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### Brodifacoum

370/TC/M/-



*ISO Common Name:* Brodifacoum

*Chemical Name:* 3-[3-(4'-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin

*CAS-Number:* 56073-10-0

*Molecular mass:* 523.4

*Empirical formula:* C31H23BrO3

*m.p.* 228-232oC

# 1 Sampling. Take at least 5 g.

# 2 Identity test

# 2.1 Infrared spectroscopy. Use either ATR technique or prepare KBr discs of Brodifacoum standard and TC. Scan from 4000 to 650 cm -1. The spectrum recorded from the sample should not differ significantly from that of the standard.



**Fig. 1** IR spectrum of Brodifacoum technical (ATR)

**2.2 HPLC.** Use the reversed phase HPLC method described below in section 3.1. The relative retention time of the Brodifacoum peaks in the sample solution should not deviate by more than 2% from those of the calibration solution.

**3 Brodifacoum**

OUTLINE OF METHOD

Brodifacoum is a compound consisting of two diastereomers (cis/trans) relative to the six membered ring. The reversed phase high performance liquid chromatography conditions described below will separate the two diastereomers. The content of brodifacoum is determined as sum of both diastereomers using UV detection at 266 nm and external standard calibration.

**3.1 Determination of Brodifacoum by reversed phase HPLC**

REAGENTS

*Brodifacoum* reference standard of known content

*Phosphoric acid (85%),* analytical grade

*Water* HPLC grade

*Acetonitrile,* HPLC grade

*Mobile Phase A,* 0.1% v/v aqueous phosphoric acid (mix 1 ml phosphoric acid (85%) with 1000 ml water)

*Mobile Phase B,* acetonitrile HPLC grade

*Calibration solution*. Weigh in duplicate (to the nearest 0.01 mg) 15-25 mg of the Brodifacoum reference standard (s mg) in a 100 ml volumetric flask. Add approx. 80 ml acetonitrile and treat for 10 minutes with ultrasound. Allow to reach ambient temperature and make up to volume with acetonitrile. Mix thoroughly (calibration stock solutions CAL-A1 and CAL-A2). Transfer 5.00 ml of the calibration stock solution in a 10 ml volumetric flask and dilute to volume with acetonitrile. Mix thoroughly. (Call these calibration solutions CA1 and CA2).

###### APPARATUS

*High performance liquid chromatograph* equipped with a temperature controlled column compartment capable of maintaining 50 ± 2 °C, a detector suitable for operation at 266 nm (UV-detection) and an injection system capable to inject 5 µl.

*Liquid chromatographic column,* stainless steel, 50 x 4.6 mm (i.d), packed with Zorbax Eclipse XDB-C18  (1.8 µm) Agilent Technologies or equivalent with the same selectivity.

*Electronic integrator or data system*

*Ultrasonic bath*

PROCEDURE

1. *Chromatographic conditions* (typical):

*Column temperature* 50°C

*Flow rate* 1.5 ml/min

*Gradient Time Mobile Phase A Mobile Phase B*
 0 min 50% 50%

 7 min 5% 95%

*Equilibration* 7.1–10 min 50% 50%

*Detector wavelength* 266 nm

*Injection volume* 5 µl

*Retention time Brodifacoum,* approximately 5.0 / 5.2 minutes (cis/trans isomers)

*(b) Equilibration of the system.* Pump sufficient mobile phase through the column to equilibrate the system. Inject 5 µl portions of the calibration solution CA1 and repeat the injections until retention times and peak areas deviate by no more than ± 1 % from the mean for three successive injections.

*(c)* *Sample preparation.* Weigh in duplicate (to the nearest 0.01 mg) sufficient sample to contain 15 - 25 mg Brodifacoum (w mg) into 100 ml volumetric flasks. Add approx. 80 ml acetonitrile and treat for 5 minutes with ultrasound. Allow reaching ambient temperature and making up to volume with acetonitrile. Mix thoroughly. Transfer 5.00 ml of this solution in a 10 ml volumetric flask and dilute to volume with acetonitrile. Mix thoroughly. (Call these sample solutions S1 and S2)

*(d)* *Determination.* Inject 5 µl portions of the second calibration solution (CA2) for two successive injections. The mean response factor for this solution should deviate by no more than 1% from those for the first calibration solution (CA1) (see paragraph (b) *Equilibration of the system*), otherwise the calibration solutions should be prepared again.

