**ORIGINAL ARTICLE**

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# Characterization and cytotoxicity of a benzocaine inclusion complex

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## Abstract

Benzocaine (BZC), is a local anesthetic widely used in topical formulations as well as in throat pastilles. A disadvantage is that the compound presents low aqueous solubility. The present work describes the preparation and characterization of an inclusion complex between BZC and β-cyclodextrin (β-CD), followed by cytotoxicity assays. The association constant (Ka) was calculated using solubility isotherms, at different temperatures, and an HPLC procedure, at room temperature, employing a reverse phase C18 column, with a mobile phase consisting of water/acetonitrile. Ka obtained with solubility isotherms at temperatures of 25, 35, and 45 °C were 229.8, 317.1, and 520.3 $M^{-1}$, respectively. Employing HPLC, Ka was 38.0 $M^{-1}$. The difference in the Ka value could be explained because HPLC analyses were conducted using organic solvent, which affected the host–guest interaction. Moreover, the continuous flow could have altered the degree of association of the drug with β-CD. The BZC/CD inclusion complex was characterized using infrared spectroscopy, thermogravimetry, and X-ray diffraction. Analysis showed a good agreement with literature, suggesting that the complex was established. Cytotoxicity assays using fibroblast V79 cells showed that BZC/CD formulation was not cytotoxic, demonstrating its potential to reduce the toxicity of the anesthetic. The assays demonstrated an effective interaction between BZC and CD, and that the inclusion complex was less toxic to V79 cells than the plain BZC, turning it a good alternative to decrease its toxicity when administered to patients.

**Keywords** Cyclodextrin · Inclusion complex · Local anesthetic · Benzocaine · Cytotoxicity

## Introduction

Local anesthetics (LAs) are compounds that promote loss of sensation in a restricted area of the body, without loss of consciousness, by depression of the excitability of nerve endings or inhibition of conduction in peripheral nerve tissue [1, 2].

Benzocaine (BZC), a local anesthetic belonging to the ester family, is widely used in formulations for topical use on skin and mucous membranes [1–3]. However, the parenteral administration of BZC is hindered by its low aqueous solubility. Unlike other LAs used in clinical practice, BZC does not possess an ionizable amine group (Fig. 1), and is always in a deprotonated form at physiological pH. It is therefore an exception to the general rule that the protonated forms of LAs are responsible for the anesthetic effect [1, 2].

In addition to long duration of action and selectivity for sensory rather than motor blockade, a desirable feature of an anesthetic molecule is that it should present low local and/or systemic toxicity. A technique that has recently been shown to promote these benefits is the use of inclusion complexes of anesthetics with cyclodextrins [2].

Cyclodextrins (CDs) are cyclic oligosaccharides composed of 6, 7, or 8 glucose units linked by α-(1,4) glycosidic bonds, and are produced from the microbial hydrolysis of starch by the cyclodextrin glycosyltransferase enzyme [4]. They have a hydrophilic exterior and a hydrophobic cavity [5, 6], and their interaction with a variety of molecules can modify the physicochemical characteristics of the latter [4, 6–8]. CDs have been used as complexation agents to

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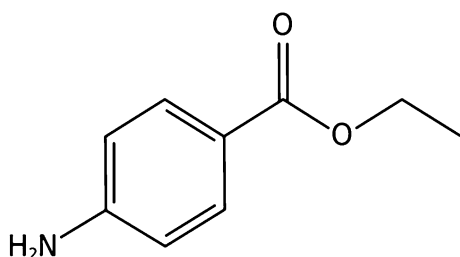
**Fig. 1** Chemical structure of benzocaine (ethyl p-aminobenzoate)

increase the aqueous solubility of relatively insoluble drugs, and can also increase the bioavailability and stability of the active principle [9].

The effects of complexation of LAs with cyclodextrins have been investigated previously, with outcomes including improved solubility, increased duration of anesthetic effect, and decreased toxicity [2, 3, 10–15].

An improvement in the solubility profile of BZC could provide a means of enhancing its bioavailability and anesthetic efficacy. The aim of this work was therefore to prepare and characterize inclusion complexes of BZC and β-CD, and compare different methodologies for determination of the association constant of the complex. The cytotoxicity of the complex was evaluated using fibroblast cell cultures.

