解剖学教研室，梁铼琚脑研究中心，西安 710032，中国）

关键词 脊髓；受体；地西泮；神经活性留类；戊巴比妥；P物质；1-氨基环戊基-1,3-二羧酸；膜片箝技术

目的：急性分离大鼠骶髓后连合核神经元。**方法**：采用酶消化结合机械性分离技术分离神经元，以制霉菌素穿孔膜片箝技术检测其机能状态。**结果**：分离的神经元对兴奋性和抑制性氨基酸具有良好的反应。P物质(SP)和反式-氨基环戊基-1,3-二羧酸(tACPD)明显增强其 NMDA 反应。而地西泮、孕烯诺龙和戊巴比妥存在下，GABA 反应被显著加强。**结论**：急性分离的大鼠 SDCN 神经元为探索 SDCN 参与痛和镇痛的机制提供了理想模型。

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Analysis of multidrug effects by parameter method[1]

ZHENG Qing-Shan[2], SUN Rui-Yuan[3]
(*Institute of Clinical Pharmacology, Yijishan Hospital, Wannan Medical College, Wuhu 241001*; [3]*Institute of Materia Medica, Wannan Medical College, Wuhu 241001, China*)

KEY WORDS drug synergism; drug antagonism; combination drug therapy; biometry

AIM: To set up a new analytic method for multidrug effects. **METHODS**: Based on the principles of the target site kinetics and the equieffective test, a new mathematical model was set as $Q = (E_o - E_e)/|E_e \cdot W - s_{\bar{x}} \cdot T|$ ($-1 < Q < 1$ addition, $Q \leqslant -1$ antagonism, $Q \geqslant 1$ synergism) where E_o = a fitted value of the observed effect of a combination, E_e = an expected value of combined effect, W = an equieffective criterion decided by a special field, $s_{\bar{x}}$ = a common standard error of E_o and E_e, and T = a value of one-sided $t_{0.05}$. All the calculations were completed with computer. Dose-effect data from different types of experiments were fitted by the new model and the results were compared with those of other methods. **RESULTS**: This parameter method dealt with different types of data well fitted with the Hill equation, and was not limited to analyze receptor interaction of drugs, or the number of combined drugs. A series of Q values was obtained from all levels of dose-effect for a systematic analysis. The analysis took the criterion of a special field and laboratory error into account. **CONCLUSION**: This parameter method can effectively analyze the multidrug effects.

Quantitative analysis of the combined effects of multidrugs, such as synergism, antagonism, and addition, is not fully solved, especially quantitative data with more than 2 drugs in combination, or in large animal experiment and clinical trials. To qualitative data, Chou-Talalay combination index method[1, 2], Xu's method[3] and Jin's method[4] are widely used on basis of different background (mechanism or empirical), but those methods may yield different or even opposite results. Now, it becomes imperative to solve the problem to decide new multidrug clinical therapeutic design.

It is generally accepted that dose-effect curve of sigmoid or hyperbola shape can be expressed with Hill equation and is characterized by its pharmacodynamic parameters. The parameters in different combinations must vary with the results of the combined effect. In this paper, we try to find a new analytic method from the regularity of the parameters changes.

ANALYSIS OF THE PARAMETER METHOD

Formula

$$Q = \frac{E_o - E_e}{|E_e \cdot W - s_{\bar{x}} \cdot T|} \quad (1)$$

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[2] Correspondence to ZHENG Qing-Shan, MS.
Phn 86-553-587-7855. Fax 86-553-586-3079.
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($-1 < Q < 1$ addition, $Q \leqslant -1$ antagonism, $Q \geqslant 1$ synergism)

where E_o is a fitted value of the combined effect and E_e is an expected value of the combined effect. W, an equieffective criterion decided by a special field, generally, equals 0.1 (10 %) in clinical trial and whole body animal experiment, 0.05 (5 %) *in vitro*, and 0.2 (20 %) in bioavailability trial according to new drug biological statistics. $s_{\bar{x}}$ is a common standard error of E_o and E_e. T is a value of one-sided $t_{0.05}$.

