Since the maximum error in substituting ϵ_N' for ϵ_N is ~15% for the hydroxybenzoic acids, it may be assumed that this value is the order of maximum error in substituting ϵ_N' for ϵ_N for the aminobenzoic acids.

In the present study, the literature values of the macroequilibrium constants K_1 and K_2 (2) are used and Eq. 9 is rearranged to solve for ϵ_U for each of the aminobenzoic acids, using the absorptiometric titration data represented in Fig. 1, according to:

$$\epsilon_U = \frac{(a - \epsilon_C C_T l)[H^+]^2 + (a - \epsilon_A C_T l)K_1K_2 + aK_1[H^+]}{C_T l K_1[H^+]} \quad \text{(Eq. 18)}$$

The values of ϵ_U thus obtained are presented in Table I. These values, along with those of ϵ_N' (Table I), are employed in Eq. 17 to calculate the value of K_{NZ}' which approximates the true tautomeric equilibrium ratio K_{NZ} of each aminobenzoic acid. The values of K_{NZ}', calculated in this manner, as well as those calculated from the literature K_1, K_2, and K_{CN}' values, are also presented in Tables III and IV for comparison.

The agreement between the microequilibrium constants calculated by both methods is quite good for the *meta*-isomer. However, in the *ortho*-isomer, the spectroscopic method indicates that the neutral molecule and the zwitterion comprise almost equal fractions of the population of uncharged molecules, while the method using the K_{CN}' of the ester suggests that the neutral molecule is predominant over the zwitterion by about 8:1. Although it is difficult to establish unequivocally which approach is more accurate, the disparity between the results obtained in this case for the *para*-isomer is more definitive. The spectroscopic method indicates that the neutral molecule predominates over the zwitterion in *p*-aminobenzoic acid by 12.3:1. However, the K_{CN}' of the methyl ester yields a negative value for the tautomeric ratio, a result that is physically impossible and is transmitted into the calculations of the remaining microconstants.

The K_{CN}' of the ethyl ester of *p*-aminobenzoic acid was determined. Use of this value to calculate the microconstants of the *para*-isomer gives microconstants in reasonably good agreement with those obtained by the spectroscopic method. Hence, the microconstants obtained by using the K_{CN}' of the ester are substantially dependent on the inductive effect (and other chemical effects) of the esterifying group. The absorptivities of the methyl and ethyl esters of *p*-aminobenzoic acid are, however, virtually identical; therefore, the spectroscopic method is, barring unusual steric interferences, free of uncertainties imposed by the nature of the esterifying group.

It is concluded, therefore, that the spectroscopic method described in this paper for estimating microequilibrium constants of prototropic reactions is, when applicable, simpler, faster, and more accurate than the conventional method, employing the dissociation constant of an alkylated derivative as equivalent to one microequilibrium constant of interest.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XXXIII: Frequency Distribution of Dicumarol Total Clearance in Rats

CHII-MING LAI and GERHARD LEVY ˣ

Received March 31, 1977, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Amherst, NY 14260.* Accepted for publication May 12, 1977.

Abstract □ The total clearance of dicumarol was determined in 172 adult male Sprague–Dawley rats. Clearance values ranged from 1.46 to 27.0 ml/hr/kg. Statistical analysis of a histogram of the total clearance values indicated a trimodal distribution, with modes at 6.28, 14.8, and 23.7 ml/hr/kg. The percentage of animals in each of these components was 60.5, 33.7, and 5.8. A previous study had shown that the total clearance of dicumarol was proportional to the fraction of nonprotein-bound drug in serum (serum free fraction) and that interindividual differences in total clearance of dicumarol in rats were due almost entirely to corresponding differences in the serum free fraction. Therefore, it is likely that the observed trimodal frequency distribution of total clearance values reflects a similar distribution of serum free fraction values of dicumarol. The frequency distribution curve for dicumarol total clearance is very similar to the trimodal frequency distribution curve for warfarin serum free fraction values in rats. This observation is consistent with the previously demonstrated strong correlation of serum free fraction values of dicumarol and warfarin in individual animals.

