$$Ns = Ns_0 \cdot e^{k_{app}(t-L_1)} \quad \text{(Eq. 6)}$$

$$Nr = Nr_0 \cdot e^{k_g(t-L_2)} \quad \text{(Eq. 7)}$$

Replacement of Ns and Nr in Eq. 3 by Eqs. 6 and 7 gives Eq. 2.

Although the observed points were scattered around the simulation curve generated from Eq. 1 by incorporating the appropriate parameters (Table I), it appears that the apparent generation curves can be reasonably well described by the present mathematical model.

Garrett and Won (2) have reported the development of penicllin-resistant organisms and proposed the following possible explanation for the regrowth phenomenon: (*a*) the consumption or degradation of drugs, (*b*) production of an inhibitor or inactivator of antibiotic action, (*c*) preexistence of drug-resistant bacteria, and (*d*) acquisition of drug resistance through mutation and/or adaptation by exposure to the drug. Besides these factors, the cell cycle (*e*) may be an important factor in regrowth after the first addition of drug.

In the present study, the drug concentration was kept constant during the experiment by using a dialysis membrane tube containing drug solution. Therefore, possibility *a* is ruled out.

The regrowth pattern appears to depend on the drug concentration (Figs. 2 and 3). The organism, once it acquired resistance after the first drug addition, retained resistance to concentrations below that of the first addition (Figs. 2 and 3), and this persisted for at least 48 h during subculture (Fig. 4). These observations and the population diagram of the resistant organisms (Fig. 5) seem to support the preexistence of insensitive bacteria with different degrees of resistance, although possibilities *b*, *d*, and *e* cannot be excluded completely on the basis of the results shown in Figs. 2–4 and 7.

The subcultured organism from a single colony showed similar apparent regrowth (Fig. 8). The possibility of picking up an organism originally resistant to the ampicillin concentration of 1.5 μg/mL is very small, because the number of organisms which may manifest resistance to 1.5 μg of ampicillin/mL is expected to be <10, judging from the population diagram (Fig. 7).

The possibility of mutation and/or adaptation may be ruled out by the MIC determinations on subcultured organisms. If the resistance was acquired by mutation and/or adaptation, the MIC should increase gradually during repeated subcultures, but in fact it did not change. Furthermore, when the drug was added just at the doubling time (DNA synthesis stage) of the synchronous culture, a similar generation curve was obtained (Fig. 10); so, possibility *e* was ruled out.

The possibility of β-lactamase production still remains; therefore, we assayed β-lactamase activity by using resistant organisms generated with 8 μg of penicillin G/mL, but β-lactamase activity was not detected in the crude preparation. This result excludes the possibility that β-lactamase may have been induced by contact with the drug, leading to the acquisition of resistance.

The remaining and most probable explanation for the bacterial regrowth after the addition of the drug is a selection process and/or change of membrane permeability caused by contact with the drug at various concentrations.

Zimmermann (10) and Sawai *et al.* (11, 12) have reported an indirect method for measuring the outer membrane permeability of β-lactam antibiotics, but it is necessary to use β-lactamase-producing organisms. To confirm the difference in degree of permeability between sensitive and resistant organisms, a direct measurement of membrane permeability is desirable; however, a direct method for such measurement is not available at present.

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Spectrofluorometric Determination of Captopril Plus Captopril Disulfide Metabolites in Plasma

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Abstract □ Captopril disulfides and the drug covalently bound to proteins were reduced with tri-*n*-butylphosphine. After sample purification on an XAD-2 column, captopril was treated with 1-(7-dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-yl)-1*H*-pyrrole-2,5-dione to form a fluorescent derivative. After acidification, the fluorescent derivative was extracted into toluene and purified on a C_{18} cartridge. The fluorescence of the dimethylformamide eluate was measured at an excitation wavelength of 380 nm and a fluorescence wavelength of 440 nm.