As all dose-effect curves have the same curvature parameters ($H_c = H_1 = H_2 \cdots H_n$) and the values of maximal effects ($E_{max\text{-}c} = E_{max\text{-}1} = E_{max\text{-}2} \cdots E_{max\text{-}n}$) according to statistical analysis, E_e can be calculated by Eq 2, otherwise, by Eq 3.

$$E_e = \frac{E_{max-c}\left(\frac{D_1}{K_1} + \frac{D_2}{K_2} \cdots + \frac{D_n}{K_n}\right)^{H_c}}{1 + \left(\frac{D_1}{K_1} + \frac{D_2}{K_2} \cdots + \frac{D_n}{K_n}\right)^{H_c}} \tag{2}$$

$$E_e = \frac{E_{max-1} \cdot \left(\frac{D_1}{K_1}\right)^{H_1} + E_{max-2} \cdot \left(\frac{D_2}{K_2}\right)^{H_2} \cdots + E_{max-n} \cdot \left(\frac{D_n}{K_n}\right)^{H_n}}{1 + \left(\frac{D_1}{K_1}\right)^{H_1} + \left(\frac{D_2}{K_2}\right)^{H_2} \cdots + \left(\frac{D_n}{K_n}\right)^{H_n}} \tag{3}$$

$$E_o = \frac{E_{max-c} \cdot \left[\sum_{i=1}^{n} D_i\right]^{H_C}}{K_c^{H_c} + \left[\sum_{i=1}^{n} D_i\right]^{H_C}} \tag{4}$$

$$s_{\bar{x}} = \sqrt{s_{\bar{x}-c}^2 + \sum_{i=1}^{n} s_{\bar{x}-i}^2} \tag{5}$$

$$f = N - n - 1$$

where n is the number of combined drugs ($i = 1, 2 \cdots n$). With pharmacodynamic parameters of combined drugs, $E_{max\text{-}c}$ = maximal effect, K_c = equilibrium dissociation constant, and H_c = curvature parameter in dose-effect curve. With pharmacodynamic parameters of drugs used alone, $E_{max\text{-}i}$ = maximal effect, K_i = equilibrium dissociation constant, and H_i = curvature parameter. f is the degree of freedom. N equals the sum of data points on dose-effect curves in single drug and in combined drugs. $s_{\bar{x}-c}$ or $s_{\bar{x}-i}$ is a standard error of a fitted effect ($\hat{E}_j$) at certain dose (D), which can be calculated by

$$s_{\bar{x}-c} \text{ or } s_{\bar{x}-i} = \sqrt{\frac{\sum_{j=1}^{M}(E_j - \hat{E}_j)}{M-2}\left(1 + \frac{1}{M} + \frac{(D-\bar{D})^2}{\sum_{j=1}^{M}(D_j - \bar{D})^2}\right)} \tag{6}$$

where M is number of data points on a dose-effect curve of separate drug or combined drugs, $\bar{D}$ is the mean dose.

Principle Certain effect of one drug is exhibited only when the drug enters certain effective sites, such as receptor, enzyme, biological membrane, biochemical metabolic link, plasma protein, DNA, *etc*. To take these special terms as a more general concept, we define the effective site as a target site[5], which is regarded as a fundamental unit for combining with a drug molecule and exhibiting an effect. When drugs are used alone and in combination at a fixed proportion, the number of bound target sites is increasing along with the effect increasing at the dose change from low to high level. When all the target sites are bound, the effect reaches a maximal value, which will keep unchanged or little change despite the dose increase, especially for the *in vitro* data. The dose-effect relationship can be expressed as Clark or Hill equation. Each of the drugs in combination has a contribution value to combined effect. At certain combined effect level, if the effect of a drug is strong, the effect of other drugs must be weak, which is like the competitive antagonism in the kinetics of drug-receptor interactions[6]. So, we set up a new theory about the target site kinetics described as following.

$$D_1 + R \underset{K_1}{\rightleftharpoons} (D_1R) \xrightarrow{\alpha_1} E_1, \tag{7}$$

