Rapid Electroconcentration in a Tube of 25-40 Microliters under a Relatively Low Applied Voltage

Shinya KITAGAWA*, Tsutomu ARAGAKI*, Takao TSUDA*† and Takatoshi MATSUMOTO**

**Department of Applied Chemistry, Nagoya Institute of Technology, Gokiso, Showa, Nagoya 466, Japan*
***Nagoya Second Red Cross Hospital, Myoken, Showa, Nagoya 466, Japan*

Electroconcentration can be performed using a very simple apparatus and a simple operational procedure. The apparatus is composed of one tube, two electrodes and a power supply. The total sample solution employed for electroconcentration was just 25 - 40 μl. After a tube of silicone rubber (i.d. 1 mm, 30 - 50 mm long) was filled with a sample solution, an electric voltage was applied along the tube, and a charged solute was forced to migrate to the end of the tube. With an application of 200 - 400 V for only 2 - 4 min, the concentration of the solute was enhanced up to 10 times. In the present electroconcentration, the sample is concentrated in a few microliters. Electroconcentration by electrode transfer is also proposed.

Keywords Electroconcentration, preconcentration, electrode transfer, electric field, electrophoretic mobility, micro volume

Today, the role of preconcentration has become very important in the development of micro-separation analysis, such as analysis using micro-liquid chromatography (MLC) and capillary zone electrophoresis (CZE). In these micro-separation methods, although the absolute sensitivity of a solute is very high, the concentration-sensitivity is generally lower compared to that obtained with conventional liquid chromatography. Also, the injection amount is generally quite small, for example, submicro liter for MLC using a packed column of 0.5 mm inner diameter and several nano-liters for CZE, in which a fused-silica capillary of 50 - 100 μm inner diameter is used as a separation tube. Therefore the preconcentration method for obtaining a highly concentrated sample in a very micro volume from a dilute sample solution is necessary for micro-separation analysis. The preconcentration should be performed within a short operational period, and the fraction volume concentrated should be sufficiently small to fit the following analysis.

There are several concentration methods using an electric voltage[1,2], such as electrodiffusion[1], electrodeposition[3], electrodissolution, electrodialysis[2], moving boundary electrophoresis[4], isotachophoresis[5,6], electrochromatography[2,7,8], countercurrent electroconcentration[2,9,10] and sample stacking for CZE.[11-13]

The principal of the moving-boundary method proposed by Tiselius can be compared to frontal analysis in chromatography. The charged-sample species to be separated, mixed with a buffer solution, fills the lower part of a U-tube electrophoretic cell, while the upper part is filled with the buffer. When an electric current is passed through such a system, the ionic species migrate to an electrode and, after some time, a partial separation occurs.[3] The sample volume used is on the order of several milliliters.

The physical principle of sample stacking in CZE is based on isotachophoresis, in which an independent zone of a solute is formed in the initial stage. The sample plug introduced in a capillary of 50 - 100 μm inner diameter is several centimeters long or more, and the electrolyte concentration in the sample medium is to be kept low in order to obtain a higher local potential gradient under a low electric current and a short operational period. The charged species can be focused in a narrow zone. The sample volume used in this method is on the order of several hundred nanoliters or less. With sample stacking in CZE, although high concentration enhancement is obtained, it is difficult to take up its zone for the following analysis using an off-line method, partly due to its too minute volume.

In the present study, we aim to develop a concentration method for 25 - 40 μl of the original sample solution. Namely, electroconcentration is performed in a tube (i.d. 1 mm, 3 - 5 cm long). This tube is filled with a sample solution, and an electric field is applied along the tube. An anion (or cation) is forced to migrate to an anode (or cathode), and, after some time, its local concentration becomes higher around the anode (or cathode). This scheme is very simple, and the process is similar to

† To whom correspondence should be addressed.

separation methods, such as moving-boundary and zone electrophoresis, except that the tube is filled only with the sample solution. The present method is able to obtain a few microliters concentrate from 25 – 40 μl of the original sample solution. The applied voltage used is relatively low, for example 200 – 400 V (potential gradient 40 – 60 V/cm), and the operational period is quite short, such as 2 – 4 min. In addition we have attained a high concentration enhancement with electrode transfer during the operational period. We call this newly proposed method "Electroconcentration with Electrode Transfer (EET)".

