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Research Article

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Structurally similar compounds separation and validation in benzisoxazol derivatives by HPLC

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ABSTRACT

Chromatographic separation was achieved on Purospher STAR RP-18e 250 mm long, 4.6 mm inner diameter and 3µm particle size column. Perchloric acid buffer and acetonitrile was used as mobile phase at the flow rate of 1.0 ml/min with gradient composition. Injection volume was set as 20 µl and UV detection was made at 238 nm. Proposed method was validated as per ICH Q2A guidelines. Inter and intra-day precision of the method was studied and found the method is repeatable and reproducible. Solution stability was carried out up to 24 h. Proposed method LOD was <0.007% and LOQ was <0.012%. Linear response were observed against the respective concentration and regression coefficient of the linear curve r^2 was >0.999. Accuracy was studied in four different concentrations in triplicate (LOQ,50, 100&150% with respect to sample concentration).Accuracy of LOQ level was observed between 91 and 112% of recovery, and the other level recovery was between 93 and 105%. Five common impurities of Paliperidone and Risperidone was separated and validation was demonstrated.

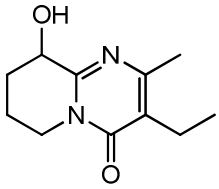
Key words: Benzisoxazol derivative, Paliperidone, 9-Hydroxy Risperidone, Reverse phase HPLC, Keto impurity separation.

INTRODUCTION

Paliperidone is chemically known as (±)-3-[2-[4-(6-fluoro-1,2 benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-9-hydroxy-2-methyl-4Hpyrido[1,2-a]pyrimidin-4-one, which is used for psychotropic agent belongs to the chemical class of benzisoxazol derivative. Paliperidone offers distinctive treatment and valuable option for patients with schizophrenia [1]. Paliperidone extended release drug is proved as a superior drug than risperidone [2]. Many metabolites had been reported in risperidone and paliperidone in existing literatures. Paliperidone (9-Hydroxy risperidone) is the active metabolite in risperidone, 9-keto {3-[2-[4-(6-fluorobenzo-[d]isoxazol-3-yl)-1-piperidinyl]ethyl]-2-methyl-7,8-dihydro-4H-pyrido[1,2-a]pyrimidin-4,9(6H)-dione} is the active metabolite in Paliperidone. Hence, metabolite identification and separation is the important phenomenon to be considered during the method development of risperidone and paliperidone. Recently Patteet *et al* [3] reported a method for the quantification of 16 antipsychotics and 8 major metabolites in serum using UHPLC with tandem mass spectrometry. Paliperidone positional isomer is the carryover impurity from key starting material (benzisoxazol). Isomer separation and identification is quite challenge in starting material and paliperidone, because of identical spectra, polarity and mass profile. Paliperidone N-oxide was reported as a very common impurity during oxidative stress study. Thus, separation of paliperidone, risperidone, isomer, metabolite and N-oxide is essential during the method development. Sawant *et al* identified and characterised degradants of paliperidone [4]. Bindu *et al* [5] reported a short UPLC method for related compounds estimation in Paliperidone. Hence, reported method was not demonstrated about the separation of isomer and risperidone. These impurities are common and expected impurities

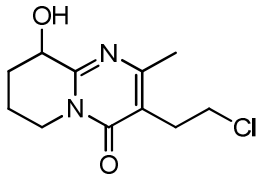
in paliperidone. Few literatures were reported to describe the related compounds estimation in Paliperidone and risperidone.

During the process development of Paliperidone, 12 impurities were identified in crude stage and their structures are shown in fig.1. Since there is no report existing in the literature to describe the separation of all the 12 impurities, the present study was planned for method development and validation.



