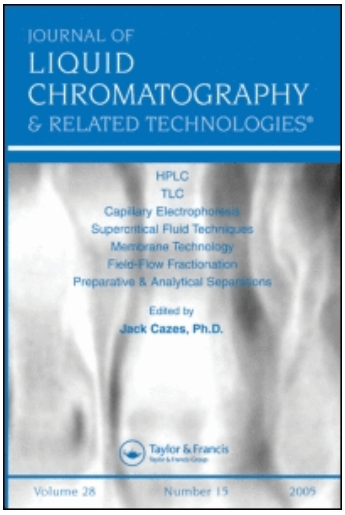

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Determination of Bicozamycin and Its Benzoylester Derivative in Yellowtail Tissues by High Performance Liquid Chromatography

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DETERMINATION OF BICOZAMYCIN AND ITS BENZOYLESTER DERIVATIVE IN YELLOWTAIL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A unique and practical method was developed for quantitative analysis of Bicozamycin(BCM) and its benzoylester(BCM-BZ) from yellowtail(Seriola quinqueradiata) tissues(blood,muscle,liver,kidney) by High Performace Liquid Chromatography(HPLC). Tissues were homogenized and then deprotenized with acetonitrile or a mixture of acetonitrile and water. The extract was evaporated, and the residue was dissolved in water and partitioned by addition of chloroform or carbontetrachloride. The aqueous solution was taken up into a BOND ELUT C_{18} cartridge column, after washing with water, BCM was eluted by methanol then the eluent was evaporated to dryness. After that the residue was dissolved in methanol/ethyl acetate/hexane (0.3:3:10) and the solution was applied to a BOND ELUT DIOL(or SEP PAK-DIOL)column, BCM eluted with acetone was dissolved in the mobile phase and analyzed by HPLC on two C_{18} reversed phase columns. BCM-BZ extracted from tissues was also cleaned up with BOND ELUT DIOL, and analyzed by HPLC on a C_{18} reversed phase column.

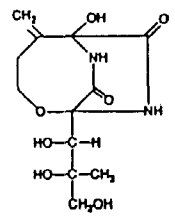
INTRODUCTION

Bicozamycin is a commercially important antibiotic that is being produced from the fermentation harvest of Streptomyces sapporonensis at Fujisawa

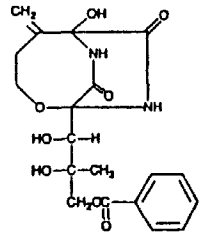
pharmaceutical company central research laboratories (1,2) and it is an effective agent against Pseudotuberculosis in yellowtail caused by Pasteurella piscicida(3). Antibiotic residues in fish or animal tissues are commonly detected by microbiological assays but these methods need more than several grams of tissues because of their inferior sensitivity. Chromatographic methods offer a promising analysis to detect and identify the residual antibiotic in small amounts of biological tissues(4). HPLC has been used for determination of a number of antibiotics in biological tissues(5).

However, bicozamycin does not have any particular absorption in the ultraviolet (UV) wave range except the absorption at 190~220nm.

In the case of HPLC analysis, there is likely to be cosiderable interference resulting from the presence of unknown compound in the test samples at the short UV wavelength range. In order to overcome this problem, we devised two analytical methods for the assay of bicozamycin from the tissues of yellowtail by HPLC. The procedure described herein, involving removal of proteins by disposable cartridge columns offers precise determination of BCM and BCM-BZ. The recovery of BCM and BCM-BZ in tissues at the level of 1ppm were 70.4 ~80.8% and 85.0~90.7%(n=6) and the sensitivity limits of BCM and BCM-BZ were 0.05ppm and 0.04~0.05ppm respectively in all tissues.



Bicozamycin



Bicozamycin benzoylester

MATERIALS

Acetonitrile, methanol, chloroform, hexane, carbon tetrachloride, ethyl acetate, acetone, sodium perchlorate, potassium dihydrogen phosphate and perchloric acid(70%) were purchased from WAKO PURE CHEMICAL INDUSTRIES,LTD. All chemicals used were Guaranteed Reagent(GR).

SEP-PAK sample preparation cartridges were purchased from Waters Chromatography Division (Milford. Ma.). Analytichem Bond Elut sample preparation products were purchased from Varian (Harbor City, CA.), Sepacol-mini-pp(filters) were purchased from SEIKAGAKU KOGYO CO.,LTD.

