

# Investigation of calcium antagonist–L-type calcium channel interactions by a vascular smooth muscle cell membrane chromatography method

Hui Du · Jianyu He · Sicen Wang · Langchong He

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**Abstract** The dissociation equilibrium constant ( $K_D$ ) is an important affinity parameter for studying drug–receptor interactions. A vascular smooth muscle (VSM) cell membrane chromatography (CMC) method was developed for determination of the  $K_D$  values for calcium antagonist–L-type calcium channel (L-CC) interactions. VSM cells, by means of primary culture with rat thoracic aortas, were used for preparation of the cell membrane stationary phase in the VSM/CMC model. All measurements were performed with spectrophotometric detection (237 nm) at 37°C. The  $K_D$  values obtained using frontal analysis were  $3.36 \times 10^{-6}$  M for nifedipine,  $1.34 \times 10^{-6}$  M for nimodipine,  $6.83 \times 10^{-7}$  M for nitrendipine,  $1.23 \times 10^{-7}$  M for nicardipine,  $1.09 \times 10^{-7}$  M for amlodipine, and  $8.51 \times 10^{-8}$  M for verapamil. This affinity rank order obtained from the VSM/CMC method had a strong positive correlation with that obtained from radioligand binding assay. The location of the binding region was examined by displacement experiments using nitrendipine as a mobile-phase additive. It was found that verapamil occupied a class of binding sites on L-CCs different from those occupied by nitrendipine. In addition, nicardipine, amlodipine, and nitrendipine had direct competition at a single common binding site. The studies showed that CMC can be applied to the investigation of drug–receptor interactions.

**Keywords** Vascular smooth muscle · Cell membrane chromatography · Frontal analysis · Dissociation equilibrium constant · Binding site

H. Du · J. He · S. Wang · L. He (✉)  
Key Laboratory of Environment and Genes Related to Diseases,  
Ministry of Education, School of Medicine,  
Xi'an Jiaotong University,  
Xi'an 710061, China  
e-mail: helc@mail.xjtu.edu.cn

## Introduction

Drug–receptor interactions always play an important role in many biological processes, such as signal transduction, physiological regulation, and gene transcription [1, 2]. The interactions are usually investigated by means of radioligand binding assay (RBA) [3–5], receptor affinity chromatography [6–10], fluorescence resonance energy transfer [11, 12], affinity nuclear magnetic resonance [13], and computer-aided simulation design [14]. These techniques have been used for determination of the equilibrium dissociation constant ( $K_D$ ), the maximum adsorption quantity ( $q_{\max}$ ), and other characteristic parameters [15, 16]. For affinity chromatography, frontal analysis (FA) has been applied widely in studies on the interactions of saccharides with concanavalin A [17], cytochalasin B with membrane proteins [18], and drugs with plasma proteins [19]; the  $K_D$  value of inhibitor binding to the human red cell nucleoside transporter [20]; as well as quantitative description of protein adsorption [21]. There have also been some reviews of frontal chromatography in theory [22] and in applications [23–25]. However, it is still an experimental problem to use FA to measure accurately the  $K_D$  and  $q_{\max}$  values for drug–receptor interactions.

The cell membrane chromatography (CMC) method has been demonstrated to be a new bioaffinity chromatographic system in which the cell membrane stationary phase (CMSP) has bioactivity and chromatographic separation [26–28]. As a bionic chromatographic method, it has been used widely in investigations of drug–receptor interactions [29–33]. Nevertheless, few studies have measured the  $K_D$  values for drug–receptor interactions by using frontal CMC. L-type calcium channels (L-CCs) as a target for calcium antagonists [34, 35] are enriched mainly in cardiac or vascular smooth muscle (VSM) cell membrane [36, 37];

therefore, VSM cells have been used to study the interactions between calcium antagonists and L-CCs by a traditional RBA technique [4, 5]. Verapamil (VP) and the dihydropyridines, including nitrendipine (NT), bind to and block L-CCs on the inside and outside of the membrane, respectively, thus stopping ecto- $\text{Ca}^{2+}$  inflow and relaxing VSM. As a result of the different affinities of VP and NT binding to L-CCs, the retention characteristics of the ligands differ from each other on the VSM/CMC column (Fig. 1, part A).

