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Competition between phenylalanine and acetic acid in a chromatographic column as indicated by their adsorption isotherms

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ABSTRACT

    Competition between phenylalanine and acetic acid was investigated through their adsorption isotherms when both compounds were used as mobile phase additives. Acetic acid was the acid component of an acetate buffer of pH 3.7. The system peak corresponding to phenylalanine was used for measurement of its adsorption isotherms at four different buffer concentrations, during detection at 254 nm. The adsorption isotherms showed a considerable influence of the acetic acid on the retention of phenylalanine. Therefore, acetic acid was considered to be a mobile phase additive that modifies the stationary phase and competes with the phenylalanine. The solubility of the phenylalanine was measured at four different buffer concentrations, and appeared to be constant. Therefore, a decrease in retention due to solubility changes was ruled out. The system peak corresponding to the acetic acid was used for the determination of the adsorption isotherm of acetic acid using detection at 220 nm. Phenylalanine enabled the visualization of the same system peak at 254 nm. Comparison of the capacity factor of the acetic acid system peak, obtained in the presence of phenylalanine in the mobile phase, with that of the same peak without phenylalanine showed very small differences over a wide range of concentrations. Therefore, it was concluded that distribution of acetic acid was not much affected by the presence of the other component in the mobile phase. Phenylalanine was injected at overload concentrations into mobile phases containing the acetate buffer only. The peak shapes and retentions behaved generally as expected from the adsorption isotherms.

INTRODUCTION

    The chromatographic separation of ionizable solutes such as amino acids is usually optimized by changing the buffer concentration in the mobile phase. Acetate buffer is commonly used for the separation of ionizable compounds[1]. The buffer is used to maintain a constant degree of ionization of the solute and to maintain a constant ionic strength. However, acetic acid can also modify the stationary phase

and change the retention of solutes. In this work we studied the influence of the buffer concentration on the capacity ratio of ionizable solutes as a result of competition between them. The ionizable component chosen for this purpose was phenylalanine, an amino acid that shows absorption at 254 nm.

A mobile phase that contains buffer is multi-component. It has at least three components in addition to water, the acid or base in their molecular form and the cations and anions of the salt. In the case of the acetate buffer in our system, the pH was 3.7, and it contained acetic acid and sodium acetate in the ratio of 9:1. Molecular acetic acid is adsorbed on the stationary phase to some extent[2–4] and therefore it modifies the surface of the column. At high concentrations and a constant pH, the acetic acid can be seen as a component that plays the role of an organic solvent modifier, at least in terms of competition with the solute on the stationary phase. The interaction between acetic acid and phenylalanine was studied here by using both compounds in the mobile phase at various compositions. Competition was investigated by studying the system peaks of phenylalanine and acetic acid in the vacancy mode.

System peaks appear in the chromatograms in addition to solute peaks when a sample different from the mobile phase is injected, under the following circumstances: the mobile phase is multi-component; some or all the mobile phase components show a high detector background; and part or all of the mobile phase components are adsorbed on the stationary phase. All types of chromatographic systems display these peaks: reversed- and normal-phase[5–10], ion-pair with UV detection[1,11–27], ion-exchange[28–31] and even gel permeation[32–36] systems.

Some background on the origins, significance and behaviour of system peaks has been described previously[2–5,22–27,37–39,40–42]. When a mobile phase component is adsorbed on the stationary phase, its corresponding system peak has a capacity factor greater than zero. Identification and quantitative treatment of system peaks permitted the calculation of the capacity factors of mobile phase components[2–4]. It has also been shown that the adsorption isotherm of a particular mobile phase component can be calculated from the corresponding system peak[3].

A theoretical and experimental treatment of system peaks in non-linear chromatography was undertaken by Golshan-Shirazi and Guiochon[37–39], using conditions in which competition between the solute and mobile phase components occurs. It was shown that injection of a solute into a binary mobile phase was accompanied by two system peaks in addition to the solute peak. One of the two system peaks was eluted according to the capacity factor of the corresponding mobile phase component and the other accompanied the solute peak. Therefore, in principle, a mobile phase component can show up in more than one system peak.