Sartorius balance, METTLER balance, Eppendorf pipet, BIOTRON homoginizer, EYELA rotary vacuum evaporator, TOMY centrifuge, HORIBA digital pH meter, SHARP ultrasonic bath, Milli-XQ(MILLIPORE) and NEOCOOL(YAMATO) were used for sample preparation.

Shimadzu balance, POLYTRON homogenizer, AUTOMATIC LABO-MIXER NS-8(Iuchiseieido), TOMY centrifuge, VAC ELUT SPS 24(Analytichem International), Milli-Q (MILLIPORE), Air pump(IWAKI GARAS), pH meter(IWAKI GARAS) and Vortex mixer were also used for sample preparation.

Hitachi HPLC system L-4000,4200(UV-detector), L-6200(pump), AS-2000 (automaticampler), Chromatocorder(SIC.), NEOCOOL(cooler,YAMATO), Pasolina 100T(cooler, Iuchiseieido) and CB-500TS(incubator,RIKOKAGAKU) were used for method A of BCM.

Shimadzu HPLC system SPD-6A(detector), LC-6A(pump), LC-9A(pump), CTO-6A (column oven), SIL-6A(auto injector), SIL-6B(auto injector), SCL-6A(system controller), SCL-6B(system controller) and C-R4A(data processor) were used for method B of BCM and BCM-BZ.

1)Analytical procedure of BCM

Method A

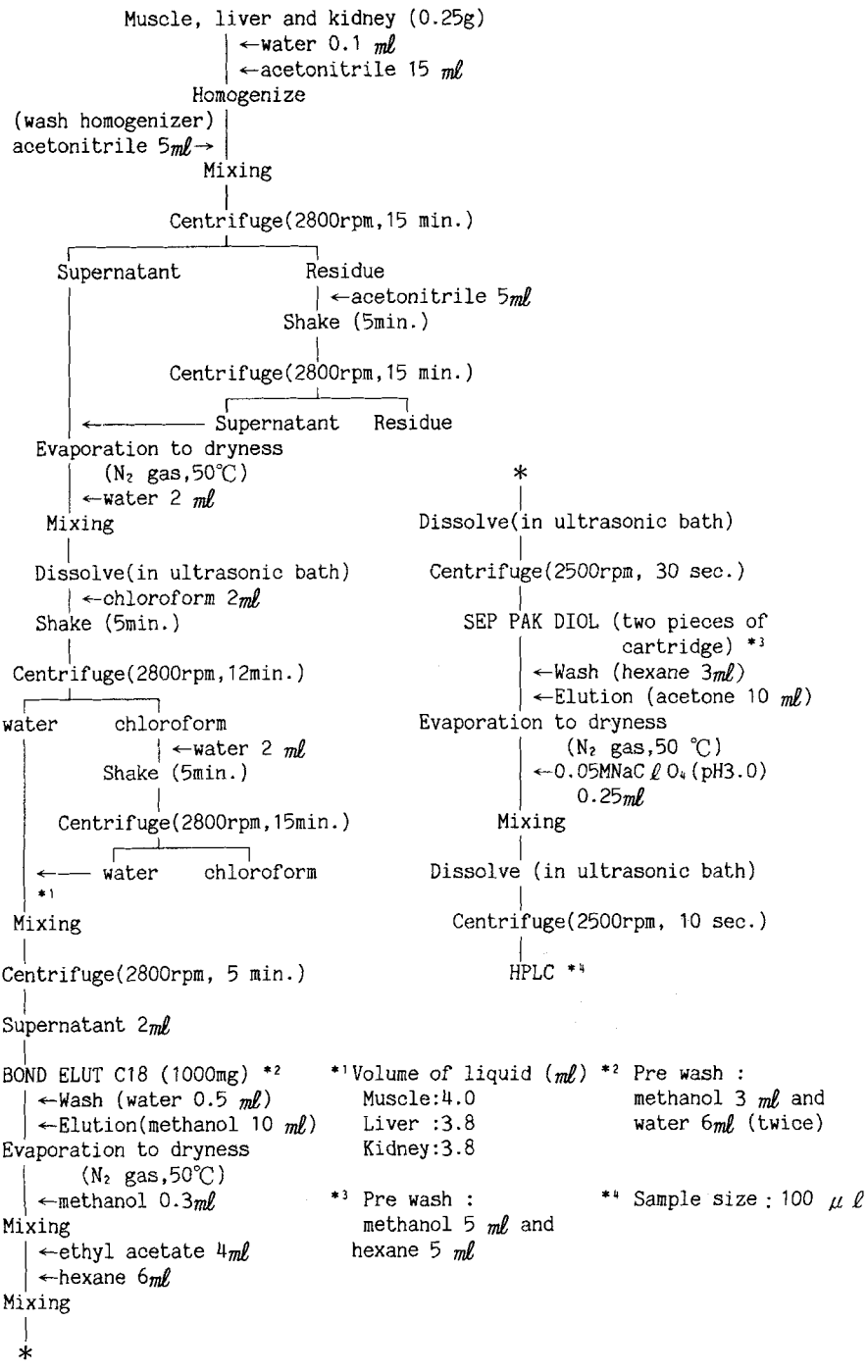
*1 Pre wash : methanol 3 ml and water 6ml (twice)