Keyphrases □ Dicumarol—total clearance in rats, frequency distribution □ Clearance, total—dicumarol in rats, frequency distribution □ Pharmacokinetics—total clearance of dicumarol in rats, frequency distribution □ Coumarin anticoagulants—dicumarol, total clearance in rats, frequency distribution □ Anticoagulants—dicumarol, total clearance in rats, frequency distribution

Pronounced differences exist in the elimination kinetics of dicumarol in animals and humans. Vesell and Page (1) found that the dicumarol biological half-life ($t_{1/2}$) in 28 healthy adult humans not taking other drugs ranged from 7 to 74 hr and that these values were reproducible upon subsequent administration of a second dose. Studies in this laboratory revealed a $t_{1/2}$ range of 5.1–27.9 hr and a total clearance range of 2.6–24.0 ml/hr/kg in 30 adult male Sprague–Dawley rats (2). Such differences have been found repeatedly and are also well reproducible (2, 3).

The dicumarol $t_{1/2}$ was determined in human subjects after administration of a standard oral dose, and these

Table I—Evaluation of Different Types of Frequency Distribution Curves for Dicumarol Total Clearance in Rats

Type of Distribution	Means, ml/hr/kg	*SD*'s	Number of Rats in Each Component	χ^2	Degrees of Freedom	*p*
Normal	9.97	5.75	172	54.0	11	<0.00001
Log-normal	8.51	+6.51, −3.69	172	18.0	10	≃0.055
Bimodal	6.28	2.07	96	16.3	9	≃0.057
	16.7	3.65	76			
Log-bimodal	6.27	+2.25, −1.65	108[a]	7.34	6	≃0.285
	14.7	+4.51, −3.44	63			
Trimodal	6.28	2.07	104	4.46	7	≃0.709
	14.8	2.67	58			
	23.7	1.45	10			

[a] One rat with a total clearance of 1.46 ml/hr/kg was excluded due to a large discontinuity in the histogram.

values were corrected for presumed differences in dicumarol absorption since its elimination kinetics are dose dependent in humans (4). A histogram of these $t_{1/2}$ values is apparently unimodal. Solomon and Schrogie (5) reported that the frequency distribution of dicumarol $t_{1/2}$ values in rabbits is apparently bimodal, but details were not provided. On the other hand, Millar *et al.* (6) found that rabbits can be divided arbitrarily into four groups on the basis of the change in prothrombin time produced by a standard dicumarol dose. However, it appears that the prothrombin times in that study may be trimodally distributed.

The $t_{1/2}$ and total clearance of dicumarol in 172 rats were determined in this laboratory over the last several years as a screening procedure for the selection of animals for studies of dicumarol and warfarin pharmacokinetics. The frequency distribution of the dicumarol total clearance in these rats has now been characterized and related to other relevant information obtained previously in this series of investigations.

EXPERIMENTAL

Five groups of adult male Sprague–Dawley rats[1], 187–280 g, were given unrestricted access to food[2] and water before and during the pharmacokinetic study. None received any drugs previously. The studies were carried out in different seasons between 1973 and 1975.

A single dose of ^{14}C-dicumarol, 8 mg/kg iv, was injected, and 0.45-ml blood samples were obtained from the tail artery every 3–6 hr for 24 hr. Plasma was separated and extracted, and the extract was analyzed for total (free and bound) unmetabolized dicumarol as previously described (7, 8). The total dicumarol clearance, *i.e.*, the product of the apparent first-order elimination rate constant and the apparent volume of distribution, was calculated from the slope and intercept of the least-squares regression line of a plot of log dicumarol concentration *versus* time.

Histograms to describe the frequency distribution of the total clearance or log total clearance values were constructed by iteratively changing the class interval to maximize the number of bars in the respective histogram while minimizing the number of regional reversals (modes and antimodes), as suggested by Martin *et al.* (9). For the multimodal characterizations, the mean values, standard deviations, and percentages of the total number of animals in each Gaussian component were estimated as described by Bhattacharya (10). The logarithm of the clearance values was used in these calculations when indicated. Distribution curves were fitted to the histograms (11), and the expected frequencies for overlapping components were summed prior to evaluating the agreement of the distribution model with the histogram by χ^2 test.

RESULTS

The total clearance of dicumarol in the 172 rats ranged from 1.46 to 27.0 ml/hr/kg. These determinations were made at different times of the year, between May 1973 and March 1975, on groups of 26–56 animals. Histograms of the results obtained from each of the five groups are shown in Fig. 1. There was no statistically significant difference between the frequency distribution of the data from each group of animals, as determined by the Kruskall–Wallis nonparametric one-way analysis of variance (12). Consequently, all data were combined for determination of the frequency distribution characteristics of the total clearance values.

