Determination of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) in Milk and Fresh Cheese Based on the Inhibition of Cyclooxygenase

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Summary

A biosensor for rapid determination of nonsteroidal anti-inflammatory drugs (NSAIDs) is described based on the inhibition of cyclooxygenase enzyme (both isoforms) by NSAIDs. The results show the full validity of the method, which has also been optimized by comparing the inhibition of two enzyme isoforms, COX-1 and COX-2, in the presence of different tested pharmaceutical drugs (diclofenac, naproxen, ibuprofen, tolmetin). Also, recovery trials were performed in milk and fresh cheese adulterated with known quantities of NSAIDs, always obtaining recovery values >88 %.

Key words: food analysis, COX, inhibition biosensor

Introduction

The term NSAID indicates a class of drugs known as nonsteroidal anti-inflammatory drugs. This is an important therapeutic class of drugs used to suppress pain and inflammatory states in cases of relevant diseases such as rheumatoid arthritis. The mechanism of action of NSAIDs involves reduction of prostaglandin synthesis by inhibition of cyclooxygenase (COX) enzyme through competitive antagonism towards arachidonic acid that binds to the COX enzyme (*1,2*). COX enzyme has 2 sub-types: COX-1 and COX-2, the former existing throughout all the biological human systems, while the latter is scarcely present in the stomach. In order to be an effective competitive inhibitor of arachidonic acid that binds to COX, a drug must possess both high lipophilic and acid properties to mimic a natural substrate. This is clearly apparent in the chemical structures of all NSAIDs, which contain carboxylic group of propionic acid (*e.g.* ibuprofen, ketoprofen, *etc.*), or carboxylic group of acetic acid (*e.g.* diclofenac), or an enolic group (*3*).

It has been demonstrated that NSAIDs exhibit adverse effects on the gastrointestinal tract including nausea, vomiting and diarrhoea. Ulcerogenic properties of NSAIDs stem from organic acids they contain, which are responsible for irritating the gastric mucosa, and from their inhibitory effects on prostaglandin biosynthesis (*4–7*).

For this reason, in order to protect the health of the consumer, European Union has ruled Maximum Residual Limits (MRLs) for the veterinary medicinal products in alimentary stocks of animal origin. On 17th September 2003 the Committee for Veterinary Medicinal Products adopted an Opinion recommending the establishment of MRLs respecting the Council Regulation (EEC) No 2377/90, as amended, for diclofenac, for bovine and porcine species. These limits still have not been established for naproxen, ibuprofen and tolmetin, which are the other three tested pharmaceutical drugs in our research.

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Comparability of monitored data is essential for any meaningful assessment and for the management of environmental risks of emerging pollutants. The reliability and comparability of data at European level is often limited, because analytical methods for emerging pollutants are often not fully validated, not harmonized or not suitable for all the relevant analysed matrices. Recent years have seen an increase in monitoring pharmaceutical residues in food (*8*) and in environmental water samples (*9*). Recently, diclofenac has been found as an environmental contaminant in sewage, surface, ground and drinking water samples (*10*). These drug residues may be released into the environment through many sources like for instance urban domestic, hospital and industrial waste effluents, and aquaculture plant or livestock farms (*11*).

The determination of NSAIDs in environmental samples is performed by reversed-phase liquid chromatography (RPLC) with UV detection (*10*) or by RPLC combined with diode-array detection and mass spectrometry (MS) (*12*). These analytical methods are characterized by high levels of precision and sensitivity, but they are very expensive and require preliminary sample treatments by solid phase extraction (SPE) (*12*) or as a free suspended droplet of an aqueous solvent, which prolong the analysis time.

Therefore, in the field of chemical analysis of these drugs, there is the need for rapid, reliable and low cost analytical methods, and the design and setting up of a sensor can represent an efficient screening method (*11*, *13–17*).

Sensors and biosensors, especially of electrochemical type, are particularly suitable for resolving various analytical problems regarding several matrices, so their use is increasing in many fields (*18–21*). To such scope a biosensor operating in aqueous solution for the determination of NSAIDs in foods (fresh cheese and milk) and based on the inhibition of COX by this drug was envisaged, designed and applied. The optimization of the procedure was carried out by testing four NSAIDs: diclofenac, naproxen, ibuprofen and tolmetin.

