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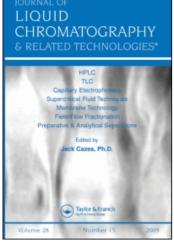
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DETERMINATION OF BICOZAMYCIN AND ITS BENZOYLESTER DERIVATIVE IN YELLOWTAIL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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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 Cls 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 Cls reversed phase columns. BCM-BZ extracted from tissues was also cleaned up with BOND ELUT DIOL, and analyzed by HPLC on a Cls reversed phase column.

INTRODUCTION

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

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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

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.

```
    Analytical procedure of BCM

    Method A
            Blood (0.25 mℓ)
                   \leftarrow water 0.1 m\ell
                                                             \leftarrow Wash (water 0.5 m\ell)
                    ←acetonitrile 15 ml
                                                             ←Elution(methanol 10 ml)
                Homogenize
                                               Evaporation to dryness (N<sub>2</sub> gas,50°C)
(wash homogenizer)
                                                            ←methanol 0.3ml
acetonitrile 5ml→
                                                          Mixing
                 Mixing
                                                            ←ethyl acetate 4ml
                                                            ←hexane 6ml
              Centrifuge(2800rpm, 15 min.)
                                                          Mixing
    Supernatant
                                                  Dissolve (in ultrasonic bath)
                           Residue
                            ←acetonitrile 5ml
                         Shake (5min.)
                                                   Centrifuge(2500rpm, 30 sec.)
              Centrifuge(2800rpm, 15 min.)
                                                       SEP PAK DIOL(500mg×2)
                                                             ←Wash (hexane 3m\ell)
      ← Supernatant
                                 Residue
                                                             ←Elution(acetone 10ml)
  Evaporation to dryness
                                                     Evaporation to dryness
         (N₂ gas ,50°C)
                                                              (N₂ gas,50 °C)
      ←water 2 ml
                                                             ←water/methanol(95:5)
                                                                 0.25ml
   Mixing
                                                         Mixing
  Dissolve(in ultrasonic bath)
                                                      Dissolve (in ultrasonic bath)
Centrifuge(2800rpm,5min.)
                                                      Centrifuge(2500rpm, 10 sec.)
Supernate 1.9 ml
                                                          HPLC *3
 BOND ELUT C18 (1000mg) *1
    *
            *1 Pre wash :
                                        *2 Pre wash :
               methanol 3 ml and
                                           methanol 5 m\ell and
                water 6ml (twice)
                                           hexane 5 ml
            *3 Sample size : 55 \mu \ell
```

Fig. 1 Assay procedure for BCM in blood of yellowtail

Method A

```
Muscle, liver and kidney (0.25g)
                      ←water 0.1 ml
                      ←acetonitrile 15 ml
                  Homogenize
 (wash homogenizer)
 acetonitrile 5ml→
                   Mixing
                Centrifuge(2800rpm, 15 min.)
                             Residue
     Supernatant
                              | ←acetonitrile 5ml
                           Shake (5min.)
                Centrifuge(2800rpm, 15 min.)

    Supernatant

                                    Residue
   Evaporation to dryness
                                                 *
         (N_2 \text{ gas}, 50^{\circ}\text{C})
       ←water 2 ml
                                        Dissolve(in ultrasonic bath)
    Mixing
   Dissolve(in ultrasonic bath)
                                         Centrifuge(2500rpm, 30 sec.)
     | ←chloroform 2ml
                                             SEP PAK DIOL (two pieces of
   Shake (5min.)
                                                            cartridge) *3
 Centrifuge(2800rpm, 12min.)
                                                   \leftarrowWash (hexane 3mL)
                                                   \leftarrowElution (acetone 10 m\ell)
water
          chloroform
                                        Evaporation to dryness
                                                      (N₂ gas,50 °C)
             ←water 2 ml
                                                   \leftarrow0.05MNaC \ell O<sub>4</sub> (pH3.0)
         Shake (5min.)
                                                        0.25mℓ
     Centrifuge(2800rpm, 15min.)
                                                Mixing
                   chloroform
                                        Dissolve (in ultrasonic bath)
        - water
                                           Centrifuge(2500rpm, 10 sec.)
 Mixing
Centrifuge(2800rpm, 5 min.)
                                                 HPLC **
Supernatant 2ml
                                *'Volume of liquid (ml) *2 Pre wash :
BOND ELUT C18 (1000mg) *2
  \leftarrow Wash (water 0.5 ml)
                                  Muscle:4.0
                                                            methanol 3 ml and
  \leftarrowElution(methanol 10 m\ell)
                                   Liver:3.8
                                                             water 6ml (twice)
                                  Kidney:3.8
Evaporation to dryness
       (N₂ gas,50°C)
                              *3 Pre wash :
                                                          ** Sample size: 100 \mu \ell
   ←methanol 0.3ml
                                  methanol 5 m\ell and
Mixing
                                 hexane 5 ml
   ←ethyl acetate 4ml
   ←hexane 6ml
Mixing
 *
```