*2 Pre wash : methanol 5 ml and hexane 5 ml

*3 Sample size : 55 μ ℓ

Fig.1 Assay procedure for BCM in blood of yellowtail

Method A



*1 Volume of liquid (ml)
Muscle:4.0
Liver :3.8
Kidney:3.8

*2 Pre wash : methanol 3 ml and water 6ml (twice)

*3 Pre wash : methanol 5 ml and hexane 5 ml

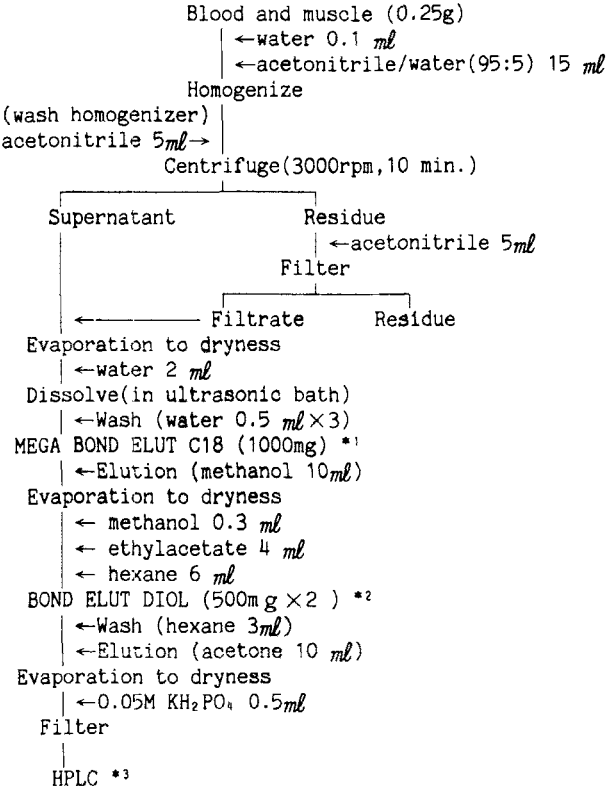
*4 Sample size : 100 μl

Fig.2 Assay procedure for BCM in muscle, liver and kidney of yellowtail

HPLC conditions (Method A for BCM)

Detection : Ultraviolet (at 210nm)
Column : Hypersil ODS-5 (4.6mm $\phi \times$250mm) (Chemco)
Guard column : Hypersil ODS-5 (4.6mm $\phi \times$10mm)
Mobile phase : A: 0.05M sodium perchlorate (pH3.0, adjusted with dil. perchloric acid)
B: 0.05M sodium perchlorate (pH3.0, adjusted with dil. perchloric acid) • Methanol(95:5)
C: Water • Methanol(1:1)
(Gradient elution of A,B and C)
Flow rate : 1.0 ml/min.
Column temperature : 12 ~40℃

Method B



*1 Pre wash :
methanol 5 ml
water 5ml

*2 Pre wash :
ethylacetate/hexane
(2/3) 5ml

*3 Sample size : 100 μl

Fig.3 Assay procedure for BCM in blood and muscle of yellowtail

HPLC conditions (Method B for BCM)
Detection : Ultraviolet (at 210nm)
Column : Capcell Pak C_{18} SG120 (4.6mm $\phi \times 250$mm) (SHISEIDO COMPANY,LTD.)
+ TSKgel ODS-80T_M (4.6mm $\phi \times 250$mm) (TOSOH CORPORATION)
Mobile phase : 0.05M potassium dihydrogen phosphate (pH3.0)
Flow rate : 1.2 ml/min.
Column temperature : 55 ℃