System peaks were used in ion-pair chromatography with UV detection of solutes with no chromophore[21–27]. An ion-pairing UV-absorbing agent was added to the mobile phase to manipulate the selectivity and to reveal the solutes. The sample components were eluted according to their particular capacity factors, and could show up in the chromatogram owing to the presence of the detection agent in the mobile phase. Peaks of solutes with no chromophore could be revealed in this chromatographic system owing to the presence of an excess or a lack of mobile phase component in each of the solute zones. An excess or lack of a mobile phase component, exhibited as a peak, is actually a system peak. As the detector was selective only for the mobile

phase additive, the recorded chromatograms displayed only system peaks. Thus, in chromatography with UV detection, where the solute is detected via the detection agent owing to ion pairing or competition, the peak recorded in the chromatogram as the solute peak is actually a system peak.

Phenylalanine was used as the detection agent in this work; the detector, operating at 254 nm, was selctive for phenylalanine only. The strongly adsorbed additive in the mobile phase was phenylalanine and acetic acid served as the weakly adsorbed component. Various buffer and phenylalanine concentrations were used, with different degrees of interference or competition between them.

THEORY

When the mobile phase is introduced into the chromatographic column, acetic acid and phenylalanine are partitioned between the two phases according to their adsorption isotherms. Equilibrium is obtained when the ratio between the amounts of the components in the mobile and the stationary phases remains constant. At that point, the composition of the effluent is constant, the concentration of the mobile phase component is C_{eq} and the amount on the stationary phase is Q_{eq}. At low mobile phase additive concentrations, the dependence of the amounts of phenylalanine and acetic acid in the stationary phase, $Q_{eq,P}$ and $Q_{eq,A}$, respectively, on their concentrations in the mobile phase, $C_{eq,P}$ and $C_{eq,A}$, respectively, is described by the individual adsorption isotherm of each component. However, at high concentrations, $Q_{eq,P}$ may also depend on the concentration of the acetic acid, $C_{eq,A}$, and $Q_{eq,A}$ might depend also on $C_{eq,P}$. In this case a competitive adsorption isotherm can describe the mutual dependence[39].

In this work we used system peaks in the vacancy mode of chromatography to study competitive conditions. A pure solvent with no additives is injected in this mode, *i.e.*, the injection volume becomes vacant of phenylalanine and acetic acid relative to the equilibrium state. The relative vacancy induces desorption of net amounts of dQ_P and dQ_A moles from the extraction layer on the stationary phase for re-equilibration. The amount on the stationary phase within the injection volume after re-equilibration is Q_i, rather than $Q_{eq,i}$, so that

$$Q_{eq,i} - Q_i = dQ_i \qquad i = A, P \tag{1}$$

In the vacancy mode, $dQ > 0$, the amount being released from the stationary phase into the injection volume to compensate for the vacancy. The difference dC_i between the equilibrium concentration and the new concentration within the injection volume is minute when the sample volume is very small:

$$C_{eq,i} - C_{m,i} = dC_i \tag{2}$$

The small disturbance dC_i of the particular component moves down the column at a constant velocity, dictated by its adsorption properties $[u_0/(1 + dQ_i/dC_i)]$. This disturbance is called the system peak. As mentioned above, Golshan-Shirazi and Guiochon[39] showed that injection of a solute can result in more than one system peak for a mobile phase component. Nevertheless, there is always one system peak that

elutes according to a particular component's capacity factor, k'_i, which can serve for the measurement of its adsorption isotherm.

The ratio dQ_i/dC_i of each solute is actually its k'_i, which is directly proportional to the slope of its adsorption isotherm. Hence the adsorption isotherm can be measured from k'_i of the respective system peak, using the following equation:

$$Q_{eq,i} = \int_0^{C_{eq,i}} k'_i \, dC_i \qquad (3)$$

This is the principle on which the minor perturbation or system peak method for adsorption isotherm measurements is based. The concentration of the particular component in the mobile phase is changed, then the respective k' of the system peak is measured experimentally according to the equation $k' = (t_R - t_0)/t_0$. The capacity factor corresponding to the particular components k'_i is multiplied by the difference in its concentration, dC_i, in each step, and summation is done over the range of concentrations from 0 to the particular point $Q_{eq,i}$.

