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Research Article

Characterization of a Sulfadiazine-Induced Lithiasis Calculus by Physicochemical Techniques

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Abstract. Currently available information on drug lithiasis usually describes the calculi based on the prescriptions given to the patient, but without a physicochemical characterization of the calculi themselves. We here have applied different, complementary, physicochemical techniques for a complete characterization of an unusual urolithiasis calculus. The calculus was characterized using powder X-ray diffraction, infrared spectroscopy, mass spectrometry, ^{1}H-NMR spectroscopy, and scanning electron microscopy. The precise nature of the calculus was identified, being formed by *N*4-acetylsulfadiazine, so being related to the drugs prescribed to the patient. Analytical techniques widely used in laboratories of Materials Chemistry have proven to be useful tools for characterizing the chemical nature of unusual urolithiasis.

KEY WORDS: calculus; lithiasis; *N*4-acetylsulfadiazine; physicochemical characterization; sulfadiazine.

INTRODUCTION

Urinary lithiasis is as ancient as mankind. Vesicular calculi of uric acid have been found in Egyptian mummies and pre-Columbian tombs. In France, one out of 50 skeletons found below a dolmen dated on 3,000 years has a calculus of calcium phosphate in the pelvis (1,2). In the nineteenth century, vesicular lithiasis was very frequent in Europe, generally composed of urates or phosphates, mainly affecting children, while in adults, the uric lithiasis was predominant. This situation continues currently in non-industrialized countries, while in developed countries, lithiasis affects mainly adults, the more frequent composition being calcium oxalate with different hydration degrees.

A few decades ago, renal lithiasis was very frequent, even in children and teenagers. Now, vesicular lithiasis is much less common and is generally restricted to old men and associated to prostatic or urethral pathologies. On the other hand, lithiasis of the upper urinary conducts is still very common, mainly that of calcium oxalate. This is the dominant type of calculus (up to 80% in some reports (2–4), 72.2% in our hospital over more than 6,200 cases), followed by calcium phosphate; as of carbonate apatite, it is 10–18% in general reports, 11.9% in our hospital.

Concerning iatrogenic lithiasis, that is, those provoked by the direct or indirect action of medical treatments, medication lithiasis is a particular and low frequent type. Using infrared techniques, Jungers *et al.* (2) have reported a 2% incidence, while Hidalgo *et al.* (5) have reported a single triamterene calculus. In our hospital, the case reported in the present work is the first we have ever found.

This work is centered in several red-colored renal calculi from a 50-years-old male with HIV infection in C3 stage, hepatitis C, brain toxoplasmosis, and tuberculosis. This patient received medical treatment between May 1999 and May 2005 with retrovirals stavudine, lamivudine, and nevirapine. In May 2005, medication was suspended up to August 2006, when stavudine, lamivudine, lopinavir–ritonavir, sulfadiazine, pyrimethamine, and folic acid were prescribed, up to November 2007. The macroscopic aspect of the calculi, mainly the color, suggested that this was not a usual lithiasis.

EXPERIMENTAL METHODS

The calculus was ground to fine particles before the study. Well-defined signals were obtained both in X-ray diffraction and Fourier transform infrared spectroscopy (FT-IR) spectroscopy, the two techniques that may be more influenced by the particle size.

The powder X-ray diffraction pattern of the sample was recorded in a Siemens D-500 diffractometer, working at 40 kV and 30 mA with a scanning speed of 2°/min, with filtered Cu$K\alpha$ radiation (λ=1.5418 Å).

Scanning electron microscopy (SEM) was performed on the ground sample at Servicio General de Microscopía Electrónica, Universidad de Salamanca, using a Digital Scanning Microscope DSM 940 Zeiss, connected to a Scan Converter DSC-1024 G Sony, which digitalizes the images. The sample was coated with a thin gold layer (about 20 nm) by metallization using a Bio-Rad ES100 SEN coating system. Chemical analysis was carried out by means of an energy dispersive X-

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ray spectroscopy (EDX) system, which allows to analyze elements with atomic number higher than 10 and concentrations larger than 0.5 wt%.

The infrared absorption spectra were recorded in a Fourier-Transform Perkin-Elmer 1739 spectrometer, equipped with a He-Ne laser radiation source (λ=632.8 nm), and Spectrum for Windows software. The KBr pellet technique was used, with a sample/KBr mass ratio close to 1:300.