In the present study, a frontal VSM/CMC method was established based on the theory of frontal chromatography. The  $K_D$  values and competitive characteristics of calcium antagonist–L-CC interactions were measured.

## Theory

According to the Clark receptor theory [38], the affinity interactions between ligand and receptor can be represented by the following reversible equation:



where L is the ligand, R is the receptor, and LR is a ligand–receptor complex. At equilibrium, the  $K_D$  value of this complex is given by the following equation:

$$K_D = \frac{[L][R]}{[LR]}. \quad (2)$$

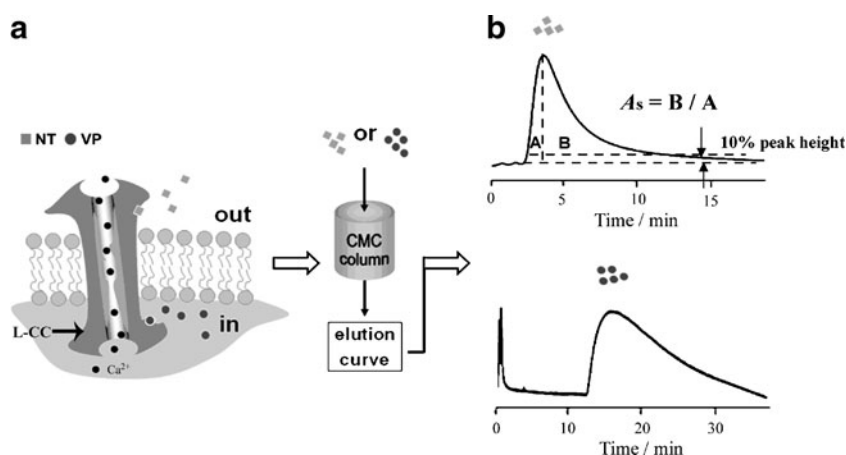
In the VSM/CMC model, the  $K_D$  value in Eq. 2 is investigated by using FA as the following equation:

$$K_D = \frac{[L]_m([R]_s - [LR]_s)}{[LR]_s}. \quad (3)$$

Equation 3 can be transformed to

$$\frac{1}{[LR]_s} = \frac{K_D}{[L]_m[R]_s} + \frac{1}{[R]_s}, \quad (4)$$

**Fig. 1** The analytical process of calcium antagonist–L-type calcium channel (L-CC) interactions using a vascular smooth muscle (VSM) cell membrane chromatography (CMC) method (a) and measurement of  $A_s$  (b). Squares nitrendipine (NT) domain on the outside, circles verapamil (VP) domain on the inside



where  $[L]_m$ ,  $[R]_s$ , and  $[LR]_s$  are the molar concentration of ligands in the effluent and the total concentrations of immobilized receptors and the complex at the surface of the stationary phase, respectively. The value of  $[LR]_s$  can be calculated by Eq. 5, and then the plot of  $1/[LR]_s$  versus  $1/[L]_m$  yields a straight line:

$$[LR]_s = (V_D - V_m)([L] - [L]_m), \quad (5)$$

where  $V_D$  is the effluent volume when the breakthrough curve reaches the inflection point,  $V_m$  is the dead volume of the column, and  $[L]$  is the total concentration of ligands in the mobile phase.

The capacity factor ( $k'$ ) of the chromatographic peak in the elution curve was calculated as follows:

$$k' = (t_R - t_0)/t_0, \quad (6)$$

where  $t_R$  is the retention time of the analyte, and  $t_0$  is the dead time of nonretained solvent.

In displacement studies, Eq. 7 [25, 39] is used to examine the location of the binding region on L-CCs:

$$\frac{1}{k' - X} = \frac{K_A[R]_s}{K_L V_M} [L]_m + \frac{K_A[R]_s}{V_M}, \quad (7)$$

where  $K_A$  and  $K_L$  are the equilibrium dissociation constants for the analyte and the ligand in the mobile phase, respectively.  $X$  is equal to  $k'$  at a competitor concentration above which the value of  $k'$  remains independent of  $[L]_m$  [40]. A linear regression of  $1/(k' - X)$  versus  $[L]_m$  is obtained.

