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Originalarbeit Original paper

Proteolytic degradation of cardiac troponin I does not influence the TOSOH AIA-PACK cTnI 2G assay, a second generation test for the determination of troponin I

Der proteolytische Abbau des kardialen Troponin I hat keinen Einfluss auf den TOSOH AIA-PACK cTnI 2G Assays, ein Test der zweiten Generation zur Bestimmung des Troponins

Thomas Brinkmann*, Christian Götting and Knut Kleesiek

Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Bad Oeynhausen, Germany

Abstract

We have evaluated the cardiac troponin I (cTnI) enzyme immunoassay AIA-PACK cTnI 2G (Tosoh Bioscience). The interassay of precision at different cTnI levels (0.30–6.70 ng ml^{-1}) showed coefficients of variation (CV) between 5.00% and 4.71% on the AIA 600 II instrument. These data are in accordance with the functional sensitivity (0.04 ng ml^{-1}) and analytical sensitivity (0.02 ng ml^{-1}) specified by the manufacturers.

Comparative troponin I measurements in serum samples were performed on the AIA-600 II analyser (Tosoh Bioscience) and the Dimension RxL analyser (Dade Behring). The cTnI concentration of serum samples (n = 226) ranged from 0 ng ml^{-1} to 114 ng ml^{-1}. Regression analysis of the cTnI values obtained showed a close correlation between both results (RxL = 0.96 AIA + 0.38; r = 0.960).

To induce proteolytic in vitro degradation of the cTnI molecule, patient serum samples (n = 20) were incubated at 37°C for 24 h and the cTnI concentration was determined before and after the incubation with both analysers. The cTnI concentration measured with the AIA-600 II analyser showed nearly identical values before and after the incubation period. However, cTnI measured with the Dimension RxL analyser showed decreased cTnI values in a range of 10 to 50% after the incubation period.

The AIA-PACK cTnI 2G assay uses two different monoclonal antibodies against two epitopes in the proteolytic stable region of the cTnI molecule (between position 40 and 90 of the cTnI amino acid sequence). Therefore, measurement of cTnI in serum remains precise and specific in spite of in vitro proteolysis.

Keywords: troponin I; immunoassay.

Zusammenfassung

Wir haben den Enzymimmunoassay AIA-PACK cTnI 2G (Tosoh Bioscience) zur Quantifizierung des kardialen Troponins I evaluiert. Die Interassay-Präzision bei verschiedenen cTnI-Konzentrationen (0,30–6,70 ng ml^{-1}) zeigte Variationskoeefizienten (CV) zwischen 5,00% und 4,71%, gemessen an dem AIA 600 I-Meßgerät.

Zur vergleichenden Bestimmung der cTnI-Konzentration wurden Serumproben sowohl an dem AIA 600 II (Tosoh Bioscience) als auch an dem Dimension RxL (Dade Behring) gemessen. Die cTnI-Konzentration verschiedener Patientenseren (n = 226) variierte von 0 ng ml^{-1} bis 114 ng ml^{-1}. Die Regressionsanalyse der bestimmten Troponin-Wertepaare ergab eine gute Korrelation der Messergebnisse (RxL = 0,96AIA + 0,38; r = 0,960).

Zur Induzierung der proteolytischen Degradation in vitro wurden Patientenseren (n = 20) für 24 Stunden bei 37°C inkubiert. Die cTnI-Konzentration wurde vor und nach der Inkubationszeit mit beiden Messverfahren bestimmt. Die gemessene cTnI-Konzentration vor der Inkubation war an beiden Systemen nahezu identisch. Nach der temperaturinduzierten Proteolyse ergaben sich am Dimension RxL deutlich niedrigere cTnI-Werte, die 10–50% niedriger als der Messwert vor der Inkubation ausfiel.

Der AIA-PACK cTnI 2G-Assay setzt zur Bestimmung der cTnI-Konzentration zwei unterschiedliche anti-cTnI-Antikörper ein, die gegen die proteolysestabile Region des cTnI-Moleküls gerichtet sind (zwischen Aminosäure

*Correspondence: Priv.-Doz. Dr. Thomas Brinkmann, Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Georgstraße 11, D-32545 Bad Oeynhausen, Germany
Fax: +49-5731-972013
E-mail: tbrinkmann@hdz-nrw.de

40 und 90). Daher bleibt die Messung der cTnI-Konzentration in Serumproben, unabhängig von der auch in vitro ablaufenden Proteolyse des Moleküls, sehr spezifisch und sensitiv.