The electrospray ionization mass spectrum was recorded on a Waters ZQ 4000 spectrometer; positive and negative ion mass spectra were obtained by signal averaging of 25 consecutive shots. The ^{1}H NMR spectrum was acquired at Servicio General de Resonancia Magnética Nuclear (Universidad de Salamanca) in a Bruker Avance DRX 400 MHz instrument, equipped with a 5-mm multinuclear inverse probe with Z-gradients (BBI-Z). Other experiment parameters are included in the own NMR figure. The sample was dissolved in dimethyl sulfoxide (DMSO).

RESULTS AND DISCUSSION

SEM (Fig. 1) and EDX analyses, together with the morphological *de visu* data, provided important information about the nature of the calculi. Sulfur was the only element detected by EDX. The complete absence of phosphorus and calcium rules out a great number of the usual calculi and suggests a purely organic composition for the present one.

Concerning the presence of sulfur, this element may be in the calculi in several forms, depending on their precise nature. The first possibility is an ionic calculus, probably ritonavir sulfate. The second possibility, considering the medical history of the patient, is the formation of a calculus composed of sulfadiazine or any of the metabolites resulting from its degradation, but keeping the sulfur-containing group. This second possibility opens new ones: sulfadiazine may be able to form the calculus as a neutral molecule itself, but its amine groups may be easily protonated within the organism, giving rise to ionic calculi formed by cationic protonated sulfadiazine and organic anions (carbonate, oxalate, *etc.*). Even more, in this last case, sulfadiazine may gain one or several protons, leading to changes in the stoichiometry of the compound forming the calculus.

To discern the nature of the calculus, it was submitted to several physicochemical analyses. Unfortunately, element chemical analysis of C, N, and S could not be carried out because no reliable results could be obtained with the available amount of sample.

Among the drugs ingested by the patient, those prone to form calculi are sulfadiazine and ritonavir, as well as, in a lesser extent, lopinavir, stavudine, lamivudine, and nevirapine. Lopinavir and ritonavir are two retrovirals from the indinavir sulfate family, whose high incidence on lithiasis is well known; in the study from Nadler *et al.* (6), up to 28.6% of the patients treated with this compound developed lithiasis. It has been also reported that formation of ritonavir calculi takes 10–18 months, and in the present case, the calculi were formed 14 months after beginning of the treatment, which again suggests the possible formation of ritonavir sulfate.

The X-ray diffraction pattern of the calculus was recorded and compared to the reported diffractograms for ritonavir. The diffractogram is included in Fig. 2. Two polymorphs of ritonavir have been widely studied by Bauer *et al.* (7); however, the diffractogram of our calculus was different than those reported by Bauer for both polymorphs. However, this does not allow to definitely rule out that the calculus may be formed by ritonavir. This is dissolved after ingestion and may recrystallize after arriving to the kidneys, and although the molecule is the same, the final crystalline form may be completely different to the initial one. Moreover, ritonavir may have been degraded within the organism, and the calculus being formed by a part of the molecule, not the complete molecule.

The FT-IR spectrum of the calculus is included in Fig. 3. It shows significant bands at 3,360, 3,313, 3,113, 3,042, 2,939, 2,872, 1,678, 1,587, 1,536, 1,331, 1,264, and 1,162 cm^{-1}. The main bands in the spectrum of ritonavir, reported by Sinha *et al.* (8), are recorded at 3,480, 3,380, 2,964, 2,720, 1,735, 1,716, 1,645, 1,622, 1,522, and 1,108 cm^{-1}.

It is obvious that the main infrared bands of our calculus do not coincide with those of ritonavir, and consequently we should rule out its presence, as well as of their metabolites, in our sample. However, the large number of bands recorded and their sharpness points out to the predominantly (or even exclusive) organic nature, suggesting again a medication origin. The spectrum of sulfadiazine (9,10) is included in the same figure; a rather important similarity can be observed, especially in the low wavenumbers region, below ca. 2,000 cm^{-1}.

