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Establishment and application of a new method for the determination of kinetic parameters by plug-plug kinetic capillary electrophoresis (ppKCE)

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A novel plug-plug kinetic capillary electrophoresis method was established, which can be used to simultaneously determine the kinetic parameters k_{on} and k_{off} in interaction systems. The method is comparatively simple and some restrictions in conventional ppKCE methods can be effectively avoided. The requirements for resolution and detection sensitivity in this method are much lower than those of conventional ppKCE. The successful determination of the kinetic parameters and the binding constant K_b between citalopram and BSA showed availability of this method. The results were confirmed by using the time ratio method. The application field of kinetic capillary electrophoresis can be expanded with this new method.

interaction, capillary electrophoresis, plug-plug kinetic capillary electrophoresis (ppKCE), kinetic parameter

1 Introduction

The non-covalent interactions between drug and protein include two reversible kinetic processes: binding and dissociation. In blood plasma, the bound-drug hardly passes through the wall of capillary vessels while the free drug can reach the target to exert pharmacological action. It can be seen that the kinetic process of the interactions between drug and plasma protein play an important role in pharmacokinetics and pharmacodynamics, so the quantitative study of the binding degree is necessary for the study of pharmacokinetics and medication[1−3].

The parameter K_b describing the quantitative binding of drug and protein is determined by the ratio of the binding rate constant k_{on} and the dissociation rate constant k_{off}. However, K_b only describes the equilibrium state in binding of drug and protein, while kinetic parameters k_{on} and k_{off} are important parameters influencing the efficacy and pharmacological activity, thus, they are essential for understanding dynamics of biological processes, determining the pharmacokinetics of target-binding drugs and designing quantitative affinity analyses[4]. Conventional methods for directly finding k_{on} and k_{off} are stopped-flow spectroscopy, surface plasmon resonance, etc. In stopped-flow spectroscopy, the insignificant changes of spectroscopy limit its application in the study of non-covalent interactions because changes of spectroscopy are essential for the determination of k_{on}. In surface plasmon resonance (SPR), which is a heterogeneous reaction, nonspecific binding and adsorption on the surface make it hard to determine the binding parameters accurately in some fields. In addition, the immobilization procedure of targets or ligands is time consuming and relatively expensive. All those mentioned above give the limitation on development and application of SPR. Kinetic capillary electrophoresis (KCE) is a powerful tool for the determination of kinetic parameters. It can facilitate the simultaneous determina-

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tion of k_{on} and k_{off}. The interaction of two species in KCE is a dynamic and homogeneous process.

ppKCE[4,5] is one mode of KCE. k_{on} and k_{off} can be obtained simultaneously by ppKCE. In this method, short plugs of materials B and A are sequentially injected into the capillary if the migration velocity of substance A is faster than B. When the voltage is applied, A passes through B to form complex C; when A has overpassed B and the two species have been separated, complex C begins to dissociate into A and B again. The kinetic parameters can be calculated according to the memory function of electropherograms for the kinetics process. There are two assumptions in ppKCE: 1. the stoichiometry of the interaction between A and B is 1∶1; (2) only the binding process or dissociation process occurs when the zone of A overlaps the zone of B or after the zones of A and B are separated. But conventional ppKCE is mainly used in the study of interactions between biomacromolecules such as DNA and protein. In the calculation of kinetic parameters using peak areas, the mathematical modeling based on partial differential equations of mass transfer is complex to derive. Also it requires that the quantum yield of DNA-bound fluorescein does not change upon DNA binding to protein in the experiment.

With the same sampling way and assumed conditions of conventional ppKCE, a new ppKCE method was developed in this work. It was established based on the definition of kinetic parameters k_{on} and k_{off} and the relationship between peak heights and concentrations in capillary electrophoresis. It shows simplicity on calculations and operation. With this novel method, the application of ppKCE can be extended to the study of quick interactions between small molecules and protein. Moreover, the detection of complex C is not as essential since only the peak height of one reactant is needed in the calculation. Therefore, the detection sensitivity and electrophoretic resolution are not as important as in the conventional methods. Based on the established new method of ppKCE, the interaction between citalopram, which is a long term antidepressant, and albumin bovine (BSA) was studied. The kinetic parameters k_{on} and k_{off} and the binding constant K_b were determined and the result was verified based on a method called the time ratio method[6].

