

CONFIRMATION OF AMPHENICOLS IN HONEY USING HPLC-RECEPTORGRAM

Julio Quintana-Rizzo*, Robert Salter, Steven Saul

Charm Sciences Inc. 659 Andover St., Lawrence, Ma 01843-1032, USA
Tel: 978-687-9200 Fax: 978-687-9216, e.mail: julio@charm.com

Abstract

A determinative method was developed for the confirmation and identification of chloramphenicol in honey. The method, termed "HPLC receptorgram" combines a separation step by High Pressure Liquid Chromatography (HPLC) with the Charm II amphenicol assay. The method is published for sulfa drugs, β -lactams and tetracyclines in milk samples. A time window for the collection of the chloramphenicol peak is established using a standard. Different amphenicols, for example, florfenicol and thiamphenicol have different time windows for collection. Ten HPLC fractions are collected from one minute to about 20 minutes and are dried, dissolved in buffer and assayed by the Charm II screening assay for amphenicols. A blank sample is run between every sample to ensure no crossover in activity. Honey found as presumptive positive for amphenicols on the screening method is extracted on a C-8 solid phase extraction column and then is subjected to HPLC receptorgram analysis. Honey spiked as low as 0.075 ppb gives a 25% drop in activity in the chloramphenicol time window. Honey samples found positive for chloramphenicol have been found to contain an earlier eluting chloramphenicol metabolite, which has an accumulative effect with chloramphenicol on the screening assay. Chloramphenicol is determined with a gradient HPLC Buffer system. Buffer A is 78% of 10 mM ammonium sulfate, pH 4.6 and 22% acetonitrile and Buffer B is 75 % acetonitrile and 25 % water. The HPLC receptorgram takes 20 minutes for fractionation, 5 minutes for a drying step, and 15 minutes for the screening analysis.

Key words: chloramphenicol/ honey/ HPLC/ immunoassay

Introduction

Chloramphenicol is a highly effective broad-spectrum antibiotic that was originally isolated from *Streptomyces Venezuelae* in 1948 [1]. Its worldwide use has varied due to the association of aplastic anemia, an often-fatal disease, with chloramphenicol even at low exposure levels [2-4]. Chloramphenicol usage for food producing animals is banned in the United States as well as other countries. Recently Chloramphenicol residues have been found in shellfish and honey produced in Asia which has resulted in some food recalls and the banning of some imported food products. A rapid detection and confirmation method for chloramphenicol is needed to ensure the safety and adequacy of the food supply as well as for compliance with governmental regulations.

The HPLC Receptorgram method combines the separation capabilities of HPLC with the specificity of an immunoassay [5-7]. The method requires a simple extraction and purification

step. After a concentration step by drying, the sample is dissolved and subjected to HPLC fractionation. A time window for collection of the chloramphenicol peak is established with a known chloramphenicol standard. Samples with activity corresponding with the chloramphenicol time window are identified as positive for chloramphenicol.

Material and Methods

Apparatus

Liquid Chromatograph: A Waters HPLC system using 515 HPLC pumps, a Waters 996 photodiode array detector, Rheodyne 7725 injector with 200 μ l loop and Millennium software system (Waters, Milford, MA); Foxy 200 fraction collector (Isco, Lincoln, NE).
LC Column: Adsorbosil C-8 column (10 μ m, 250 mm x 4.6 mm) (Alltech, Deerfield, IL) with pre-column filter.

Filters: Acrodisc 0.2 μ m LC 13 PVDF filters (Gelman Sciences, Ann Arbor, MI), Fisherbrand Glass Fiber Filter Circle G6 (Fisher Scientific, Pittsburg, PA) using Swinnex 25 filter holder (Millipore Corp., Bedford, Ma); 0.45 μ m, 47 mm nylon filter, Osmonics, Westborough, MA).

Extraction columns: Bond elut C8 500 mg columns (Varian, Harbor City, CA).

Extraction System: Vac elut sps 24 (Varian, Harbor City, CA).

Assay system: Charm II system for assay of amphenicols (Charm Sciences Inc. Lawrence, MA).

Reagents

Solvents: LC grade acetonitrile and methanol (Fisher Scientific, Pittsburg, PA), HPLC grade water system (Zenon Environmental, Randolph, MA).

Mobile Phase: Buffer A contains 22% acetonitrile and 78% 10 mM ammonium acetate at pH 4.6. Add 0.7708 g of ammonium acetate to 900 ml water and adjust pH to 4.6 with glacial acetic acid. Bring volume to 1 liter. Combine 780 ml of the ammonium acetate Buffer with 220 ml acetonitrile and filter under vacuum through the 0.45 μ m filter. Buffer B contains 75% acetonitrile and 25% HPLC grade water.