## Materials and methods

### Determination of the association constant by phase solubility studies

Phase-solubility isotherms were generated according to the methodology described by Higuchi and Connors [16]. An excess amount of BZC (20 mM) was added to flasks together with increasing amounts of β-CD (0–15 mM). The flasks were sealed and the suspensions were stirred at 25, 35, and 45 °C until equilibrium was achieved. The samples were then centrifuged for 1 h at 280x*g*, and filtered through 0.22 μm membrane filters (Millipore). The concentration of BZC was determined spectrophotometrically at 284 nm, and the association constant (Ka) was calculated from the linear plot obtained for the isotherm, using

$$Ka = \frac{Slope}{S_0 \, (1 - Slope)} \tag{1}$$

where $S_0$ is the solubility of BZC in water in the absence of β-CD.

### Determination of the association constant by chromatography

Solutions of BZC (1 mM) and β-CD (0–15 mM) were prepared in ultra-pure water (MilliQ) and then filtered through a 0.22 μm pore size nylon membrane (Millex GP).

Chromatographic analyses were carried out using a Varian high performance liquid chromatograph (HPLC) equipped with a Model 9012 pump, an auto-injector (Model 9050), and a UV–Vis detector (Model 9300). The eluent flow rate was 1 mL min$^{-1}$ (isocratic), and the detector wavelength was set at 284 nm. A Varian C18 SI column (250 × 4.6 mm; 5 μm) was used, at room temperature, and the system was controlled with Star Toolbar® software. The mobile phase was a mixture of 30% (v/v) acetonitrile (HPLC grade, Merck) and 70% (v/v) β-CD solution.

The retention factor for BZC was obtained for increasing concentrations of β-CD. The Ka value was then determined using

$$\frac{1}{k'} = \frac{1}{k_0} + \frac{K_a \, [CD]^x}{k_0} \tag{2}$$

where k' is the retention factor of the analyte, $k_0$ is the retention factor of the analyte without β-CD in the mobile phase, [CD] is the β-CD concentration, and x is the stoichiometry of the complex [10].

### Preparation and characterization of the BZC/CD complex in the solid state

Inclusion complexes were prepared using a 1:1 BZC/CD molar ratio, as described by Pinto and co-workers [2]. Briefly, appropriate amounts of BZC (Sigma Chemical Co) and β-CD (Roquette) were mixed in ultra-pure water (MilliQ) at room temperature (25 °C). After reaching equilibrium, the solution was freeze-dried and stored at −20 °C for subsequent use.

The formation of the inclusion complex was investigated using Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), and X-ray powder diffractometry (XRPD). Samples of plain BZC, plain β-CD, and the BZC/CD (1:1 molar ratio) inclusion complex were analyzed in these experiments. Physical mixtures were also prepared and used as controls.

The infrared spectroscopy measurements were performed with an Excalibur FTS 3000 instrument, using a wave number range from 4000 to 400 cm$^{-1}$, 16 overlaps, and a resolution of 4 cm$^{-1}$. The samples were homogenized with KBr and filled into the holder using a compression gauge.

Thermogravimetric analyses employed a Shimadzu DTG-60AH simultaneous DTA/TG instrument. Scans were

carried out from room temperature up to 200 °C, with a heating rate of 10 °C min$^{-1}$, under a nitrogen atmosphere.

A Rigaku Geigerflex diffractometer was used for the XRPD measurements (at the Soil Sciences Department of UFLA). The samples were analyzed at room temperature, using Co Kα radiation, a current of 20 mA, and a voltage of 40 kV. The scanning speed was 1°θ min$^{-1}$, and the angular variation was 2°–60°.

## Cytotoxicity assays

Chinese hamster V79 lung fibroblasts were grown in DMEM containing antibiotics (100 U mL$^{-1}$ penicillin G; 100 μg mL$^{-1}$ streptomycin) and supplemented with 10% fetal calf serum, at 37 °C under a humidified atmosphere containing 5% $CO_2$. For the cell viability assays, 96-well tissue culture plates were inoculated with $3 \times 10^4$ cells mL$^{-1}$ and incubated at 37 °C for 48 h. The medium was removed and replaced with treatment medium containing different doses of the test material. The cells were then incubated for 24 h and evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [17]. Different concentrations of BZC, β-CD, and BZC/CD inclusion complex were tested. Dimethyl sulfoxide (at concentrations not exceeding 0.1%, which is not toxic to the cells) was used to solubilize benzocaine. All the experiments were performed using eight replicates.

