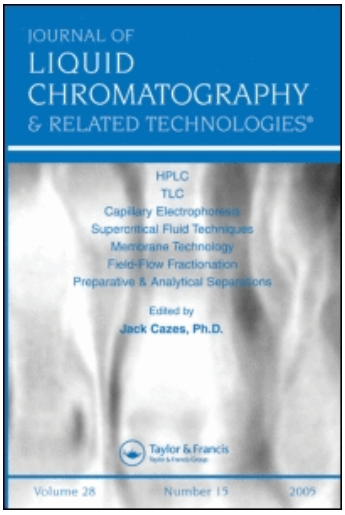

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DETERMINATION OF BENZYLPENICILLIN AND OTHER BETA-LACTAM ANTIBIOTICS IN PLASMA AND TISSUES USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY FOR RESIDUAL AND PHARMACOKINETIC STUDIES

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ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method for the simultaneous determination of four β-lactam antibiotics in kidney, liver, meat, and plasma is described. The samples were extracted with acetone-trichloroacetic acid (4 mL), the organic layer then being evaporated and cleaned-up using LMS solid phase extraction columns. The method is simple, requires only small quantities of chemicals reagents and involves minimal manual work-up procedures. The lower limit of quantification was 15, 20, 40, and 60 ng/g, for kidney, liver, and meat and 40, 50, 80, and 100 ng/g for plasma, for benzylpenicillin (Penicillin G), phenoxymethyl penicillinic acid (Penicillin V), cloxacillin, and dicloxacillin, respectively.

INTRODUCTION

β-lactam antibiotics, especially benzylpenicillin, are commonly used in veterinary medicine for the treatment of a range of bacterial infections. Their wide application represents a potential hazard to consumers due to residues when used in food producing animals. Despite their low toxicity, β-lactam antibiotics have, nevertheless, been reliably shown to pose a danger to human health, as residual concentrations of penicillins in food lead to allergenic reactions in some sensitive people.[1,2]

Microbiological assays are commonly used for the determination of penicillins, being extremely sensitive to benzylpenicillin (PG) and many other β-lactam antibiotics,[3,4,5] and it is difficult to obtain comparable sensitivity with physico-chemical procedure. They are however less sensitive for cloxacillin and dicloxacillin. Like immunological or receptor tests, microbiological assays are well suited as screening procedures. With the possible exception of immunoassay, none of the screening procedures can differentiate between the various β-lactam antibiotics.

To achieve such differentiation chromatographic methods are necessary. A few papers describing instrumental methods for more specific analysis of penicillin residues in meat and kidney, based on high performance liquid chromatography have been published.[6,7,8,9,10] A capillary gas chromatographic method[11] has also been described. These methods are, however, time-consuming. They require the use of large quantities of chemicals reagents, and involve extensive manual work-up procedures.

The purpose of the present study was to develop a rapid, simple, and specific method, for the determination of beta-lactam antibiotics, in meat and different organs. The sensitivity should at least meet the requirement of quantitative detection at the MRL level, as set by the FAO/WHO Codex Alimentarius.

EXPERIMENTAL

Materials and Reagents

Samples of fresh kidney, liver, plasma, and meat from healthy cows obtained from a local slaughterhouse, were used as control material and also for spiking with a mixture of the four penicillins, to conduct recovery experiments. The samples were stored frozen (-20°C).

Benzylpenicillin (PG), phenoxymethyl penicillinic acid (PV), cloxacillin (CL), dicloxacillin (DCL) and oxacillin (OXA) was supplied by Sigma Co. (St. Louis, Mo, USA). All chemicals and solvents were of analytical and HPLC grade.

PG, PV, CL and DCL stock solution and working standards were prepared fresh weekly by dilution with distilled water. The stock solution and working standards were stored in the refrigerator.

Solid phase extraction (SPE) columns Bond Elut (1cc/25 mg) LMS, were purchased from Varian (Harbor City, CA, USA).

Solution A, consisting of 0.15 % trichloroacetic acid (TCA) in acetone was prepared by dissolving 85g TCA in 15g water (85% TCA in water is stock solution). The stock solution was stored in a refrigerator. 150 µL stock solution was diluted with acetone to 100 mL (solution A).