Materials

Reagents and chemicals

Naproxen (sodium salt), diclofenac (sodium salt), ibuprofen, tolmetin (sodium salt) (Fig. 1), Trizma® hydrochloride, arachidonic acid (acid from porcine liver), cyclooxygenase-1 (from sheep), cicloxygenase-2 (from human recombinant), and dialysis membrane were from Sigma-Aldrich (Milan, Italy). Lead nitrate and cadmium nitrate were from Carlo Erba Reagenti SpA (Milan, Italy). Mercury nitrate (monohydrate) was from Sigma-Aldrich (Milan, Italy).

Apparatus

Amperometric measurements were carried out in a 25 mL-glass cell and thermostated at room temperature under continuous stirring. The oxygen electrode, model 332/P, was connected to a dissolved oxygen meter (Mod. 360, AMEL Milan, Italy) and to a recorder (Mod. 868, AMEL Milan, Italy). A potential of –650 mV was applied to the platinum cathode of the oxygen electrode, which is the value at which oxygen reduction occurs.

Methods

Principle of the method

The prostaglandins are produced from the oxidation of arachidonic acid catalysed by cyclooxygenase enzyme (COX), as shown in Eq. 1:

$$Arachidonic\ acid + O_2 + H_2O \xrightarrow{COX} prostaglandins + H_2O_2 \quad /1/$$

NSAIDs exert a reversible inhibition on the cyclooxygenase (COX) by means of a competitive action with the arachidonic acid so that the kinetics of the reaction is affected. This inhibition is related to the oxygen consumption: in the presence of NSAIDs oxygen consumption is lower than in their absence.

COX-1 and COX-2 are isoenzymes, different proteins able to catalyze the same reaction: COX-1, or constituent enzyme, is in charge of the basic level of pro-
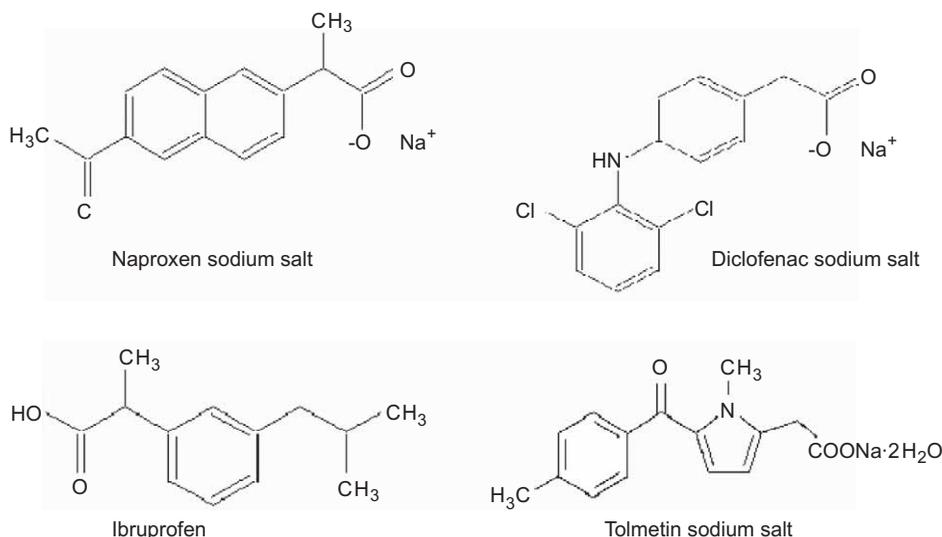

Fig. 1. Pharmaceutical drugs tested

staglandins; COX-2, or inducibile enzyme, is activated by various stimuli mediated by the inflammatory reactions.