## Results and discussion

### Determination of the association constant by phase solubility studies

At the temperatures tested, the solubility of BZC in water increased linearly with the concentration of β-CD. The solubility increased approximately threefold at 25, 35, and 45 °C, in the ranges 5.5–15.7, 6.7–20.8, and 7.5–19.5 mM, respectively (Fig. 2). The values obtained from linear regressions of the solubility isotherms were used to calculate the apparent association constants (Ka) for the BZC/CD complexes at each temperature. The values obtained were 229.8, 317.1, and 520.3 M$^{-1}$, for temperatures of 25, 35, and 45 °C, respectively.

Thermodynamic parameters of the complexation were calculated according to the van't Hoff equation ($\ln K = -\Delta H / RT + \Delta S / R$), where T is the temperature in Kelvin and R is the gas constant. Values of ΔH and ΔS for the complexation were determined as −32.48 KJ mol$^{-1}$ and 0.153 J K$^{-1}$ mol$^{-1}$ respectively.

The negative enthalpy demonstrates that the process is exothermic, as normally observed in inclusion complexes with cyclodextrins, due to non-formal interactions such
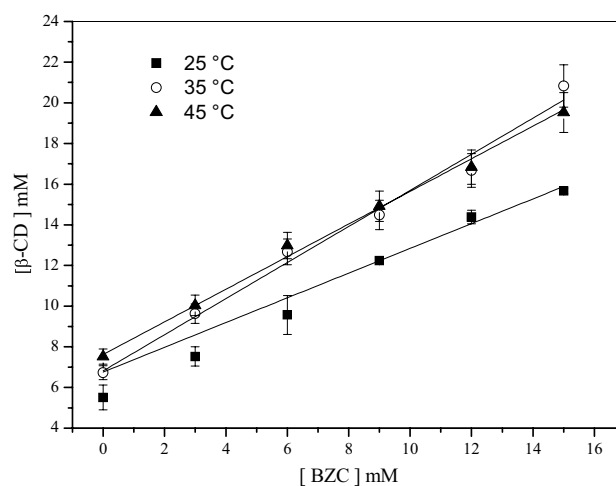


**Fig. 2** Solubility isotherms obtained for BZC in the presence of β-CD at different temperatures

as the hydrophobic and van der Waals forces. Regarding the entropy of these systems, negative or slightly positive values can be found, such as the result found in this study, indicating an orderly state of the formed complex. This demonstrates that the inclusion of the molecule was not accompanied by a very strong desolvation, being the process influenced primarily by the enthalpic processes [10, 18, 19].

The Gibbs free energy (ΔG) was calculated from the enthalpy and entropy values as − 78.07 KJ mol$^{-1}$, demonstrating that the process of forming the complexes in aqueous solution is spontaneous.

The isotherms obtained were classified as type $A_L$ [16], suggesting that for a 1:1 molar ratio the inclusion complexes were formed in a first-order process, and were highly soluble (the solubility limit was governed by the solubility of CD).

The increase in the Ka value at higher temperatures was indicative of greater interaction between the guest molecule (BZC) and the host (β-CD). This can be explained by the fact that the kinetic energy of the molecules was greater at higher temperatures, which accelerated the dynamics of the complexation.

In clinical practice, the solubility increase observed should be reflected in higher bioavailability of the drug in the body.

### Determination of the association constant by chromatography

The retention of BZC on the chromatographic column, governed by its distribution between the mobile and stationary phases, was modified according to the extent of its complexation with cyclodextrin (see Supplementary Material). The solute retention factors were calculated after establishing

the retention time and lag time for each β-CD concentration tested [10].

The retention time decreased as the concentration of β-CD in the mobile phase was increased, since formation of the BZC/CD inclusion complex (Table 1) resulted in greater solubility of the guest molecule in the mobile phase [10, 20].