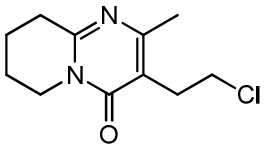
3-Ethyl-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-A



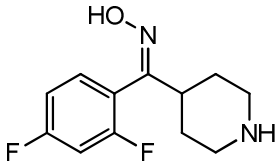
3-(2-Chloroethyl)-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-B



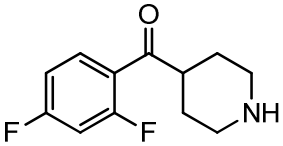
3-(2-Chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-C



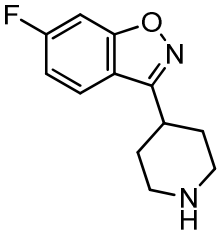
(*Z*)-(2,4-Difluorophenyl)(piperidin-4-yl) methanone oxime

Impurity-D



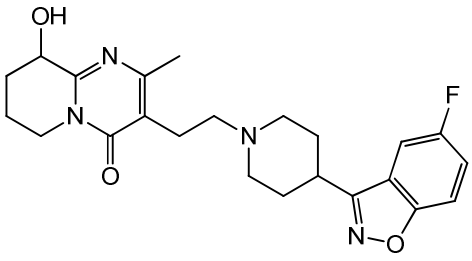
(2,4-difluorophenyl)(piperidin-4-yl)methanone

Impurity-E



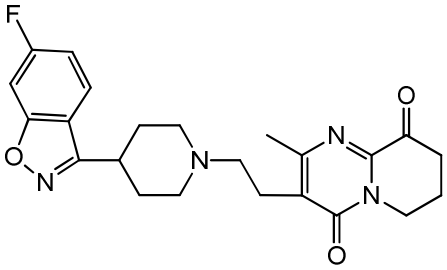
6-Fluoro-3-(piperidin-4-yl)benzo[*d*]isoxazole

Impurity-F



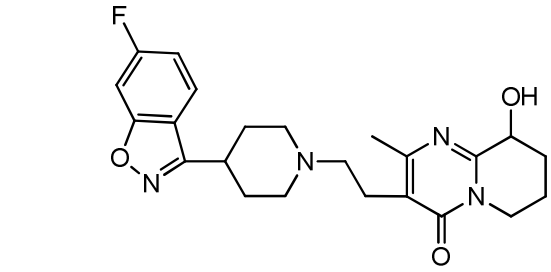
3-(2-(4-(5-fluorobenzo[*d*]isoxazol-3-yl) piperidin-1-yl)ethyl)-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-G



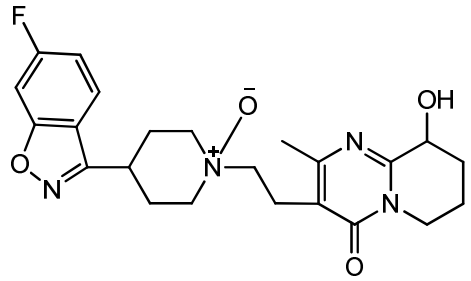
3-(2-(4-(6-fluorobenzo[*d*]isoxazol-3-yl) piperidin-1-yl)ethyl)-2-methyl-7,8-dihydro-4*H*-pyrido [1,2-*a*]pyrimidine-4,9(6*H*)-dione

Impurity-H



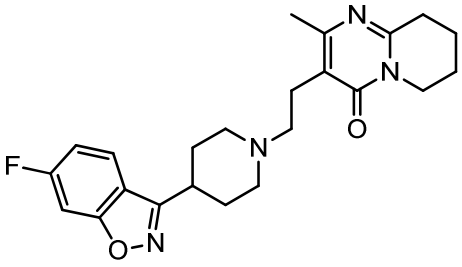
3-(2-(4-(6-fluorobenzo[*d*]isoxazol-3-yl) piperidin-1-yl)ethyl)-9-hydroxy-2-methyl -6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Paliperidone



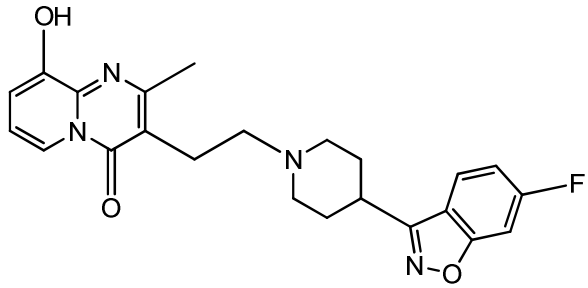
4-(6-fluorobenzo[*d*]isoxazol-3-yl)-1-(2-(9-hydroxy-2-methyl-4-oxo-6,7,8,9-tetrahydro -4*H*-pyrido[1,2-*a*]pyrimidin-3-yl)ethyl) piperidine 1-oxide

