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Application of ion-exchange cartridge clean-up in food analysis III. Determination of benzylpenicillin, phenoxymethyl-penicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in bovine liver and kidney by liquid chromatography with ultraviolet detection

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Abstract

A multiresidue analytical method was developed for the simultaneous determination of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC) in bovine liver and kidney. The method involves the use of an ion-exchange cartridge for sample clean-up followed by ion-pair high-performance liquid chromatography with ultraviolet detection. The recoveries of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC from bovine liver spiked at levels of 0.5 mg/kg and 0.1 mg/kg were in the range of 73–91% and 83–96% with coefficients of variation of 1.4–4.2% and 3.4–8.7%, respectively. For bovine kidney spiked at levels of 0.5 mg/kg and 0.1 mg/kg, the recoveries of these compounds were 79–92% and 82–92% with RSDs of 1.8–5.9% and 2.7–7.8%, respectively. The detection limits for the six penicillins were 0.02–0.05 mg/kg in bovine liver and kidney.

Keywords: Food analysis; Sample handling; Antibiotics; Penicillins

1. Introduction

Penicillin antibiotics have been widely used for livestock as veterinary drugs to prevent and treat infectious diseases. Such use may lead to problems with residues in the livestock products.

In Japan, a zero tolerance has been established for penicillins residues in edible animal tissues to protect the consumer [1]. One of the major roles of public health agencies is to provide safe products for consumers through the monitoring of these residues in the livestock products.

Usually, the liver and kidney are used as the target tissues for the monitoring of penicillins and their monitoring are exclusively carried out by bioassays which are complicated, time consuming, and non-specific. On the other hand, as high-performance liquid chromatography (HPLC) is a fast and reliable technique with high sensitivity, many HPLC methods have been reported for the analysis of residual penicillins in milk and meat [2–15]. However, only a few methods have been reported for the simultaneous analysis of penicillins in liver and kidney [16–21] and most of them require derivatization using toxic

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reagents or special instruments. Therefore, there is a great need for a simple and reliable analytical method which permits the simultaneous determination of penicillins in the liver and kidney.

We have reported in our previous studies [22–27] the applicability of sample clean-up by an ion-exchange cartridge in combination with ion-pair HPLC for the analysis of ionable compounds. Using this technique, we have already developed an analytical method for the quantitative determination of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC), all of which are ionable substances, in meat. Although this method is simple, rapid and reliable, it did not yield satisfactory results for the residual analysis of the penicillins in animal liver and kidney due to the lack of recovery and clean-up effects. However, these problems can be overcome by careful consideration of the following: the extraction of the penicillins from animal liver and kidney and washing the cartridges used for clean-up. Investigation of the above points has led to more simple, rapid and reliable analytical methods for the residual penicillins in bovine liver and kidney. This paper describes a method for the simultaneous analysis of six penicillins in bovine liver and kidney using an ion-exchange cartridge clean-up followed by ion-pairing HPLC determination with UV detection.

2. Experimental section

2.1. Chemicals and reagents

Methanol, acetonitrile and distilled water were HPLC grade reagents. Sodium chloride (NaCl), sodium tungstate, sulfuric acid, acetic acid, disodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate and other chemicals were analytical reagent grade reagents. Cetyltrimethylammonium chloride was the ion-pairing reagent obtained from Tokyo Kasei Kogyo (Tokyo, Japan).

Bond Elut C18 (Lot No. 070070, 070751 and 070781) had a 6 ml capacity packed with 500 mg solid-phase and was purchased from Varian (Harbor City, CA, USA). Sep-Pak Accell Plus QMA (Lot No. W8117J1, T7023G1, T8054G1) had a 3 ml capacity packed with 500 mg solid-phase and was obtained from Waters (Milford, MA, USA).

The PCG potassium salt, PCV potassium salt, MPIPC sodium salt, MCIPC sodium salt, NFPC sodium salt and MDIPC sodium salt were purchased from Sigma (St. Louis, MO, USA). Separate stock solutions of each penicillin were prepared by dissolving 50 mg of each compound in 50 ml of water. Subsequent dilutions were made using the mobile phase of the HPLC. All the working standards were prepared fresh daily.