McIlvaine/EDTA Buffer: Mix 272.5 ml 0.1 M citric acid with 227.5 ml of 0.2 M dibasic sodium phosphate. Add 37.2 g ethylenediaminetetraacetic acid (EDTA) and adjust to pH 4.5 with ammonium hydroxide or phosphoric acid. Add distilled water to make 1 liter.

Chloramphenicol standard: Prepare a stock solution of chloramphenicol (U.S Pharmacopoeia Convention, Rockville, MD) at 1 mg/ml in methanol and store at -20°C for up to 1 month. Prepare diluted stock standards for HPLC as needed.

Charm II Test for Chloramphenicol (Charm Sciences Inc. Lawrence, MA).

Zero Control Standard (Charm Sciences Inc. Lawrence, MA).

Fortification of Honey samples

Prepare fortified honey samples at 0.075, 0.150, 0.300 and 0.6 ppb for testing by the HPLC receptorgram method.

Sample Preparation

Weigh 20 g of honey in a clean disposable centrifuge tube and add 20 ml of McIlvaine/EDTA Buffer. Mix well so all the honey is dissolved. Filter through the glass fiber filter using a 20 ml syringe. Using the extraction system condition the C8 bond elut column by adding 6 ml of methanol followed by 6 ml of distilled water. Add the honey sample and wash with 6 ml water. Elute sample with 3 ml of methanol into a 13 x 100 mm test tube and dry sample under a stream of nitrogen. Dissolve sample in 75 μ l of mobile phase Buffer A and filter through the 0.45 μ m Acrodisc filter into an 1.5 ml Ependorff vial. Dissolve the remaining sample with an additional 75 μ l of mobile phase and filter through same filter. Filter another 75 μ l of mobile phase through the filter as a rinse. Centrifuge the Ependorff vial for 1 minute.

Liquid Chromatography

Prior to injecting sample, flush the sample loop with 1 ml methanol followed by 1 ml of mobile phase and repeat 2 additional times. Inject the sample. Elute chloramphenicol with a gradient starting with 100 % Buffer A at a rate of 1 ml/min. At 1 min start gradient to 20 minutes where the composition will be 50% Buffer A and 50% Buffer B. From 20 minutes to 31 minutes the composition of the gradient will change to 75 % Buffer A and 25 % Buffer B. At 32 minutes the Buffer composition will change to 100 % Buffer A. After each sample, perform a blank injection, collect fractions and assay fractions to ensure that there is no cross contamination.

Calibration of Time Windows

Inject 200 μ l of a 300 ppb Chloramphenicol standard and record the retention time. Program the fraction collector with this time for the Chloramphenicol peak and collect 2 ml fractions for non-peak fractions starting at 1 minute.

Radioimmunoassay of HPLC fractions

Place each of the ten fractions collected in an aluminum weighing dish and dry over a hot plate until they are completely dry. Dissolve dried extract in zero control standard and perform assay as outlined in the Charm II instruction manual for Antimicrobial drugs in Honey.

Results and Discussion

The Charm II test is a screening method for amphenicols in honey that uses a radioimmunoassay with a 0.3 ppb detection level for chloramphenicol. Florfenicol and thiamphenicol are 40 times and 50 times less sensitive on this assay than chloramphenicol,

respectively. The assay contains an antibody with a high specificity for chloramphenicol and] Chloramphenicol ^3H -bound as a tracer. When the antibody is added to the sample, any chloramphenicol in the sample will bind to antibody. The tracer is then prevented from binding to the antibody resulting in a reduction in the amount of tritium bound to the antibody. The amount of tritium bound to the antibody is measured and a Bs/Bo value is determined where Bs is the counts per minute (cpm) of the sample and Bo is the cpm of the control. The control point for the detection of chloramphenicol is determined by averaging the cpm from six negative honey samples and multiplying this value by 0.8. Figure 1 shows the Charm II standard curve (N=6) for chloramphenicol in honey where chloramphenicol spiked at 0.15 ppb, 0.3 ppb, 0.6 ppb, 1.2 ppb and 2.4 ppb resulted in a Bs/Bo of 0.85, 0.75, 0.65, 0.55 and 0.45, respectively. The control point for the assay is 0.8, and the Bs/Bo for 0.3 ppb chloramphenicol is 0.75 indicating that 0.3 ppb chloramphenicol can be detected in honey.

The HPLC receptorgram combines HPLC fractionation of the sample with the amphenicol screening method for confirmation and identification of chloramphenicol in honey samples. Before HPLC fractionation, the sample is first extracted and concentrated using a C-8 solid phase extraction column. The HPLC chromatogram of thiamphenicol, chloramphenicol and florfenicol is shown in figure 2. From the chromatogram a time window of approximately 1.1 ml is established centering on the retention time for chloramphenicol and 2 ml of mobile phase are collected for all but one of the other fractions. After drying each fraction the activity of each peak is determined by the Charm II screening method for amphenicols. A value under a Bf/Bo of 0.8 where Bf is the cpm of the fraction indicates a positive fraction for amphenicols.