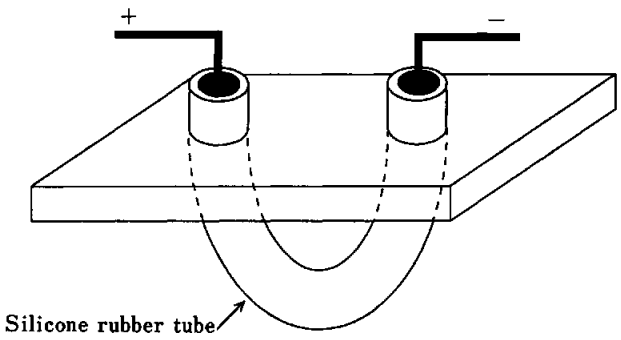

Fig. 1 Schematic diagram of the apparatus for electroconcentration.

Experimental

Apparatus and procedure of electroconcentration

A schematic diagram of an apparatus for concentration is shown in Fig. 1. The apparatus was composed of a U-silicone rubber tube (i.d. 1.0 mm, 30 – 50 mm long, volume 25 – 40 μl), two platinum electrodes (o.d. 0.3 mm) and a power supply (R-2, Toyo Roshi Ltd., Tokyo).

The procedure of electroconcentration was as follows. After filling the tube with the sample solution, electrodes were set at both ends and a voltage was applied to the micro tube for a few minutes. Then the two electrodes were taken off, and the concentrated fraction located at the end of the tube was separated by closing off the tube with a home-made clip. The separated fraction was taken up by using a micro syringe (minimum scale: 0.1 μl), and subjected to analysis by liquid chromatography.

Chromatographic system

A pump (LC-9A, Shimadzu, Kyoto, Japan), a glass column (Pre-packed Column, inner diameter 4 mm, 20 cm long, packed with octadecylsilane-modified silica gel of particle diameter 10 μm, C.I.G. Kusano Scientific, Tokyo), an on-column injection port and a detector (SPD-6AV, Shimadzu, Kyoto, Japan) were used. The injection was performed directly by piercing the septum of the injection port with the needle of a syringe. This injection method is not common nowadays, but is convenient because one can inject any volume. Effluents for the separations of PQQ, UMP and pyridoxamine were a 50 mM borate buffer at pH 7.4, a mixture of 0.1 M KH_2PO_4 aqueous solution and acetonitrile (98 : 2), and a mixture of 0.1 M phosphate buffer (KH_2PO_4–H_3PO_4) at pH 3.2 and methanol (98 : 2), respectively.

Solute distribution

The experimental solute distribution curve was obtained by the following procedure. After the first fraction was taken up for analysis, the other end of the tube was closed with a small clip. Then the second fraction was separated again with a clip. The third fraction was separated after taking up the second fraction. This procedure was repeated. The solute concentration of each fraction was measured by liquid chromatography.

Electroconcentration with electrode transfer

The typical operational procedure was as follows: After the application of a voltage for a few minutes between both ends of a 5 cm long tube, we released the voltage. Then one electrode was transferred 2 cm closer to the other electrode, so that the distance between both electrode became 3 cm. An electrovoltage was again applied for a few minutes. When the solute is an anion, the transferring electrode should be cathode.

Optimum and experimental concentration enhancement

The optimum concentration enhancement, CV_{opt}, is given as follows:

$$CV_{opt} = V_{total}/V_{sampling} \quad (1)$$

where V_{total} and $V_{sampling}$ are the volume of the solution in the silicone rubber tube and the volume of the concentrated fraction, respectively. When we use a total solution volume of 40 μl (V_{total}), and the concentrated fraction is 2 μl ($V_{sampling}$), the optimal concentration enhancement is 20 times.