$K_1 = D_1 \cdot R/(D_1R)$,
hence $R/(D_1R) = K_1/D_1$

$$D_2 + R \underset{K_2}{\rightleftharpoons} (D_2R) \xrightarrow{\alpha_2} E_2, \tag{8}$$

$K_2 = D_2 \cdot R/(D_2R)$,
hence $(D_2R) = D_2 \cdot R/K_2$

$$D_n + R \underset{K_n}{\rightleftharpoons} (D_nR) \xrightarrow{\alpha_n} E_n, \quad (9)$$

$K_n = D_n \cdot R/(D_nR)$,
hence $(D_nR) = D_n \cdot R/K_n$

$$\alpha_i = \frac{E_i}{(D_iR)} = \frac{E_{max-i}}{R_T} \quad (10)$$

$$R_T = R + (D_1R) + (D_2R) + \cdots (D_nR) \quad (11)$$

where R is the free target site, R_T is the total target site, (D_iR) is the bound target site ($i = 1, 2, \cdots n$). D_i is a dose, E_i is the corresponding effect, and α_i is a proportional constant between effect and target site. E_{max-i}, K_i and n are the same as above-mentioned. According to Eq 10, we have

$$E_1 = E_{max-1}\frac{(D_1R)}{R_T} \quad (12)$$

Put Eq 11 into Eq 12, Eq 13 is obtained,

$$E_1 = E_{max-1}\frac{(D_1R)}{R + (D_1R) + (D_2R) + \cdots (D_nR)} \quad (13)$$

Put Eq 7-9 into Eq 13, we have

$$E_1 = \frac{E_{max-1} \cdot D_1}{K_1 + D_1 + \frac{D_2 \cdot K_1}{K_2} + \cdots \frac{D_n \cdot K_1}{K_n}}$$

$$= \frac{E_{max-1}\frac{D_1}{K_1}}{1 + \frac{D_1}{K_1} + \frac{D_2}{K_2} + \cdots \frac{D_n}{K_n}}$$

Similar to above,

$$E_2 = \frac{E_{max-2}\frac{D_2}{K_2}}{1 + \frac{D_1}{K_1} + \frac{D_2}{K_2} + \cdots \frac{D_n}{K_n}}$$

$$E_n = \frac{E_{max-n}\frac{D_n}{K_n}}{1 + \frac{D_1}{K_1} + \frac{D_2}{K_2} + \cdots \frac{D_n}{K_n}}$$

$$E_e = E_1 + E_2 + \cdots E_n =$$

$$\frac{E_{max-1}\frac{D_1}{K_1} + E_{max-2}\frac{D_2}{K_2} + \cdots E_{max-n}\frac{D_n}{K_n}}{1 + \frac{D_1}{K_1} + \frac{D_2}{K_2} + \cdots \frac{D_n}{K_n}} \quad (14)$$

If a dose-effect relationship is expressed as the Hill equation, curvature parameter can be obtained and Eq 14 is expressed as Eq 3. To qualitative data, E_{max-c}, E_{max-1}, E_{max-2}, $\cdots$ E_{max-n} equal 1 (100 %). As dose-effect curves in combination and alone have the same curvature parameters and values of maximal effects according to statistical analysis, addition is regarded as different doses of one drug in combination and we have Eq 2.

According to traditional methods, we can get a formula:

$$Q = E_o/E_e \quad (15)$$
($Q = 0$ addition, $Q > 1$ synergism, $Q < 1$ antagonism)

But it is untrustworthy for Eq 15 to just only consider the quotient of an expected effect of multidrug with an observed effect, and the criterion of a special field and laboratory error was not taken into account. For example, the influence of one drug alone on blood pressure is + 5 % or − 5 % higher than that in combination and maybe $Q > 1$ or $Q < 1$. But clinicians take it as clinical equivalency. So the equieffective criterion should be decided by special field. On the other hand, number of data points and experimental error must be taken into account in statistics. According to the principle of equieffective test[7], such as the two one-sided t test or the equivalent limit method, equieffective cutoff (L) of $P < 0.05$ is decided in the parameter method:

$$L = |E_e \cdot W - s_{\bar{x}} \cdot T|$$

$E_o - E_e$ is compared to L, and Eq 1 is got.