## Experimental

### Materials and reagents

Imperatorin (IMP), nifedipine (NF), nimodipine (NM), NT, nicardipine (NC), amlodipine (AL) and VP as standard

drugs were provided by the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Gefitinib (GEF) was purchased from Nanjing Ange Pharmaceutical (Nanjing, China). Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical (Qingdao, China). All other reagents and solvents were of analytical reagent grade and were used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

#### Apparatus and conditions

CMC analysis was performed with a Shimadzu LC-20A apparatus that consisted of two LC-20 AD pumps, a DGU-20A3 degasser, an SIL-20A autosampler, a CTO-20A column oven, and an SPD-M20A diode-array detector (Shimadzu, Kyoto, Japan). The data were acquired by the LCSolution program (Shimadzu).

The mobile phase consisted of phosphate-buffered saline (PBS; 5 mM, pH 7.4) and was delivered at a flow rate of 0.2 mL min<sup>-1</sup>. The detection wavelength was 237 nm, and the column temperature was 37°C.

The molar extinction coefficients of six calcium antagonists at 237 nm were measured using a UV-2450 spectrophotometer equipped with the UVProbe program (Shimadzu, Kyoto, Japan), and the relative values were 1.72 (NF), 3.08 (NM), 2.91 (NT), 3.69 (NC), 2.20 (AL), and 1.00 (VP).

#### Preparation of standard solutions

The stock solutions (5 × 10<sup>-3</sup> M) of GEF, IMP, NF, NM, NT, NC, AL, and VP were prepared by separately dissolving the standard drugs in methanol. Standard solutions at various concentrations were prepared by diluting the stock solutions with the mobile phase for IMP, GEF, NF, NM, and NT and with methanol for NC, AL, and VP.

#### Preparation of VSM/CMSP

Sprague–Dawley rats (5–7 weeks old, 250 g) were supplied by the Experimental Animal Centre of Xi'an Jiaotong University (Xi'an, China). All experimental protocols involving animals were reviewed and approved by the Institutional Animal Experimentation Committee of Xi'an Jiaotong University. The tissue-ticking method was used to obtain primary cultures of VSM cells from thoracic aortas. The VSM cell membrane was prepared as described previously [27]. The denatured VSM cell membrane was also prepared by adding 10 gL<sup>-1</sup> trichloroacetic acid to a normal cell membrane suspension (1:4, v/v), and heating the mixture at 90°C for 10 min. Briefly, 0.04 g silica was activated at 105°C for 60 min and used as a carrier. It was

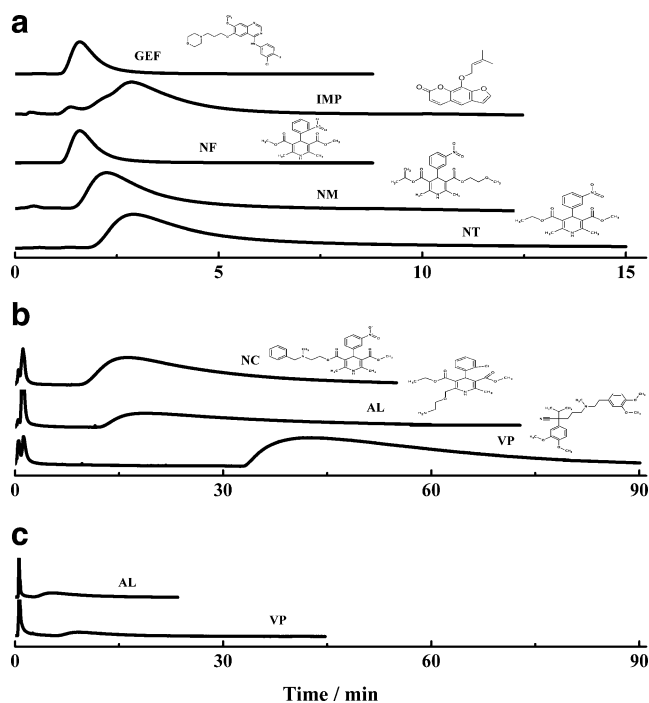
then homogenized with the VSM cell membrane suspension under a vacuum and with agitation at 4°C. The next day, the CMSP was washed with 5 mM PBS ten times, and the mixture obtained was packed into the column by a wet method (10 MPa, 3 min) to yield a VSM/CMSP column (10 mm × 2.0 mm, 5 μm).