Impurity-I



3-(2-(4-(6-fluorobenzo[*d*]isoxazol-3-yl) piperidin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-J



3-(2-(4-(6-fluorobenzo[*d*]isoxazol-3-yl) piperidin-1-yl)ethyl)-9-hydroxy-2-methyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one

Impurity-K

Figure 1: Structure of Paliperidone and its impurities

EXPERIMENTAL SECTION

Reagents and chemicals

ACS grade perchloric acid (70%) was purchased from Merck specialties private Ltd, Mumbai, India-400 018. HPLC grade acetonitrile and sodium hydroxide procured from S.D. fine chem Ltd, Mumbai, India-400 030. ACS grade hydrochloric acid purchased from Sigma Aldrich co, St Louis, Mo 631 03, USA-314-771-57615.Water used for mobile phase preparation and diluent was purified in Millipore Mill Q water system. The investigated samples of Paliperidone and known impurities are prepared in Research and development department, Cipla Ltd, Virgo nagar, Bangalore, India-560 049.

Equipments and instruments

Shimadzu make HPLC system LC-2010 CHT and Agilent 1200 series was used for method development and validation, Agilent technologies 1290 infinity coupled with AB Sciex QTRAP 5500 mass spectro meter was used for MS studies.

Chromatographic conditions

Chromatographic separation was achieved on a Purospher STAR RP-18e (250mm x 4.6mm, 3µm) column. Mobile phase was a gradient mixture of solution A (Buffer: 13 ml of 70% perchloric acid and 4.5 g of Sodium hydroxide dissolved in 1000 ml of water , pH of the solution was adjusted to 2.60 with dilute sodium hydroxide solution) and mobile phase B (Acetonitrile) pumped at flow rate of 1.0 ml/min. Gradient programme was as follows [Time (min)/% A(v)/%B(v)] 0/77/23, 7/77/23, 35/72/28, 45/65/35, 60/25/75, 62/77/23, 67/77/23. The injection volume was set as 20 µl and UV detection was made at 238 nm, Mobile phase A and B in the ratio of 3:1 v/v was used as a diluent. Sample concentration was 1 mg/ml. 50 mg of the examined sample was transferred in to 50 ml volumetric flask and 15 ml of 0.1 M hydrochloric acid added, sonicated to dissolve and diluted to the volume with diluent.

Method validation

Specificity, System suitability, Precision, Limit of Detection (LOD), Limit of Quantification (LOQ), Linearity & Range, Solution stability, Accuracy and Robustness was studied according to ICH Q2[6].

Specificity and system suitability
There should not be any interference due to mobile phase gradient, diluent at the retention time of impurities and Paliperidone. 0.15% of the impurity solutions were prepared individually and injected. All the impurities are spiked in the level of 0.15% in Paliperidone and injected for system suitability. The limit set for system suitability criteria was not less than 5000 theoretical plates for the peak of Paliperidone. Resolution between any two adjacent peaks should not be less than two.

System Precision
System precision was checked by injecting six replicates of limit level concentration of impurities and Paliperidone. The percentage relative standard deviation (%rsd) for the peak area responses of impurities and Paliperidone from six replicate injections of standard solution should not be more than 5% for each and 1% for retention time.

Method Precision
Method precision was evaluated by injecting six replicates of fresh test preparation. Impurities observed in replicate injection between the results should not be more than the limit specified below. Impurities observed in the sample were less than the LOQ level %rsd need not to be calculated. Observed results are ≤0.10%, >0.10% and <1.0%, the %rsd of the limit not more than15.0%, 10.0% and 5.0% respectively.