Schlüsselwörter: Troponin I; Enzymimmunoassay.

Introduction

Cardiac Troponin I (cTnI) has been known as a highly specific diagnostic marker of myocardial infarction for more than ten years [1, 2]. At present cTnI is considered to be one of the most specific and sensitive markers for myocardial cell death [3–6]. In the year 2000 myocardial infarction was newly defined during a consensus conference of the European Society of Cardiology and the American College of Cardiology. The new WHO definition of an Acute Myocardial Infarction (AMI) is: increased serum concentration of troponin in combination with either chest pain or electrocardiographic changes [7].

Serum concentrations of cTnI increase 4–6 h after myocardial infarction and remain elevated for up to 7 days [8]. After myocardial necrosis, troponin I is released into the blood in complexed forms with cTnC and cTnT and in a free form cTnI [9]. Furthermore, cTnI is released in a phosphorylated and non-phosphorylated form. It has been shown that cTnI is phosphorylated at different sites by a cAMP-dependent protein kinase [10] and by a Ca^{2+} -phospholipid-dependent protein kinase [11, 12]. The phosphorylation in position Ser^{23} and Ser^{24} changes the conformation of the cTnI molecule and therefore affects interaction of cTnI with monoclonal antibodies (mAbs) used for enzyme immunoassays [13]. In addition, human cTnI contains two Cys residues [14]. Oxidation of the Cys-SH groups could also affect the interaction of the troponin molecule with binding mAbs [15].

Necrosis of cardiac tissue caused by infarction is accompanied by liberation of proteolytic enzymes from lysosomes. Cardial TnI is highly susceptible to proteolysis and therefore a rapid degradation of the molecule occurs in both the ischemic myocardium [16–18] and the circulation [19]. Blood contains varying quantities of proteolytic enzymes, which interact with the cTnI leading to its fragmentation [20]. The amino acid region most resistant to proteolysis is the sequence located between amino acid 30 and 110 [20]. In contrast, the N- and C-terminal regions are highly vulnerable to proteolytic degradation [21]. Therefore, for a sensitive and reproducible measurement of cTnI in serum, antibodies directed against the stable region of the cTnI molecule should be used [20].

The aim of our study was to evaluate the stability of the cTnI determination of two different enzyme immunoassays under the influence of induced proteolytic degradation of the cTnI molecule. We compared the AIA-PACK cardiac troponin I second generation (cTnI 2G) immunoassay on the AIA 600 II (Tosoh Bioscience) with the cTnI enzyme immunoassay on the RxL Dimension (Dade Behring).

Materials and methods

AIA 600 II analyser

The AIA 600 II is an automated immunoassay analyser with a bichromatic fluorescence kinetic measurement. Each test cup contains magnetic beads with antibodies to the specific antigen. The conjugate is lyophilised and contains the signal antibody labelled with alkaline phosphatase in each cup. The antigen-antibody reaction begins when sample and diluent are added. Unbound reactants are removed by a washing step after 10 min of incubation at 37°C. The signal is generated by adding the substrate 4-methylumbelliferyl phosphate to the test cup being measured using a fluorescence rate determination.

The evaluated Tosoh AIA-PACK cTnI 2G assay is a one-step enzyme immunoassay based on a “sandwich” principle using two different monoclonal antibodies specific for epitopes of the cTnI molecule. These epitopes are located away from the regions susceptible to proteolysis around position 50 and 90 on the molecule (Figure 1). The functional sensitivity is considered to be 0.04 ng ml^{-1} and the analytical sensitivity 0.02 ng ml^{-1} [22].

Dimension RxL analyser

The cTnI method on the Dimension RxL analyser is a one-step enzyme immunoassay based on the sandwich principle. The sample is incubated with chromium dioxide particles coated with a monoclonal antibody specific for cTnI, and a conjugate reagent (alkaline phosphatase) labelled monoclonal antibody specific for cardiac cTnI, to form a particle/cTnI/conjugate sandwich. After washing, the sandwich bound alkaline phosphatase triggers an amplification cascade which leads to a coloured end product that absorbs at 510 nm. The colour change measured is directly proportional to the concentration of cTnI.