2.2. Sample preparations

2.2.1. Preparation of crude extracts of bovine liver and kidney

Bovine liver was sliced and homogenized with a mixer. A portion (5 g) of a homogenized sample was weighed in a 250 ml centrifuge tube and blended with 50 ml of 2% NaCl aqueous solution, 5 ml of 5% sodium tungstate aqueous solution and 5 ml of 0.17 *M* sulfuric acid aqueous solution using a high-speed blender. After centrifugation (3100 rpm, 15 min), the supernatant was transferred to another 250 ml centrifuge tube. The residual plug was reextracted twice with 40 ml of 2% NaCl aqueous solution, then the supernatants were combined in the centrifuge tube. The tube was allowed to stand for 15 min and then centrifuged for 15 min at 3100 rpm.

A homogenized bovine kidney (5 g) was first blended with 55 ml of 2% NaCl aqueous solution, 2.5 ml of 5% sodium tungstate aqueous solution and 2.5 ml of 0.17 *M* sulfuric acid aqueous solution. The second and third extractions were then done in same way.

2.2.2. Purification of crude extract using precleanup cartridge (Bond Elut C18)

All of the supernatants (about 140 ml) from the liver and kidney extracts were passed through a Bond Elut C18 cartridge preconditioned with 5 ml of methanol followed by 5 ml of water at a flow-rate of ca. 1 ml/min. The Bond Elut C18 cartridge was washed with 10 ml of water, 5 ml of 15% aqueous methanol containing 2% NaCl, and 5 ml of water, and then air-dried under vacuum for 10 min. The elution was carried out with 5 ml of 55% aqueous methanol.

2.2.3. Purification of eluate from Bond Elut C18 by clean-up cartridge (Sep-Pak Accell Plus QMA)

The eluate from the Bond Elut C18 cartridge was applied to a Sep-Pak Accell Plus QMA cartridge preconditioned with 5 ml of methanol, 5 ml of water, and 5 ml of 55% methanol. The Sep-Pak Accell Plus QMA cartridge was washed with 3 ml of 55% methanol and 3 ml of 10 m*M* acetic acid methanolic solution followed by 3 ml of water, and then air-dried under vacuum for 10 min. The retained penicillins were then eluted with 2 ml of the HPLC mobile phase. A 20 μl aliquot of the eluate was injected into the HPLC.

2.3. Apparatus

The HPLC system consisted of an LC-10AD pump, an SIL-10AXL auto injector, an SCL-10A system controller, an SPD-10A UV–Vis absorbance detector, and a CR-6A recorder (Shimadzu, Kyoto, Japan).

2.4. Chromatographic conditions

The separation was performed on a TSKgel ODS-80Ts column (5 μm, 150×4.6 mm I.D.; TOSOH, Tokyo, Japan) at 30°C. The mobile phase consisted of acetonitrile–0.02 *M* phosphate buffer, pH 6.2, (4.3:5.7, v/v) containing 12 m*M* cetyltrimethylammonium chloride. The flow-rate of the mobile phase was 0.8 ml/min and detection was carried out at 220 nm.

2.5. Quantitation

Calibration curves were obtained by plotting the absolute peak heights of the penicillins versus the concentration of the standard solutions. Quantification of the penicillins in the bovine liver and kidney samples was calculated from the calibration curves and reported in grams of sample weight (mg/kg). Recoveries were calculated as the ratio of the peak heights of the analytes from the fortified samples to the peak heights of the standard solutions. The calibration curves were linear over the ranges of 0.01–2 μg/ml (for PCG, PCV and MDIPC) and 0.005–1 μg/ml (for MPIPC, MCIPC and NFPC) with correlation coefficients over 0.999.