After applying a voltage, the concentration of the fraction, C_{exp}, becomes higher compared to the original concentration of the solute, C_{org}. The experimental concentration enhancement, CV_{exp}, is given as follows:

$$CV_{exp} = C_{exp}/C_{org} \quad (2)$$

We define the recovery ratio, CR, as follows:

$$CR = (CV_{exp}/CV_{opt}) \times 100 \quad (3)$$

Results and Discussion

Without the application of a voltage, the solute concentration remains constant at every point in the micro tube. However, after applying a voltage along the tube, the concentration of the sample solute becomes uneven; it becomes highest around a positive electrode if the solute has a negative charge, and lowest around a negative electrode. In the optimum case, the solute

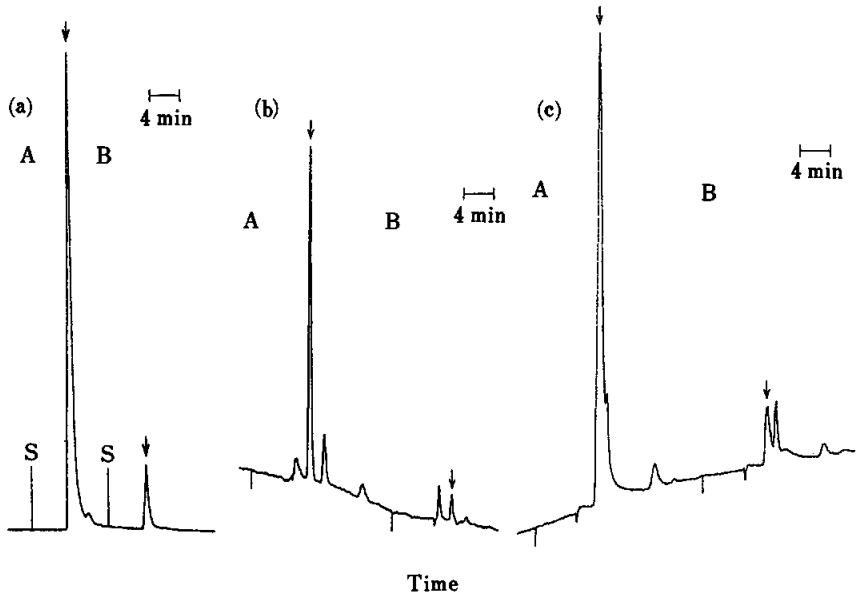

Fig. 2 Electroconcentration for three different solutes. The chromatograms of the concentrate (A) and the original solution (B) are shown for pyrroloquinoline quinone (chromatogram a), uridine 5'-phosphoric acid (b) and pyridoxamine (c). A solute is marked with (↓).

accumulates in a very small volume around the positive electrode.

If the charged solute is highly concentrated, diffusion broadens its zone. Namely, the diffusion of the solute becomes a force which is counter to the concentration. If we assume that the electrophoretic mobility of a solute is constant at any point of a tube during electroconcentration, the mass transfer can be described as follows:

$$\frac{\partial C}{\partial \theta} = D\frac{\partial^2 C}{\partial x^2} - \frac{\mu_{mob} E}{L}\frac{\partial C}{\partial x} \quad (4)$$

In Eq. (4) C, θ, D, x, μ_{mob}, L and E are the concentration of the solute, period, diffusion constant, position on the tube, electrophoretic mobility of the solute, length of the tube and applied voltage, respectively. The first term on the right side of Eq. (4) is the mass transfer, which depends on the diffusion, and the second is the mass transfer depending on the electrophoretic mobility. If we assume that L is unity (1) and the solute migrates from 0 to 1 at x, the boundary conditions are $C=0$ at $x=0$ and $(dC/dx)=0$ at $x=1$. The contribution of diffusion to the concentration process depends, for example, on the operational period and solute concentration in the original solution. Therefore in the case of a long operational period, we cannot ignore the contribution of diffusion. However, diffusion may work only to a limited extent in the present study, since the period of applying an electro voltage is just 2 – 5 min.

Electroconcentration is attained both with and without electrode transfer. We first show the results of electroconcentration without electrode transfer, for which the principles of electroconcentration are clearly demonstrated; secondly, we deal with the concentration using electrode transfer.

Typical examples of electroconcentration without electrode transfer

Two anions and one cation were concentrated. Pyrroloquinoline quinone (PQQ), a coenzyme originating in fungi, was used as one of the typical solutes. PQQ has three carboxylic groups, and has negative charges in a medium of pH 8. Fifteen micromolar PQQ in 10% pyridine aqueous solution in a 5 cm long silicone tube (Fig. 1) was subjected to electroconcentration with the application of 300 V (the potential gradient was 60 V/cm), and an operational period of 4 min. After electroconcentration, a concentrated fraction of 1.8 μl around the anode end of the silicone tube was analyzed using liquid chromatography, as shown in Fig. 2a. Chromatograms A and B in Fig. 2 were obtained from the concentrate and the original, respectively. The concentration enhancement of PQQ, CV_{exp}, is 7.8 times, and the recovery ratio, CR, is 35%.