To determine the detection level for chloramphenicol by the HPLC receptorgram method, a negative honey sample was fortified with 0 ppb, 0.075 ppb, 0.15 ppb, 0.3 ppb, 0.6 ppb and 1.2 ppb of chloramphenicol. Samples were run in triplicate by the HPLC receptorgram method (table I). Honey spiked at 0.075 ppb gives a Bf/Bo of 0.75 ± 0.03 for the chloramphenicol time window demonstrating that a sample containing as low as 0.075 ppb chloramphenicol can be detected by the HPLC receptorgram method. A sample spiked with 0.15 ppb chloramphenicol gives a Bf/Bo in the chloramphenicol peak of 0.61 ± 0.01 . So far in 2002, we have received 97 honey samples for analysis. Of these, 25 samples or 26% were found to be negative for chloramphenicol and 72 samples or 74% were found to be presumptive positives by the screening method. Eleven of the presumptive positives were subjected to HPLC receptorgram analysis with all eleven samples confirmed as positive for chloramphenicol. A typical HPLC receptorgram is shown in figure 2. The chloramphenicol peak for this sample showed a Bf/Bo of 0.2 and an earlier eluting metabolite showed a Bf/Bo peak of 0.68. All the honey samples that showed a positive peak for chloramphenicol also showed a positive peak for the chloramphenicol metabolite. The highest concentration in a sample was 8 ppb Chloramphenicol, as determined by sample dilution and quantitation with the screening method.

Reference:

- [1]. Ehrlich, J., Smith, R.M., and Penner, M.A., Process for the Manufacture of Chloramphenicol. US patent 2,483,892. 1949

- [2]. Settepani, J.A., The hazard of using chloramphenicol in food animals. *J Am Vet Med Assoc.* 184(8) (1984), 930-931
- [3]. Allen, E.H., Review of chromatographic method for chloramphenicol residues in Milk, eggs, and tissues from food-producing animals. *J Assoc Of Anal Chem.* 68 (5) (1985), 990-999
- [4]. Page S.W., Chloramphenicol 1. Hazards of use in the current regulatory environment. *Aust Vet J.* 68(1) (1991) 1-2
- [5]. Zomer, E., Saul, S., and Charm, S.E., HPLC Receptorgram: A Method for Confirmation and Identification of Antimicrobial Drugs by Using Liquid Chromatography with Microbial Receptor Assay. I. Sulfonamides in Milk. *J Assoc Of Anal Chem.* 75 (6) (1992) 987-993
- [6]. Zomer, E., Quintana, J., Saul, S., and Charm, S.E. LC-Receptorgram: A Method For Identification and Quantitation of β -Lactams in Milk by Liquid Chromatography with Microbial Receptor Assay. *J. Assoc Off Anal Chem.* 78 (5) (1995) 1165-1172
- [7]. Zomer, E., Quintana, J., Scheemaker, J., Saul., S, and Charm S.E. High-Performance Liquid Chromatography-Receptorgram: A Comprehensive Method for Identification of Veterinary Drugs and Their Active Metabolites in: *Veterinary Drug Residues*, American Chemical Society, Oxford (1996) 149-160

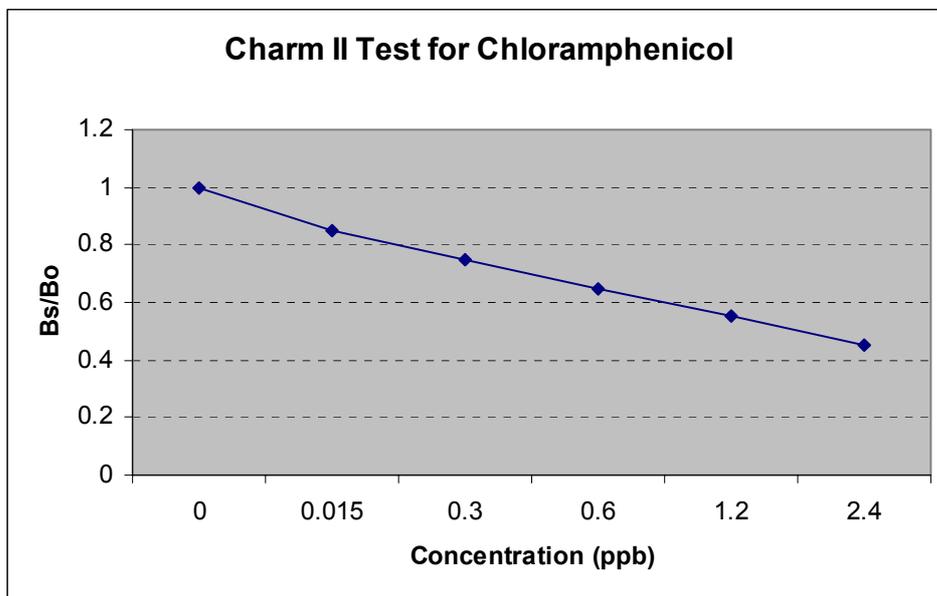


Figure 1. A negative honey is spiked at 0, 0.15, 0.3, 0.6, 1.2 and 2.4 ppb chloramphenicol and each sample assayed with the Charm II amphenicol assay (N=6) for each concentration.