EXPERIMENTAL

Materials

The mobile phase was prepared by dissolving the appropriate amounts of phenylalanine (Merck, Darmstadt, F.R.G.) in acetate buffer (pH 3.7). The acetate buffer consisted of analytical-reagent grade sodium acetate (Merck)–acetic acid (Frutarom, Haifa, Israel) (1:9).

Instrumentation

The high-performance liquid chromatographic (HPLC) system that was used in most of the experiments included a Model 880-UP single-line high-pressure pump (Jasco, Tokyo, Japan) with a Rheodyne injection valve using a 20-μl loop. The chromatograms were detected with a UV-1 single-path monitor (Pharmacia, Bromma, Sweden) at 254 nm in most instances and at 280 nm in some of the adsorption isotherm measurements when the background of the mobile phase was too high for the detector to balance. An SP8300 UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.) operated on 254 nm was used when high concentrations of phenylalanine were injected. All the chromatograms were recorded on a single-pen Unicorder U-228 recorder (Pantos, Nippon Danshi Kagashu, Japan).

Adsorption isotherms of acetic acid were measured using another chromatographic system: the solvent delivery system was a Hitachi L-6200 (Merck) and the detector was an HP series 1050 (Hewlett Packard, Palo Alto, CA, U.S.A.) operating at 220 nm, with an HP 3396A integrator (Hewlett Packard).

The temperature was kept constant to within ± 0.5°C using a circulating water-bath.

Procedure

All the chromatographic runs were done with a LiChrosorb RP-18 cartridge (250 × 4 mm I.D.) (Merck). The temperature was kept constant at 30°C throughout

the experiments and the flow-rate was 1.5 ml/min. The system was thoroughly washed with acetonitrile–water occasionally, especially between changes in the mobile phase composition. At least 15 column volumes were eluted until a stable baseline was obtained, when phenylalanine was added to the mobile phase.

Measurements of adsorption isotherms

Adsorption isotherms of phenylalanine and sodium acetate were measured by integration of the system peak capacity factors. The concentration of phenylalanine in the mobile phase, C_{eq}, was gradually changed at each buffer concentration and the system peak capacity factors were measured. The calculation is explained in the Theory section.

Solubility of phenylalanine

An excess amount of phenylalanine (0.005 mol) was added to 25 ml of the four buffer concentrations, 0.01, 0.1, 0.5 and 1 mol/l, to obtain precipitates. The saturated solutions were stirred overnight, filtered, diluted (1:40) and re-injected into the HPLC system, with a 1 M buffer concentration in the mobile phase. Peaks areas were converted to concentrations by a calibration graph.

RESULTS AND DISCUSSION

Measurements of adsorption isotherms from system peaks

The chromatographic system described here consisted of a reversed-phase column and an aqueous solution of acetate buffer and phenylalanine as the mobile phase. The chromatograms obtained at four buffer concentrations are shown in Fig. 1. Detection was effected at 254 nm, so phenylalanine could serve for the detection of all three system peaks, marked A, B and C in Fig. 1. Peak A corresponds to an unretained species, whereas B and C can both be related to species adsorbed on the stationary phase. The retention times of peaks A and B can be related to acetate salt and acetic acid, respectively, in agreement with a previously described system, which included acetate buffer[3]. Peak C eluted at a capacity factor corresponding to phenylalanine. The peaks could appear negative or positive. Peaks A and B at buffer concentrations 0.5 and 1 M had a derive-like shape, probably due to interference from the acetate or to refractive index perturbations in the flow cell at the extremely high concentration. A peak appears negative when the detector response to the sample zone is lower than that to the mobile phase. At low acetate concentrations peaks A and B were small because the interference of phenylalanine with the other components was small.

Phenylalanine. Adsorption isotherms of phenylalanine were calculated from the capacity factors of system peak C at increasing phenylalanine concentrations in the mobile phase. Four adsorption isotherms, measured at different concentrations of the acetate buffer in the mobile phase, are shown in Fig. 2. In addition, the adsorption isotherm of the acetic acid was calculated from peak B capacity factors at increasing buffer concentrations with no other additives. The parameters a and b for phenylalanine and acetic acid, which were calculated according to the Langmuir expression:

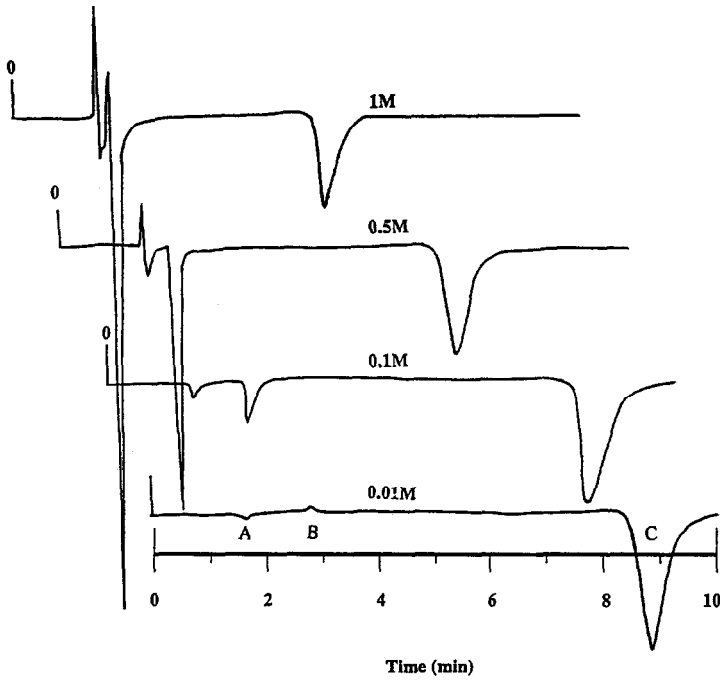$$Q_{eq,i} = a_i C_{eq,i}/(1 + b_i C_{eq,i}) \qquad (4)$$

Fig. 1. Chromatograms obtained when water was injected into the mobile phase. The buffer concentrations are marked on the chromatograms. The phenylalanine concentration was 0.005 M and full-scale was 0.04 absorbance in all four instances.
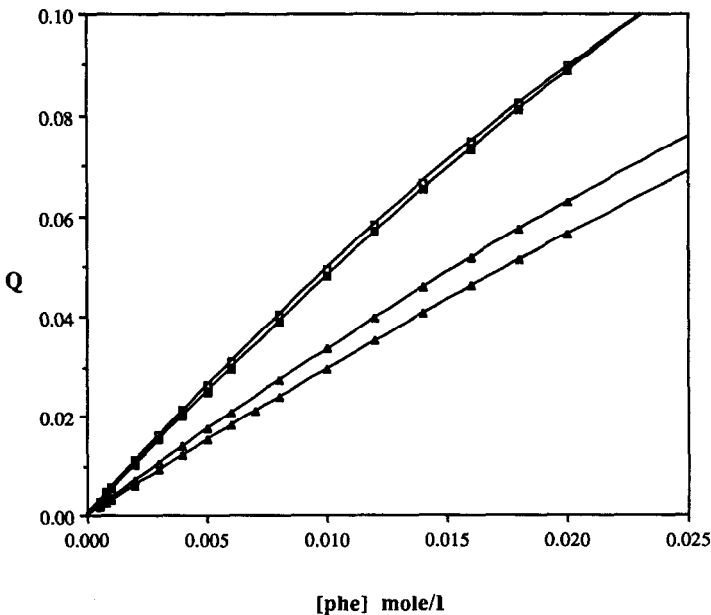

Fig. 2. Adsorption isotherms of phenylalanine at four different phenylalanine concentrations in the mobile phase. Buffer concentrations: $\square$ = 0.01; $\blacksquare$ = 0.1; $\triangle$ = 0.5; $\blacktriangle$ = 1.0 M.

TABLE I

ADSORPTION ISOTHERM PARAMETERS OF PHENYLALANINE AT FOUR DIFFERENT
ACETATE BUFFER CONCENTRATIONS AND THE INDIVIDUAL ADSORPTION ISOTHERM
OF ACETIC ACID