The Ka value for the BZC/CD inclusion complex, calculated from Eq. (2) using the linear relationship between $1/k'$ and [β-CD] (Fig. 3), was 38.1 M$^{-1}$. The value determined spectrophotometrically, using solubility isotherms, was 229.8 M$^{-1}$ at 25 °C (the same temperature used in the chromatographic analyses). In previous work, Moraes et al. [10] also found that a smaller association constant was obtained for a system containing the local anesthetic S(-) bupivacaine and 2-hydroxypropyl-β-cyclodextrin when the HPLC method was used, compared to the value obtained from spectrophotometric assays [11]. This difference may be explained by the fact that the complexation was studied in water when solubility isotherms were used, without the presence of an organic solvent that could interfere in the host–guest interaction. The addition of acetonitrile or

methanol has been reported to have a negative impact on the formation of complexes with cyclodextrins [10, 20].

The presence of organic solvent (H$_2$O/ACN: 70/30) resulted in the mobile phase becoming less polar, so that it became more attractive to BZC, hence decreasing the affinity of the drug for the hydrophobic cavity of the β-CD. This competition therefore resulted in weaker binding of BZC within the cavity of the β-CD [10, 20], which explains the disparity in the Ka values.

The use of a continuous flow, mimicking administration of the formulation by the systemic route, could have also contributed to decreased binding of the drug to the β-CD. The inclusion complexes formed between cyclodextrin and guest molecules are maintained by non-covalent interactions, in a dynamic equilibrium where the guest molecule is constantly associating with, and disassociating from, the cyclodextrin cavity [10, 21]. Redistribution during the chromatographic run, due to the presence of a less polar solvent, could have shifted the balance towards release of the drug from the CD cavity. Even low percentages of organic solvent in the mobile phase can enhance this dissociation [10].

## Characterization of the BZC/CD complex in the solid state

The BZC/CD inclusion complex was analyzed using infrared spectroscopy in order to identify any changes in the absorption bands of the guest molecule after its inclusion in the β-CD cavity. Figure 4 illustrates the main vibrational frequencies for BZC, β-CD, BZC/CD, and a physical mixture of the two components. The plain BZC spectrum showed characteristic absorption bands in the 3423–3213 cm$^{-1}$ region, associated with asymmetric and symmetric N–H stretching, and C–H stretching bands in the 2981–2897 cm$^{-1}$ region. There was also ester C=O stretching at 1687–1633 cm$^{-1}$, and C–N stretching at 1272–1178 cm$^{-1}$.

The spectrum of plain β-CD showed characteristic bands in the regions 3250–3450 and 1000–1100 cm$^{-1}$, associated with O–H and C–O–C, respectively [22].

The spectrum of the physical mixture analyzed using infrared spectroscopy showed no significant differences, compared to the spectra of the individual components. Slight variations in the spectra were not indicative of inclusion of BZC within the β-CD cavity [7]. However, inspection of the spectrum obtained for the BZC/CD inclusion complex revealed modifications in the vibrational modes of BZC and β-CD. Changes in position and relative intensities of some characteristic peaks of BZC, indicated that the molecule was included in the CD cavity [26]. It was observed a narrowing in the regions around 3300 and 1000–1100 cm$^{-1}$, possibly due to the breaking of hydrogen bonds after interaction with

**Table 1** HPLC retention times obtained using different β-CD concentrations

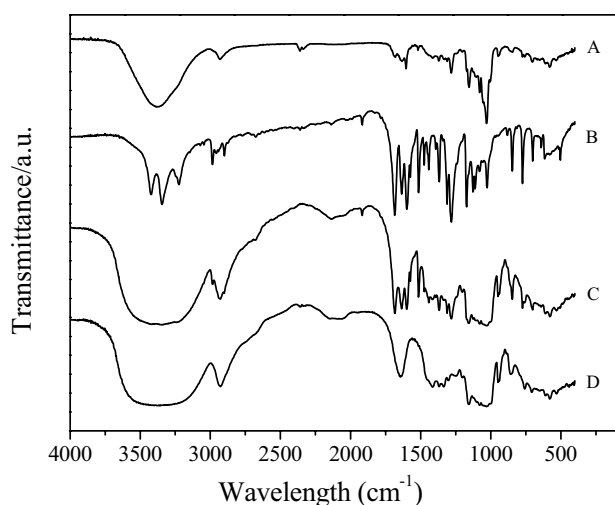| β-CD concentration (mM) | Retention time (min) |
| --- | --- |
| 0 | 12.060 ± 0.036 |
| 3 | 9.988 ± 0.310 |
| 6 | 9.727 ± 0.354 |
| 9 | 9.256 ± 0.723 |
| 12 | 9.158 ± 0.405 |
| 15 | 8.813 ± 0.403 |