Solution B, consisting of 0.5 M $Na_2HPO_4 \cdot H_2O$, was made by dissolving 44.5 g $Na_2HPO_4 \cdot H_2O$ in ca. 450 mL of water. The pH was then adjusted to 6.0 with 85% phosphoric acid, and the solution further diluted up to volume (0.5 l) with water, and the pH again adjusted to 6.0 with 85% phosphoric acid.

Solution C, was prepared in the same manner as solution B (pH 6.0), but the Na_2HPO_4 concentration was 0.01M, and the phosphoric acid concentration used to adjust pH to c. 6.2 and 6.0 was 5M and 1M, respectively.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 200 quaternary pump and a Series 200 autosampler. The acquired data were processed with either Multiview 1.3 and MacQuan 1.5 software packages, which concern spectral information data processing and quantification data processing, respectively (Perkin-Elmer), and operated on a Model 8500 Apple Power Macintosh. An API 100 LC/MS system (PE SCIEX) single quadruple mass spectrometer with a standard API-Ion Spray ionization source, was employed for this study. The LC-MS was set to collect multiple single-ion data in negative ion mode for the ions at *m/z* 333, 348.8, 434, and 468 for PG, PV, CL, and DCL, respectively. The entrance electrode voltages were adjusted to provide the optimum overall intensities for the four molecular ions. The optimum sensitivities for PG, PV, CL, and DCL were obtained with N2 nebulizer gas (NEB) 10 l/min and curtain gas (CUR) 10 l/min. The ion source (IS) was -4000 V. The orifice (OR) was -10 for PG and DCL, and -25 and -15 V for PV and CL, respectively. The ring (RNG) was -330, -350, -300, and -380 V, for PG, PV, CL, and DCL, respectively, while the Quadrupole 0 (Q0) was +2

for PG, PV, CL, and DCL. The analytical column (stainless steel, 250 x 4.6 mm I.D.) was packed with 5-μm particles of Supelcosil LC-C18 DB (Supelco, Bellefonte, PA, USA). The guard column was connected to an A. 318 precolumn filter with an A-102X frits (Upchurch Scientific, USA).

The mobile phase consisted of an aqueous solution of 50% methanol (channel A) and 50% 10 mM (0.77g/l) ammonium acetate (channel B). The flow rate was 1 mL/min.

The LC eluent was split post-column approximately 1:20 so that c.50 μL flowed into the Ion-Spray ion source. The samples were injected at intervals of 15 min for the determination of all penicillins.

Sample pretreatment

Volumes of 1 mL water (or standard) and 4 mL solution A were added to 3 g kidney, liver, or meat. The mixture was homogenized for approximately 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, F.R.G.). After centrifugation for approximately 7 min. (5000 rpm), 2 mL of the supernatant (corresponding to 0.75 g kidney/liver/meat) was pipetted into a conical centrifuge tube, 1 mL solution B was added, and the mixture then blended. The homogenate was centrifuged for 3 min. (4000 rpm). The upper layer was transferred into a graduated (12 mL) conical glass-stoppered centrifuge tube.

The sample was evaporated to approx. 0.75 mL under a stream of air, using a Reacti-Term heating module at 35°C and a Reacti-Vap evaporating unit (Pierce, Rockford. IL, USA). After adding 1 mL of solution B, the sample was mixed and loaded onto a conditioned 1 cc/25 mg LMS column.

Clean-up on SPE-column

The column was conditioned with 1 mL methanol, followed by 2x1 mL solution C. The aqueous extract was applied onto the column. The column was washed with 2x100 μL solution C and then suctioned to dryness for c. 10 sec., (at a vacuum of -10 inches Hg, using a Vac Master system from International Sorbent Technology), prior to eluating with 400 μL methanol-water (6+4).

After the eluting solvent had passed through, the column was suctioned to dryness for 5 sec. and the volume adjusted to 600 μL with 200 μL water. The sample was mixed and filtered through a microcentrifuge filter (0.2 μm nylon filter, Spin-X, Costar, USA).