COX biosensor

The biosensor used to determine the NSAIDs was obtained by coupling an amperometric electrode (Clark type) for oxygen (Pt cathode at a constant potential of –650 mV with respect to Ag/AgCl/Cl⁻) as electrochemical transducer, and 25 µL of COX enzyme (1000 U) immobilised in a gel-like κ-carrageenan membrane. The gel containing the enzyme was in contact with the gas permeable membrane of the PTFE cap of Clark electrode transducer. A nylon net and a PTFE O-ring were used to fix the gel-like enzymatic membrane to the cap itself, which was closed at one end by a PTFE gas permeable membrane (Fig. 2). The procedure followed to obtain the gel-like κ-carrageenan membrane and the immobilization of the enzyme was described in details in previous papers (*14–16*).
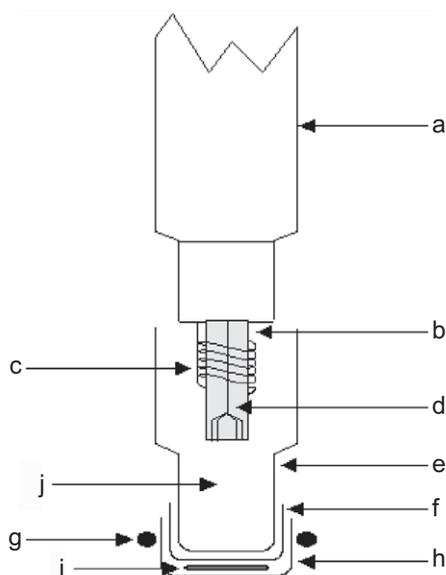

Fig. 2. a: body electrode; **b:** dielectric; **c:** Ag⁺/AgCl anode; **d:** Pt cathode; **e:** cap of a Clark electrode; **f:** gas permeable membrane in PTFE; **g:** O-ring; **h:** nylon net; **i:** κ-carrageenan containing immobilised COX enzyme; **j:** internal solution of KCl 0.1 M

Measurement procedure

The electrode was placed in a glass cell thermostated at 25 °C containing 10.0 mL of Tris-HCl buffer (0.1 mol/L at pH=8.0) and allowed to stabilise under constant magnetic stirring. Once the signal has become constant, 1.0 mL of arachidonic acid aqueous solution of $7.5 \cdot 10^{-4}$ mol/L was added and the current was measured in terms of $\Delta i(O_2)$/ppm (it is of the order of 0.400 $\Delta i(O_2)$/ppm). The measuring cell was rinsed and dried and a new measurement was performed by dipping the biosensor in 10.0 mL of one of the various aqueous solutions containing different concentrations of the four considered NSAIDs. After adding the substrate solution, the change in oxygen concentration was recorded and compared with the one previously obtained.

Percent oxygen value (Δi) is a function of the NSAID concentration in the cell:

$$\Delta i = (Sc - Sd)/Sc \cdot 100 \qquad /2/$$

where Sc and Sd represent a current intensity in the absence or presence of NSAIDs, respectively, due to the addition of arachidonic acid solution.

Treatment of samples

The mass of 5.0 g of fresh cheese was homogenised (at 10 000 rpm for 5 min) in 5.0 mL of distilled water. A volume of 1.0 mL of the homogenate was used in the analysis and added to 9.0 mL of Tris-HCl buffer (0.1 mol/L) at pH=8.0. In the case of milk, 1.0 mL of the sample was added to 9.0 mL of Tris-HCl buffer.

Results and Discussions

Of the two previously mentioned COX isoenzymes, COX-1 was used to optimize the substrate concentration used in the measurements and to test its effect on the inhibition degree in a 45-minute incubation. The data in Fig. 3 present the effect of the substrate concentration on the response after incubation with the tested NSAIDs. It can also be seen that the best sensitivity and accuracy are achieved at substrate concentrations higher than $7.5 \cdot 10^{-4}$ mol/L.

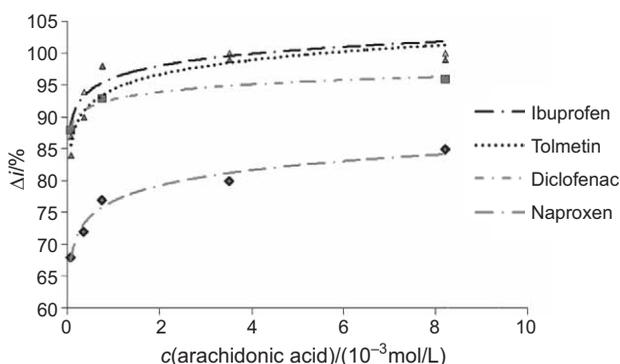**Fig. 3.** Response dependence of the amperometric COX-1 biosensor after incubation with $1 \cdot 10^{-6}$ mol/L naproxen, diclofenac, ibuprofen and tolmetin, on arachidonic acid concentration (0.1 mol/L Tris-HCl buffer, pH=8.0, inhibition time 30 min, *N*=3)

To select other optimal conditions for enzyme inhibition, the immobilised COX-1 biosensor inhibition *vs.* incubation time dependence was recorded in the presence of different NSAIDs. Fig. 4 shows the effect of incubation time. The percentage of inhibition increased with incubation time for all the tested pharmaceutical drugs, probably due to an increase of the interaction time between the inhibitor and the enzyme.