2 Fundamental theory for parameter calculation

According to the hypothesis in the ppKCE methods, the interaction process during the electrophoresis can be represented as Figure 1. The migration velocity of A is faster than that of B and there are two processes, binding and dissociation, during the electrophoresis.

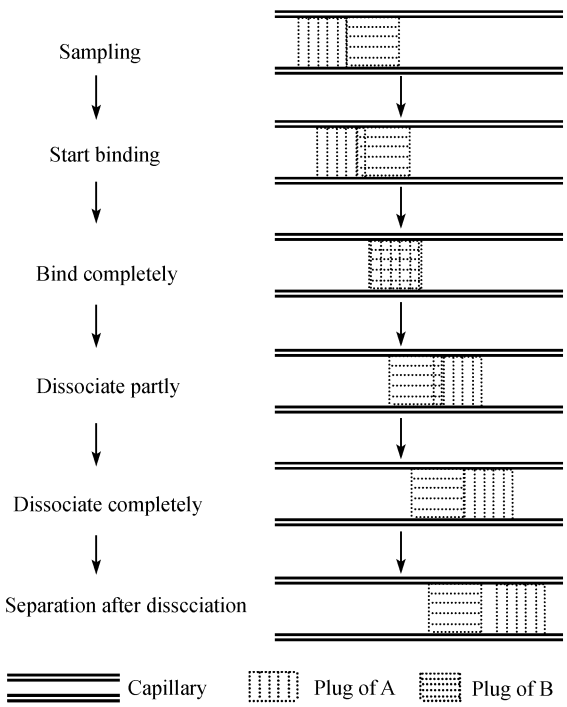

Figure 1 Sketch map of the interaction process in ppKCE.

If the concentration of A in the sample is a and the concentration of B is b, when the two species are mixed and interact with each other, the concentrations of A and B will change into a_0 and b_0, respectively. The calculation formulae of a_0 and b_0 are as follows:

$$a_0 = \frac{l_A}{l_A + l_B} a, \tag{1}$$

$$b_0 = \frac{l_B}{l_A + l_B} b, \tag{2}$$

where l_A and l_B are the zone lengths of species A and B injected. They can be calculated by eq. (3).

$$l_{in} = \frac{gr^2\rho}{8\eta l_{total}} h t_{in}, \tag{3}$$

where g is the acceleration of gravity; r is the inner diameter of capillary; ρ is the density of the solution; η is the liquid viscosity; l_{total} is the total length of capillary; h

is the height in gravitational sampling; t_{in} is the injection time.

In a typical binding process, A and B bind to result in the formation of C when A passes B and this process can be represented as:

$$\begin{array}{cccc} & \mathrm{C} \xleftarrow{k_{on}} \mathrm{A}+\mathrm{B} & & \\ t=0 & 0 & a_0 & b_0 \\ t=t_1 & c_1 & a_1 & b_1 \end{array} \quad \left(t_1 = t_{pass} = \frac{l_A + l_B}{v_B - v_A}\right). \tag{4}$$

The value of k_{on} can be calculated based on the definition of k_{on}[7] as follows:

$$k_{on} = \frac{1}{t_1(a_0 - b_0)} \ln \frac{b_0 a_1}{a_0 b_1} = \frac{1}{t_1(a_0 - b_0)} \ln \frac{b_0(a_0 - c_1)}{a_0(b_0 - c_1)}, \tag{5}$$

where a, b and c are the concentrations of species A, B and C, respectively; v_A and v_B are the migration velocities of species A and B in electrophoresis, respectively, which are equal to l_{total} divided by the corresponding retention time.