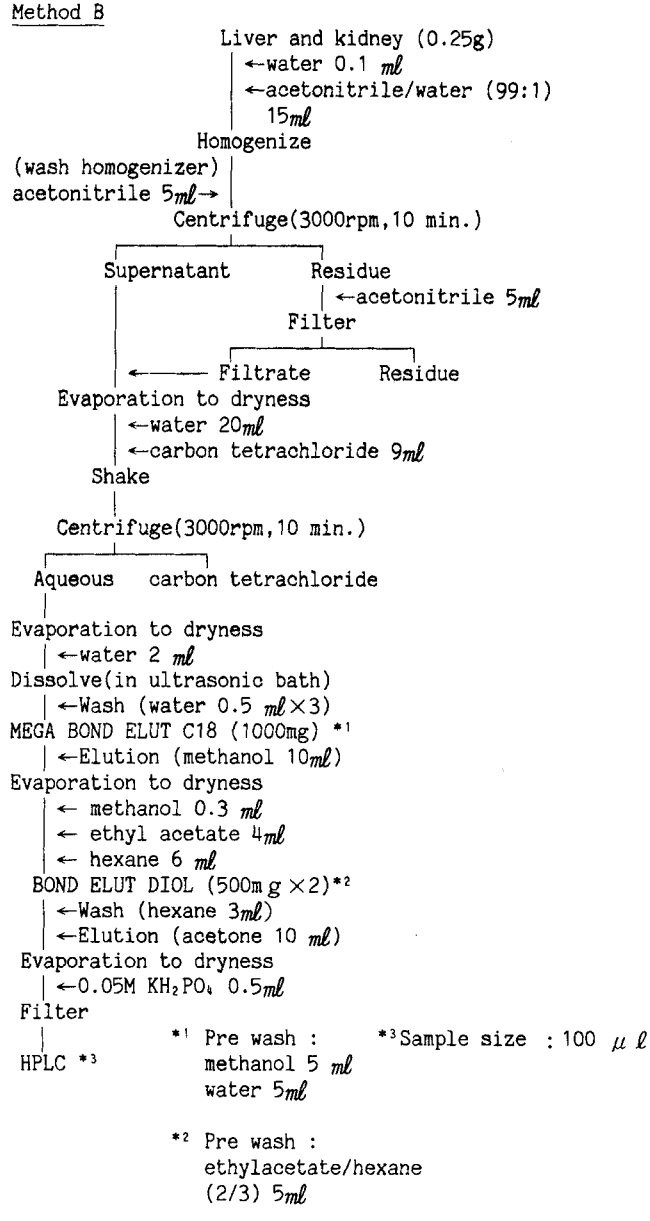


Fig.4 Assay procedure for BCM in liver and kidney of yellowtail

HPLC conditions for BCM-BZ
Detection : Ultraviolet (at 230nm)
Column : TSKgel ODS-80T$_M$ (4.6mmϕ × 150mm) (TOSOH CORPORATION)
Guard column : TSKgel ODS-80T $_M$ (4.6mm ϕ × 10mm)
Mobile phase : Water • methanol (62:38)
Flow rate : 1.0 ml/min.
Column temperature : 45 ℃