Uridine 5'-phosphoric acid (UMP) in the medium pH 10 (2.7 μM UMP in 5.7 mM L-arginine aqueous solution) was concentrated using 400 V (potential gradient 80 V/cm) and an operational period of 3 min. The concentrated fraction of 1.2 μl was taken up for an analysis. This result is shown in Fig. 2b. CV_{exp} and CR of UMP are 13.6 and 47%, respectively.

As an example of positive-charged solutes, we used pyridoxamine of 20 μM in 1% acetic acid aqueous solution. Pyridoxamine migrates to a cathode after

applying a voltage. The applied voltage and concentration period were 200 V (40 V/cm) and 5 min, respectively. The fraction of 2.4 μl around the cathode end was analyzed. CV_{exp} and CR of pyridoxamine are 7.8 and 41%, respectively, as shown in Fig. 2c.

In Figs. 2 and 6, the peaks of the impurities are also concentrated. It is suggested that these solutes have charge of same sign as that of the main peak.

There is some possibility that the solute and buffer will be chemically changed due to electron transfer and/or a reaction at the electrode. This is unavoidable for the process of electroconcentration. However, in the experimental results, shown in Figs. 2 and 6, there are no peaks of byproducts except (b) in Fig. 2. Therefore the possibility of byproducts at the electrode might be decreased by more than first order of the concentration of the solute (9).

It is clear that electroconcentration can be applied to both positively and negatively charged solutes.

The effect of operational period

The relationship between the experimental concentration enhancement and the operational period is shown in Fig. 3. Twelve experimental runs with different operational periods in a tube of 5 cm long were performed using 15 μM PQQ in 10% aqueous pyridine solution as a sample under 300 V. The sampling volumes of the first fractions at operational periods of 0, 10, 20, 30, 40, 50, 60, 90, 120, 180, 240 and 300 s were 2.4, 2.4, 2.6, 2.6, 2.4, 2.6, 2.4, 2.4, 2.8, 2.2, 2.4 and 2.8 μl, respectively. As shown in Fig. 3, the experimental concentration enhancements given by the first fraction become larger when the operational period is longer; after 200 s, it becomes constant. Therefore an operational period of 4 min is sufficient for concentration using a 5 cm long tube.

Most of the solutes included in a sample solution can be focused in a small fraction of 2.2–2.8 μl around the electrode.

Distribution of a solute after electroconcentration

The distribution pattern of a solute along the tube is directly related to the recovery of the solute. The fractional distributions of the solute's concentration are shown in Fig. 4.

A 1.5 μM of PQQ in 10% pyridine aqueous solution in a 3 cm tube was concentrated by applying 180 V (60 V/cm) over a 4 min operational period. The vertical axis is the molarity of PQQ of each fraction, and the horizontal axis is the sampling position, on which the positions of the anode and cathode end of the tube are defined as 0 and 1, respectively. The horizontal line corresponds to the concentration of the original solution. In Fig. 4, about 60% of the total PQQ molar quantity is focused in the first fractions. A similar experiment had been performed using a tube of 5 cm long with an applied voltage of 300 V; nearly the same distribution profile was obtained. Although the first and second fractions show a higher concentration than the original, the other fractions are less, and the molarities of the solute are nearly constant in the range from 0.6 to 1 of the x-axis. Despite a very short operational period, the effective concentration into a small fraction around an anode can be achieved.

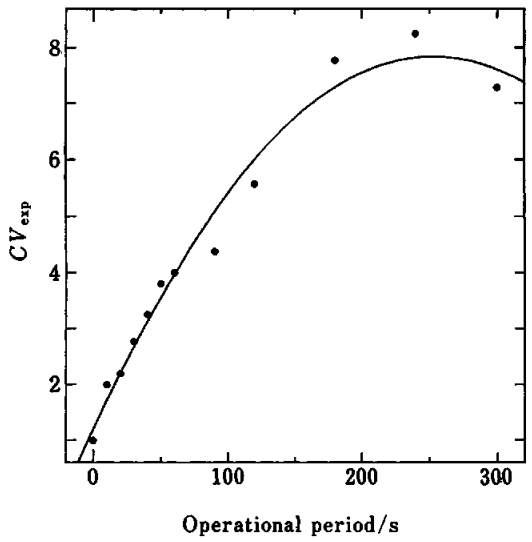

Fig. 3 Relationship between the experimental concentration enhancement (CV_{exp}) and the operational period. Tube length, 5 cm; applied voltage, 300 V; sample, 15 μM pyrroloquinoline in 10% pyridine solution. The volumes of the first fraction taken up after electroconcentration were 2.2–2.8 μl.