The characteristics of the different types of frequency distribution curves fitted to the data and the results of their statistical evaluation are summarized in Table I. There was a highly statistically significant difference between the normal distribution curve and the histogram and a borderline difference between the log normal and bimodal curves and the respective histograms. A reasonably good fit was obtained with the log-bimodal distribution curve, but the best fit was obtained with the trimodal distribution curve.

According to the trimodal distribution model, 60.5% of the animals were in the component with the lowest clearance (mean ± *SD* = 6.28 ± 2.07 ml/hr/kg), 33.7% were in the component with intermediate clearance (14.8 ± 2.67 ml/hr/kg), and 5.8% were in the component with the highest clearance (23.7 ± 1.45 ml/hr/kg). The combined histogram for all 172 animals and the trimodal distribution curve fitted to the data are shown in Fig. 2.

There was no statistically significant correlation between the total clearance and either the apparent volume of distribution of dicumarol (*milliliters per kilogram*) or the body weight (indicative of age) of the animals.

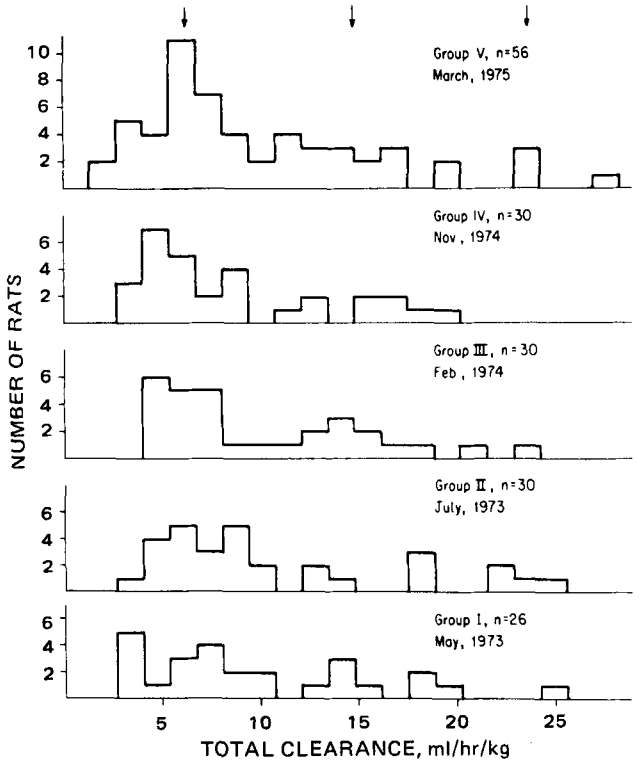

Figure 1—*Frequency distribution of dicumarol total clearance values in each of five groups of adult male Sprague–Dawley rats. The arrows on the abscissa indicate the means of the Gaussian components of the trimodal distribution curve shown in Fig. 2.*

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Charles River Formula 4RF.

Table II—Comparison of the Frequency Distribution Characteristics of Dicumarol Total Clearance and Serum Free Fraction of Warfarin in Rats

Parameter	Relative Mean of Each Component[a]			Percentage of Animals in Each Component		
	Component I	Component II	Component III	Component I	Component II	Component III
Total clearance of dicumarol	0.265	0.624	1.0	60.5	33.7	5.8
Serum free fraction of warfarin[b]	0.239	0.675	1.0	53.6	27.2	19.2

[a] Relative to the mean of Component III. [b] From Ref. 17.

DISCUSSION

The total clearance of dicumarol in rats is proportional to the serum free fraction of the drug (13). This result is consistent with theoretical considerations (14) and was demonstrated previously with respect to warfarin in rats (15) and humans (16). Intersubject differences in serum protein binding are largely responsible for the observed differences in the total clearance of these anticoagulants (13–16). Thus, it is reasonable to assume that the frequency distribution characteristics of dicumarol total clearance determined in this investigation reflect a corresponding frequency distribution of serum free fraction values of dicumarol.