Method comparison

226 serum samples with elevated cTnI concentration from patients after mycardial infarction or open heart operation were analysed for cTnI on the AIA 600 II and on the RxL Dimension, each analysis being performed according to the manufacturer’s instructions.

Calibration and quality control

Both cTnI assays were calibrated with calibrators obtained from the manufacturers (AIA 600 II and Dimension RxL). Three internal quality controls were run every day. Precision and accuracy were controlled daily according to the obligatory German quality control regulations. The material was stored as aliquots at −20°C. Mean values, standard deviation and %CV of the concentrations were calculated.

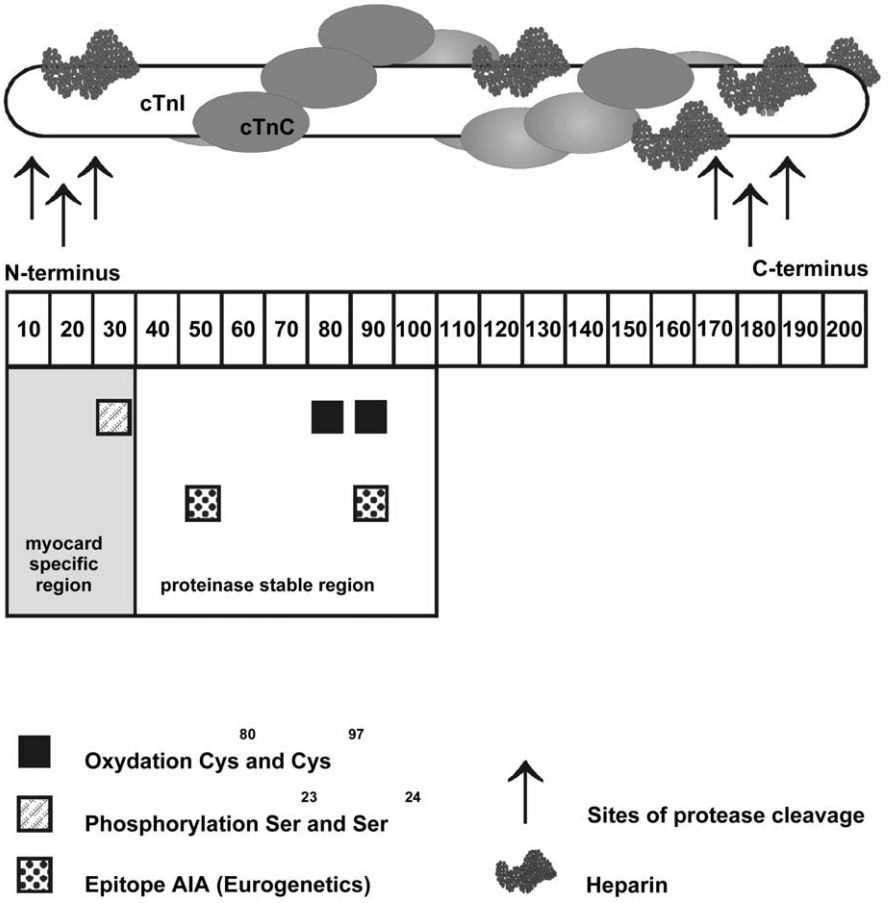

Figure 1 Schematic presentation of the troponin I molecule. The N- and C-terminal regions are myocard-specific but vulnerable to proteolytic degradation. Two serine residues in position 23 and 24 can be found in a phosphorylated or dephosphorylated form. The cystein residues in position 80 and 97 are either oxidated or reduced. (Eurogenetics, now Tosoh Bioscience).

