regression technique. The general method developed is anticipated to be applicable to other nonlinear problems although appropriate numerical techniques for solving simultaneous nonlinear equations must be developed for the particular model under examination.

[1] The programs were written using the Statistical Analysis System (SAS) version 79.5 run utilizing North Dakota State University Computer System.

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Comparison of UV and Fluorescence Spectrophotometry for the Quantification of a Potent Myotonia Inducer: Anthracene-9-carboxylic Acid, in Plasma, Urine, and Saline Perfusion Fluids

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Abstract □ UV and fluorescence spectrophotometry were used to establish the analytical profile of a potent myotonia inducer, anthracene-9-carboxylic acid (I). UV spectrophotometry is useful for the determination of I when it is dissolved in physiological solutions (Ringer's, Tyrode's, *etc*). In these fluids there is a linear relationship between UV absorption and I concentration between 500 and 2000 ng/ml ($2.25–9.0 \times 10^{-6}$ *M*). However, in biological fluids there are interferences in the UV absorption due to organic substances. On the other hand, fluorescence spectrophotometry is more sensitive than UV for determinations in plasma and urine. Within the range of 200–1000 ng/ml ($0.9–4.5 \times 10^{-6}$ *M*) fluorescence intensity increases linearly with concentration. Furthermore, when both emission and excitation spectra are combined there are no interferences due to organic substances normally present in those fluids. An extraction procedure of I from plasma and urine is also described, and the importance of I determinations in relation to the problem of this myotonia-inducing aromatic monocarboxylic acid is discussed.

Keyphrases □ Fluorescence spectrophotometry—comparison of UV for quantification of a potent myotonia inducer

Several classes of chemical agents can produce, both *in vivo* and *in vitro*, changes in the function of mammalian skeletal muscle resembling the condition known as myotonia congenita in humans and in goats (1, 2). These agents include the veratrum alkaloids (3), the substitution of chloride ion by other ions that do not cross the muscle membrane (4, 5), a group of hypocholesterolemic substances (6, 7), clofibric acid that reduces the triglyceride levels (8), and other aromatic monocarboxylic acids (9–11). All these substances act directly on the membrane of mammalian skeletal muscle. However, an increasing number of studies in recent years (12–14) have confirmed the early proposal of Bryant and Morales-Aguilera (15) that aromatic monocarboxylic acids induce in mammalian muscle fibers a state that more closely resembles naturally occurring myotonia congenita than do the other agents. Bryant and Morales-Aguilera (15) and Palade and Barchi (16) showed that anthracene-9-carboxylic acid (I) is the most potent inducer of myotonia among the tested aromatic monocarboxylic acids and also that these substances very specifically block the chloride channel. The availability of chemical agents whose effects resemble the naturally occurring condition is important in the development of animal models that can give information as to the mechanisms of the disease. With the exception of clofibric acid (17), there is a lack of quantitative studies regarding the *in vivo* kinetics of the myotonia-inducing aromatic monocarboxylic acids. Also, methods are not available for their chemical determination in biological fluids. In this paper we report UV and fluorescence spectrophotometric methods for I determination and compare them in blood, plasma, urine, and saline perfusates, covering most of the present analytical needs of biomedical researchers interested in the study of myotonia induced by I.

EXPERIMENTAL

Determination of I by UV Spectrophotometry—*Sodium Bicarbonate Buffer*—Fifty milligrams of I[1] and 1.5 g of sodium bicarbonate were placed in a 100-ml volumetric flask and brought to volume with water. Since I is almost insoluble in water (the concentration of a saturated water solution is 1.88×10^{-4} *M*), it was necessary to alkalinize the medium with sodium hydroxide to pH 8–9 (22°)[2] and stir vigorously for at least 20 min. This solution has 320 mosm[3]. By successive dilutions of the isotonic alkaline solution of I the following concentrations were obtained: 0.5, 1.0, 1.5, and 2.0 μg/ml. The absorbance of these solutions was measured relative to an isotonic solution blank at 255 nm[4]. These solutions are unstable in light, so it is necessary to proceed immediately; the solutions must be protected from light.

Chloroform—A 50-mg volume of I was placed in a 100-ml volumetric flask, and brought to volume with chloroform. The dilutions to 0.5, 1.0, 1.5, and 2.0 μg/ml were made with the same solvent. Their absorbance was measured *versus* a chloroform blank at 255 nm.

Excitation/Emission Spectrum of I in Chloroform—In a 50-ml volumetric flask, 25 mg of I was added, brought to volume with chloroform, and diluted to a final concentration of 10 μg/ml. The spectra of excitation and emission were obtained in a spectrophotofluorometer[5], and the results are shown in Fig. 1. The excitation spectrum exhibits a clear absorption peak at 355 nm. This wavelength was used subsequently for excitation of the samples. The fluorescence spectrum manifests an enhanced emission at 470 nm, and the peak at this wavelength was used to quantify I in blood, plasma, and urine.

[1] Aldrich Chemical Co.
[2] Coleman Instruments Model 37-A Ph Meter.
[3] Wescor Model 5100 Osmometer.
[4] Unicam Sp 800 Spectrophotometer.
[5] Aminco-Bowman.

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