Keyphrases □ Captopril—spectrofluorometric analysis, plasma □ Angiotensin-converting enzyme inhibitors—captopril, spectrofluorometric analysis, plasma

Captopril (1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline; I) a potent, specific, and orally active inhibitor of angiotensin-converting enzyme, has been shown (1) by *in vitro* and *in vivo* metabolism studies to exist in blood as unchanged drug, symmetrical disulfide, captopril–cysteine and captopril–glutathione mixed disulfides, and as captopril covalently bound to plasma albumin by S—S linkages. It has been reported that the plasma proteins and mixed disulfides with endogenous thiol compounds may act as a reservoir from which captopril is liberated over time to exert pharmacological effects (2, 3).

Several methods for determining captopril have been re-

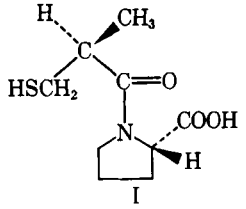

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Table I—Fluorescence of Captopril–Reagent as a Function of Concentration

Captopril, μg/mL of Plasma	Fluorescence at 380/440 nm
10.0	110[a]
8.0	88
6.0	64
4.0	46
2.0	22
0	0
1.0	100[b]
0.8	81
0.6	59
0.4	40.5
0.2	20.0
0	0

[a] Sensitivity 1, for concentrations of 0–10 μg/mL. [b] Sensitivity 4, for concentrations of 0–1 μg/mL.

ported, including GC–MS (4), GC (5), and HPLC (6–8). However, total captopril in plasma can be determined only after reduction of captopril disulfides and drug covalently bound to proteins. Tri-*n*-butylphosphine, used for reduction of captopril disulfides in urine (6), is also effective in plasma because it can penetrate the hydrophobic regions of proteins (9).

A rapid and simple analytical method is needed for measuring microgram levels of captopril plus disulfide metabolites in the plasma of patients during prolonged therapy (8), when the drug accumulates. Since all published methods for determination of captopril require expensive instrumentation not available to small hospital laboratories, a fluorometric method for measuring captopril plus disulfide metabolites in plasma has been developed.

EXPERIMENTAL SECTION

Apparatus—Fluorometric measurements were performed on a spectrofluorometer[1]. Samples were shaken on a heavy-duty shaker[2]. A centrifuge[3], 150- and 200-mm test tubes and plastic screw caps with polytef-faced rubber liners[2], 20-mL scintillation vials[2], C_{18} cartridges[4], 10-mL plastic syringes with 21-gauge needles, XAD-2 columns[5], and a water bath[6] set at 50°C were used.

Reagents—Acetone, toluene, hydrochloric acid, phosphoric acid, dimethylformamide, Tris buffer, urea, dibasic sodium phosphate heptahydrate, EDTA, and diethylenetriaminepentaacetic acid were reagent grade. 1-(7 - (Dimethylamino)-4-methyl-2-oxo- 2*H* -1-benzopyran-3-yl)-1*H*-pyrrole-2,5-dione reagent[7] (10 mg dissolved in 10 mL of acetone) was stable in a refrigerator for at least 1 year. A 20% v/v solution of tri-*n*-butylphosphine[8] was prepared in a fume hood by diluting 2 mL of the reagent with 8 mL of acetone.

A 0.1 M, pH 8.2, buffer was made by dissolving 12.1 g of Tris in 980 mL of distilled water; the pH was adjusted with phosphoric acid, and the buffer was diluted to 1 L with distilled water. Urea buffer, for reduction, was prepared daily by dissolving 20 g of urea in 0.1 M, pH 8.2, Tris buffer and diluting to 50 mL. HCl (0.1 M) with EDTA was prepared by adding 100 mg of the disodium salt per liter of acidic solution. A 0.05 M, pH 6.9, phosphate–dimethylformamide buffer was made by dissolving 20 g of dibasic sodium phosphate heptahydrate and 100 mg of diethylenetriaminepentaacetic acid in 650 mL of distilled water and adding 350 mL of dimethylformamide; the pH was adjusted with phosphoric acid. Column activator concentrate[5], 35 mL, was diluted to 500 mL with distilled water and kept in a refrigerator. Plasma was prepared from whole blood using citric acid as anticoagulant. A captopril disulfide[9] standard solution (20 μg/mL) was prepared in methanol and kept in a refrigerator no longer than 3 months.