2) Analytical procedure of BCM-BZ

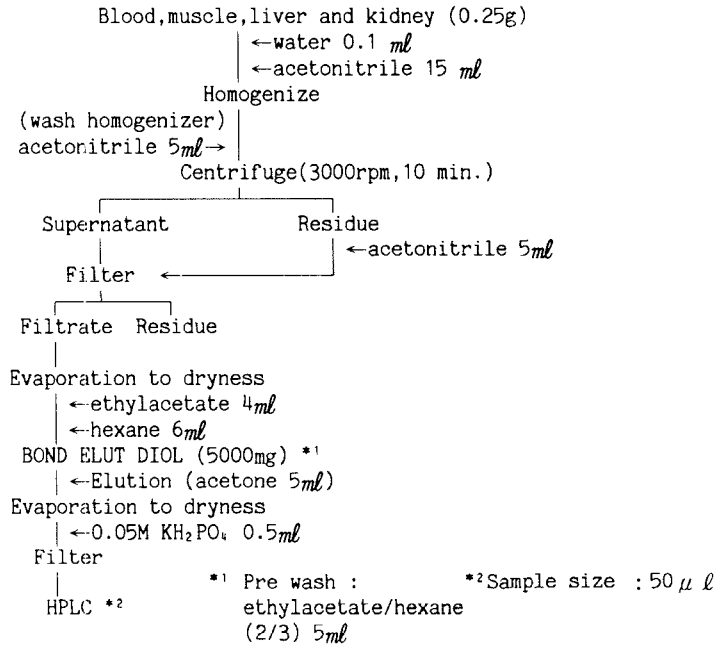


Fig.5 Assay procedure for BCM-BZ in blood,muscle,liver and kidney of yellowtail

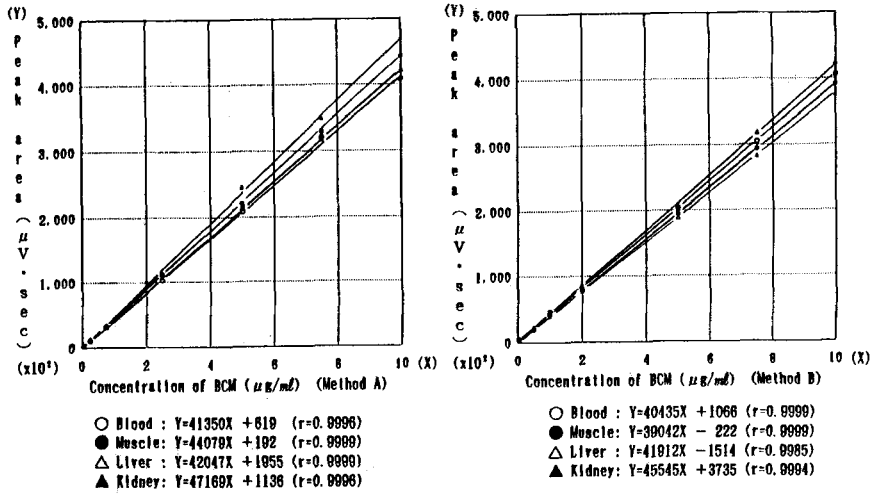


Fig.6 Calibration curve of BCM

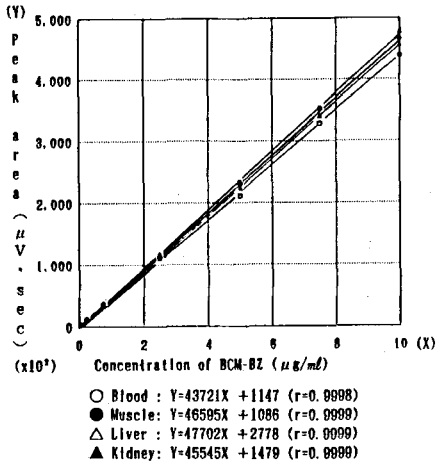


Fig.7 Calibration curve of BCM-BZ

Table 1 Recovery rate(%) of BCM (added amount:1ppm)

Tissue	Repetition	Method A		Method B	
Blood	1	77.8		69.7	
	2	72.1		71.1	
	3	74.4	$\overline{X}$= 75.5	77.1	$\overline{X}$= 74.3
	4	76.7	CV= 2.7%	72.4	CV= 4.9%
	5	75.5		78.1	
	6	76.3		77.2	
Muscle	1	77.9		81.1	
	2	70.4		80.5	
	3	70.3	$\overline{X}$= 70.4	81.1	$\overline{X}$= 80.8
	4	66.0	CV= 5.9%	80.4	CV= 1.1%
	5	70.5		82.2	
	6	67.1		79.6	
Liver	1	74.6		73.3	
	2	73.8		69.9	
	3	69.2	$\overline{X}$= 74.8	70.7	$\overline{X}$= 72.9
	4	78.1	CV= 5.5%	74.9	CV= 2.9%
	5	80.7		73.5	
	6	72.4		74.8	
Kidney	1	76.2		77.0	
	2	71.0		73.5	
	3	72.2	$\overline{X}$= 73.1	73.6	$\overline{X}$= 73.9
	4	77.4	CV= 4.9%	77.3	CV= 4.5%
	5	67.6		74.0	
	6	74.0		68.0	

Table 2 Recovery rate(%) of BCM-BZ (added amount:1ppm)