However, longer incubation time means longer analysis time as well, and a shorter lifetime of the biosensor. Therefore, incubation time was chosen as a compromise among the inhibition, analysis time and lifetime values. It can be seen in Fig. 4 that the optimal inhibition time for naproxen, diclofenac, ibuprofen and tolmetin was determined to be 30 min.
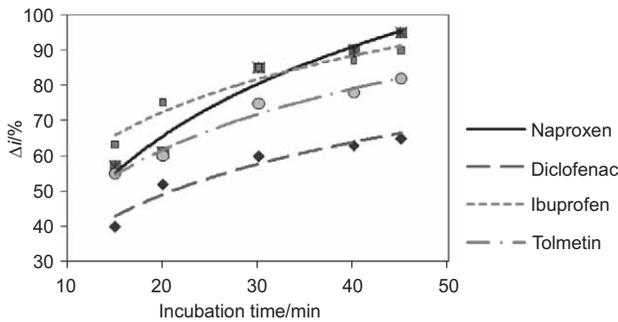
Fig. 4. Dependence of the inhibition level of COX-1 biosensor on incubation time in solution of $1 \cdot 10^{-5}$ mol/L naproxen, diclofenac, ibuprofen and tolmetin (0.1 mol/L Tris-HCl buffer, pH= 8.0, inhibition time 30 min, $N=3$)

In order to evaluate which one of the two enzymatic isoforms had better characteristics (sensitivity, resistance) for the arrangement of the COX biosensor, calibration curves of both isoenzymes (response *vs.* concentration of each tested pharmaceutical drug) were determined (Figs. 5 and 6). The shown trends are logarithmic and have been linearized.

It is shown that the activities of both enzymatic isoforms are more inhibited by naproxen and diclofenac than by ibuprofen and tolmetin. Detection limits determined as the concentration of the inhibitor giving a decrease of the signal corresponding to three times the variability of the blank value were: $5.0 \cdot 10^{-8}$ mol/L for naproxen and diclofenac and $0.5 \cdot 10^{-8}$ mol/L for ibuprofen and tolmetin (Table 1), and thus validating that the pro-
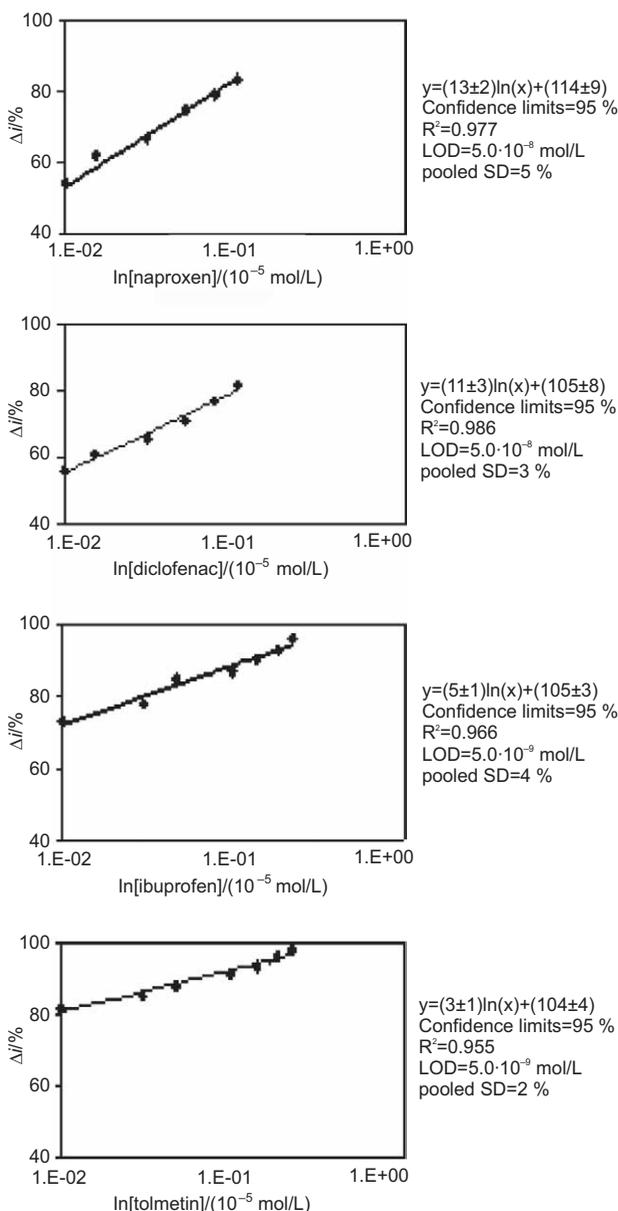