We find from Eq 1 that

(1) if D_1, D_2, $\cdots D_n$ are different doses of one drug in combination, it is real additon ($E_e = E_o$ and $Q = 0$).

(2) In the kinetics of drug-receptor interaction, a dose-effect curve is shift to right or down in the presence of competitive or noncompetitive antagonist. To target sites kinetics, $Q \leqslant -1$ calculated by the parameter method indicated antagonism, which is not

further divided into competitive and noncompetitive antagonism. So a conclusion given by the parameter method is a comprehensive. For example, if the combined effect of 3 drugs (ABC) exhibits addition by paramters method, it is possible that A synergizes B and B antagonizes C.

(3) If a drug has no effect or has opposite effect in single use, E_{max} and K should be equal 0, and not be put into any equation to calculate.

(4) According to Eq 1, a series of doses at a fixed proportion can give a series of Q values for systematic analysis of combined effect.

EXPERIMENTS

Influence of epinephrine (Epi) and isoprenaline (Iso) used alone and in combination on systolic blood pressure (SBP) of a dog An adult mongrel dog weighing 12 kg was anesthetized with sodium pentobarbital (ip). NaCl 0.15 mol·L^{-1} solution was infused into femoral vein at 1 mL·min^{-1} and all other drugs were injected into femoral vein. The series of doses of Epi and Iso were prepared separately and in combination (proportion fixed as 1:4.44). Propranolol was given to block β receptor 30 min before Epi and Iso administration. SBP of carotid artery was measured as described[8].

Influence of acetylcholine (ACh), $BaCl_2$ and atropine (Atr) used alone and in combination on guinea pig ileum contraction The ileum segments were in length 2-2.5 cm. Tension of segment contraction in Krebs-Henseleit solution bubbled with gas was recorded by 2-channel physiograph. The volume of medication was 0.1 mL. Basal tension was approximately 2 g. The series of concentrations of the 3 drugs were prepared separately and in combination of 2 drugs or 3 drugs at a fixed proportion. Atr was given at first when it was combined with other drugs. After the effect of a concentration was observed, the segment was washed twice before the next concentration was given.

Inhibitory effect of 5-fluorouracil (5-FU), vincristine (Vin) and cyclophosphamide (Cyc) used alone and in combination on human gastric adenocarcinoma cells Gastric adenocarcinoma cells and blood 5 mL were taken during operation. The plasma was used. The procedures of the experiment were done according to the manual of the tumor drug sensitivity experimental box (Taixi Biochemical Factory, No 941201). The cells (1×10^{16} per bottle) with drugs were cultured in bottles for 48 h. Then the inhibitory rate was measured according to the decreased rate of brown dot in 100 cells.

Data analysis The simplex method was used to fit curves of dose-effect. All parameters were estimated by computer and all curve fittings were of statistical significance ($P < 0.01$).

RESULTS AND DISCUSSION

Combined effect of Epi and Iso on SBP of dog The pharmacodynamic parameters of the two drugs used alone: (1) Epi: E_{max} = 7.57 kPa, K = 6.22 nmol·L^{-1}, H = 0.79; (2) Iso: E_{max} = 6.06 kPa, K = 14.65 nmol·L^{-1}, H = 1.10. At the same proportion (Epi : Iso = 1:4.44), the combined effect of Epi (7.98 - 12.00) nmol·L^{-1} and Iso (35.43 - 53.46) nmol·L^{-1}, analyzed by the parameter methods, exhibited addition. It exhibited synergism above the range and antagonism below the range at the same proportion (Fig 1).