3. Results and discussion

3.1. Sample extraction

The extraction procedure for the bovine muscle used in our previous study [22], the repeated (three times) extractions of the penicillins with 2% NaCl aqueous solution (60, 40, 40 ml), could not be applied to bovine liver, because the obtained extract was bubbly and viscous which caused the precleanup cartridge (C18 cartridge) to become clogged. In order to avoid these problems, it is necessary to develop an extraction procedure with deproteinization for bovine liver. The addition of acetonitrile or sodium tungstate and sulfuric acid to the extraction solution is the general deproteinization method for the analysis of β-lactams in biological samples [2,7,10–12,14,19–21]. Both deproteinizations were very effective to control the bubbling and viscosity of the extract from bovine liver, however, the extract containing acetonitrile cannot be directly applied to the C18 cartridge. Accordingly, we decided to add sodium tungstate and sulfuric acid to the extraction solution (2% NaCl) as the deproteinization reagents.

Since the mixture of sodium tungstate with sulfuric acid was unstable, we could not store the extraction solution containing both reagents for a long time. One must add the reagents to the extraction solution (2% NaCl) just before the extraction. As mentioned above, the three extractions (60, 40, 40 ml) were effective in extracting the penicillins from a sample, so we used the three extractions in the present study. However, the addition of the reagents to the extraction solution at each extraction step is very complicated. Consequently, we decided that sodium tungstate and sulfuric acid should only be added to the extraction solution (60 ml) during the first extraction step and are not contained in the extraction solutions (40 ml each) used for the second and third extraction steps.

Next, the effect of the deproteinization reagent volume (5% sodium tungstate aqueous solution–0.17 *M* sulfuric acid aqueous solution, 1:1) in the first extraction solution (60 ml) was examined in the range from 5 ml to 30 ml on the deproteinization and the recoveries of the penicillins from bovine liver spiked with the six penicillins (0.5 mg/kg). With an increase in the volume of the reagent, the effect of

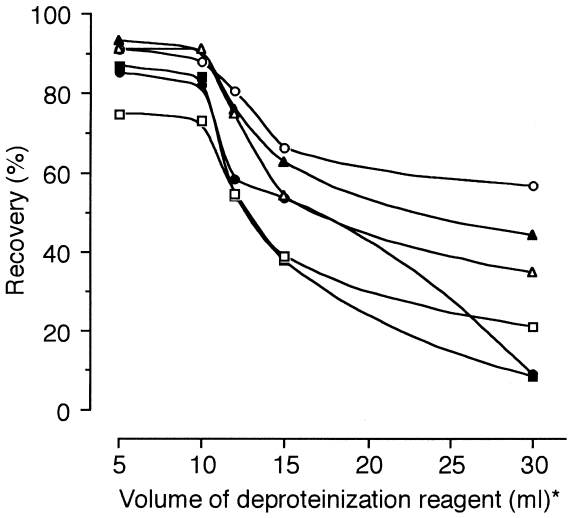

Fig. 1. Effect of volume of the deproteinization reagent in the first extraction solution for bovine liver. *Total volume of the first extraction solution is 60 ml. The deproteinization reagent; 5% sodium tungstate aqueous solution–0.17 *M* sulfuric acid aqueous solution (1:1). Results of three replicates. (●) PCG, (○) PCV, (▲) MPIPC, (△) MCIPC, (■) NFPC, (□) MDIPC.

deproteinization was increased, however, the recoveries of each penicillin decreased (Fig. 1). The larger volume (>10 ml) caused the recovery to decrease (<85%) and the smaller volume (<10 ml) caused the effect of the deproteinization to decrease. Judging from the results of these preliminary experiments, we decided to use 50 ml of 2% NaCl aqueous solution and 10 ml of the deproteinization reagent (5% sodium tungstate aqueous solution–0.17 *M* sulfuric acid aqueous solution, 1:1) as the first extraction solution.

We considered that it is necessary to allow the combined extract to stand for a while to obtain sufficient deproteinization, because the second and third extraction solutions do not contain the deproteinization reagent. The time for allowing the combined extract to stand was examined. When we allowed it to stand over 15 min, good effects of deproteinization were obtained and the recoveries of the penicillins did not decrease. Therefore, we selected a stand time of 15 min for the extract and finally a simple and reliable extraction procedure for bovine liver was established as described in Section 2.2.1.