Repetition	Blood	Muscle	Liver	Kidney
1	88.0	83.8	82.5	90.0
2	91.3	89.1	86.6	91.5
3	85.7	91.1	85.2	90.9
4	90.4	87.6	84.5	91.1
5	88.9	93.4	87.8	90.2
6	92.0	91.2	83.2	90.4
$\overline{X}$	89.4	89.4	85.0	90.7
CV(%)	2.6	3.7	2.4	0.6

Table3 Detection limit of BCM and BCM-BZ in tissues or blood (μg/g)

Tissues	BCM		BCM-BZ
	Method A	Method B	
Blood	0.050	0.050	0.040
Muscle	0.049	0.050	0.040
Liver	0.047	0.050	0.040
Kidney	0.047	0.050	0.040

METHODS

Analysis of BCM and BCM-BZ in yellowtail tissues were carried out according to the following analytical procedures. Standards of BCM and BCM-BZ were diluted into concentrations ranging from 0.05 ~50 μg/ml using the HPLC mobile phase as diluent.

RESULTS

1) Calibration curve

12.5ng~2.5 μg of BCM or BCM-BZ was added to each tissue or blood sample and the analysis was performed by described procedures. 50~100 microliter samples of these standards were injected onto the column using the automatic injector. Seven point calibration curves of BCM and BCM-BZ were developed for each tissue or blood sample using peak area.

2) Recovery of added BCM and BCM-BZ

BCM and BCM-BZ were added at the indicated levels (based on original tissue weight) to tissues or blood and carried through the procedures.