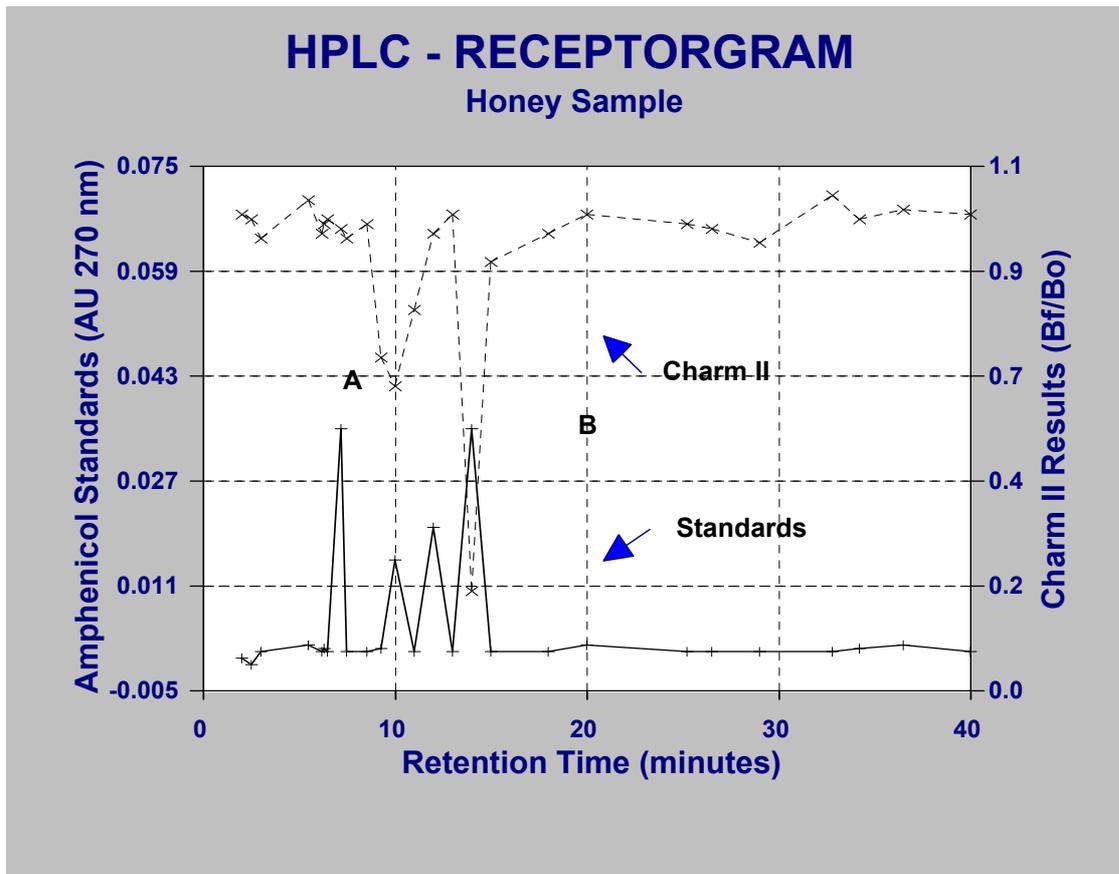


Figure 2. LC Chromatogram showing retention times of the amphenicol standards and HPLC receptorgram of a presumptive positive honey sample. The drop in the Bf/Bo for the receptorgram is matched with the retention times of the amphenicol standards to identify the contaminating drug residue.

Table I. HPLC Receptorgram results for the chloramphenicol time window of honey spiked at varying concentrations of chloramphenicol.

| Concentration (ppb) | Average Bf/Bo | Standard Deviation | CV (%) |
|---------------------|---------------|--------------------|--------|
| 0 | 1.0 | 0 | 0 |
| 0.075 | 0.75 | 0.03 | 3.9 |
| 0.15 | 0.61 | 0.01 | 2.5 |
| 0.3 | 0.52 | 0.05 | 10.0 |
| 0.6 | 0.52 | 0.02 | 5.0 |
| 1.2 | 0.50 | 0.02 | 3.2 |

N=3