Secondly, we investigated the extraction of the penicillins from bovine kidney based on the same procedure as that of the liver. Since the kidney extract was not so bubbly and viscous compared with that of the liver, a half volume of the deproteinization reagent used in the case of liver gave a satisfactory extraction efficiency (the effect of deproteinization and the recoveries of each penicillin), namely, we chose to use 55 ml of 2% NaCl aqueous solution, 2.5 ml of 5% sodium tungstate aqueous solution and 2.5 ml of 0.17 *M* sulfuric acid aqueous solution as the first extraction solution.

3.2. Purification of crude extracts

Our previous study demonstrated the applicability of the double cartridge clean-up using a Bond Elut C18 (C18 cartridge) as a preclean-up cartridge and a Sep-Pak Accell Plus QMA (QMA cartridge) as a clean-up cartridge for the determination of six penicillins (PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC) in bovine muscle. In this clean-up procedure, the washing C18 cartridge and QMA cartridge with 15% methanol containing 2% NaCl and 55% methanol is very effective, respectively, to provide satisfactory chromatograms without interfering peaks from the sample. However, the purification used for the muscle extract did not work well for the liver or kidney extract. Accordingly, we tried to further wash both cartridges in order to improve the clean-up effect. On the basis of the chemical properties of the C18 cartridge and the QMA cartridge, the following washing solutions were investigated for each cartridge; *n*-hexane, ethyl acetate and 5 m*M* hydrochloric acid aqueous solutions were for the C18 cartridge and ethanol, 5 m*M* hydrochloric acid methanolic solution and 0.5% ammonium methanolic solution were for the QMA cartridge. The bovine liver extract spiked with the penicillins (0.5 mg/kg) was analyzed after the double cartridge clean-up with an additional washing using 3 ml of the above solvents. The obtained results were compared with respect to the effect of the clean-up and recoveries of each penicillin. Among them, only the washing QMA cartridge with 5 m*M* hydrochloric acid methanolic solution showed a satisfactory clean-up effect, namely, no interfering peaks appeared around the

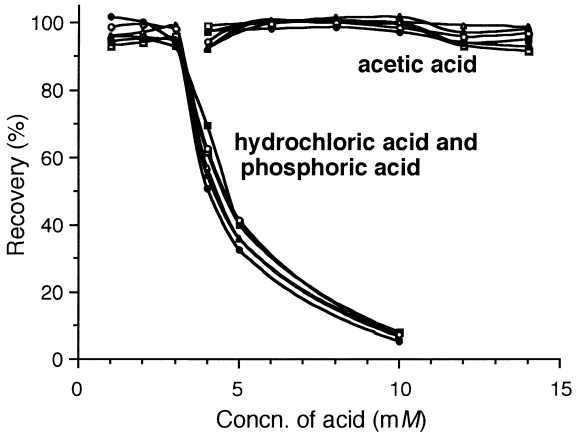

Fig. 2. Effects of concentrations of acids in methanol as a washing solution for Sep-Pak Accell Plus QMA. Results of three replicates. For symbols, see Fig. 1.

retention times of the penicillins, although the recovery was about 40%. We considered that this effect was caused by the washing QMA cartridge under acidic conditions. Therefore, the effects of the various concentrations of several acids (hydrochloric acid, acetic acid and phosphoric acid) in methanol on the recoveries of the penicillins were examined as the washing solution for the QMA. The obtained recoveries are shown in Fig. 2. In the cases of hydrochloric acid and phosphoric acid, the concentrations lower than 3 m*M* gave unsatisfactory results for clean-up, and at the concentrations greater than 3 m*M*, the recoveries of all penicillins were dramatically decreased (<70%). Only the 3 m*M* methanolic solutions had good results for the clean-up and recoveries. These experimental results mean that a slight difference in the concentration influences the recoveries so dramatically that they are not able to be practically used. Next, the concentrations of acetic acid were examined in the range from 4 m*M* to 14 m*M* in methanol. They only slightly influenced the recoveries of the penicillins, e.g. the recoveries were over 90% for all the concentrations. The interfering peaks that appeared on the chromatograms of the bovine liver and kidney overlapped the peaks of PCG and PCV when 8 m*M* acetic acid in methanol was used. Accordingly, we decided to use a 10 m*M* acetic acid methanolic solution for the QMA cartridge.