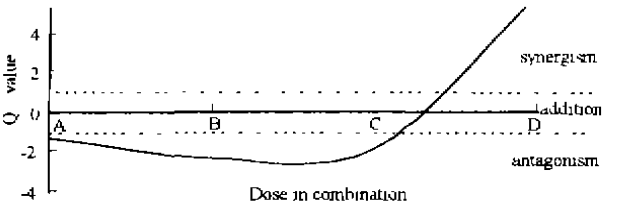

Fig 1. Systematic analysis of actions of Epi (nmol·L^{-1}) and Iso (nmol·L^{-1}) in combination (Epi:Iso = 1:4.44) on SBP of dog Iso + Epi: 3.03 + 0.68 (A), 10.12 + 2.28 (B), 30.32 + 6.83 (C), 101.23 + 22.8 (D).

Combined effect of ACh, $BaCl_2$, and Atr on guinea pig ileum contraction The pharmacodynamic parameters of the 3 drugs used alone: (1) ACh: E_{max} = 325 mg, K = 300.8 μmol·L^{-1}, H = 0.90; (2) $BaCl_2$: E_{max} = 400 mg, K = 8.8 mmol·L^{-1}, H = 1.40; (3) Atr: E_{max} = 0, K = 0, H = 0. According to the parameter method, ACh combined with $BaCl_2$ ($BaCl_2$: ACh = 1 mmol·L^{-1} : 35 μmol·L^{-1}) indicated synergism. As ACh and $BaCl_2$ combined with Atr, the synergism (Q values) was reduced and became antagonism, which indicated that Atr was an antagonist. $BaCl_2$

(0.86 – 1.42 mmol·L^{-1}), ACh (30 – 50 μmol·L^{-1}), and Atr (17.1 – 28.6 nmol·L^{-1}) in combination at fixed proportion (1 mmol·L^{-1}: 20 nmol·L^{-1}:35 μmol·L^{-1}) exhibited addition. At the same proportion, synergism was exhibited below the range and antagonism above the range.

Inhibitory effect of 5-FU, Vin and Cyc in combination on human gastric adenocarcinoma cells The pharmacodynamic parameters of the 3 drugs used alone: (1) 5-FU: $E_{max} = 1$ (100 %), $K = 53.93$ μmol·L^{-1}, $H = 2.48$; (2) Vin: $E_{max} = 1$ (100 %), $K = 2.27$ μmol·L^{-1}, $H = 2.05$; (3) Cyc: $E_{max} = 1$ (100 %), $K = 8.85$ μmol·L^{-1}, $H = 2.22$. The combined effect of 5-FU, Vin and Cyc (107:3:11) was from addition to synergism, again to addition along with the increase of their doses. At the same proportion, the combined effect indicated synergism as 5-FU was in the range of 9.17 – 59.15 μmol·L^{-1}, Vin was in the range of 0.25 – 6.09 μmol·L^{-1}, and Cyc was in the range of 0.94 – 6.09 μmol·L^{-1}. Above or below the range, the effect would be of addition (Tab 1).

The data of 5-FU combined with Vin belong to mutually non-exclusive case according to the median effect principle originated by Chou[1, 2]. Change trend and size of Q or CI values were different by different methods in Tab 1. At the first point of data, the effect levels of 5-FU and Vin used alone were very low and their standard errors were comparatively big. But Jin's method[4] did not take the errors into account so that Q value became so big (6.8). On the other hand, it was questionable for $Q = 1.1$ indicating synergism in Jin's method and $CI = 1.1$ indicating antagonism in Chou-Talalay combination index method without statistical and equalient tests.

The parameter method compared with other methods The parameter method could effectively analyze different types of data well fitted with the Hill equation (Tab 2).

Evaluation of the method The parameter method can effectively deal with different types of data well fitted with the Hill equation, which is not limited to receptor action of drugs only. Q values can be got from all levels of dose-effect for a systematic analysis. The results of analysis are obtained by taking the criterion of a special field

Tab 1. Inhibitory effect of different dose of 5-FU, Vin and Cyc used in combination on human gastric adenocarcinoma cells (equieffective criterion: 5 %)