**Fig. 3** $1/k'$ versus [β-CD] plot for BZC, obtained from the retention time

**Fig. 4** FTIR spectra obtained for (*A*) BZC/CD inclusion complex, (*B*) BZC, (*C*) physical mixture, and (*D*) β-CD



**Fig. 5** TG thermograms obtained for (*A*) BZC, (*B*) β-CD, (*C*) BZC/ CD inclusion complex, and (*D*) physical mixture

the guest molecule, and complete release of water from the interior of the CD cavity. There was also a decrease in the intensity of the ester C=O vibrational modes, in agreement with earlier findings [7, 8]. However, some bands that could be attributed to BZC were obscured by the bands from CD [7].

Thermal analysis was used to identify formation of the inclusion complex (Fig. 5). β-CD showed endothermic peaks at 40–100 °C, due to the evaporation of water from within the cavity, and at 320–350 °C, corresponding to thermal decomposition of the cyclodextrin. BZC presented a single mass loss peak 150–220 °C, corresponding to its decomposition. The physical mixture showed three decomposition peaks, at 40–100 °C (water evaporation), 140–180 °C (decomposition of the β-CD), and 320–350 °C (decomposition of BZC) [23].

The curve obtained for the inclusion complex in the thermal analysis was similar to that of the physical mixture; however, a diminution of the peak corresponding to the decomposition of plain BZC indicated that the inclusion complex was thermally more stable. Furthermore, the higher temperature required for degradation of complexed BZC, compared to the free drug, suggests that there was a substantial improvement in the thermal stability of BZC after its complexation with the β-CD [24].

The X-ray powder diffractometry technique can be used to detect changes in crystallinity indicative of inclusion complex formation, as well as to characterize the structure of the complex and the interactions between the cyclodextrin and the guest molecule [25]. The results obtained for the different materials are shown in Fig. 6.

In their crystalline states, some CDs form intra- and inter-molecular hydrogen bonds that stabilize the conformation
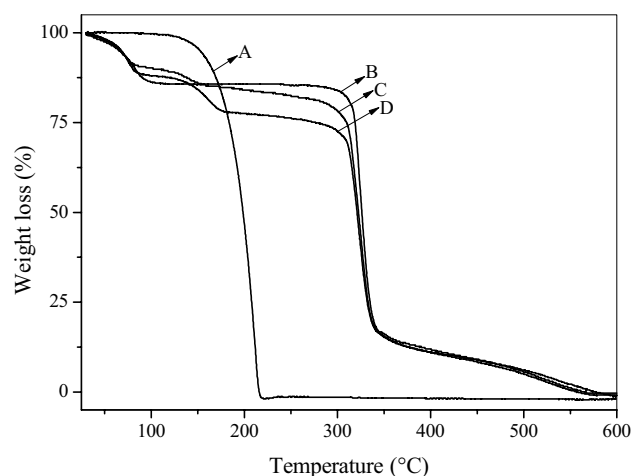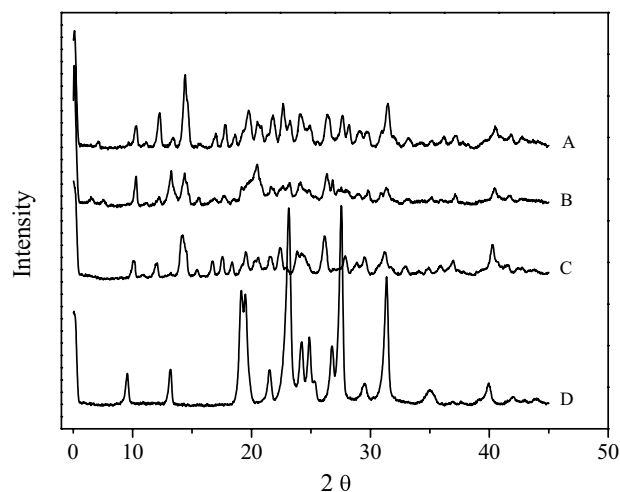