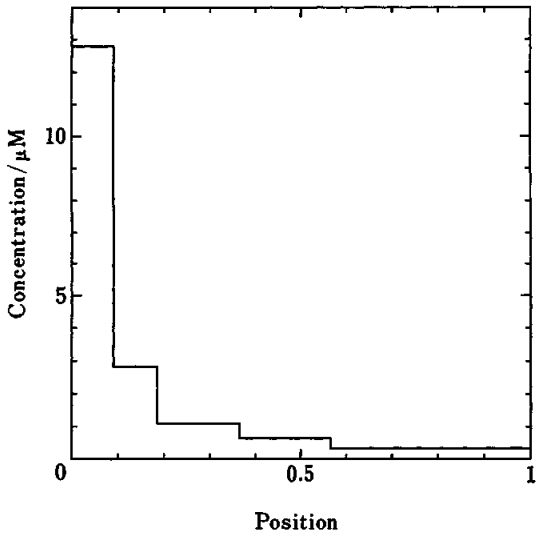

Fig. 4 Distribution profile of a sample solute after electroconcentration. Tube length, 3 cm; sample, 1.5 μM pyrroloquinoline quinone in 10% pyridine solution; operational period, 4 min; applied voltage, 180 V.

Table 1 Effect of the operational period on the recovery ratio

Operational period/min	Recovery ratio[a], %
1	56
2	60
3	61
5	67

The total sample volume used was 40 μl. a. A fraction of 8 μl around the anode was used for the estimation.

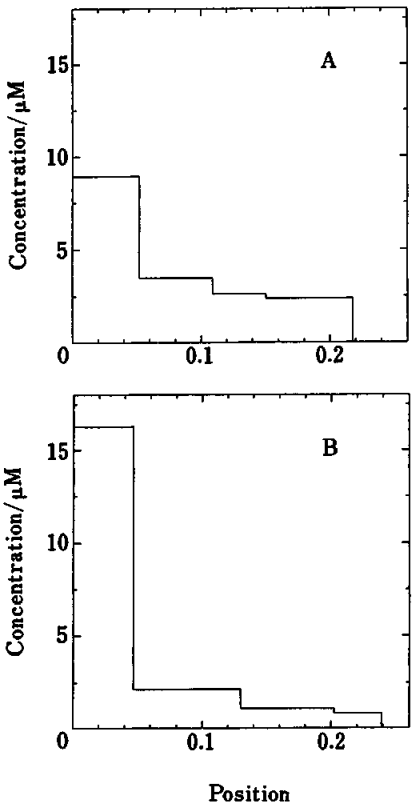

Fig. 5 Effect of operational periods of 1 min (A) and 5 min (B) on the distribution profiles. Tube length, 5 cm; sample solution, 1.5 μM pyrroloquinoline quinone in 10% pyridine solution; applied voltage, 300 V.

Effect of the operational period on the distribution pattern around an electrode

We examined *ca.* 25% of the length of the tube. We separated it into several fractions, and obtained recovery ratios and distribution profiles with operational periods of 1, 2, 3 and 5 min. The recovery ratios are listed in Table 1, and the typical distribution profile obtained with operational periods of 1 and 5 min are shown in Fig. 5. Immediately after applying 300 V per 5 cm length (A in Fig. 5), the solute concentration along the tube becomes uneven, and finally the fractions near to the anode are highly concentrated (B in Fig. 5). From Table 1, only 1 min after the start, over 50% of the total PQQ molar quantity is focused in 20% of the total volume. After 5 min operation, about 67% of the total PQQ is focused. Thus the electroconcentration process proceeds very rapidly within an initial short period, and then becomes rather slow. The contribution of electrophoretic migration to electroconcentration is quite clearly shown in Fig. 5.