Conditioning, application of the sample, washing and eluting the column all took place under gravity flow. Only at the beginning of the eluting process was it necessary to apply a little positive pressure with a syringe. It is important not to allow the sorbent to dry out during the conditioning step. Aliquots of 100 μL (75 μL for liver) were injected onto the column at intervals of 15 min. for the determination of PG, PV, CL, and DCL.

Plasma

Volumes of 100 μL water (or standard) and 600 μL solution A were added to 500 μl of plasma samples. The mixture was vortex-mixed and centrifuged (4 min. 4000 rpm). 200 μL water were added to 400 μl supernatant and blended and centrifuged through a Spin X filter tube. Aliquots of 100 μL were injected into the column at intervals of 15 min. for the determination of all penicillins.

Calibration Curves and Recovery Studies

The precision, recovery and linearity, for PG, PV, CL, and DCL were determined by spiking kidney, liver, and meat samples with standard solutions to yield 20, 40, 100, 200, 300, and 400 ng/g sample for PG and PV, and 40, 100, 200, 300, and 400 ng/g sample for CL and 60, 100, 200, 300, and 400 for DCL. For plasma, spiking was performed to yield 50, 100, 200, 500, and 1000 ng/mL for PG, V, and CL, and 100, 200, 500, and 1000 ng/mL for DCL. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked kidney and meat, with those of standard solutions. The linearity of the standard curves for PG, PV, CL, and DCL in kidney, liver, meat, and plasma were calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean samples of kidney and meat from cow and corresponding samples spiked with PG, PV, CL, and DCL are shown in Figures 1, 2, 3, and 4.

The standard curves were linear in the investigated areas 20 - 400 ng/g for PG and PV, 40 - 400 ng/g for CL, and 60 - 400ng/g for DCL in liver. For kidney and meat, the figures were 20 - 400 ng/g for PG and PV and 40 - 400 ng/g for CL and DCL, respectively. In plasma, the standard curves were linear in the area 50-1000 ng/mL for PG, PV, and CL, and 100 to 1000 ng/mL for DCL. The corresponding correlation coefficients were 0.999 for PG, PV, and CL and 0.998 for DCL in kidney and meat, respectively.

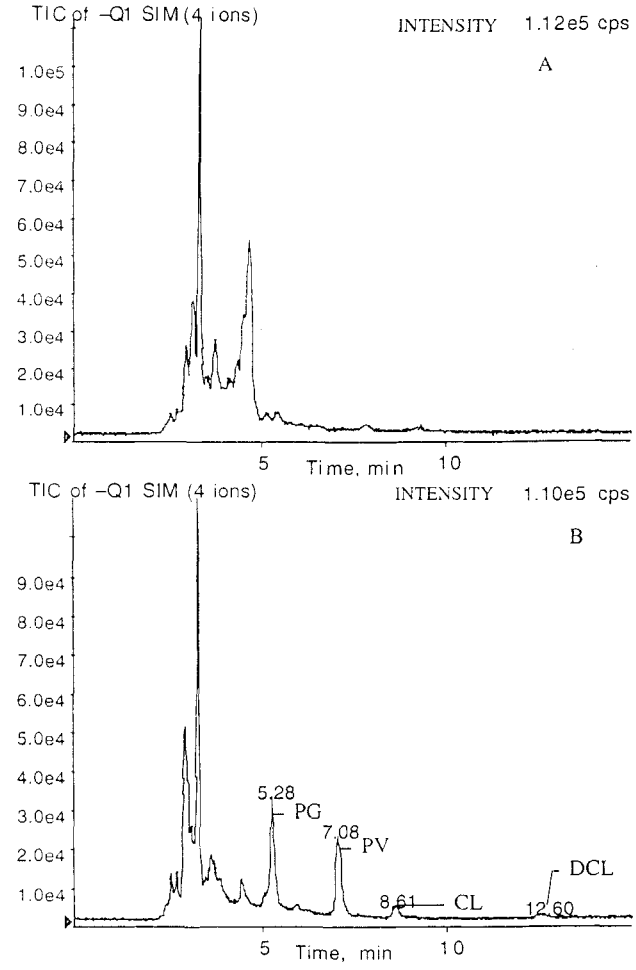

Figure 1. Chromatograms of extracts from cow liver. **A:** drug-free liver, **B:** liver spiked with PG, PV, CL, and DCL (100ng/g).