#### Measurement of the asymmetry factor ( $A_s$ )

The  $A_s$  value was defined as the distance from the center line of the chromatographic peak to the back slope divided by the distance to the front slope, with all measurements made at 10% of the maximum peak height (Fig. 1, part B), and was employed to evaluate the reversibility of the affinity action of calcium antagonists binding to the L-CCs in the VSM/CMC system.

#### Determination of $K_D$ values

$K_D$  values of calcium antagonists were determined by means of the VSM/CMC system as follows. Standard solutions of each drug were separately added to the mobile phase to obtain a set of new mobile phases with a



**Fig. 2** Elution profiles of gefitinib (GEF), imperatorin (IMP), nifedipine (NF), nimodipine (NM), and NT (a) and nicardipine (NC), amlodipine (AL), and VP (b) on the VSM/cell membrane stationary phase (CMSP) column and AL and VP on the denatured VSM/CMSP column (c). VSM/CMSP column 10 mm × 2.0 mm; flow rate 0.2 mL min<sup>-1</sup>; column temperature 37°C; detection wavelength 237 nm; mobile phase 5 mM phosphate-burred saline, pH 7.4

**Table 1** Affinity characteristics of calcium antagonist–L-type calcium channel interactions obtained by a vascular smooth muscle cell membrane chromatography (CMC) method ( $n=5$ )

Ligands	$A_s$	$[R]_s$ (mol)	$K_D$ (M)	$R$
Nifedipine	1.90±0.10	$(1.52±0.23) \times 10^{-10}$	$(3.36±0.28) \times 10^{-6}$	0.9988
Nimodipine	2.94±0.16	$(3.21±0.35) \times 10^{-10}$	$(1.34±0.15) \times 10^{-6}$	0.9995
Nitrendipine	3.80±0.19	$(3.25±0.24) \times 10^{-10}$	$(6.83±0.48) \times 10^{-7}$	0.9991
Nicardipine	6.91±0.23	$(3.57±0.31) \times 10^{-10}$	$(1.23±0.16) \times 10^{-7}$	0.9878
Amlodipine	7.28±0.31	$(6.97±0.47) \times 10^{-10}$	$(1.09±0.09) \times 10^{-7}$	0.9991
Verapamil	7.35±0.35	$(1.21±0.19) \times 10^{-9}$	$(8.51±0.61) \times 10^{-8}$	0.9880

concentration range of  $2.22 \times 10^{-8}$ – $2.96 \times 10^{-7}$  M. This mobile phase with different concentrations of each drug was separately pumped through the VSM/CMC column at a flow rate of  $0.2 \text{ mL min}^{-1}$  under the gradient program and the breakthrough curves of the drug with different concentrations were recorded. In accordance with Eq. 4, the corresponding graph of the reciprocal value of  $[LR]_s$  versus  $[L]_m$  was obtained. Moreover,  $K_D$  could be determined by calculating the ratio of the intercept to the slope, and  $[R]_s$  was obtained from the inverse of the intercept.

### Competitive binding assay

A competitive binding assay was studied by using NT as a mobile-phase additive at different concentrations ranging from  $2.83 \times 10^{-8}$  to  $5.66 \times 10^{-7}$  M, and small quantities of calcium antagonists ( $10^{-6}$  M) were injected onto the column. The void time of the VSM/CMSP column was determined by making similar injections of a nonretained compound (GEF). The values of  $k'$  and  $X$  were evaluated over a series of competitor concentrations and, in accordance with Eq. 6, a linear relationship was obtained by plotting  $1/(k' - X)$  versus  $[L]_m$ .