On comparing these spectra, it is concluded that the spectrum of the calculus contains almost all the characteristic bands of sulfadiazine; for instance, the N–H vibrations

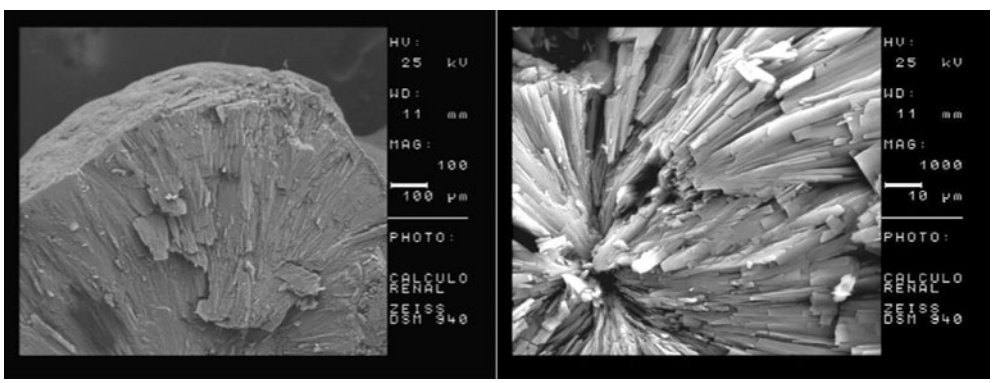

Fig. 1. Micrographs of the calculus. The *scale bar* corresponds to 100 µm (*left*) or 10 µm (*right*)

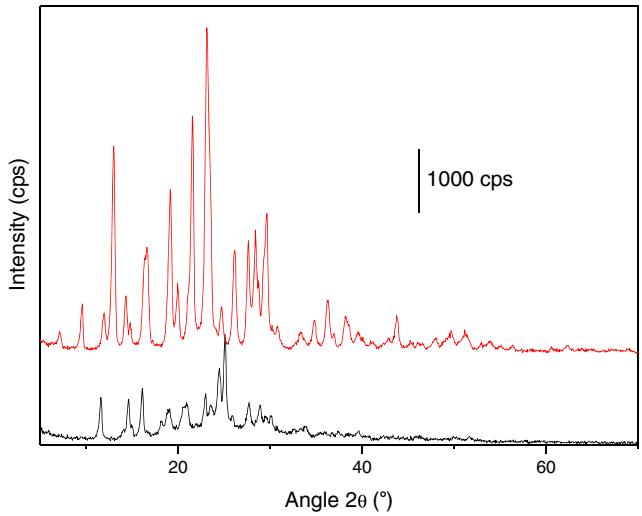

Fig. 2. Comparison between the X-ray diffractograms of the calculus (*below*, *black*) and sulfadiazine (*top*, *red*)

between 3,500 and 3,100 cm^{-1}, those due to C–H bonds stretching at 2,800–3,050 cm^{-1}, C–C vibrations at 1,300–1,500 cm^{-1} and C=C at 1,600–1,700 cm^{-1}, sulfonamide group, R–SO_2–N, at 1,336, 1,162, and 571 cm^{-1}, and C=N bonds at 1,590 cm^{-1}.

However, some significant differences can be also observed. In the 3,500 cm^{-1} region, the bands are similar, but with a small variation in position and, mainly, in intensity. This difference suggests that the terminal $-NH_2$ group of the neutral molecule may have been modified, probably protonated, in the calculus, which might thus be formed by a sulfadiazine derivative. The very intense peak at 1,092 cm^{-1} is typical of sulfate anions, suggesting that the calculus may be formed by sulfate of protonated sulfadiazine. However, this vibration may also correspond to the group –SO_2–, located in the central part of the sulfadiazine molecule. In order to sort out his problem, a very small amount of the calculus was dissolved in water containing a drop of 2 M HCl, and the further addition of Ba^{2+} immediately led to the formation of a white precipitate of $BaSO_4$. Unfortunately, this cannot be considered conclusive for the presence of sulfate, because in the acidic medium (needed to dissolve the calculus), the group –SO_2– hydrolyzes giving rise to sulfate anions, and leading to a "false positive" on the presence of this anion. On the other hand, comparing very carefully both spectra, it is observed that a band at 1,374 cm^{-1} in the spectrum of the calculus is absent in that of pure sulfadiazine. This band may be ascribed to carboxylate anions, which suggests that the calculus may be formed by a sulfadiazine carboxylate. In such a case, oxalate, alone or combined with other anions, may be obviously the first candidate because of its presence in many lithiasis.