Since there is a strong correlation between the serum free fraction values of dicumarol and warfarin in individual rats (2), it may be expected that the frequency distribution characteristics of dicumarol total clearance and warfarin serum free fraction are quite similar in the same strain of rats. The frequency distribution of serum free fraction values of warfarin determined in this laboratory in rats from the same strain and source as those used in the present investigation was trimodal (17). A quantitative comparison of the dicumarol and warfarin frequency distribution characteristics is presented in Table II. Considering the fact that these studies were performed with different animals and by different investigators, the similarity of the frequency distribution characteristics is striking[3].

Multimodal frequency distribution characteristics can occur as artifacts caused by combining different groups of animals. These groups may differ in age, environmental history (*e.g.*, exposure to enzyme-inducing pesticides), or physiological status related to the season of the year. These types of artifacts were ruled out to the best of our ability in this investigation.

If the trimodal frequency distribution of dicumarol total clearance values in rats is a genetic effect, it would be ideal to carry out multigeneration studies. Motulsky (4) pointed out that when a population can be categorized into clearly distinct classes by some measurement and a multimodal frequency distribution curve is obtained, the involvement of a single gene may be suspected. The trimodality of the dicumarol total clearance data in rats suggests the existence of three genotypic classes, consisting of two homozygous groups and one heterozygous group and representing small, intermediate, and large clearance phenotypes. Since the large intersubject variation of dicumarol and warfarin serum free fraction values cannot be explained by corresponding variations in the concentration of serum albumin or total proteins, it may be due to quantitative differences in the serum concentration of endogenous inhibitor(s) of drug binding to proteins or qualitative differences in albumin (15, 18). Intersubject variations in the serum concentration of endogenous inhibitors could be due to genetically determined differences in the formation rate or clearance of these compounds.

The results of studies in rabbits performed more than 30 years ago were interpreted to indicate that recessive genes play a role in the biotransformation of dicumarol (19). More recently, Motulsky (4) found a sibling–sibling correlation of dicumarol half-life values, but no parent–offspring correlation, and concluded that these findings are indicative of the operation of recessive genes in the elimination of dicumarol. Subsequently, Vesell and Page (1) determined the dicumarol biological half-life in seven sets of identical twins and seven sets of fraternal twins who were not taking other drugs. The half-life values were almost identical in each set of identical twins but significantly different in each set of fraternal twins. These investigators concluded that the dicumarol biological half-life is under genetic control.

None of these studies revealed the biochemical site of the genetic effect, and in none was the serum free fraction of the drug determined. On the other hand, some rats in this study were used to determine the serum free fraction value of dicumarol, and a linear relationship between this parameter and the total clearance of dicumarol was found (13). Vesell and Page (1) observed a strong correlation between the half-life values of the extensively (>99%) serum protein-bound drugs dicumarol and phenylbutazone in the same subjects and no apparent correlation between the half-life values of either of these drugs and that of the poorly (<10%) serum protein-bound drug antipyrine. They speculated that the correlation of dicumarol and phenylbutazone half-life values may be related to their extensive serum protein binding. It would be of interest, therefore, to determine the serum protein binding of dicumarol and phenylbutazone in the same sets of identical and fraternal twins. One such twin study, carried out with nortriptyline, revealed that the plasma protein binding of this drug in humans is partly under genetic control (20).

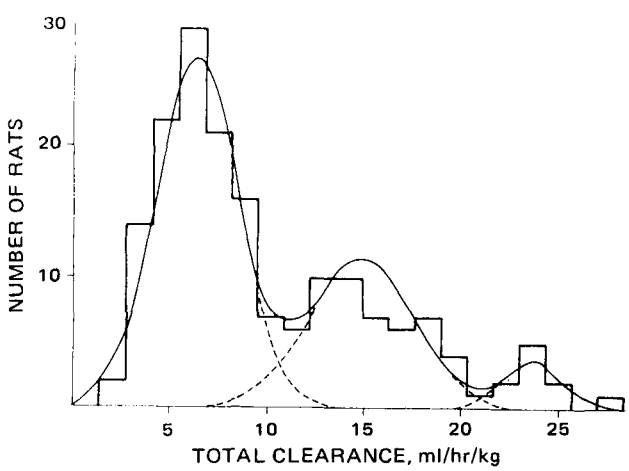

Figure 2—*Composite histogram showing the frequency distribution of dicumarol total clearance values for all 172 rats. Also shown is the trimodal distribution curve fitted to the data, with the dotted lines indicating the overlapping portions of the individual Gaussian components.*

[3] An explanation for the use of total clearance as a screening parameter for dicumarol studies and serum free fraction as a screening parameter for warfarin experiments is in order. Theoretically, either parameter is suitable. We know now (but did not know when these investigations were started) that either drug can be used to screen for rapid and slow eliminators of one or the other anticoagulant. It is technically easier, and more rapid, to determine serum free fraction values, but the dicumarol free fraction is so small that these determinations are relatively imprecise. Therefore, total clearance was used as a screening parameter for dicumarol.