Intermediate Precision
Intermediate precision was carried out in different instrument by different analyst on different day by injecting six test preparations in fresh injection. Limit set for intermediate precision was same as per method precision additionally cumulative %rsd was calculated between repeatability and reproducibility. However the limits are same as per repeatability study.

Limit of Detection (LOD) and Limit of Quantification (LOQ)
A serial dilution of known impurities and Paliperidone were diluted and injected in chromatographic system. The LOD & LOQ was determined based on S/n ratio and precision. S/n ratio ≥3 and ≥10 was considered as LOD and LOQ respectively. Precision at LOQ level should be determined; the %rsd for peak area responses of impurities and Paliperidone from six replicates of LOQ solution should not be more than 5% for each and 1% for retention time.

Linearity and range
Linearity was studied in six different levels from LOQ to 150% of working level concentration, such as LOQ, 50%, 80%, 100%, 120% and 150%. Calibration curve was computed between concentrations versus response. From the calibration curve regression co-efficient r^2 was determined. Regression co-efficient should not be less than 0.999.

Solution stability
Stability of analytical solution was checked in seven different intervals from 0 to 24 h. Initial and each interval chromatogram were compared, if any extraneous peaks of impurities or degradation are present in the chromatogram of sample and standard solution, solution stability is considered as unstable.

Accuracy
Accuracy of the proposed method was determined by the standard addition method on API, known amount of six impurities have been added at four different concentrations (LOQ, 50%, 100%, and 150%). The accuracy was calculated as percent recovery, amount of analyte added to the sample versus recovered. The acceptance criteria set for % recovery for LOQ and other level was 85to115% and 90 to110% respectively.

Robustness
To evaluate robustness of the method, experimental factors that might cause variability in the method responses were examined. Two factors (pH of buffer, column temperature) were investigated. For this test one lower & higher value of the factors were used. The acceptance criteria set for robustness study was as per intermediate precision.

RESULTS AND DISCUSSION

Eleven impurities separation was demonstrated in the specificity study. However, impurity-A, B, F, H, I and J standards were used in method validation. Impurity-C, D, E were not present in our sample, though this impurities standards were used only for specificity study. Impurity-G and K was present in our crude sample in the range of <0.05%. Probable structure of these two impurities was determined by mass spectra; however these impurities are not prepared and characterized. In mass spectra same mass were observed for impurity-G and Paliperidone {Positive mode: m/z $[M^{+1}]$ = 427.21}, for better understanding Paliperidone and impurity-G was further fragmented. Similar fragmentation pattern were observed for both the analytes. Same kind of precursor mass and fragmentations pattern

was observed in starting material such as benzisoxazole. Impurity-K mass was identified as {Positive mode: m/z} $[M^{+1}]$ = 423.18, based on the fragmentation pattern probable schema was drawn. For better understanding probable schema were presented in below.