### Data analysis

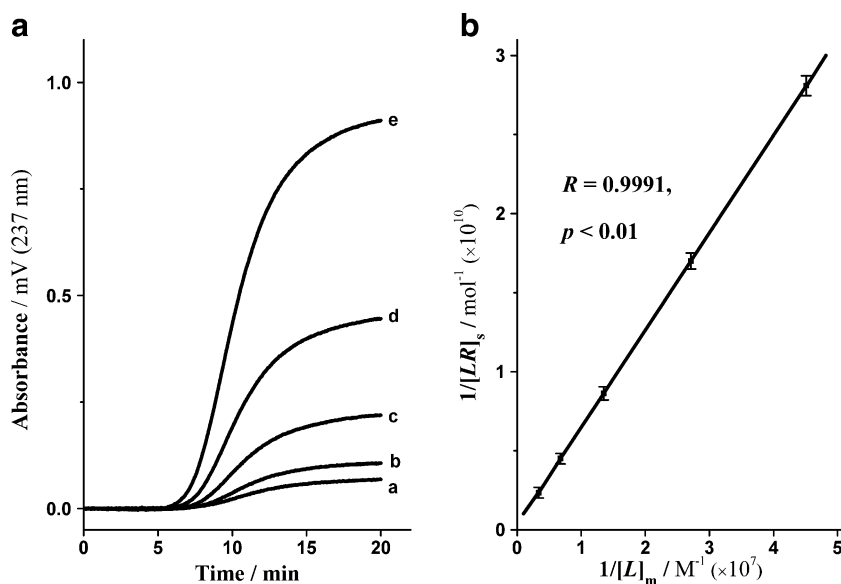
The affinity parameters  $A_s$ ,  $[R]_s$ , and  $K_D$  were obtained from the elution profiles and breakthrough curves of the interactions of six calcium antagonists with L-CCs. The SPSS software package (version 13.0 for Windows; SPSS; Chicago, IL, USA) was used in the calculations. All data were expressed as means  $\pm$  the standard error of the mean ( $n \geq 3$ ) and values of  $P < 0.01$  (two-tailed) were regarded as indicative of statistical significance.

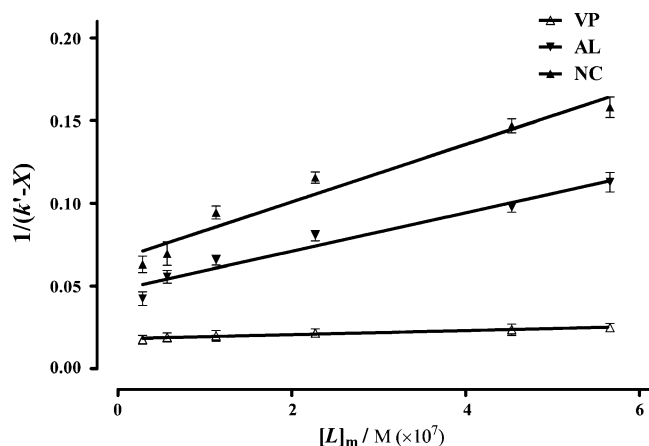
## Results and discussion

### Specificity and reversibility on the L-CCs

The elution profiles of eight ligands on the VSM/CMSP column are shown in Fig. 2 (profiles A, B). It was found that the retention times of the ligands differed on the column, and the  $k'$  value of VP was maximal among the eight ligands. As shown in Fig. 2, profiles A, GEF, a specific antagonist acting on epidermal growth factor receptor, having a structure similar to that of NC, had no

**Fig. 3** VSM/CMC breakthrough curves of NT (a) and a regression curve achieved by plotting  $1/[LR]_s$  versus  $1/[L]_m$  (b). Each point with a bar represents the mean  $\pm$  the standard error of the mean (SEM) ( $n=5$ ). In a the NT concentrations (a–e) were  $2.22 \times 10^{-8}$ ,  $3.70 \times 10^{-8}$ ,  $7.40 \times 10^{-8}$ ,  $1.48 \times 10^{-7}$ , and  $2.96 \times 10^{-7}$  M, respectively. VSM/CMSP column  $10 \text{ mm} \times 2.0 \text{ mm}$ ; flow rate  $0.2 \text{ mL min}^{-1}$ ; column temperature  $37^\circ\text{C}$ ; detection wavelength 237 nm