In a typical dissociation process, C formed in the binding process starts to dissociate when A has passed B and they have been separated. This process can be expressed as follows:

$$\begin{array}{cccc} & \mathrm{C} \xrightarrow{k_{off}} \mathrm{A}+\mathrm{B} & & \\ t=t_1 & c_1 & a_1 & b_1 \\ t=t_2 & c_2 & a_2 & b_2 \\ t=t_3 & c_3 & a_3 & b_3 \end{array} \tag{6}$$

where t_2 and t_3 are the retention times of species A or B at different applied voltages, which can be obtained by changing voltages under the condition that the binding process of A and B is kept consistent. According to the definition of k_{off}, it can be expressed as following:

$$k_{off} = \frac{1}{t_2 - t_1} \ln \frac{c_1}{c_2}, \tag{7}$$

$$k_{off} = \frac{1}{t_3 - t_1} \ln \frac{c_1}{c_3}. \tag{8}$$

Since the critical value c_1 is difficult to find, the calculation formula of k_{off} can be converted to eq. (9) by the iteration of eqs. (7) and (8).

$$k_{off} = \frac{1}{t_3 - t_2} \ln \frac{c_2}{c_3}. \tag{9}$$

Because the increased quantity of C equals to the decreased quantity of A or B, according to the following three rules: (1) the stoichiometry of the interaction between A and B is 1∶1 in hypothesis 1; (2) the principle of mass conservation retains; (3) the relationship between the peak height and the concentration of one species in the capillary electrophoresis can be described as $h = \varepsilon c$ (h is the peak height; ε is the absorption coefficient; c is the concentration), and the values of c_2 and c_3 can be respectively obtained through the peak heights of species A or B as shown in eqs. (10) and (11).

$$c_2 = \frac{h_{a_0} - h_{a_2}}{h_{a_0}} a_0 \quad \text{or} \quad c_2 = \frac{h_{b_0} - h_{b_2}}{h_{b_0}} b_0, \tag{10}$$

$$c_3 = \frac{h_{a_0} - h_{a_3}}{h_{a_0}} a_0 \quad \text{or} \quad c_3 = \frac{h_{b_0} - h_{b_3}}{h_{b_0}} b_0, \tag{11}$$

where h_{a_0} is the peak height when the concentration of A is a_0 and the meanings of h_{a_2}, h_{a_3}, h_{b_0}, h_{b_2} and h_{b_3} can be analogized in the same way. Then a clearer expression of k_{off} can be seen as follows:

$$k_{off} = \frac{1}{t_3 - t_2} \ln \frac{h_{a_0} - h_{a_2}}{h_{a_0} - h_{a_3}} = \frac{1}{t_3 - t_2} \ln \frac{h_{b_0} - h_{b_2}}{h_{b_0} - h_{b_3}}. \tag{12}$$

From all the above equations, kinetic parameters k_{on} and k_{off} in the interaction system can be calculated by the following method. First, the applied voltage is changed to obtain the retention times and the peak heights of species A or B at different dissociation times. Then the k_{off} value is calculated by eq. (12). Second, after the value of c_1 is obtained by making use of the obtained k_{off} and eqs. (7) or (8), the k_{on} value can be determined by eq. (5). Finally, the binding constant K_b can be obtained by the eq. (13):

$$K_b = \frac{k_{on}}{k_{off}}. \tag{13}$$

3 Experimental

3.1 Instrumentation and reagents

The instruments used included a self-assembled CE system comprised of a 30 kV high voltage power supply and an absorption detector of variable wavelengths in the visual-ultraviolet region, an HW-2003 work station (Nanjing Qianpu Software Ltd.), an uncoated fused-silica capillary (the total length of 56.5 cm, the effective length of 43.0 cm and the inner diameter of 50 μm. Hebei Yongnian Reafine Chromatography Ltd., China). Reagents included Na_2HPO_4 (AR, Chongqing Beibei Chemical Reagent Factory), NaH_2PO_4 (AR, Xi'an Chemical Reagent Plant), citalopram (purity>98.5%, Chongqing Lummy Pharm Techn Co., Ltd.), bovine serum albumin (BSA, purity>98%, Beijing Dingguo Biotechnology Co. Ltd.); acetone (AR, Chongqing Chuan-

dong Chemical (Group) Co., Ltd.)

3.2 Operation conditions and procedure

The running buffer used was 20 mmol·L^{-1} Na_2HPO_4-20 mmol·L^{-1} NaH_2PO_4 (pH=7.0). The stock solutions of citalopram and BSA were made with this running buffer. The experimental temperature was 18℃; the applied voltage was kept at 20 kV when h_{a_2} and t_2 were determined, and then was changed into 15 kV after 20 kV was applied for 0.5 min to keep all the binding process in the experiment to be the same when h_{a3} and t_3 were determined. In order to eliminate the influence of changing voltage on peak heights, 20% acetone solution (volume ratio) was used as the reference in the experiment. The sampling order was a follows: the 20% acetone solution, BSA and the citalopram solution (the plug of BSA was replaced by the running buffer when h_{a0} was determined). Three plugs were all gravitationally injected for 20 s and the injection height was 15 cm. The detection wavelength was 240 nm.