Compound	Buffer concentration (mol/l)	a	b
Phenylalanine	0.01	5.85	19.65
	0.1	5.35	13.76
	0.5	3.66	9.6
	1	3.14	6.89
Acetic acid	No phenylalanine	1.04	1.91

are given in Table I. Parameter a is actually the slope of the isotherm close to the origin,
where it is linear. Parameter b is proportional to the saturation concentration in the
column, namely the concentration where the isotherm levels off. It can be seen that
both a and b were affected by the buffer concentration. The capacity factor of
phenylalanine decreased at the origin and over the whole range of concentrations, in
addition to the saturation concentration. These effects were caused by the increase in
the acetic acid concentration. The acetic acid served as an organic modifier that
competes with the phenylalanine on the stationary phase partition sites. In order to
verify that the decrease in the retention of phenylalanine was not due to the effect of
ionic strength on the solubility, a test was made. Solubility was measured by preparing
saturated solutions of phenylalanine at the four buffer concentrations. The phenyl-
alanine solubility, as shown in Table II, was not significantly changed, hence it
certainly could not be responsible for the considerable change in the retention.

The isotherm parameters a_i and b_i used in eqn. 4 should actually be expressed in
mathematical terms to depict the competition between the solutes over the whole range
of compositions. For that purpose, there is a need for measurements of an appropriate
set of adsorption isotherms at various mobile phase compositions to represent the
whole range of competitive conditions. A systematic measurement of k' of the system
peaks of phenylalanine and acetic acid at various concentration ratios is the key for the
overall competitive isotherm. A competitive adsorption isotherm was used by
Golshan-Shirazi and Guiochon[39]:

$$Q_1 = a_1 C_1/(1 + b_1 C_1 + b_2 C_2) \qquad (5)$$

TABLE II

SOLUBILITY OF PHENYLALANINE IN FOUR ACETATE BUFFER CONCENTRATIONS (pH
3.7) AT ROOM TEMPERATURE

Buffer concentration (mol/l)	Phenylalanine solubility (mol/l)
0.01	0.158
0.1	0.135
0.5	0.141
1.0	0.153

In our system component 1 was acetic acid and 2 was phenylalanine. Here, the terms a and b from each individual adsorption isotherm, measured with no competitors, should be used. We took the parameters a and b obtained at 0.01 M buffer, $i.e.$, the conditions with the minimum competition, as the parameters of the individual isotherm of phenylalanine. Q values were calculated at buffer concentrations of 0.1, 0.5 and 1 M according to eqn. 5 using the isotherm parameters for acetic acid in Table I. Poor agreement was found between the calculated values and $Q_{eq,P}$ values measured by integration of the system peak capacity factor. The measurement of the individual isotherm of phenylalanine and the fit of eqn. 5 to the experimental isotherms obtained at various mobile phase compositions is currently under study.

   Acetic acid. The capacity factor of peak B was not affected much by changes in the phenylalanine concentration in the mobile phase, as can be seen from Fig. 3. This figure shows the curves for k' of peak B as a function of acetic acid concentration with and without phenylalanine. Integration of the curve with no phenylalanine gave the individual adsorption isotherm of acetic acid from which the a and b values in Table I were calculated. The other curves show k' for peak B as function of the concentration of acetic acid at four concentrations (0.009, 0.09, 0.45 and 0.9 M). Each curve corresponds to a different phenylalanine concentration in the mobile phase as indicated.

   The curves lie very close together, indicating that the presence of phenylalanine had almost no effect on the distribution of acetic acid. This observation is in agreement with a previous publication, in which the column void volume was calculated from the
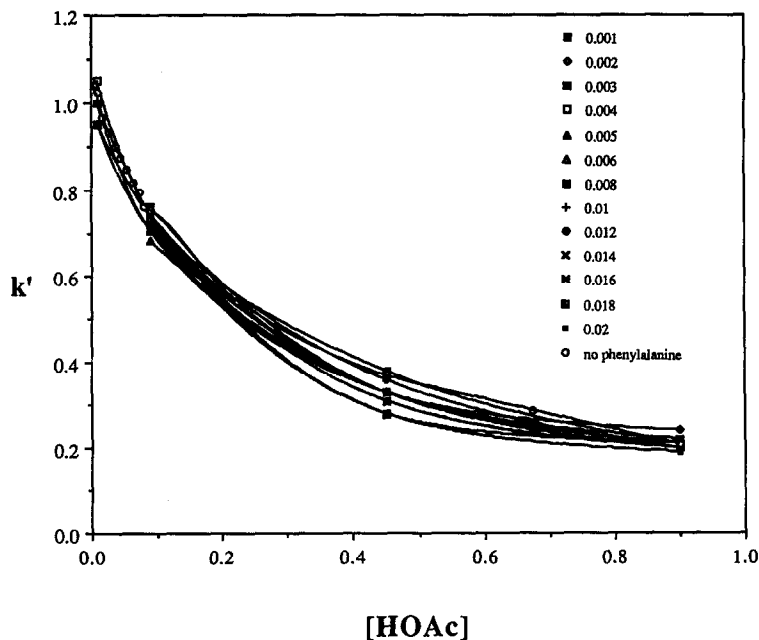