[1] Model 204; Perkin-Elmer.
[2] Fisher Scientific.
[3] International IEP2741.
[4] SEP-PAK; Waters Associates, Inc.
[5] Brinkmann Instruments.
[6] Organomation Associates, Inc.
[7] Polysciences, Inc.
[8] Strem Chemicals, Inc.
[9] The Squibb Institute for Medical Research.

Table II—Recovery of Captopril and Disulfides from Plasma

Drug or Metabolite	Amount Added, μg/mL	Amount Found, μg/mL	Recovery, %
Captopril	5.0	4.90	98.0
	2.5	2.43	97.2
	1.0	0.98	98.0
	0.5	0.48	96.0
	0.25	0.26	104.0
Captopril Disulfide	5.00	4.96	99.2
	2.5	2.42	96.8
	1.0	0.97	97.0
	0.5	0.49	98.0
	0.25	0.25	100.0
Captopril–Glutathione	12.0	11.70	97.5
	6.0	5.70	95.0
	3.0	2.96	98.7
Captopril–Cysteine	8.0	7.80	97.5
	4.0	3.90	97.5
	2.0	1.93	96.5
Plasma Blank	0	0	—

Preparation of Columns—The protective cotton was removed from an XAD-2 column, and the column was placed on a 200-mm test tube. The diluted column activator (5 mL) was passed through the column, and the column was washed with 5 mL of 0.1 M HCl containing EDTA. Before use, a 21-gauge syringe needle was placed on the tip of the column. Columns were prepared on the day used.

Reduction—Plasma samples and one plasma blank were thawed, and 1.0 mL was transferred to a 20-mL scintillation vial. Urea buffer (5 mL) was added to each vial, followed by 0.1 mL of 20% tri-*n*-butylphosphine solution. Captopril disulfide standard solution (0.10 mL) was added to the plasma blank. The scintillation vials were covered with tin-lined plastic caps, and the samples were mixed on a vortex mixer. The vials were placed in a 50 ± 3°C water bath with the water level at half the height of the vials. The reduction was allowed to take place in the dark for 1 h.

Isolation, Purification, and Derivatization—The samples and standard were removed from the water bath and 5 mL of 0.66 M phosphoric acid was added immediately to each vial. The vials were capped and gently inverted four times to mix the acid. The solutions were decanted onto separate activated XAD-2 columns. Five milliliters of 0.1 M HCl containing EDTA was added to each vial, and the vials were then gently inverted four times.

After each sample passed through a column, the 0.1 M HCl wash was decanted onto the appropriate column. After all the wash had passed through the columns, the syringe needles were removed. The columns were washed with 10 mL and then 20 mL of 0.1 M HCl, placed on 150-mm screw-cap culture tubes, centrifuged at ~1000 rpm for 4 min, and then placed on clean 150-mm screw-cap culture tubes containing 0.1 mL of the reagent. To each column, 10.0 mL of 0.05 M pH 6.9 buffer was added. From time to time, the eluates were gently mixed with the reagent during elution[10]. The reagent blank was prepared in a 150-mm screw-cap culture tube by adding 10 μL of the reagent solution to 1.0 mL of 0.05 M pH 6.9 buffer and mixing.

Extraction of Captopril Derivative—After 10 min, 1.0 mL of each sample and the standard were transferred to 150-mm screw-cap culture tubes. One milliliter of 1.98 M phosphoric acid and 10.0 mL of toluene were added to each sample, standard, and blank. The tubes were shaken on a shaker for 5 min and centrifuged at ~1800 rpm for 5 min.

Purification on C_{18} Cartridges—A C_{18} cartridge was attached to a 10-mL syringe with the plunger removed. The syringe with the cartridge was placed on a 200-mm test tube, and 8.0 mL of toluene extract was transferred to it. The solution passed through the column by gravity; occasionally it was necessary to initiate flow through the cartridge with a plunger. After all the extract passed through the cartridge, 5 mL of toluene was added. At the end of the washing, the remaining toluene was forced out with the plunger and discarded. Each cartridge and syringe was placed on a fresh 200-mm test tube. Dimethylformamide (5.0 mL) was added to each syringe. The solvent was allowed to pass through the column by gravity, and at the end of the elution, the remaining solvent was forced out with the plunger. The eluates were mixed on the vortex mixer.