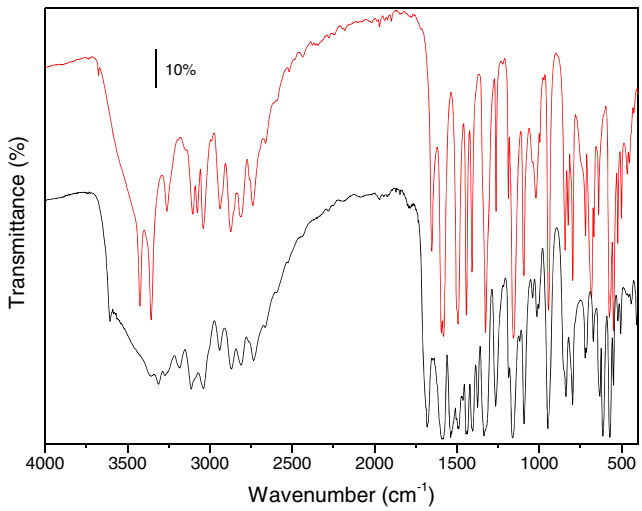

Fig. 3. FT-IR spectra of the calculus (*below*, *black*) and of sulfadiazine (*up*, *red*)

A comparison was done between the X-ray diffractogram of the calculus and that of sulfadiazine (Fig. 2). As it can be observed, both diagrams are very different, *i.e.*, the calculus is not sulfadiazine in its original form. Moreover, if the calculus is formed by a compound derived from sulfadiazine, or by molecular fragments formed during its metabolism, the corresponding X-ray diffractograms may also be completely different from that of sulfadiazine.

Thus, in order to know if the calculus was formed by molecular sulfadiazine or by a derived ionic compound, it was studied by mass spectrometry. The spectrum obtained (Fig. 4) indicates that it is composed by a single compound with molecular mass of 292.0. The most intense signal in the spectrum, at 293.0 units, corresponds to the usually called "M + H" or molecular ion, that is, a cation formed by protonation of the molecule composing the sample, and thus it can be deviated by the electric field. From the difference between the molecular mass found and the molecular mass of sulfadiazine, the only organic species that can combine with sulfadiazine to produce a molecule with a molecular mass of 292.0 is the acetyl group (CH_3–CO). In this sense, all the signals in the spectrum perfectly match, both their positions and relative intensities, with those in the spectrum simulated for the molecular ion with the formula $C_{12}H_{13}O_3N_4S$. This simulation was carried out using the free software available at Sheffield University (http://winter.group.shef.ac.uk/chemputer), which considers both the composition of the molecule and the natural isotopic abundance of each of its elements.