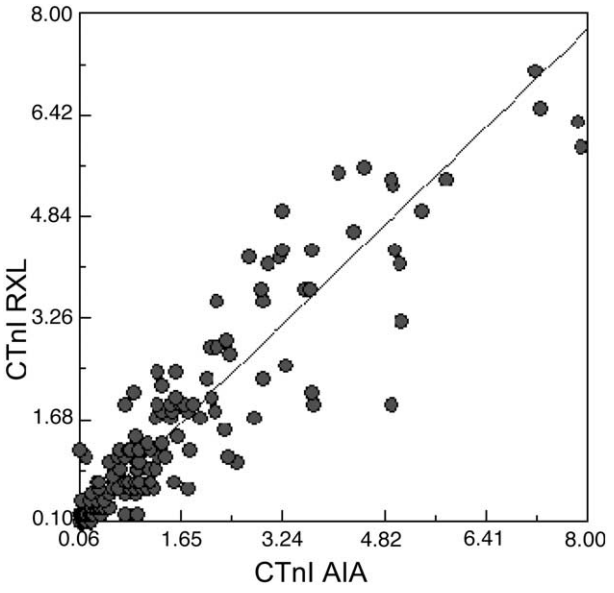

Figure 2 Linear regression analysis of cardiac troponin I (cTnI) values between 0.06 and 8.0 $\mu g\ l^{-1}$ measured with the AIA-PACK 2nd Gen (Tosoh Bioscience) and the Dimension RxL (Dade Behring) cTnI enzyme immunoassay ($y = 0.96x + 0.49$; $r = 0.96$).

Proteolytic degradation of the cTnI molecule

Serum samples from 20 patients with elevated cTnI concentration after myocard infarct or open heart surgery were measured twice within 24 h at room temperature with the AIA-PACK assay on the A 600 II analyser in order to analyse the influence of proteolytic degradation of the cTnI molecule. These sera were also measured twice on both analysers, the A 600 II and the Dimension RxL within 24 h after incubation at 37°C.

Results

Method comparison

Comparing the cTnI results obtained with the AIA 600 II automated immunoassay system with the Dimension RxL system ($n = 226$) we observed a strong correlation with a coefficient of 0.96 (Figure 2 and Figure 3).

Imprecision of the AIA 600 II immunoassay

Three different levels of internal quality controls (0.30–6.50 ng ml^{-1}) were run every day on the AIA 600 II. The

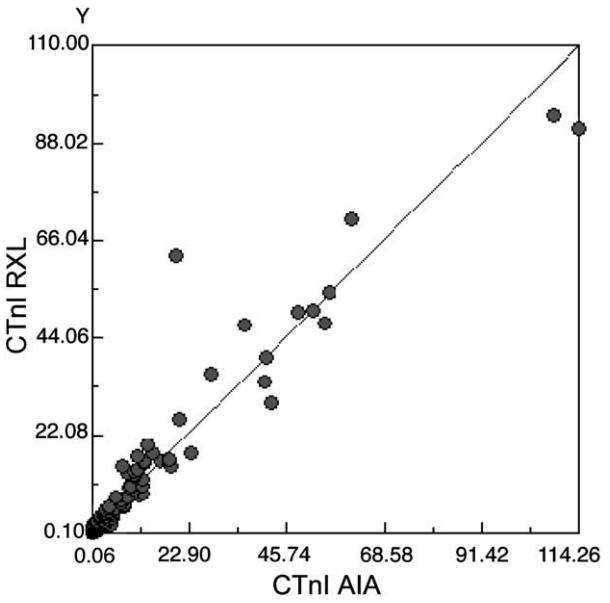

Figure 3 Linear regression analysis of cardiac troponin I (cTnI) values between 0.06 and 114 μg l^{-1} measured with the AIA-PACK 2nd Gen (Tosoh Bioscience) and the Dimension RxL (Dade Behring) cTnI enzyme immunoassays ($y = 0.96x + 0.04$; $r = 0.91$).

results obtained show an imprecision of 4.89%, 5.00% and 4.71% (Table 1).

Stability after proteolytic degradation of the cTnI molecule

The cTnI concentration was determined in 20 different serum samples with elevated cTnI concentration which had been incubated at 37°C for 24 h. The cTnI concentration was determined at the beginning (t_0) and at the end (t_{24}) of the incubation period on the AIA 600 II and on the Dimension RxL.The cTnI values obtained with the AIA 600 II were nearly constant before and after incubation at 37°C. However, the values obtained with the Dimension RxL were decreased by up to 50% of the initial value after 24 h (Figure 4 and Table 2).