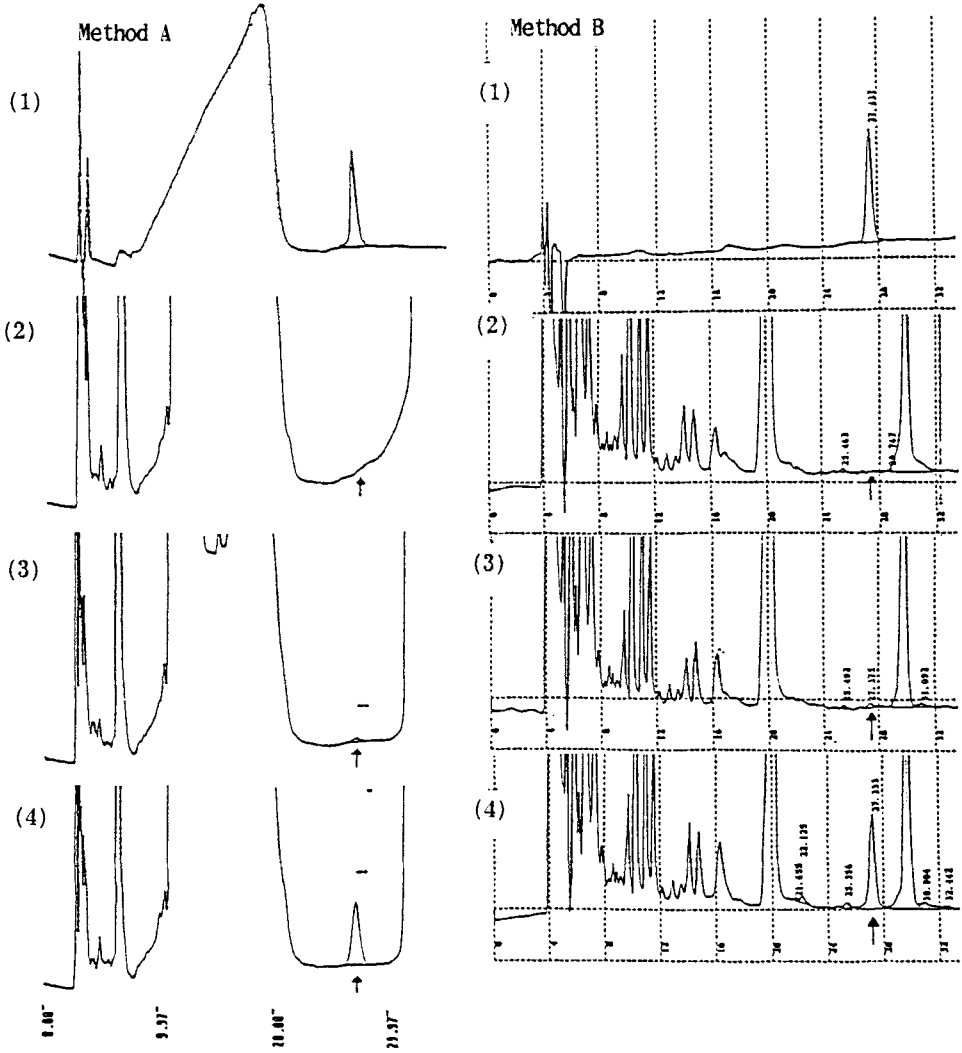


Fig.8 Chromatograms of BCM standard and BCM in tissue extracts
(1) standard :0.5ppm (3) muscle extracts (spiked with 0.05 μg/g of BCM)
(2) muscle extracts (blank test)(4) muscle extracts (spiked with 1.0 μg/g of BCM)

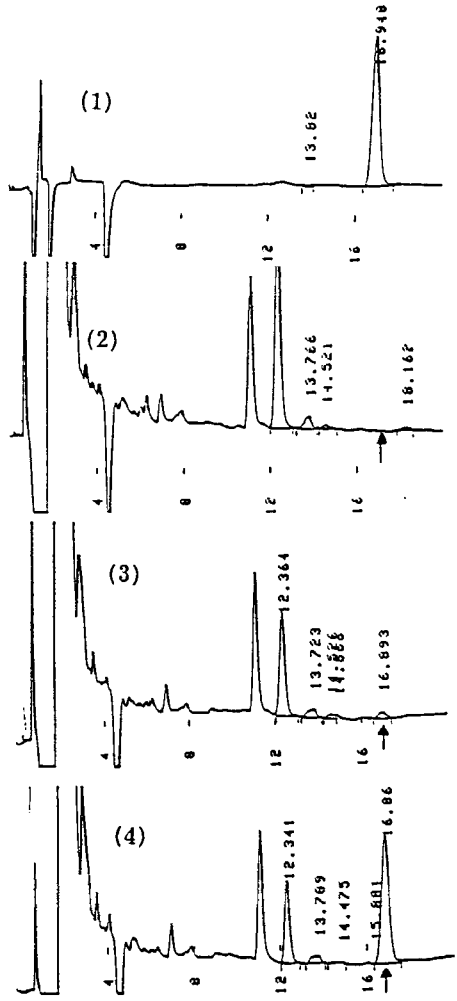


Fig.9 Chromatograms of BCM-BZ standard and BCM-BZ in tissue extracts
(1) standard :0.5ppm
(2) muscle extracts (blank test)
(3) muscle extracts (spiked with 0.05 μg/g of BCM-BZ)
(4) muscle extracts (spiked with 1.0 μg/g of BCM-BZ)

Quantitation was based on linear extrapolation from standards.

Recoveries of BCM and BCM-BZ are summarized in Table 1 and Table 2.

3) Detection limit

In order to determine the detection limit, 10.0~12.5ng of BCM and BCM-BZ were added to tissues or blood, and analysis was carried as described. The results are summarized in Table3.

4) Chromatography

Typical chromatograms of BCM and BCM-BZ in extracts of yellowtail muscle, blank muscle and standards are shown in Fig.8 and Fig.9. The peak of BCM and BCM-BZ in every chromatogram were well separated from the endogeneous peaks. The retention times of BCM in method A and method B were ca. 27 min., and the retention time of BCM-BZ was ca. 17 min.

DISCUSSION

In recent years, there has been increasing interest in the development of HPLC for the analysis of various biological substances. However, sensitivity and selectivity are critical for the detection of residual drugs or compounds in complex matrices such as biological specimens, pharmaceutical preparations, etc. Although the chemical structure of BCM is similar to amino acids we could not find a suitable chemical derivatization strategy in HPLC from the view point of the separation in biological matrix. Therefore, we have improved sample clean-up and HPLC conditions relating to sensitivity and selectivity for consistently quantifying BCM as low as 0.05 ~10ppm. We used two types of clean-up columns, one is a reversed phase C_{18} (Bond Elut C_{18}), the other is a

diol (Bond Elut 2-OH or Sep pak 2-OH). After sample clean-up, to avoid the interference of matrix, we set up two HPLC conditions. Method A is a gradient elution, and Method B is an isocratic elution system.

These method could be used to determine BCM or BCM-BZ in other biological tissues from animals or poultry such as swine, cow, chicken etc.

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