In liver, the correlation coefficients were 0.997 for PG and CL, 0.998 for PV and 0.999 for DCL. For plasma, the correlation coefficients were 0.999 for PG and PV, and 0.996 and 0.998 for CL and DCL, respectively. The recovery and repeatabilities for PG, PV, CL, and DCL from kidney, liver, meat, and plasma from cows are shown in Table 1.

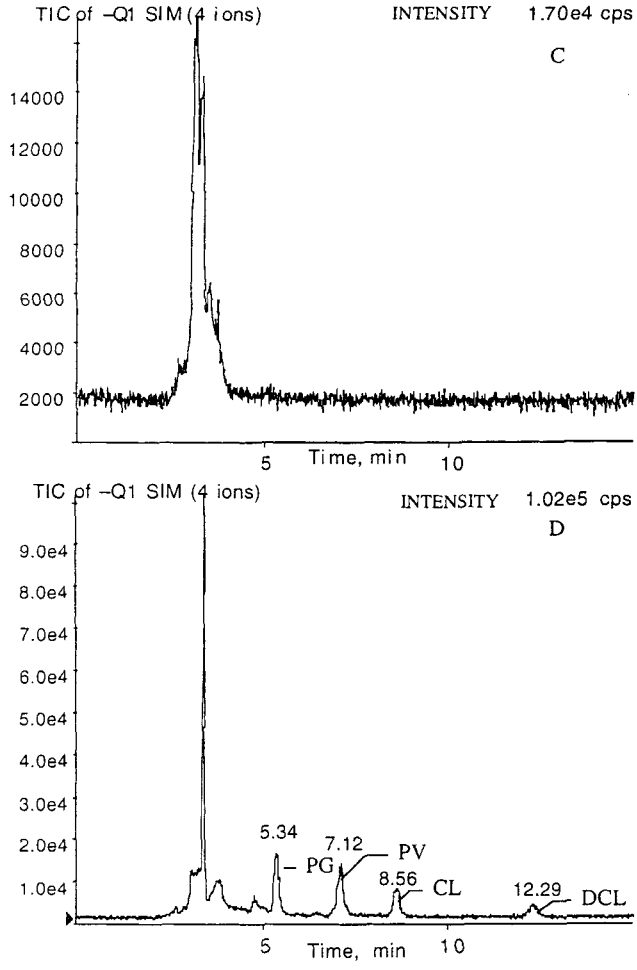

Figure 2. Chromatograms of extracts from cow meat. **C:** drug-free meat, **D:** meat spiked with PG, PV, CL and DCL (100 ng/g).

In our preliminary studies, non-polar sorbent materials such as tC18 SPE-cartridges from Waters, Bond Elut Certify and Certify II, C18, C8, C2, and LMS from Varian, were tested. The best results were obtained for most β-lactams with kidney, liver, and meat pretreated on LMS. The capacity of LMS sorbent material was also tested, and 25 mg showed good reproducibility.