Fig. 5. Calibration curves of COX-1 biosensor measuring naproxen, diclofenac, ibuprofen and tolmetin with equation R^2, LOD, SD (0.1 mol/L Tris-HCl buffer, pH=8.0, inhibition time 30 min, $N=3$)

Fig. 6. Calibration curves of COX-2 biosensor measuring naproxen, diclofenac, ibuprofen and tolmetin with equation R^2, LOD, SD (0.1 mol/L Tris-HCl buffer, pH=8.0, inhibition time 30 min, $N=3$)
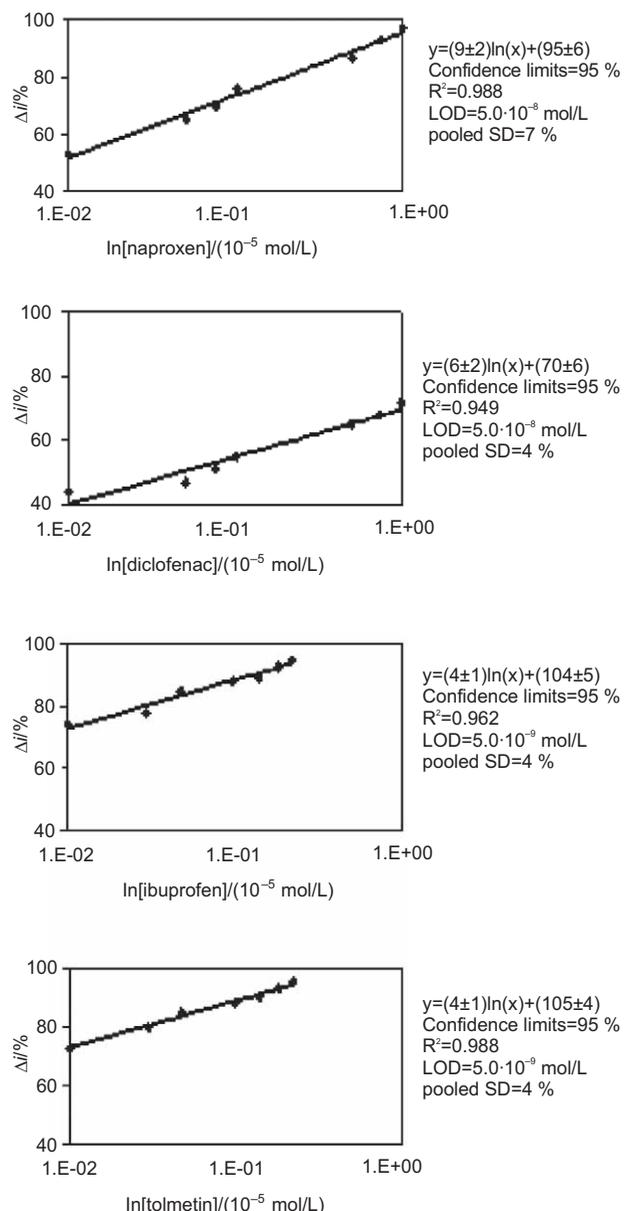
Table 1. Limit of detection (LOD) and values of the current signal for the pharmaceutical principles tested by a biosensor