When the confirmation of the proposed method was carried out by using the time ratio method the voltage was set at 20, 15, 12 and 10 kV, respectively, after 20 kV was applied for 0.5 min and other conditions and operation were kept the same as those mentioned above for the interaction between BSA and citalopram with a specific concentration.

Before the first run, a new capillary was normally conditioned by being flushed with 0.1 mol·L^{-1} NaOH and water for 10 min each, and finally being flushed for 30 min with the running buffer. Between runs, the capillary was flushed with 0.1 mol·L^{-1} NaOH, water and the running buffer for 3 min each to eliminate the adsorbed serum albumin.

4 Results and discussion

4.1 The determination of kinetic parameters

The citalopram solutions of 7.00, 6.00, 5.00, 4.00 and 3.00 mmol·L^{-1} diluted from the stock solution interacted with BSA of 1.00 mmol·L^{-1}, respectively. Plug-plug kinetic capillary electrophoresis was carried out according to the CE procedure. The data were obtained by averaging the measured values of each sample with 3 replicates and the relative peak heights of citalopram compared to that of acetone were used in the calculation. Figure 2 shows the electropherogram of the interaction between citalopram and BSA. The value of parameter h_{a0} can be obtained from Figure 2(a), while those of h_{a2} and t_2, can be obtained from Figure 2(b) and those of h_{a3} and t_3 from Figure 2(c) at different dissociation times. All of the data are shown in Table 1 (assume that A represents citalopram and B represents BSA in the formulae).

The mean and the standard deviation of k_{off} were calculated as 1.19×10^{-3} s^{-1} and 0.20×10^{-3} s^{-1}, respectively,

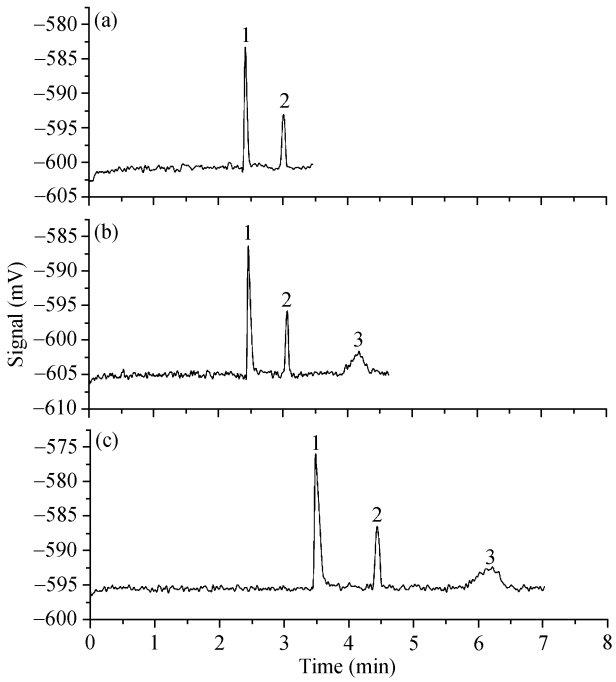

Figure 2 The electropherogram of the interaction between citalopram and BSA. Peak 1, Citalopram; peak 2, acetone; peak 3, BSA. Running buffer: 20 mmol·L^{-1} Na_2HPO_4-20 mmol·L^{-1} NaH_2PO_4 (pH 7.0). Sampling form: gravitation sampling 15 cm×20 s. Sampling order: (a) 20% acetone solution-running buffer-citalopram solution; (b) and (c), 20% acetone solution-BSA-citalopram solution. Applied voltage: (a) and (b) 20 kV; (c) the voltage was changed to 15 kV after 20 kV was applied for 0.5 min.