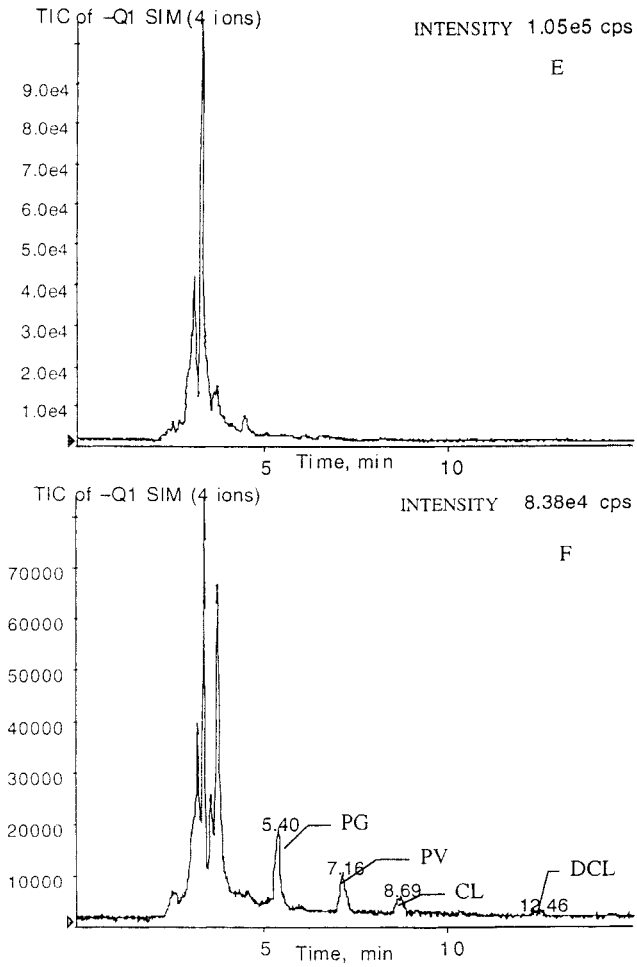

Figure 3. Chromatograms of extracts from cow kidney. **E:** drug-free kidney, **F:** kidney spiked with PG, PV, CL and DCL (100ng/g).

When eluting the LMS column, it may be necessary to apply a brief positive pressure. We do not recommend gentle vacuum because of difficulties with the flow control, since a satisfactory eluting process (and good recovery) is dependent on slow flow. We, therefore, used gravity flow. To the eluate (400 μL) from the SPE cartridge was added 200 μL water, because 60% methanol in water will change the baseline resolution, especially for DCL. The same is true for plasma.

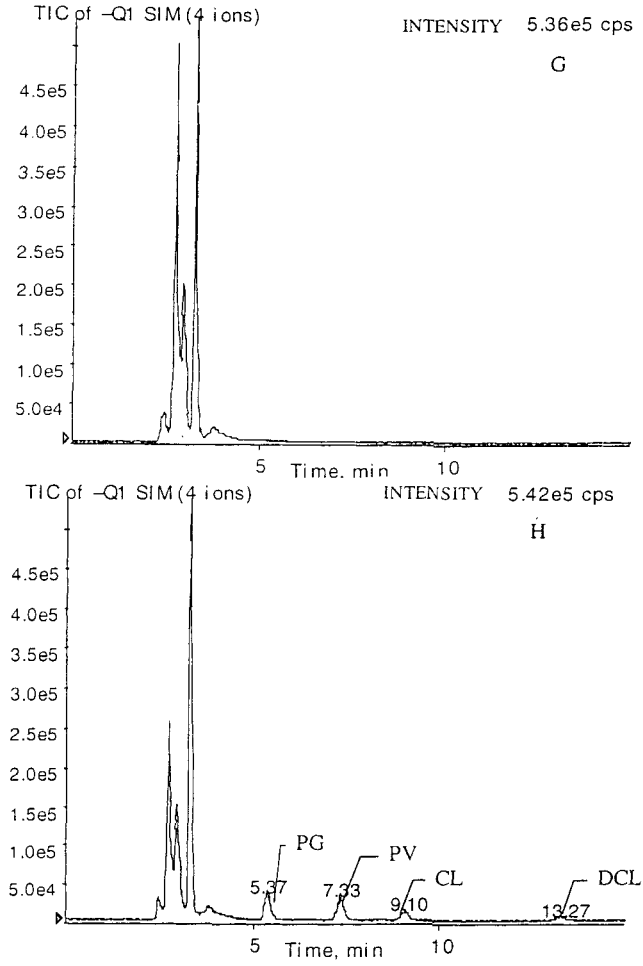

Figure 4. Chromatograms of extracts from cow plasma. **G:** drug-free plasma, **H:** plasma spiked with PG, PV, CL and DCL (500 ng/mL).