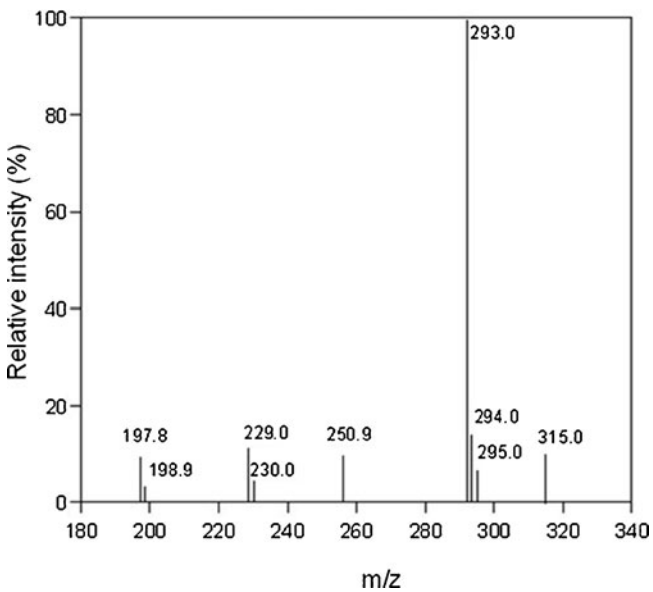

Fig. 4. Mass spectrometry spectrum of the calculus

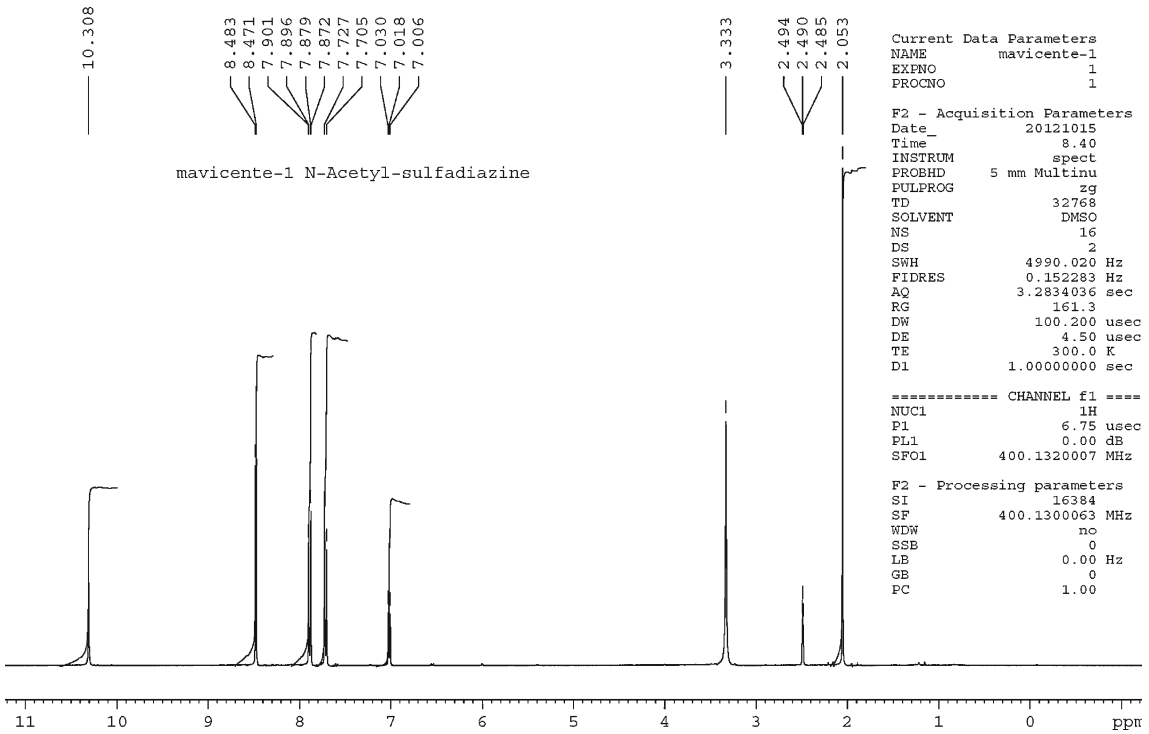

Fig. 5. ^{1}H NMR spectrum of the calculus

Thus, the calculus should be formed by an acetyl derivative of sulfadiazine, formed by an amide bond between the sulfadiazine and the acetyl group; this may come from acetic acid or any of their derivatives (ester, amide, *etc.*). This compound is named *N*4-acetylsulfadiazine, which is a known metabolite of sulfadiazine (11). Moreover, this compound perfectly matches with the FT-IR signals and with the other experimental data here reported.

In order to definitely confirm the composition of the calculus, it was submitted to ^{1}H NMR spectroscopy. The spectrum obtained is shown in Fig. 5 and perfectly matches with the theoretical spectrum of *N*4-acetylsulfadiazine. Only the signal corresponding to the proton in NH groups is not observed, which can be expected because of the interaction with the DMSO used as solvent. No signals from other compounds were found, demonstrating that *N*4-acetylsulfadiazine is the only component of the calculus. The structure of this compound is shown in Fig. 6.