Fluorescence Measurement and Calculations—Fluorescence of the samples and standard was measured at an excitation wavelength of 380 nm and a fluorescence wavelength of 440 nm using the reagent blank to set the instru-

[10] Sometimes it is necessary to initiate flow through the columns by pressure from the palm of the hand or a rubber bulb.

Table III—Reproducibility of Results [a]

Run	Fluorescence at 380/440 nm
1	54.0
2	53.5
3	53.8
4	55.0
5	56.0
6	54.2
Mean	54.4
SD	0.93
CV	1.7%

[a] Replicate assay of 2 μg of captopril disulfide/mL of plasma.

ment to zero fluorescence. The concentration of captopril per milliliter of plasma was calculated from the concentration and fluorescence of a captopril disulfide standard with the same instrument settings used for the sample and standard measurements.

RESULTS AND DISCUSSION

Captopril has no native fluorescence and a very low absorbance in the UV region. Fluorescence can be obtained by reacting captopril with maleimide derivatives such as *N*-(*p*-2-benzimidazolyl)phenylmaleimide, *N*-(3-pyrene)-maleimide, *N*-[*p*-(2-benzoxozolyl)phenylmaleimide, and 1-(7-(dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-yl)-1*H*-pyrrole-2,5-dione which react with the sulfhydryl group. Because of low water solubility, none of these reagents can be used for reaction of captopril in blood.

Among fluorescent-labeling sulfhydryl reagents, 1-(7-(dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-yl)-1*H*-pyrrole-2,5-dione was selected for analysis of captopril in serum. It provides good sensitivity and is stable during all steps of the procedure. The synthesis of this reagent was described by Machida *et al.* (10).

Captopril reacts with the reagent on a mole per mole basis. Excess reagent was used to speed up the reaction and to account for some impurities in column eluates, which can consume the reagent. The reaction of captopril with the reagent under the assay conditions was complete in ~15 min. The reaction product is stable in the mixture for at least 1 h. When solutions are acidified and stored in a refrigerator, the product is stable for at least 24 h.

The fluorescent adduct can be extracted from acidified solutions with many water-immiscible solvents such as ethyl acetate, toluene, and dichloromethane. Toluene was chosen because of its selectivity; it extracts very few impurities from aqueous reaction solutions. The captopril–reagent derivative is stable in toluene for at least 24 h, and the adduct can be absorbed quantitatively from toluene onto C_{18} cartridges. The excitation and fluorescence maximum wavelengths of the captopril–reagent adduct vary with the solvent. In dimethylformamide it is 380/440 nm.

Standard curves were prepared in the ranges of 0–1 μg and 0–10 μg of captopril/mL of plasma. Concentrations in the ranges tested produced straight lines that passed through the origin. Data for the standard curves are given in Table I.

The sulfhydryl group of captopril is very reactive. It can react with blood components or be oxidized to captopril disulfide and mixed disulfides. If the sulfhydryl group is not protected immediately after blood is drawn from the patient, the free captopril is oxidized to disulfides. A considerable amount of captopril is covalently bound to plasma proteins.

In vitro experiments show that when ^{14}C-labeled captopril, symmetrical disulfide, and captopril–cysteine mixed disulfide were added to plasma, after 1 week of storage at 5°C, 32% of captopril was covalently bound to plasma proteins and 68% remained as disulfides. In samples stored at −20°C, 68% of the captopril was bound to plasma proteins and 32% remained as disulfides. After 4 weeks of storage, 62% of captopril was bound to plasma proteins at 5°C and 87% at −20°C.

Interchange between the —SH groups of plasma proteins and captopril disulfides can occur. After 4 weeks of storage at 5°C and −20°C, a complete interchange between captopril and captopril–cysteine occurred; 43% of captopril was interchanged for symmetrical captopril disulfide. The *S*-methyl metabolite of captopril is stable in plasma.

To determine total captopril in plasma with the reagent, samples have to be reduced. Tri-*n*-butylphosphine was chosen as the reducing agent because it is a potent agent for the cleavage of captopril disulfide, captopril mixed disulfides, and drug covalently bound to proteins. One mole of tri-*n*-butylphosphine reduces 1 mol of disulfide, and the reaction is relatively rapid and quantitative.