**Fig. 4** Competitive displacement graph of NC, AL, and VP using NT as a mobile-phase additive ranging from  $2.83 \times 10^{-8}$  to  $5.66 \times 10^{-7}$  M. Each point with a bar represents the mean  $\pm$  SEM ( $n=5$ ). VSM/CMSP column 10 mm $\times$ 2.0 mm; flow rate 0.2 mL min<sup>-1</sup>; injection volume 5  $\mu$ L; column temperature 37°C; detection wavelength 237 nm

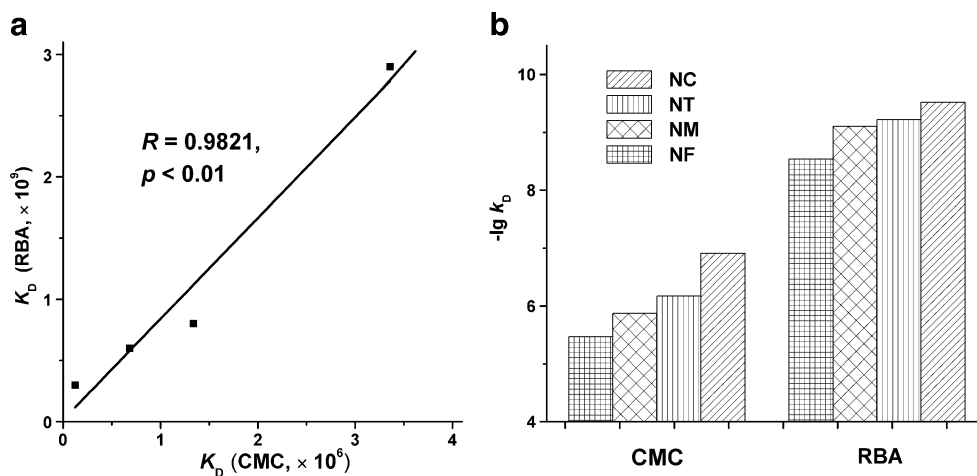
retention on the VSM/CMSP column. IMP had a retention equivalent to that of NT, and related research suggested that IMP induces vasodilatation by possible mechanisms inhibiting the voltage-dependent calcium channel [41]. The column packed with denatured cell membrane was employed as a control. As shown in Fig. 2, profiles C, the retention times of AL and VP were very short compared with those in the normal VSM/CMSP column (Fig. 2, profiles B). The results indicate that the retention on the VSM/CMSP column was mostly caused by the specificity of the interactions between the drug and the receptor. In addition,  $A_s$  was used to investigate the reversibility of ligand–receptor interactions, and the results are given in Table 1. The  $A_s$  values of AL and VP were lower, which suggests that AL and VP, with stronger binding affinity, were much more difficult to elute on the VSC/CMC

column. Therefore, the reversibility of AL or VP interacting with L-CCs was poorer than that of other ligands. Measurement of the  $A_s$  values is an effective way to screen for long-acting calcium antagonists.

#### Affinity on the L-CCs

Representative VSM/CMC breakthrough curves for NT obtained by FA are presented in Fig. 3a. The mean position of the curves shifted to shorter breakthrough times as the concentration of NT in pH 7.4 PBS was increased. According to Eq. 4, the degree of the shift is related to the concentration of the ligands in the mobile phase, the  $K_D$  values, and the total concentrations of the immobilized receptors. Plots of  $1/[LR]_s$  versus  $1/[L]_m$  were obtained using the FA data from Fig. 3a, with five replicates on five different VSM/CMC columns, and each plot for NT gave a linear relationship over all the concentrations studied ( $n=5$ ,  $r=0.9991$ ,  $p<0.01$ ) (Fig. 3b). From Eq. 4, this linear behavior suggested that NT was binding to a single type of site on the L-CCs. Graphs similar to those in Fig. 3b were obtained for other calcium antagonists over the same concentration range, and the linear behavior was again seen under the same VSM/CMC conditions. In this case, the correlation coefficients varied from 0.9878 to 0.9995 over the five different concentrations of ligands tested. As shown in Table 1, the  $K_D$  value for VP was lower than the values for the other calcium antagonists. The affinity rank order measured from the VSM/CMSP column for six ligands was NF ( $3.36 \times 10^{-6}$  M) > NM ( $1.34 \times 10^{-6}$  M) > NT ( $6.83 \times 10^{-7}$  M) > NC ( $1.23 \times 10^{-7}$  M) > AL ( $1.09 \times 10^{-7}$  M) > VP ( $8.51 \times 10^{-8}$  M), which was proportional to the  $k'$  values of the calcium antagonists. The calculated  $[R]_s$  values are also presented in Table 1. It was shown clearly that the number of sites for VP binding to the VSC/CMC column ranked first, whereas for NM, NT, and NC, the numbers were almost same.