The presence of acetyl in the living organism may come from three metabolic routes (12):

1. *Cycle of fatty acids*. Fatty acids are synthesized in the citosol by addition of dicarbonated fragments to a chain linked to a protein carrier of acyls. Then, the fatty acids are carried into the cell, to the mitochondria, where the enzymes fragment the acetyl groups. The acetyl groups are carried from the mitochondria to the citosol by the citrate–malate shuttle. These acetyls are then bonded to the coenzyme A to form acetyl coenzyme A, which enter to the Krebs cycle.
2. *Cycle of citric acid*. The common final way for the oxidation of combustible molecules (carbohydrates, amino acids, and fatty acids) occurs inside the mitochondria. Most of the combustible enters in the cycle of acetyl coenzyme A. The carbon atoms in the acetyl fragment are completely oxidized to CO_2 in the citric acid cycle, with simultaneous formation of HNAD and H_2FAD, which transfer their high potential electrons to the respiratory chain, with final formation of ATP. The complete oxidation of an acetyl unit generates one GTP, three HNAD, and one H_2FAD.
3. *Acetyl coenzyme A*. The main sources of this active dicarbonated fragment are the oxidative decarboxylation of pyruvate and the β-oxidation of fatty acids. Acetyl coenzyme A can also derivate from ketogenic amino acids. The final destination of acetyl coenzyme A, contrary to other metabolism molecules, is very restricted; among other routes, it can be completely oxidized to CO_2 in the citric acid cycle, as explained above.

N-Acetylation of aromatic amines is to be expected (13). *N*4-Acetylsulfadiazine may be formed by a reaction between

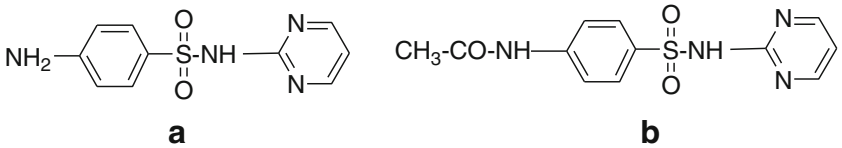

Fig. 6. Chemical structure of sulfadiazine **a** and its acetyl derivative, *N*4-acetylsulfadiazine **b**

the acetyl group (or a molecule which contains it, *i.e.*, ester, amide, *etc.*) and the nitrogen 4 of sulfadiazine. It may be remarked that N4 in the sulfadiazine is terminal, and consequently very reactive for the formation of amide derivatives. It seems not reasonable to think that sulfadiazine crystallizes in the kidneys forming the calculus as neutral molecule, and acetyl groups react later with it. It is more reasonable to think that most or a part of sulfadiazine ingested by the patient circulates through the organism as a complete, unreacted, molecule, without degradation during digestion. At a given moment, some molecules take contact with free acetyl groups or with any protein carrying acetyls, reacting and forming *N*4-acetylsulfadiazine, which is further deposited in the kidneys.

Some studies support that sulfadiazine is a component of urinary lithiasis. Among others, Daudon and Jungers (14) and Servais *et al.* (15) have indicated that drug-induced calculi represent 1–2% of all renal lithiasis. An important group of these drugs includes poorly soluble compounds with high urine excretion that favors crystallization in the urine. Among them, triamterene is the leading drug, it being currently responsible for a significant number of calculi. Drugs used for the treatment of HIV-infected patients, namely indinavir and sulfadiazine, have become the most frequent cause of drug-containing urinary calculi. Besides these drugs, about 20 other compounds, as silica and antibiotics, have been reported to induce nephrolithiasis. Although almost all drug lithiasis are observed in patients receiving long-term treatments or high doses, there are other risk factors characteristics of the patient, as urine pH and diuresis. The diagnosis of lithiasis provoked by the metabolism of the medicaments is mainly based on clinic information, and probably its incidence is being underestimated. A better knowledge of medicament solubility and patient risks may help to reduce medicament lithiasis. Usually, the formation of the calculus may be a secondary effect much less important that the medical benefits produced by the drugs and logically will not limit their use. However, the possibility of lithiasis may be taken into account in patients with long, high doses treatments.

CONCLUSIONS

Currently available information on medicament lithiasis usually describes the calculi based on the prescriptions given to the patient, but without a physicochemical characterization of the calculi themselves. We here have applied different, complementary, physicochemical techniques for a complete characterization of a calculus. We have identified its precise nature, related to the medicaments prescribed or their metabolites. Combined use of powder X-ray diffraction and FT-IR and NMR spectroscopies, together with mass spectrometry, confirm that it is formed by *N*4-acetylsulfadiazine.

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