3.3. Recoveries

Bovine liver and kidney samples were fortified with the six penicillins (0.5 or 0.1 mg/kg each), and analyzed according to the procedure described in Section 2.4 in this paper. The recoveries and corresponding RSDs are listed in Table 1. The average recoveries for the penicillins from liver ranged from 73 to 96% with the RSDs ranging from 1.4 to 8.7%.

Table 1
Recoveries of penicillins from bovine liver and kidney

Penicillins	Liver			Kidney		
	Added (mg/kg)	Recovery[a] (%)	RSD (%)	Added (mg/kg)	Recovery[a] (%)	RSD (%)
Benzylpenicillin	0.5	82	4.2	0.5	83	4.7
	0.1	86	7.4	0.1	82	5.8
Phenoxymethylpenicillin	0.5	88	1.4	0.5	82	1.8
	0.1	83	4.1	0.1	86	7.8
Oxacillin	0.5	91	1.4	0.5	92	3.2
	0.1	96	3.4	0.1	92	4.2
Cloxacillin	0.5	91	2.9	0.5	89	2.9
	0.1	92	8.7	0.1	90	2.7
Nafcillin	0.5	84	1.7	0.5	80	3.5
	0.1	84	3.8	0.1	89	3.8
Dicloxacillin	0.5	73	3.1	0.5	79	5.9
	0.1	89	6.4	0.1	89	4.3

[a] Average of 5 trials. RSD=Relative standard deviation.

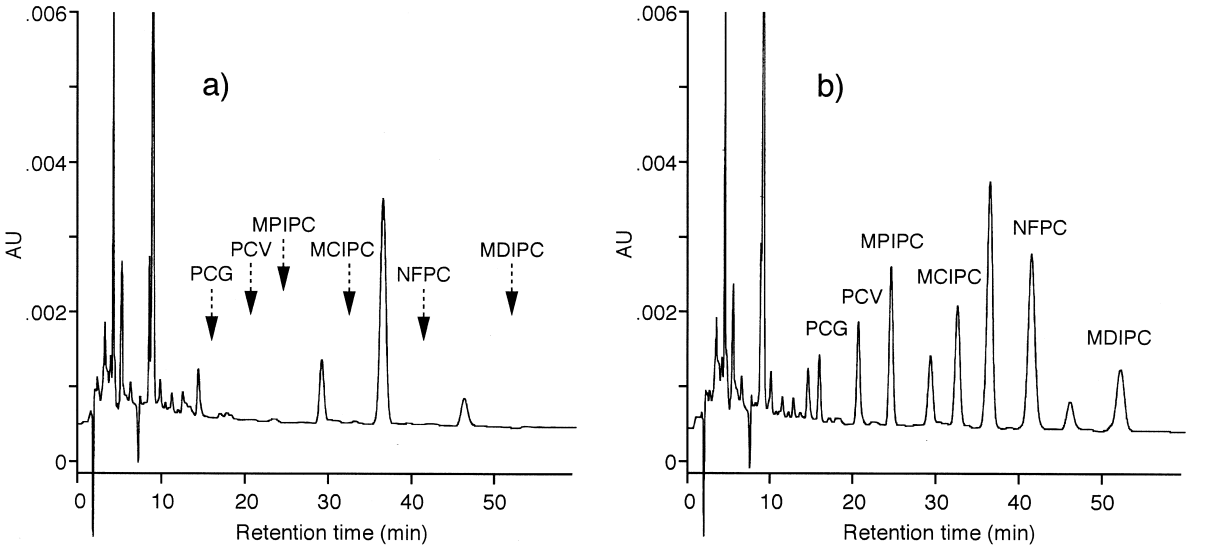

Fig. 3. Typical HPLC chromatograms of bovine liver samples. (a), Bovine liver (control); (b), added at a level of 0.5 mg/kg each of penicillins.