Increasing the injection volume with the eluate, will also lead to a broadening of the peak and consequent loss in resolution, especially for DCL. In contrast, more water in the final extract (sample) permits a larger volume to be injected into the column.[12]

Table 1

Recovery and Repeatability for PG, PV, CL and DCL from Spiked Samples of Kidney, Liver, Meat and Plasma

Sample	n	Amount of Drug Added*	PG		PV		CL		DCL	
			SD• %	Rec. %	SD %	Rec. %	SD %	Rec. %	SD %	Rec. %
Kidney	8	40	1.0	96	2.9	91	2.2	93	2.4	92
3g	8	200	0.6	98	3.6	87	2.2	90	3.8	85
Liver	8	60	0.7	97	2.2	81	0.9	69	2.0	58
3g	8	400	1.2	92	4.5	94	2.5	85	1.9	65
Meat	8	40	0.8	87	0.7	78	3.6	90	4.5	77
3 g	8	200	1.1	77	2.8	76	2.4	80	3.1	75
Plasma	8	100	1.5	98	1.6	98	1.1	95	1.0	98
0.5 mL	8	1000	0.8	98	1.6	98	0.8	99	0.9	98

* Concentration: ng/mL for plasma, ng/g for tissue.
SD• = standard deviation.
Rec. = recovery.

Lower pH leads to loss of acid-labile penicillins, while a higher value will result in lower overall recovery. A balance must therefore be achieved between good extraction efficiency and loss of the substance caused by acid degradation.[13]

The pH value, which influences the extraction from kidney, liver, and meat of those of the penicillins included in the acetone, had to be optimized by adding a small amount % TCA in acetone. We found that 0.15 % TCA in acetone was optimal for kidney, liver, and meat and that the same % of TCA in acetone gave good recovery from plasma.

The detection limit of the assay depends mainly on the sensitivity of the LC-MS. This in turn could be influenced by such factors as the position of the ion spray inlet, the position of the silica tubing onto the sprayer, the composition of the mobile phase, and the flow-rate of mobile phase into the ion source. API-100 maintained a stable sensitivity for more than 96 hours without interruption, during the analysis of β-lactam samples from meat and kidney, and for 48 hours for liver and plasma. Afterwards, the ion spray inlet was cleaned with a mobile phase of MeOH - water (1 : 1) for 5 min and 100% MeOH for 5 min; the curtain plate of API-100 being cleaned with MeOH-water (1 : 1) with a paper with good absorption capacity.

We were then able to continue with the analysis. It was not necessary to adjust anything even if the ion source housing had been removed to clean the curtain plate, an operation that took four minutes. The chromatographic system appeared to be efficient for the determination of the four β-lactams in kidney, liver, meat, and plasma; the limits of quantification being 15, 20, 40, and 60 ng/g for kidney, liver and meat, and 40, 50, 80, and 100 ng/mL for plasma. The limits of detection were close to 7, 10, 15, and 20 ng/g for kidney, liver and meat and 30, 40, 50, and 80 ng/mL for plasma for PG, PV, CL, and DCL, respectively.

The detection limit of the assay was calculated to be three times the baseline noise from a drug-free tissue. No interference was seen during analysis, (with the exception of PG in liver which had a small interference) when calibrating the curves, or when performing recovery studies. The method presented in this paper is selective, robust, sensitive, and accurate. With concentrations of drugs above the standard curve, we injected a lesser quantity into the column.

This method can also be used for the determination of oxacillin (OXA). In this case the composition of the mobile phase is changed to methanol - water (60 : 40). As OXA is not used to treat livestock in Norway, the precision, recovery and linearity were not validated in this report. Kidney and meat from sheep and pigs show a similar baseline resolution to samples from cows. We compared N2 and air produced from a central air compressor (generator) for evaporating the samples of β-lactam from kidney, liver, and meat. No differences were found.

High performance liquid chromatography (HPLC) has acquired an increasingly important role in the analysis of different contaminants in food, as demonstrated by the wide variety of applications reported in recent years. The advantage of the LC-MS technique lies in a combination of the separation capabilities of HPLC and the power of MS as an identification and confirmation method with high sensitivity, selectivity, and quantitative capability.

Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. Using conventional HPLC detectors, detection may be complicated by false-positive results. In such cases, MS has proved to be a valuable technique for the unambiguous identification of contaminants in food. While conventional HPLC methods may require long complex separations, the LC-MS method requires only a simple clean-up procedure and no derivatization. Thus LC-MS seems to provide a better alternative than HPLC. The assay is suitable for use as a confirmatory method in residue testing programmes.

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