Pharmacologically active substances	LOD (10^{-8} mol/L)	$\Delta i/\%$ (COX-1)	$\Delta i/\%$ (COX-2)
Naproxen	5.0	17±5	30±4
Diclofenac	5.0	30±4	29±4
Ibuprofen	0.5	63±3	65±3
Tolmetin	0.5	72±3	65±3

posed biosensor is not specific to a particular NSAID but to the whole class of NSAIDs. The comparison between the data obtained using COX-1 and COX-2 reveals that the range of linearity, sensitivity and LOD values for each tested pharmaceutical drug are almost the same. However, COX-1 enzyme shows a longer life in use than COX-2, so it is preferred for a large scale production and to develop a sensor to perform the recovery and interference tests.

In addition to NSAIDs, cyclooxygenase is also inhibited by other organic contaminants, for instance other classes of pharmaceuticals, pesticides and surfactants. Although these organic compounds could inhibit cyclooxygenase, it was found that dimethoate, sodium dodecyl sulphate (SDS) and sulphamethoxazole at the concentration of $1 \cdot 10^{-5}$ mol/L yield a markedly lower inhibition (28 %) than the tested NSAIDs at the concentration of 10^{-8} mol/L. Therefore, these organic compounds should not cause significant interference with the analysis. The influence of heavy metal ions such as mercury, cadmium and lead, as the most relevant from the environmental point of view and well known enzymatic inhibitors, has also been considered.

The results shown in Table 2 show that heavy metals and pesticides can interfere significantly with enzymatic activity of COX enzyme, but surfactants and antibiotic drugs do not interfere with the catalytic activity of the enzyme.

Table 2. Results obtained using COX-1 biosensor in the presence of different active substances (for reference see Table 1)

Potential interfering at $c=1 \cdot 10^{-5}$ mol/L	$\Delta i/\%$
Mercury	65±5
Cadmium	89±3
Lead	77±4
Dimethoate	28±6
SDS	0±4
Sulphamethoxazole	0±3

The reproducibility of the biosensor was also investigated. Five measurements using the same biosensor were carried out at naproxen concentration of $5.0 \cdot 10^{-6}$ mol/L. Relative standard deviation (RSD) of the amperometric responses is 6 %. The enzyme electrode was stored at 4 °C in 0.1 mol/L Tris-HCl buffer (pH=8.0).

Lastly, the biosensor method using COX-1 to determine NSAIDs was tested using real samples. To this

end, different foods of animal origin (milk and fresh cheese) were chosen and specifically adulterated with known quantities of pharmaceutical drugs. The recovery of NSAIDs was evaluated by the biosensor method. Tests were also run to determine the efficiency of aqueous extraction procedure using real food samples adulterated with different pharmaceutical drugs at just one adulteration level ($1 \cdot 10^{-6}$ mol/L).

The results of both recovery and extraction efficiency are summarized in Table 3. Clearly, according to the used procedure, the percentage of unrecovered NSAIDs is of the order of 10–12 %.

Table 3. Recovery obtained using COX-1 biosensor in real food samples adulterated with known quantities of NSAIDs

Sample adulterated with [NSAIDs]/ $1 \cdot 10^{-6}$ mol/L	NSAIDs used	Nominal value/%	Experimental value/%	Recovery/%
Milk	Naproxen	62	58±3	93
	Diclofenac	69	63±2	91
	Ibuprofen	96	87±1	91
	Tolmetin	98	88±1	90
Fresh cheese	Naproxen	62	56±3	90
	Diclofenac	69	62±3	90
	Ibuprofen	96	86±2	90
	Tolmetin	98	84±2	88

Conclusions

The obtained results indicate the complete validity of the method, especially of preliminary screening for the presence of NSAIDs in food. The COX biosensor allows the determination of the presence of nonsteroidal anti-inflammatory drugs (NSAIDs) in aqueous solution in which the analytes were soluble. The use of COX-1 for the biosensor is more suitable for large production lines than COX-2, because of the longer lifetime of the enzyme. The recovery tests performed on adulterated foods with the four tested pharmaceutical drugs show that the percentage of unrecovered NSAIDs is of the order of 10–12 %. However, the method can be reliably applied only on free samples or the samples treated in order to let them be free from heavy metal ions and pesticides.

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