For the kidney, the average recoveries ranged from 79 to 92% with the RSDs ranging from 1.8 to 7.8%. Figs. 3 and 4 show typical chromatograms of the fortified liver and kidney samples (b) and their corresponding blank control (a), respectively. The detection limits have been estimated at 0.02 mg/kg for MPIPC, MCIPC and NFPC, 0.03 mg/kg for PCV, 0.04 mg/kg for PCG, and 0.05 mg/kg for MDIPC in the bovine liver and kidney (S/N ratio= 3). In order to prove the clean-up effect of the present method, we analyzed ten samples (five each of liver and kidney) which were previously found to

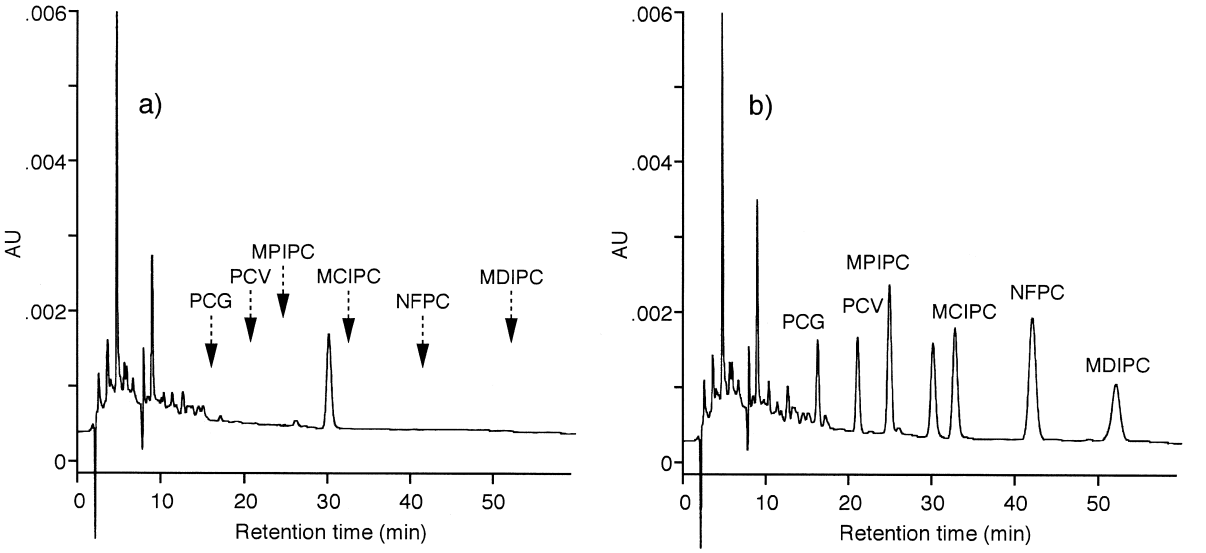

Fig. 4. Typical HPLC chromatograms of bovine kidney samples. (a), Bovine kidney (control); (b), added at a level of 0.5 mg/kg each of penicillins.

be negative for penicillins using bioassay screening. The obtained chromatograms were almost the same as in Figs. 3a and 4a except for the shorter retention time region of less than 10 min. These results clearly indicated that the method proposed here has satisfactory reproducibility, recovery and accuracy for the simultaneous determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine liver and kidney. The analysis time required is about 5 h for each liver and kidney sample, and the time is much shorter than the bioassay and combination analysis of an individual chemical analysis.

4. Conclusions

A high-performance ion-pair chromatographic method with UV detection for the determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine liver and kidney in a single assay was developed with the following characteristics: (1) Use of 2% NaCl solution containing sodium tungstate and sulfuric acid is effective for the extraction of the penicillins from the bovine liver and kidney. (2) The combined use of the above extraction solution and double cartridge clean-up using C18 and QMA cartridges provides good recoveries (73–96%) with small RSDs (1.4–8.7%). (3) Use of 10 m*M* acetic acid as the washing solution for the QMA cartridge is useful to eliminate interfering substances that originate from the bovine liver and kidney. Therefore, we recommend the present analytical method for the simultaneous determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine liver and kidney.

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