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 m\ell/\min.
    Column temperature : 12 ~40℃
    Method B
                    Blood and muscle (0.25g)
                        ←water 0.1 ml
                        ←acetonitrile/water(95:5) 15 ml
                    Homogenize
  (wash homogenizer)
  acetonitrile 5ml \rightarrow
                  Centrifuge(3000rpm, 10 min.)
       Supernatant
                               Residue
                                ←acetonitrile 5ml
                             Filter
                     - Filtrate
                                      Residue
     Evaporation to dryness
        | ←water 2 ml
     Dissolve(in ultrasonic bath)
        \mid \leftarrow Wash (water 0.5 m\ell \times 3)
    MEGA BOND ELUT C18 (1000mg) *1
        \leftarrow Elution (methanol 10m\ell)
     Evaporation to dryness
         ← methanol 0.3 ml
         ← ethylacetate 4 ml
         ← hexane 6 ml
     BOND ELUT DIOL (500m g \times2) *2
        \leftarrow Wash (hexane 3m\ell)
         \leftarrowElution (acetone 10 m\ell)
    Evaporation to dryness

| ←0.05M KH<sub>2</sub>PO<sub>4</sub> 0.5ml
      Filter
       HPLC *3
            *1 Pre wash :
                                *3 Sample size: 100 \mu \ell
               methanol 5 ml
               water 5ml
            *? Pre wash :
               ethylacetate/hexane
               (2/3) 5 ml
```

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<sub>18</sub> SG120 (4.6mm \phi \times 250mm) (SHISEIDO COMPANY,LTD.)
                + TSKgel ODS-80T<sub>M</sub> (4.6mm \phi \times 250mm) (TOSOH CORPORATION )
  Mobile phase: 0.05M potassium dihydrogen phosphate (pH3.0)
  Flow rate : 1.2 m\ell/\min.
  Column temperature : 55 °C
        Method B
                             Liver and kidney (0.25g)
                               ←water 0.1 ml
                               ←acetonitrile/water (99:1)
                                  15ml
                           Homogenize
         (wash homogenizer)
         acetonitrile 5ml→
                        Centrifuge(3000rpm, 10 min.)
                 Supernatant
                                      Residue
                                       ←acetonitrile 5ml
                                   Filter

    Filtrate

                                            Residue
             Evaporation to dryness
                    ←water 20ml
                   ←carbon tetrachloride 9ml
                Shake
             Centrifuge(3000rpm, 10 min.)
                      carbon tetrachloride
           Aqueous
        Evaporation to dryness
           ←water 2 ml
        Dissolve(in ultrasonic bath)
            \leftarrow Wash (water 0.5 m\ell \times 3)
        MEGA BOND ELUT C18 (1000mg) *1
           \mid \leftarrow Elution (methanol 10m\ell)
        Evaporation to dryness
             ← methanol 0.3 ml
             ← ethyl acetate 4ml
             ← hexane 6 ml
           BOND ELUT DIOL (500mg \times 2)^{*2}
            ←Wash (hexane 3ml)
            ←Elution (acetone 10 ml)
         Evaporation to dryness
           | ←0.05M KH<sub>2</sub>PO<sub>4</sub> 0.5ml
         Filter
                        *! Pre wash :
                                            *3Sample size : 100 \mu \ell
         HPLC *3
                           methanol 5 ml
                           water 5ml
                        *2 Pre wash :
                           ethylacetate/hexane
                           (2/3) 5ml
```