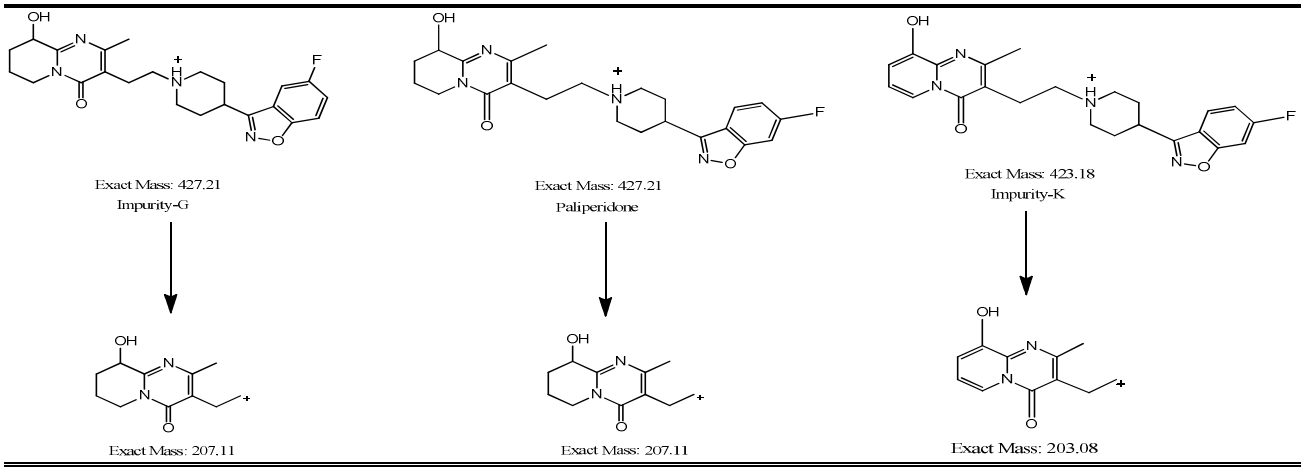


Figure 2: Fragmentation scheme of impurity-G, Paliperidone and impurity -K.

Specificity and system suitability

There is no diluent and gradient interference at the retention time of known, unknown impurities and Paliperidone. Peak purity was confirmed with PDA detector, there is no co-elution. The column efficiency for the peak of Paliperidone in system suitability solution was 29553 theoretical plates. Minimum resolution between two adjacent peaks is 2.8, maximum resolution was 18.6. Typical system suitability chromatogram was presented in fig.3.

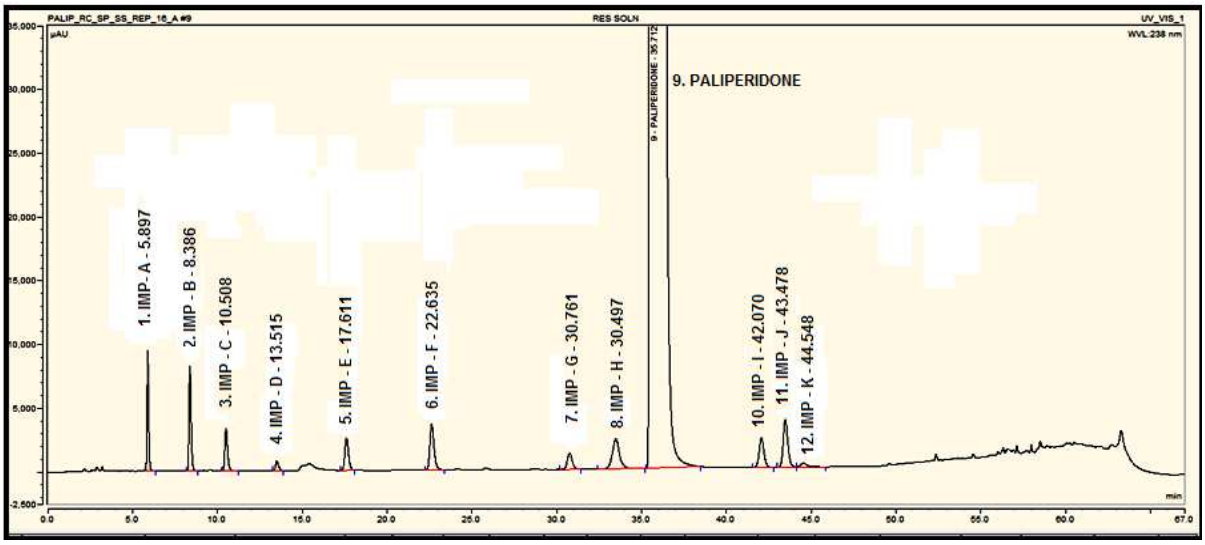


Figure 3: Typical system suitability chromatogram of Paliperidone spiked with impurities

Precision

System precision

The %rsd for the peak area responses of impurities and Paliperidone from six replicates of standard solution were <1.0% and <0.10% for retention time.

Repeatability and reproducibility

Intra and inter-day precision of the method were evaluated by content of known impurities and single maximum unspecified impurities. The %rsd of the impurities content in six determinations was well within the limit set for acceptance criteria. Intermediate precision results were evaluated and compared with repeatability study. The% rsd of the impurities content of these six determinations was well within the limit as per acceptance criteria. Cumulative %rsd between inter, intra-day precision results also well within the limit. Proposed method is repeatable and reproducible. Precision results are presented in below table.