The reduction of disulfides with tri-*n*-butylphosphine is pH and temperature dependent. Almost complete reduction of captopril disulfide is obtained in solutions at pH >8. It takes ~30 min to reduce captopril disulfide with 20 mg of tri-*n*-butylphosphine/mL of plasma at 50°C and ~45 min to reduce captopril bound to proteins. About 96% of the drug is reduced at this concentration. With 2 mg of tri-*n*-butylphosphine/mL of plasma, only 50% reduction is obtained. At room temperature, only ~85% of the plasma sample is reduced. Increasing the temperature to 70°C does not have any effect on the reaction. The volume of urea–Tris buffer up to 10 mL has no effect on the reduction efficiency. However, volumes of plasma >1 mL cause incomplete reduction of captopril disulfides.

Captopril was isolated from the tri-*n*-butylphosphine mixture and purified on XAD-2 resin before it was treated with the reagent. Captopril was retained on XAD-2 resin, while other compounds containing the sulfhydryl group passed through the column. The optimum pH for adsorption of captopril on XAD-2 resin is 0.5–2.5. The adsorptive power of the resin is optimized by the incorporation of a column prewash using an activator solution.

The unimpeded flow rate of the diluted sample through the column would be ~5 mL/min. This is too fast for complete retention of the drug on the resin. The flow rate is reduced to ~1 mL/min with a 21-gauge syringe needle.

Captopril can be quantitatively eluted from XAD resin with dioxane, methanol, ethanol, ethyl acetate, acetonitrile, acetone, isopropyl alcohol, tetrahydrofuran, dichloromethane, chloroform, and other solvents. Some of them elute interfering compounds. However, captopril does not react with fluorescent labels in any of these solvents; it reacts only in aqueous solutions. Removal of organic solvents by evaporation, even under controlled conditions, results in the oxidation of captopril. A 0.05 M phosphate buffer containing dimethylformamide proved to be a suitable eluant. It elutes captopril quantitatively from XAD-2 resin and provides a proper pH and medium for the reaction with the reagent. Up to 35% dimethylformamide does not interfere with the reaction. About 10 mL of dimethylformamide–phosphate buffer, pH 6.9, is needed for complete elution of captopril; ~2 mL of solvent is retained by the column. Therefore, before and after elution, columns must be centrifuged. Captopril is eluted from columns into tubes containing ~100 μg of reagent to prevent reoxidation of the drug. However, if needed, samples can be first eluted, gently mixed by four inversions, and 1 mL of eluate treated with 10 μg of the reagent.

Samples have to be further purified by extraction of the captopril–reagent derivative from the acidic solution with toluene. For complete extraction of the adduct, the ratio of aqueous solution to toluene should be 1:10. Excess reagent and some remaining impurities are removed with C_{18} cartridges. The

Table IV—Total Captopril in Human Plasma Measured by Fluorometric and GC–MS Methods [a]

Patient	Total Captopril, ng/ml		Δ (GC–MS − Fluorometry)	$\frac{\Delta}{\text{Mean}} \times 100$, %
	Fluorometry	GC–MS		
B439 096	769	738	−31	−4.1
100	544	558	+14	+2.5
124	213	217	+4	+1.9
150	523	499	−24	−4.7
201	715	702	−13	−1.8
229	171	157	−14	−8.5
262	539	551	+12	+2.2
301	656	658	+2	+0.3
312	512	505	−7	−1.4
313	650	673	+23	+3.5
338	600	628	+28	+4.6
380	560	554	−6	−1.1
	Mean absolute difference, %			3.05

[a] Patients received 100 mg of captopril orally.

captopril-reagent derivative adsorbs onto the cartridge packing from toluene and can be eluted with dimethylformamide.

A plasma sample with standard captopril disulfide (2 μg/mL) is run along with samples to compensate for incomplete reduction. If high (off scale) fluorescence readings from the sample are obtained, eluates from the cartridges can be diluted to proper concentration with dimethylformamide.