Table 1 Imprecision of the AIA 600 II analyser determined with three different internal quality controls over a period of ten days.

Day	control 1 [cTnI] ng ml^{-1}	control 2 [cTnI] ng ml^{-1}	control 3 [cTnI] ng ml^{-1}
1	0.30	1.86	6.37
	0.28	1.81	6.89
2	0.27	1.67	6.43
	0.28	1.70	6.45
	0.25	1.59	n.d.
5	0.28	1.88	7.06
	0.29	1.81	6.70
	n.d.	1.75	6.31
6	0.28	1.75	6.18
7	0.28	1.72	6.21
10	n.d.	1.68	n.d.
Mean	0.28	1.75	6.51
SD	0.01	0.09	0.31
CV (%)	4.89	5.00	4.71

nd, not determined.

Discussion

As cTnI measurement has not yet been standardised, troponin values obtained from different enzyme immunoassays are not comparable. The reasons for these discrepancies are the use of different anti-cTnI antibodies, liberation of different serum cTnI forms into the serum, posttranslational modification of the cTnI molecule, e.g., phosphorylation and oxidation, and the proteolytic degradation of the troponin molecule.

For some years the problem of troponin I degradation in serum by proteolytic enzymes has been a well known phenomenon in the quantification of the molecule using enzyme immunoassays [9, 19]. However, cTnI is not only degraded in blood but also in necrotic tissue. Troponin measured in serum samples with a high cTnI concentration were incubated with necrotic tissue for 20 h at 37°C. The cTnI concentration measured after incubation was less than 10% of the initial value [9], the reason being that the N- and C-terminal regions of cTnI are not resistant to proteolysis [23]. However, the central region of the molecule located between amino acid residues 30 and 110 is more stable and, in addition, dimeric complexes of cTnI with cTnC protect cTnI from proteolytic degradation. Although the main myocard specific region is determined from amino acid 1 to 30, the proteolytically stable region also shows myocard specific areas in comparison to slow or fast skeletal muscle troponin and can be used as target epitopes for cTnI assays [24–26]. Sandwich immunoassays utilising antibodies specific to the unstable region exhibit a lower sensitivity, especially in the case of late diagnosis of an acute myocard infarct (AMI), in comparison to assays based on antibodies recognising the stable region of the molecule.

One reason for troponin becoming the new golden standard for AMI diagnosis is that it is detectable in the blood of patients immediately after the infarct and over a long period of time (5–7 days) after the onset of the first symptoms of AMI. However, in cases of a slight release of troponin, e.g., after a microinfarct, enzyme immunoassays using epitopes in the unstable region may lead to a false negative cTnI concentration, as a large amount of cTnI is degraded after a short time, and the quantification of cTnI is not possible.

It is well known that a reinfarct is difficult to determine, when it occurs several days after the first episode. In this case, the concentration of cTnI can still be elevated due to the initial AMI, and only serial measurements of cTnI can confirm or negate the new diagnosis.

Another reason for difficult cTnI measurements is the progressive phosphorylation of the serine residues in position 23 and 24. This phosphorylation could change