Table 1 The determination of k_{off} in plug-plug kinetic electrophoresis

a_0 (mmol·L^{-1})	h_{a_0}	h_{a_2}	t_2 (s)	h_{a_3}	t_3 (s)	k_{off} (s^{-1})
1.50	1.68	1.37	152.4	1.38	210.3	0.99×10^{-3}
2.00	1.79	1.47	145.5	1.49	215.9	1.09×10^{-3}
2.50	1.87	1.58	149.8	1.60	210.6	1.23×10^{-3}
3.00	2.22	1.93	150.4	1.95	218.2	1.15×10^{-3}
3.50	2.42	2.10	146.9	2.15	205.2	1.48×10^{-3}

through studying the interaction of citalopram and BSA at five different concentrate ratios.

The difference in viscosity and density of solutions caused by the addition of solutes can be neglected as the quantity of citalopram and BSA is very little in the solution. The sampling lengths of citalopram and BSA were both calculated to be 0.41 cm at $\eta = 1.0\times10^{-3}$ Pa·s. Then t_1 was calculated using parameters t_a and t_b which are the retention times of citalopram and BSA at 20 kV, respectively. The value of k_{on} was determined according to the calculation step two in which the mean of k_{off} obtained in Table 1 was used for the calculation of c_1. The data are shown in Table 2.

The mean and the standard deviation of k_{on} were calculated to be 3.01×10^2 L·mol^{-1}·s^{-1} and 0.58×10^2 L·mol^{-1}·s^{-1}, respectively, through studying the interaction of citalopram and BSA at five different concentrate ratios. Then K_b was calculated to be 2.54×10^5 L·mol^{-1} by eq. (13).

4.2 The confirmation of k_{off} by time ratio method

The accuracy of the complete set of data is directly controlled by the accuracy of k_{off} which is the basis for the determination of k_{on} and K_b. The calculating accuracy of k_{off} was confirmed by the time ratio method.

A series of reaction times and the corresponding concentrations are the foundation of the time ratio method. The concentration at a specific time can be represented by the relative concentration which equals to the present concentration divided by the initial concentration. Each relative concentration corresponds to a certain value of $\tau_{p/q}$ for a certain reaction order and $\tau_{p/q}$ represents the relative time when the relative concentration reaches a value of p/q. Then the character of a reaction order can be expressed by the time ratio which is the ratio of different relative time, and the reaction order can be determined by the time ratio method. In addition, the kinetic parameters can also be calculated further and the rate constant equals to $\tau_{p/q}$ divided by the corresponding reaction time for the first order reaction.

The different concentrations of the resulting complex C at different dissociation times were determined in the calculation of k_{off}. In order to adopt the time ratio method, the dissociation process of the interaction between 5.00 mmol·L^{-1} citalopram and 1.00 mmol·L^{-1} BSA was further monitored and the data obtained are shown in Table 3.

Through adjusting the applied voltage, the relative peak height of citalopram at different dissociation time can be obtained from the electropherograms, and then the corresponding concentration of resulting complex C can be determined by eq. (10) or (11). The relative peak height of citalopram at the dissociation time of infinitude equals to h_{a0} value ($a_0 = 2.50$ mmol·L^{-1}) since the resulting complex C has completely dissociated at this time. The relative peak height and concentration of the resulting complex C at the dissociation time of 0.00 s were calculated according to the fitting equation $y = 4778.9\cdot\ln(h) - 2041.3$ ($R^2 = 0.9886$) which exactly describes the logarithmic relationship between the dissociation time (y) and the relative peak height (h). Each relative concentration can be calculated on the premise that the concentration of resulting complex C at 0.00 s is the initial concentration. The values of $t_{3/4}$ and $t_{1/2}$ which are the dissociation times when the relative concentrations are 3/4 and 1/2, respectively, were determined to be 257.70 s and 622.32 s based on the fitting equation $y = -899.2\cdot\ln(x) - 1.0$ ($R^2 = 0.9961$) about the relationship between the dissociation time (y) and the relative concentration (x). Furthermore, the ratio of $t_{3/4}$ and $t_{1/2}$ which equal to the ratio of $\tau_{3/4}$ and $\tau_{1/2}$ was calculated to be 0.414. This value is very close to the characteristic