**Fig. 6** X-ray powder diffractograms for (*A*) BZC/CD inclusion complex, (*B*) physical mixture, (*C*) β-CD, and (*D*) BZC

of the molecule as well as the crystal structure, generating a diffraction pattern with some characteristic peaks between 10 and 30 2θ [26] some of them still remaining in the complex and physical mixture. Intense and sharp peaks were observed in the diffractogram of plain BZC that became reduced in the samples of inclusion complex and physical mixture [7] probably due to the concentration of BZC in these samples.

In the physical mixture, the peaks corresponding to free crystalline BZC were broadened and of low intensity, clearly indicating a loss of crystallinity of the drug [25]. The XRPD pattern of the BZC/CD inclusion complex also showed a decrease in crystallinity, with few peaks characteristic of the drug; this indicated that the inclusion complex had been

formed [26]. Although, through this technique only weak interactions between host and guest could be observed.

## Cytotoxicity assays

Growth of the V79 cells was not inhibited by the β-CD alone, at the concentrations used (0.3–2.0 mM), according to Fig. 7. In contrast, benzocaine caused cytotoxic effects in the V79 cells, as evaluated using the MTT assay ($IC_{50} = 1.6$ mM).

The objective of the cytotoxicity tests was to evaluate the effects of uptake of the anesthetic by intact cells. The BZC/CD formulation was not cytotoxic at the concentrations used; at the same benzocaine concentration, use of the formulation avoided the decrease in cell viability observed for plain BZC. These findings confirmed that the BZC/CD inclusion complex presented lower cytotoxicity to V79 fibroblasts, compared to the plain anesthetic formulation.

## Conclusions

The construction of phase-solubility isotherms demonstrated that the solubility of BZC increased after formation of the BZC/CD inclusion complex, at the temperatures tested. The association constants determined by spectrophotometry and chromatography indicated that there was effective interaction between the LA and the β-CD cavity, and that use of

the inclusion complex should therefore be able to increase the bioavailability of the drug when administered in clinical applications.

Characterization using FTIR, TGA, and XRPD confirmed that the anesthetic was incorporated into the cyclodextrin cavity, with formation of the inclusion complex.

The results of cytotoxicity tests indicated that the BZC/CD inclusion complex formulation was less toxic towards V79 fibroblast cells, compared to the formulation containing the plain anesthetic.
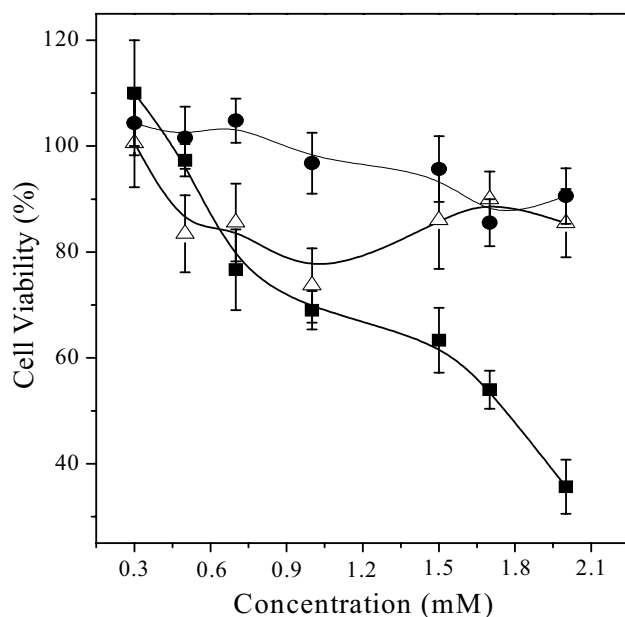
**Fig. 7** Cell viability after treatment for 24 h with β-CD (filled circle), BZC (filled square), and 1:1 BZC/CD inclusion complex (open triangle). Endpoint evaluated: MTT test

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