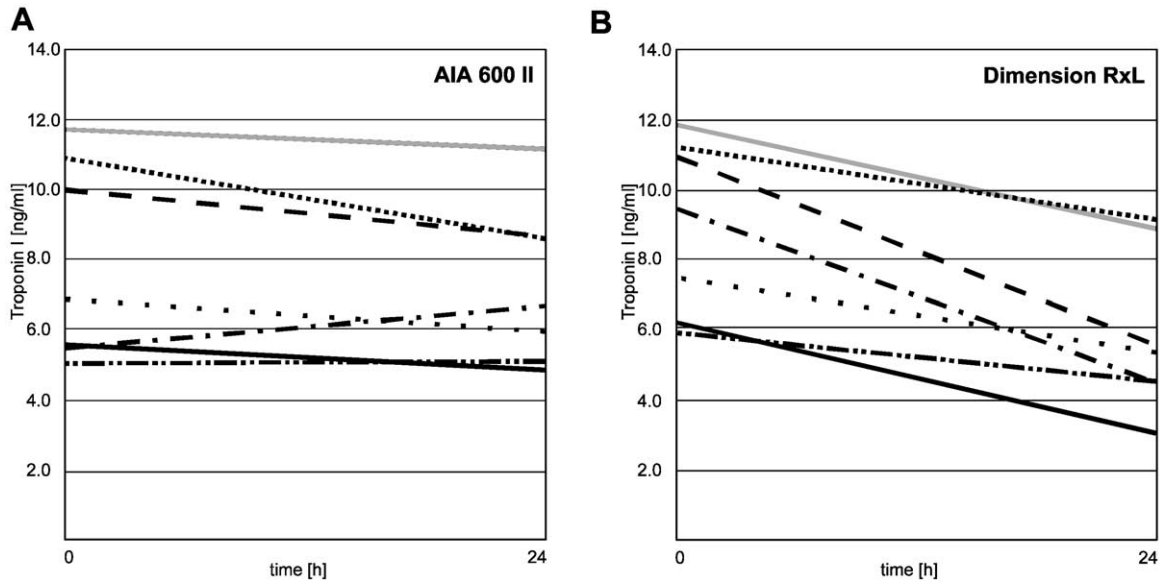

Figure 4 Graphic presentation of the measured cTnI values determined with both the AIA 600 II (A) and the Dimension RxL (B). Serum samples with elevated cTnI concentration were incubated for 24 h at 37°C and cTnI was measured before (t_0) and after the incubation period (t_{24}).

the conformation of the troponin I molecule, thereby affecting the interaction with the enzyme immunoassay antibodies directed against this protein region. This effect has been observed with some troponin immunoassays when a serum sample spiked with troponin was incubated with alkaline phosphatase for dephosphorylation prior to testing. The cTnI concentration measured in the dephosphorylated sample was much higher than in the same serum sample without alkaline phosphatase [23].

In addition, the two cTnI cysteine residues in position 80 and 97 have been shown to exist in either oxidised or reduced forms [14, 27]. The oxidation of the SH-groups affects the interaction with other troponin molecules [28] resulting in an intrachain disulphide bond. The importance of these factors with respect to the immunoreactivity detected by different cTnI immunoassays has not been fully established. Currently, there is no clear answer as to how the oxidation of the two cysteine residues affects the antibody binding.

For these reasons it is essential to use antibodies directed against the stable region of the cTnI protein. Only assays using these antibodies allow the detection

Table 2 Measurement of the cTnI concentration with both the AIA 600 II and the Dimension RxL. Aliquots of serum samples were incubated for 24 h at 25°C and at 37°C and cTnI was measured before (t_0) and after the incubation period (t_{24}).

Sample	RxL t_0	RxL t_{24} 25°C	RxL t_{24} 37°C	AIA t_0	AIA t_{24} 25°C	AIA t_{24} 37°C
1	16.4	15.0	11.7	16.3	16.7	18.3
2	10.7	10.4	8.03	8.55	9.12	10.1
3	11.1	11.5	9.08	11.6	12.1	11.5
4	19.5	19.5	8.4	30.4	32.3	33.3
5	5.60	5.62	4.47	7.46	8.03	8.54
6	11.8	10.0	8.89	3.34	5.66	5.37
7	19.4	18.5	14.9	15.3	19.5	20.8
8	6.80	6.03	5.38	5.51	5.90	6.34
9	9.10	9.11	6.85	8.34	9.26	9.18
10	11.0	9.17	5.56	10.5	9.94	8.54
11	6.50	5.74	3.68	6.23	7.27	6.42
12	10.3	10.4	5.80	12.1	11.4	9.76
13	7.50	6.06	5.37	6.65	6.55	5.99
14	6.20	4.95	4.38	5.27	5.46	5.67
15	18.0	15.9	14.5	26.3	26.5	28.6
16	9.80	8.83	8.26	6.91	7.85	9.19
17	5.90	5.02	4.51	4.49	5.15	5.43
18	9.40	7.72	4.49	9.88	9.83	8.57
19	11.2	9.49	8.45	4.80	5.15	5.25
20	6.20	5.46	3.07	5.44	5.50	6.43

of all cTnI molecules. Our data clearly demonstrate that the AIA PACK cTnI 2G is a precise and specific assay for measurement of cTnI independent of the posttranslational modification in serum.

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