Recovery experiments were carried out by addition of various concentrations of captopril, captopril disulfide, captopril-cysteine disulfide, or captopril-glutathione disulfide to plasma. These were kept at room temperature for 4 h then stored at 5°C overnight. Results are shown in Table II. Recoveries ranged from 95 to 104%.

Reproducibility was checked by performing six assays of a plasma sample spiked with captopril disulfide at 2 μg/mL (Table III). Precision at this concentration is 1.7% (*CV*).

Results obtained by the fluorometric method were compared with those obtained by the GC–MS method (Table IV). The data were correlated by linear regression analysis, which yielded an intercept of 5.6, a slope of 0.9914, and a correlation coefficient of 0.9949. Agreement between the methods is acceptable.

Although the fluorometric method is very sensitive (because as little as 2 ng of a pure captopril standard/mL of dimethylformamide solution can be accurately measured in a 1-cm cell, without HPLC separation), the method can be used for measuring captopril in plasma containing >200 ng of the drug/mL. Therefore, it is suitable for patients who accumulate captopril during extended therapy and have microgram per milliliter levels of captopril plus disulfide metabolites in plasma. It can be especially useful to the small hospital laboratory which does not have expensive instrumentation (such as GC–MS) available.

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Reverse-Phase High-Performance Liquid Chromatographic Determination of Halogenated 8-Hydroxyquinoline Compounds in Pharmaceuticals and Bulk Drugs

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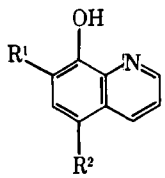
Received August 23, 1983, from the *Food and Drug Administration, 599 Delaware Avenue, Buffalo, NY 14202.* Accepted for publication December 6, 1983.

Abstract □ A reverse-phase high-performance liquid chromatographic (HPLC) method was developed for determining iodochlorhydroxyquin, 5,7-dichloro-8-hydroxyquinoline, and 5,7-diiodo-8-hydroxyquinoline in creams, ointments, shampoos, tablets, and bulk drugs. A column packed with 10-μm phenyl-silica and a mobile phase of 0.001 M $NiCl_2$ in acetonitrile–methanol–water (30:20:50) was used to separate the nickel complexes of the three drugs, with detection at 273 nm. Analysis of creams, ointments, shampoos, and tablets gave results close to the label declarations. Recovery of standard material added to samples was ≥98%. Linearity of response was shown over a range of 30–150% of label claim for standards of the three drug substances. Multiple analyses of iodochlorhydroxyquin and diiodohydroxyquinoline bulk drugs showed purities of 99.96 and 98.77% with *CV* of 1.17 and 0.73%, respectively. The HPLC method offers an alternative to current USP procedures, which lack stability-indicating and specificity characteristics.

Keyphrases □ HPLC—Halogenated 8-hydroxyquinoline compounds □ 8-Hydroxyquinoline—halogenated, HPLC

The current USP XX IR spectrophotometric procedure (1) for determining iodochlorhydroxyquin (I) in creams and ointments has many deficiencies, as reported by Gruber *et al.* (2), who used GC to determine trimethylsilyl ether derivatives of related halogenated 8-hydroxyquinolines. A colorimetric procedure (3) complexed I with nickel ion. However, other 8-hydroxyquinolines, present as impurities, would interfere to give high results. A normal-phase high-performance liquid chromatographic (HPLC) procedure (4) involved esterification of I using a mixture of pyridine–acetic anhydride, an evaporation step, and a reconstitution of the residue in the mobile phase. Procedures using anion-exchange HPLC (5, 6) and reverse-phase HPLC (7, 8) failed to adequately separate I from other 8-hydroxyquinolines.

In this study, a simple and specific reverse-phase HPLC method was developed to separate and quantitate iodochlorhydroxyquin (I), 5,7-dichloro-8-hydroxyquinoline (II), and 5,7-diiodo-8-hydroxyquinoline (III) as their nickel complexes



I, $R^1 = I$, $R^2 = Cl$
II, $R^1 = R^2 = Cl$
III, $R^1 = R^2 = I$
IV, $R^1 = H$, $R^2 = Cl$
V, $R^1 = R^2 = H$
I.S., $(C_6H_5)_2NH$

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