5-FU /μmol·L^{-1}	Vin /μmol·L^{-1}	Cyc /μmol·L^{-1}	Inhibition rate	5-FU + Vin PM Q	Result	Chou[1, 2] CI	Result	Jin[4] Q	Result	5-FU + Vin + Cyc Inhibition rate	PM Q	Result
5.35	0.15	0.55	0.213	0.4	+	0.5	#	6.8	#	0.215	0.45	+
10.70	0.30	1.10	0.322	1.2	#	0.8	#	3.8	#	0.332	1.19	#
21.40	0.60	2.20	0.764	2.1	#	0.7	#	2.4	#	0.758	1.89	#
42.80	1.20	4.40	0.950	1.8	#	0.7	#	1.8	#	0.923	1.48	#
85.60	2.40	8.80	0.954	0.7	+	1.1	–	1.1	#	0.967	0.51	+

PM: the parameter method. +: $1 > Q > -1$ addition, #: $Q \geqslant 1$ synergism; Chou: Chou-Talalay combination index method, #: $CI < 1$ synergism, –: $CI > 1$ antagonism; Jin: Jin's method, #: $Q > 1$ synergism.

Tab 2. Comparison of 4 methods.

Drugs combined	Number of drugs	Data type	Experimental type	Nature of experiment	CAM	Chou	PM	Jin
Epi + Iso	2	quantitative	*in vivo*	competitive antagonism	yes	no	yes	no
Atr + $BaCl_2$	2	quantitative	*in vitro*	non-competitive antagonism	no	no	yes	no
Atr + $BaCl_2$ + ACh	3	quantitative	*in vitro*	non-competitive antagonism	no	no	yes	no
5-FU + Cyc + Vin	3	qualitative	*in vitro*	non-exclusive	no	yes	yes	no
5-FU + Vin	2	qualitative	*in vitro*	non-exclusive	no	yes	yes	yes

PM: the parameter method; CAM: the competitive antagonism method[6]; Chou: Chou-Talalay combination index method[1, 2]; Jin: Jin's method[4]; yes: can be used to deal with the data; no: can not be used to deal with the data.

and laboratory error into account. The number of combined drugs is not limited, but when many drugs are combined, it is laborious to get dose-effect curve of every drug. The method can be used for clinical trial, especially in dealing with the data *in vitro* for getting dose-effect curves easily and precisely. Curve fitting is better than linear regression and must be of statistical significance ($P<0.01$). Some conditions using the parameter method are suggested that (1) the data of dose-effect should be well fitted with the Hill equation; (2) E_{max} be examined and estimated precisely. To qualitative data, E_{max} should be fixed as 1 (100 %); (3) A range of dose for every drug in combination should be kept in the range used separately.

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参数法分析多药物联合作用[1]

郑青山[2], 孙瑞元[3]

([2]皖南医学院弋矶山医院临床药理研究所, 芜湖 241001; [3]皖南医学院药物研究所, 芜湖 241001, 中国)

关键词 药物协同作用; 药物拮抗作用; 药物联合治疗; 生物统计学

目的: 建立一种新的分析多药物联用效果(协同, 相加或拮抗)的方法. **方法**: 根据靶体动力学原理, 引入药物等效性检验法, 建立新的数学模型:

$$Q=(E_o-E_e)/|E_e\cdot W-s_{\bar{x}}\cdot T|$$

($Q\leqslant -1$ 拮抗, $Q\geqslant 1$ 协同, $1>Q>-1$ 相加)

其中 E_o 为药物联用实测效应拟合值, E_e 为联用药效期望值, W 为专业等效标准, 大小据专业而定, 一般为 10 % ($W=0.1$). $s_{\bar{x}}$ 为 E_o 和 E_e 共同标准误. T 是单侧 t 检验 $t_{0.05}$ 值. 并设计不同实验, 取得不同类型具有多重属性的数据, 用此模型对实验结果进行分析, 并与其他方法比较. **结果**: 本法适用于能用 Hill 方程进行拟合的数据, 质反应数据 E_{max} 固定为 1 (100 %), 不局限于联用药物是否作用于受体, 联用药物数目不受限制, 可对数据进行系统分析. 所得结论为专业结论和统计结论的综合. **结论**: 参数法能有效地分析多种类型联用数据.