Electroconcentration with electrode transfer

From the solute distributions shown in Figs. 4 and 5, the solute is strongly concentrated in a few fractions located in the range between 0 and 0.2 of the x-axis; it is both much less and constant between 0.6 and 1.0. If we try to force the solute to migrate to the area around the anode in the latter range (if the solute has a negative charge), a long operational period might be required. When a concentration enhancement of 10 times is expected, it is not necessary to apply an electric voltage in the later range except during the initial short period. Therefore, after a short initial period of electro-voltage application, it may be a good idea to transfer one electrode, called the transferring electrode, toward the other. The sign of the electric voltage applied to a transferring electrode should be the same as that of the solute charge. For example, in the case of the concentration of an anion, the cathode is transferred toward the anode.

There are two possible modes of electrode transfer: continuous and step-by-step transfers. In the present studies, we adopted a step-by-step transfer. As the result of transferring, the distance between the anode and cathode became shorter during electroconcentration. As a result, it is expected that the concentration enhancement by electrode transfer is higher, and that its operational period under an applied voltage shorter compared to electroconcentration without electrode transfer.

For studying the present method of electroconcentration with electrode transfer (EET), we examined the distribution profiles of PQQ obtained with and without electrode transfer using a 5 cm long tube. A constant applied voltage was used during electroconcentration. Namely, the potential gradient was varied from 60 V/cm at the beginning to 100 V/cm after electrode transfer (the constant applied voltage was 300 V and the effective tube lengths were 5 cm and 3 cm before and after electrode transfer, respectively). Fractions of 10% of the total volume (about 4 μl) were examined. The molar quantities were estimated. The recovery ratios of PQQ of this fraction to the total are 71.8% and 48.9% for electroconcentration with and without electrode transfer, respectively.

EET was also applied for 4 μM pyridoxamine in 1% acetic acid aqueous solution. The tube length and applied voltage were 5 cm and 200 V, respectively. The CV_{exp} and CR of pyridoxamine in a first fraction of 4 μl around the cathode were 9.1 times and 91%, respectively. The chromatograms obtained before and after electroconcentration are shown in Fig. 6. When the sampling volume was limited to only 0.2 μl around the cathode, we

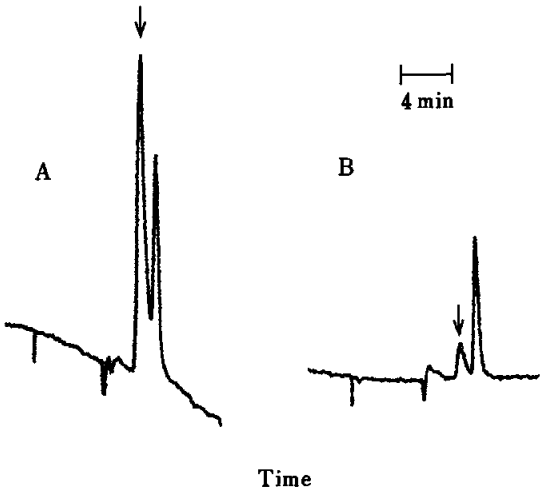

Fig. 6 Chromatograms of pyridoxamine obtained before (B) and after (A) electroconcentration with electrode transfer. The estimated concentration enhancement is 9.1 times, and the recovery is 91%. (↓) shows the solute to be concentrated.

obtained a concentration enhancement of 30 times. The proposed EET method under a constant applied voltage is quite effective. EET consumes less electric energy compared to the method without electrode transfer.

It is desirable to completely concentrate the solute by the present method. In the present art of technology in electroconcentration the rate of a maximum concentration is 91%. Therefore it is necessary to add an internal standard for the quantitative determination.

In the present study, the model sample included only one solute. However it is often encountered that the sample comprises multicomponents, in which case groups are separated; for example, a group having a relative large mobility, a group having a relative small mobility, and a group having a reverse charge. In other words, the solutes which have nearly the same pK values are concentrated at once, and their recovery ratios are almost the same.

Newly proposed methods of electroconcentration with electrode transfer may have a high ability for rapid preconcentration. The present method will be applicable as a very effective concentration method for concentrations of biologically important substrates in plasma *etc.*, and is now under investigation.

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