Table 2 The determination of k_{on} in plug-plug kinetic capillary electrophoresis

a_0 (mmol·L^{-1})	t_a (s)	b_0 (mmol·L^{-1})	t_b (s)	t_1 (s)	c_1 (mmol·L^{-1})	c_2 (mmol·L^{-1})	k_{on}
1.50	152.4	0.50	243.1	7.7	0.34	0.28	2.21×10^2
2.00	145.5	0.50	245.9	6.7	0.43	0.36	2.88×10^2
2.50	153.9	0.50	256.7	6.8	0.46	0.38	3.00×10^2
3.00	150.4	0.50	254.1	7.0	0.48	0.40	3.16×10^2
3.50	146.9	0.50	247.3	6.8	0.49	0.41	3.81×10^2

Table 3 The different concentrations of the resulting complex C at different dissociation times

Applied voltage (kV)	–	20	15	12	10	–
Dissociation time (s)	0.00	147.10	203.80	255.00	301.34	∞
Relative peak height	1.53	1.58	1.60	1.62	1.63	1.87
Concentration of C (mmol·L^{-1})	0.45	0.38	0.36	0.34	0.32	0.00
Relative concentration (p/q)	1.00	0.84	0.80	0.76	0.71	0.00

value of time ratio of 0.415 for the first order found in the literature [6]. Thus, this result illustrates that the dissociation process of the complex of citalopram and BSA accords with the law of first order reaction.

The value of $\tau_{3/4}$ for the first order reaction was 0.287 from table 1.2 in the literature [6] and k_{off} can be determined by the ratio of $\tau_{3/4}$ and the corresponding reaction time as following:

$$k_{off} = \tau_{3/4}/t_{3/4} = 1.11\times10^{-3}\ s^{-1}.$$

This value is basically consistent with the mean of k_{off} ($1.19\times10^{-3}\ s^{-1}$) determined by conventional ppKCE and it can be seen that the result obtained by the established method in this paper is accurate and reliable.

4.3 Discussion

4.3.1 The interaction between citalopram and BSA. There is no obvious peak of the complex C formed by the interaction of citalopram and BSA in the electropherogram. The possible reason for this may be because of overlapping of the peak corresponding to the complex with that corresponding to BSA completely, since the molecule weight of BSA is so great and the mobilities of the complex C and BSA are close to each other. The separation of BSA and the complex was hardly achieved under the applied electrophoresis condition. However, the calculation of the kinetic parameters will not be affected whether the complex can be detected or not. The quantity of the complex can be determined by the change of the peak height after the interaction of citalopram and BSA.

4.3.2 The determination of sampling order. A marker compound (label) was used for eliminating the influence of the change of voltage on peak heights in the experiment. The order of migration velocities is: citalopram > acetone > BSA, because citalopram, acetone and BSA are, respectively, positively charged, neutral and negatively charged in the experimental condition. Therefore, the sampling order, which was acetone solution, BSA and citalopram solution, will result in the calibration of the peak height of citalopram by the addition of acetone while the interaction between citalopram and BSA will not be affected.

4.3.3 The confirmation of the hypothesis. It was assumed that only the binding process occurs when the zone of A passes through the zone of B, and only the dissociation process occurs after the zones of A and B are separated. If this assumption is tenable, t_{pass} must be much shorter than the characteristic dissociation time of complex C, $t_{char,}$ which equals to $1/k_{off}$. t_{char} was calculated to be 840.34 s based on the value of k_{off}, then the relationship $t_{pass} = t_1 \approx 7 << t_{char}$ exists and the hypothesis can be satisfied. In other words, the actual interaction for the interaction system selected is consistent with our assumption, and the method of research and calculation is reliable.

5 Conclusion

A novel method of ppKCE, in which the kinetic parameters k_{on} and k_{off} and the binding constant K_b can be simultaneity determined, has been established for quick interaction systems. The interaction between citalopram and BSA was studied in this way and the relevant kinetic parameters and binding constant were calculated. The experimental result was verified by the time ratio method, and the similarity of the data obtained by the two different methods illustrates the accuracy and reliability of the newly established method. The new method shows the ease in manipulation and calculation and can effectively avoid the complex mathematical modeling and the strict requirement for signaling in conventional ppKCE. The requirements for separation resolution and detection sensitivity in this method are also comparatively low. It is the first application for ppKCE to the interaction between small molecules and protein, which has extended the application of kinetic capillary electrophoresis to the study of small molecules.

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