[HOAc]

Fig. 3. Capacity factor of peak B as function of the acetic acid (HOAc) concentration in the mobile phase at various phenylalanine concentrations (as specified, in M).

acetic acid system peak[4]. In this work, the presence of various alkylsulphonates had little influence on the capacity factor of the acetic acid.

Adsorption isotherms of mixtures can also be measured by frontal analysis, provided that the inflection points in the breakthrough curves are distinguishable. Comparison of adsorption isotherms obtained by system peaks with other methods are under investigation.

Peak shape under overload conditions

The relationship between adsorption isotherms and peak shape in non-linear chromatography was intensively investigated by Guiochon[43]. The chromatograms of phenylalanine at an overloading concentration (0.1 *M*) injected into four different mobile phases are shown in Fig. 4. The concentrations of the buffer in the mobile phase are specified on the chromatograms. The most important points to note are the peaks shapes and retention. The peaks have sharp fronts and diffuse rears, as expected from their adsorption isotherms.

Retention of the phenylalanine peak in Fig. 4 decreased with increase in buffer concentration. This decrease in retention agrees with the behaviour of the adsorption isotherms due to higher concentrations of acetic acid in the mobile phase.
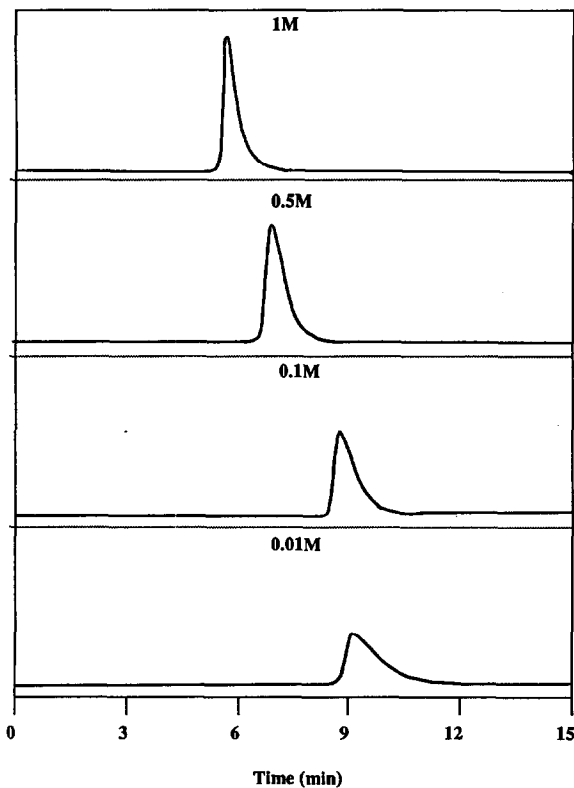

Fig. 4. Overload concentration (0.1 *M*) of phenylalanine injected into four different buffer concentrations, indicated.

CONCLUSION

When using an organic buffer in the mobile phase, it should be taken into account that the buffer can modify the stationary phase, in addition to its role in keeping the degree of ionization of the solute constant. The retention of ionizable solutes can be affected by the molecular acid or base component of the buffer, which may act like an organic modifier in the mobile phase. This point is especially important in the non-linear range of chromatography, where competition between components dictates parameters such as the separation efficiency and solute purity. Therefore, competitive adsorption isotherms are needed in order to predict the conditions and the extent of competition among the solutes, and between solutes and mobile phase components. However, the measurement of an overall competitive adsorption isotherm is not a trivial task. We suggest here that system peaks can be used for this purpose, provided that the right set of representative adsorption isotherms at various competitor compositions is chosen. The set presented in this paper is just a first example.

ACKNOWLEDGEMENTS

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