**Fig. 5** The correlation (a) and diversity (b) between  $K_D$  values obtained from the VSM/CMC and radioligand binding assay (RBA) methods



## Analysis of binding sites

Displacement studies were used to determine whether or not calcium antagonists shared a common binding site on the L-CCs. Initial studies were performed by injecting a small amount of NT ( $10^{-6}$ M) onto the VSM/CMSP column, while continuously increasing the concentration of NT in the mobile phase. A decrease in  $k'$  values was observed in the range  $2.83 \times 10^{-8}$ – $5.66 \times 10^{-7}$ M, without saturation of the binding sites. In addition, this method was employed in investigations of the displacement of NC, AL, and VP, with five replicates on five different VSM/CMC columns, respectively. The corresponding graph of the reciprocal values of ( $k'-X$ ) versus  $[L]_m$  is presented in Fig. 4. The fitting lines for VP, AL, and NC are  $y=0.01807+0.001229x$  ( $r=0.9627$ ),  $y=0.04757+0.01165x$  ( $r=0.9579$ ), and  $y=0.06612 + 0.01738x$  ( $r=0.9581$ ), respectively. The slope of the regression curve for VP was much lower than that for NC or AL, which suggested that VP interacted with L-CCs in a manner different from NT [25]. In addition, this type of response was consistent with NC, AL and NT having direct competition at a single common binding site. In fact, it has been reported that there are three different binding sites on the  $\alpha_1$  subunit of L-CCs, named DHP, Verapamil, and Diltiazem [42]. It can be concluded that the value of the slope reflected the possibility that the competitor and ligand occupied the same class of binding sites on L-CCs.

According to the investigation of the interactions between known-target calcium antagonists and L-CCs, the displacement studies can provide some useful information on binding sites with ligand–receptor interaction.

## Correlation with the RBA method

The affinity rank order of the four ligands was consistent with that reported previously [43], and the coefficient indicated that there was a strong positive correlation between  $K_D$  determined using the VSM/CMC method and the published  $K_D$  values ( $r=0.9821$ ,  $p<0.01$ ) (Fig. 5a). NC had the strongest binding affinity among the four ligands, whereas NF ranked last (Fig. 5b). The CMC system is convenient and feasible for determining the ligand–receptor  $K_D$  values, and the results obtained from the VSM/CMC model also indicate the diversity and variety of calcium antagonist–L-CC interactions.

## Reliability of the VSM/CMC method

The reproducibility of the VSM/CMC method was performed by injecting  $10^{-6}$ M standard solution of NT onto the different VSM/CMSP columns ( $n=6$ ), and the relative standard deviations of the retention times and peak areas

were 4.67 and 9.15%, respectively. In addition,  $10^{-6}$ M standard solution of NT was continually injected into the system ( $n=50$ ), and the results showed that the retention time decreased by merely 8.39%. The lifetime of these columns is about 3 days under trial conditions, all of which met the assay requirements.

## Conclusion

We developed a VSM/CMC method, which combined high-performance liquid chromatography, cytobiology, and receptor pharmacology, and simulated the interaction of calcium antagonists with L-CCs. With use of FA, the characteristic parameters were calculated, and the results correlated positively with the  $K_D$  values reported in the literature as obtained using the RBA technique. The specificity, reversibility, and competitive binding for calcium antagonist–L-CC interactions were validated successfully.

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