Inject in duplicate 5 µl portions of each sample solution (S1, S2, …, etc.) bracketing them by duplicate injections of the calibration solution (CA1) using the following sequence:

CA1, CA1, S1, S1, S2, S2, CA1, CA1, …

Determine the total peak area of Brodifacoum (sum of cis/trans isomer)

*(e)* *Calculation.* Calculate the mean value of each pair of calibration response factors bracketing the two injections of a sample, and use this value for calculating the Brodifacoum contents of the bracketed sample injections.



content of Brodifacoum =  [g/kg]

where:

*fi* = individual response factor

*f* = mean response factor

*Hs* = peak area of Brodifacoum in the calibration solution (sum of cis/trans isomer)

*Hw* = peak area of Brodifacoum in the sample solution (sum of cis/trans isomer)

*s* = mass of the Brodifacoum reference standard taken (mg)

*w* = mass of sample taken (mg)

*P* = purity of Brodifacoum reference standard (g/kg)



**Fig 2** Typical HPLC-chromatogram of Brodifacoum technical material

**BRODIFACOUM Ready to use bait**

370/RB/M/-

**1 Sampling.** Take at least 100 g.

**2 Identity test**

**2.1 HPLC.** Use the reversed phase HPLC method described below in section 3.1. The relative retention time of the Brodifacoum peaks in the sample solution should not deviate by more than 2% from those of the calibration solution.

**2.2 HPLC.** Use the reversed phase HPLC method described below in section 2.2. The relative retention time of the Brodifacoum peaks in the sample solution should not deviate by more than 2% from those of the calibration solution.

REAGENT

*Brodifacoum* reference standard of known content

*Water,* HPLC grade

*Acetonitrile,* HPLC grade

*Methanol*, HPLC grade

*Phosphoric acid (85 %),* analytical grade

*Mobile Phase A,* 0.1 % v/v aq. phosphoric acid (mix 1 ml phosphoric acid (85%) with 1000 ml water)

*Mobile Phase B,* methanol

*Calibration solution*. As for Brodifacoum RB 370/RB/M/3.

For identity test purpose only, the internal standard solution can be substituted by acetonitrile.

APPARATUS

*High performance liquid chromatograph* equipped with a detector suitable for operation at 266 nm (UV-detection) and an injection system capable to inject 5µl.

*Liquid chromatographic column*, Agilent Zorbax SB-Phenyl, 50 x 4.6 mm, 1.8 µm particle size.

*Electronic integrator or data system*

PROCEDURE

1. *Chromatographic conditions* (typical):

*Column temperature* 50°C

*Flow rate* 1.2 ml/min

*Gradient Time Mobile Phase A Mobile Phase B*
 0 min 50% 50%

 7 min 5% 95%

*Equilibration* 7.1–10 min 50% 50%

*Detector wavelength* 266 nm

*Injection volume 5* µl

*Retention time Brodifacoum,* approximately 6.8 / 6.9 minutes (cis/trans isomers)

1. *Sample preparation:* As for Brodifacoum RB 370/RB/M/3

For identity test purpose only the internal standard solution can be substituted by acetonitrile.



**Fig. 3** Typical chromatogram of the Brodifacoum Ready to use Bait Identity Test (using sample solution as described in 370/RB/M/3)

#### 3 Brodifacoum As for Brodifacoum technical 370/TC/M/3 except:

The content of Brodifacoum is determined as sum of both diastereomers using UV detection at 266 nm and internal standard calibration.

#### 3.1 Determination of Brodifacoum by reversed phase HPLC As for Brodifacoum technical 370/TC/M/3.1 except:

Add at REAGENTS

*Internal standard* Difenacoum, purity ≥ 97%, must not contain impurities with same Retention Time as Brodifacoum

*Heptane,* HPLC grade

*Acetic acid (glacial),* analytical grade

*Extracting solution,* acetonitrile/water/acetic acid 80/18/2 (v/v/v). E.g. mix 800 ml

acetonitrile, 180 ml water and 20 ml acetic acid.