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Comparison of Two Methods for Obtaining Size Distribution Characteristics of Particulate Matter in Large-Volume Parenterals

JAMES BLANCHARD ˣ, JOHN A. SCHWARTZ, DALE M. BYRNE *, and DAVID B. MARX ‡

Received October 4, 1976, from the *Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721.* Accepted for publication June 21, 1977. *Present address: Optical Sciences Center, University of Arizona, Tucson, AZ 85721. ‡Present address: College of Agriculture, University of Arizona, Tucson, AZ 85721.

Abstract □ The size distributions of the particulate matter present in six types of large-volume parenteral solutions, as determined by an automatic particle counter and a microscopic counting technique, were compared by plotting log $N_{>D}$ *versus* log D. The resulting data were analyzed individually and also as averages. The data showed a linear relationship between log $N_{>D}$ and log D over the 1–100-μm particle-size range, indicating that both methods determine a similar particle-size distribution. The data also indicated that the particle-size distributions were largely independent of the type of solution and obeyed a power law of the form $N_{>D} = N_{>1}D^K$. These observations suggest that the major source of contamination is air-borne dust particles, which fall into a solution randomly, and that it may be possible to monitor the smaller, more abundant particles with the automatic particle counter to obtain a rapid estimate of parenteral cleanliness. The automatic particle counter thus appears to be a viable alternative to the microscopic counting technique for assessing the particulate matter content of parenterals.

Keyphrases □ Size distribution—particulate matter in six large-volume parenteral solutions, automatic particle counter and microscopic techniques compared □ Distribution, size—particulate matter in six large-volume parenteral solutions, automatic particle counter and microscopic techniques compared □ Particles—size distribution in six large-volume parenteral solutions, automatic particle counter and microscopic techniques compared □ Parenterals, large volume—six types, size distribution of particulate matter, automatic particle counter and microscopic techniques compared □ Dosage forms—large-volume parenterals, six types, size distribution of particulate matter, automatic particle counter and microscopic techniques compared

Particulate matter has been defined as "extraneous, mobile, undissolved substances, other than gas bubbles, unintentionally present in parenteral solutions" (1). The problem of particulate matter has plagued the preparers of parenterals since their introduction. Although the clinical significance of particulate matter is still somewhat controversial, it is generally accepted that the safest approach is to minimize particulate matter as much as possible (2–4).

Standards set by the USP–NF (1), based on a microscopic counting procedure, limit the allowable levels of particulate contamination. While this method is the accepted standard, it suffers from several shortcomings since it is subjective, tedious, and time consuming and requires a considerable amount of practice before the operator becomes proficient. In addition, the method is destructive and, therefore, not ideally suited for the in-line monitoring of production batches of parenterals. It is highly desirable to find an alternative method that will overcome these limitations.

In a previous study (5), the ability of several methods to monitor the levels of particulate matter in parenterals was compared. The automatic particle counter warranted further investigation since it offered some advantages over the other methods tested. This instrument was first mentioned by Draftz and Graf (6) who described its characteristics. One apparent limitation of this instrument is its inability to count accurately small numbers of particles since the minimum detectable concentration is only 1 particle/ml. This limitation could be a serious drawback when monitoring particles larger than approximately 5 μm in diameter since sufficiently high counts to obtain statistically reliable data are not normally found in the relatively clean solutions available commercially.

This limitation does not necessarily preclude the use of the automatic particle counter in monitoring the particulate matter content of these solutions provided that the number of larger, less abundant particles (upon which the present compendial standard is based) can be determined from the number of smaller, more abundant particles. The major goals of this study were to determine if such a relationship exists and, if so, to characterize its nature.

EXPERIMENTAL

Large-volume parenteral solutions[1] (1000 ml) were inspected by two methods: an instrumental technique using an automatic particle counter[2]

[1] McGaw Laboratories, Glendale, CA 91201.
[2] Prototron model ILI 1000, Spectrex Corp., Redwood City, CA 94063.