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_{\rm M} (4.6mm \phi 	imes150mm) (TOSOH CORPORATION)
  Guard column : TSKgel ODS-80T _{\rm M} (4.6mm \phi \times 10mm)
  Mobile phase : Water • methanol (62:38)
  Flow rate: 1.0 m\ell/\min.
  Column temperature : 45 °C
 2) Analytical procedure of BCM-BZ
             Blood, muscle, liver and kidney (0.25g)
                           ←water 0.1 ml
                           ←acetonitrile 15 ml
                       Homogenize
     (wash homogenizer)
     acetonitrile 5ml→
                     Centrifuge(3000rpm, 10 min.)
        Supernatant
                                Residue
                                   ←acetonitrile 5ml
          Filter
     Filtrate Residue
     Evaporation to dryness
         ←ethylacetate 4ml
          ←hexane 6ml
      BOND ELUT DIOL (5000mg) *1
         \leftarrow Elution (acetone 5m\ell)
     Evaporation to dryness
         ←0.05M KH<sub>2</sub>PO<sub>4</sub> 0.5ml
       Filter
                        *1 Pre wash :
                                                *2 Sample size : 50 \mu \ell
        HPLC *2
                           ethylacetate/hexane
                           (2/3) \, 5m\ell
```

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

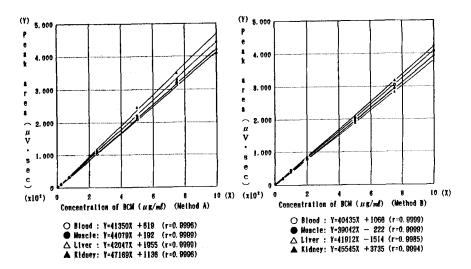


Fig.6 Calibration curve of BCM

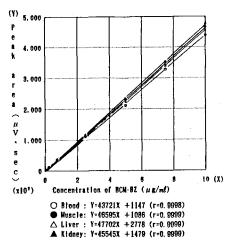


Fig.7 Calibration curve of BCM-BZ

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

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

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

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

Tissues	ВС	BCM-BZ		
1133463	Method A	Method B	DCM-DZ	
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	

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

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 \sim 50 μ g/ $m\ell$ using the HPLC mobile phase as diluent.

RESULTS

1) Calibration curve

12.5ng \sim 2.5 μ g of BCM or BCM-BZ was added to each tissue or blood sample and the analysis was performed by described procedures. $50\sim100$ 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.

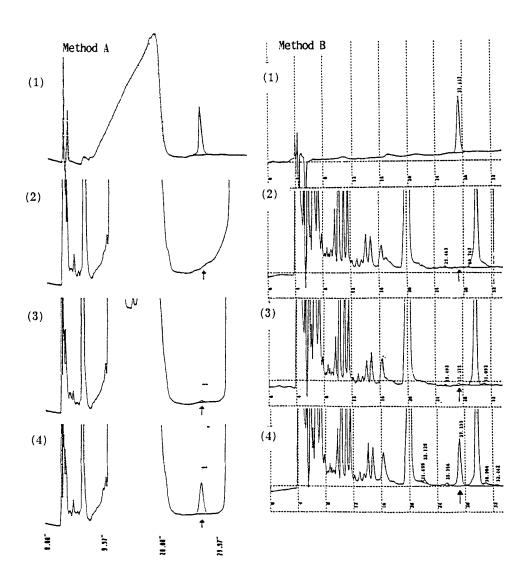


Fig.8 Chromatograms of BCM standard and BCM in tissue extracts (1) standard :0.5ppm (3) muscle extracts (spiked wi

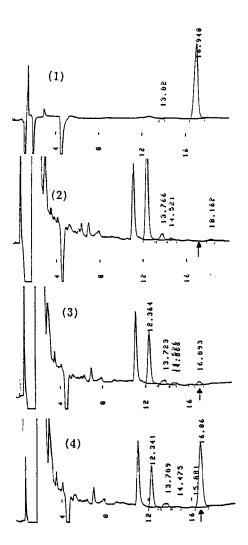


Fig. 9 Chromatograms of BCM-BZ standard and BCM-BZ in tissue extracts

- (1) standard :0.5ppm
- (2) muscle extracts (blank test) (3) muscle extracts (spiked with 0.05 μ g/g of BCM-BZ) (4) muscle extracts (spiked with 1.0 μ g/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, $10.0\sim12.5$ ng 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 C18 (Bond Elut C18), 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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