*Internal standard solution.* Prepare enough of a 0.2% Difencoum solution in acetonitrile. E.g. Weigh (to the nearest 0.1 mg) 40 - 60 mg of the Difenacoum internal standard (r mg) in a 250 ml volumetric flask. Add approx. 200 ml acetonitrile and treat for 10 minutes with ultrasound. Allow reaching ambient temperature and making up to volume with acetonitrile. Mix thoroughly.

Change *Calibration solution* to*:*

*Calibration solution*. Weigh in duplicate (to the nearest 0.01 mg) 15 - 25 mg of the Brodifacoum reference standard (s mg) into separate 100 ml volumetric flasks. Add approx. 90 ml internal standard solution and treat for 10 minutes with ultrasound. Allow to reach ambient temperature and make up to volume with internal standard solution. Mix thoroughly. Call these calibration stock solutions CAL-B1 and CAL-B2. Transfer 5.0 ml of the calibration stock solution into a 100 ml volumetric flask and dilute to volume with extracting solution. Mix thoroughly. Call these calibration solutions CB1 and CB2.

Add at APPARATUS

*Household Cheese grater*

*Magnetic stirrer*

Add at PROCEDURE

*Retention time Internal standard Difenacoum* approximately 4.1 / 4.3 minutes (cis/trans isomers)

Change *(b) Equilibration of the system* to

*(b) Equilibration of the system.* Pump sufficient mobile phase through the column to equilibrate the system. Inject 5 µl portions of the calibration solution CB1 and repeat the injections until retention times and peak areas deviate by no more than ± 1 % from the mean for three successive injections.

Change *(c) sample preparation* to:

*(c)* *Sample preparation.* Grate the blocks using a cheese grater*.* Weigh in duplicate (to the nearest 0.1 mg) enough sample to contain 0.9-1.1 mg Brodifacoum (w mg) into separate 500 ml Erlenmeyer flasks with conical joint. Add 200 ml heptane, 95 ml of extracting solution and 5.0 ml of internal standard solution IS. Close the flasks using ground-glass stoppers. Stir the suspension for 90 minutes using a magnetic stirrer. Stirring velocity should be as high as possible. Allow the phases to separate. Undissolved particles will remain between the 2 phases. Filter a portion of the lower phase using a 0.45 µm PTFE single use filter unit. Discard the first milliliter of the filtrate. In case the filtrate is still turbid repeat this step using a new 0.45 µm PTFE single use filter unit. Call these sample solutions S1 and S2.

Change *(d) determination* to:

*(d)* *Determination.* Inject 5 µl portions of the second calibration solution (CB2) for two successive injections. The mean response factor for this solution should deviate by no more than 1% from those for the first calibration solution (CB1) (see paragraph (b) *Equilibration of the system*), otherwise the calibration solutions should be prepared again.

Inject in duplicate 5 µl portions of each sample solution (S1, S2, …, etc.) bracketing them by duplicate injections of the calibration solution (CB1) using the following sequence:

CB1, CB1, S1, S1, S2, S2, CB1, CB1, …

Determine the total peak area of Brodifacoum (sum of cis/trans isomer) and the total peak area of Difenacoum internal standard (sum of cis/trans isomer)

Change *(e) calculation* to

*(e)* *Calculation.* Calculate the mean value of each pair of calibration response factors bracketing the two injections of a sample, and use this value for calculating the Brodifacoum contents of the bracketed sample injections.

 

 content of Brodifacoum [mg/kg] = 

where:

*fi* = individual response factor

*f* = mean response factor

*Hs* = peak area of Brodifacoum in the calibration solution (sum of cis/trans isomer)

*Hw* = peak area of Brodifacoum in the sample solution (sum of cis/trans isomer)

*Ir* = peak area of the internal standard in the calibration solution (sum of cis/trans isomer)

*Iq* = peak area of the internal standard in the sample solution (sum of cis/trans isomer)

*s* = mass of the Brodifacoum reference standard taken for the calibration stock solution (mg)

*w* = mass of sample taken (mg)

*P* = purity of Brodifacoum reference standard (g/kg)



**Fig 4** Typical HPLC-chromatogram of Brodifacoum Ready-to-use Bait