Table 1: Inter, intraday precision results

Analytes	% of impurity present in sample	%RSD of intra-day replicate results (n=6)	Cumulative %RSD of inter-intraday precision results (n=6+6)	Precision Limit (% RSD)
Impurity-A	0.001	BLOQ	BLOQ	BLOQ
Impurity-B	0.06	0.3	1.56	15%
Impurity-F	0.03	1.5	1.94	15%
Impurity-H	0.14	6.0	6.19	10%
Impurity-I	0.004	BLOQ	BLOQ	BLOQ
Impurity-J	0.02	4.1	3.57	15%
Unspecified impurity	0.07	0.3	9.71	15%

n-6, six replicate injection of intraday precision. n- 6+6, six replicates of intraday and 6 replicate of inter-day injection. BLOQ- obtained values are below LOQ, hence no need to calculate %rsd.

LOD and LOQ

The LOD & LOQ were determined by injecting a series of dilutions of known concentrations of the impurities and API. For better understanding sensitivity data is presented below.

Table 2: Sensitivity data

Analytes	LOD Conc.	LOD S/n ratio	LOQ Conc.	LOQ S/n ratio	%RSD of standard area at LOQ level (precision n=6)
Impurity-A	0.004%	32	0.012%	54	0.5%
Impurity-B	0.004%	19	0.012%	26	1.5%
Impurity-F	0.004%	8	0.012%	21	4.1%
Impurity-H	0.007%	7	0.022%	21	2.7%
Impurity-I	0.004%	7	0.012%	20	2.9%
Impurity-J	0.004%	9	0.012%	26	2.6%
Paliperidone	0.002%	7	0.008%	20	3.0%

Linearity

Linear responses were observed between the peak areas and the respective concentrations of the analytes. Linearity data is presented in Table 3.

Table 3: Linearity data

Analytes	Slope of Calibration curve	Regression Coefficient (r^2)	Relative response factor of analytes
Impurity-A	376207	1.000	1.04
Impurity-B	295948	1.000	0.80
Impurity-F	331586	1.000	0.88
Impurity-H	215021	1.000	0.65
Impurity-I	285393	1.000	0.85
Impurity-J	408106	1.000	1.09
Paliperidone	374691	1.000	1.0

Solution stability

The results of initial analysis and results of each time interval were compared and found to be well within the limit set for the acceptance criteria. There is no extraneous peak or degradation observed in the chromatogram of sample and standard solution. Hence the sample solution was stable up to 24 h. (Study was conducted up to 24 h)

Accuracy

The %recovery for all accuracy levels and %rsd of the studies are well within the limit set for acceptance criteria. So the developed method gave satisfactory recovery for API. Hence the method is accurate.

Table 4: Average percentage recovery

Impurities	Impurity-A	Impurity-B	Impurity-F	Impurity-H	Impurity-I	Impurity-J
LOQ (n=3)	94%	91	112	96	97	98
50% (n=3)	101	99	105	94	100	101
100%(n=6)	99	98	102	96	99	99
150%(n=6)	98	96	100	93	97	97

n=3: Triplicate preparation, n=6: six replicate preparation

Robustness

Robustness of the method was measured by making small and deliberate change in the chromatographic conditions and results are observed. Column temperature ±2 ºC and pH of the mobile phase ±0.2 was changed and measured.

Method was sensitive with mobile phase pH. Mobile phase pH should be maintained strictly during the mobile phase preparation. There is no impact on result in column oven temperature variation.

CONCLUSION

A simple, sensitive, robust and accurate method was developed to control the related compounds in Benzisoxazol derivatives (Paliperidone and Risperidone drug substance). As a significance of the proposed method, six structurally similar compounds (including isomer and metabolite) were separated with baseline separation. Five common impurities of Paliperidone and Risperidone were separated along with two new impurities. As an outcome of this study, we believe the proposed method may be a better one to monitor and control the impurities in Paliperidone and Risperidone synthesis and routine quality control release.

Acknowledgement
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