U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 20B: Rapid HPLC Determination of Sulfamethazine in Milk

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Introduction

A simple, relatively rapid HPLC procedure has been developed for determining sulfamethazine in raw bovine milk in the low parts per billion (ppb) range. A separatory funnel is used to extract sulfamethazine from milk with chloroform, the chloroform is evaporated, and the fatty residue is dissolved in hexane. The sulfamethazine is then partitioned into an aqueous potassium phosphate solution which is injected directly onto the chromatograph.

A. Equipment and materials

1. Liquid chromatograph. Series 4 or 410 pump equipped with Model LC-95 UV/Vis detector (Perkin-Elmer Corp., Instrument Div., Norwalk, CT 06056), or equivalent
2. Column heater and controller capable of maintaining 35 ± 0.2°C (Fiatron, Oconomowoc, WI 53066), or equivalent
3. Column, 250 x 4.6 mm, packed with LC-18-DB (Supelco, Bellefonte, PA)
4. Guard column, 2 cm long, LC-18-DB (Supelco)
5. Precolumn filter, 3 mm diameter frit, 0.5 m porosity (Supelco)
6. Rotary evaporator (Buchi Laboratory Techniques Ltd, Flawil, Switzerland), or equivalent
7. Vortex mixer (Genie Scientific, Fountain Valley, CA), or equivalent
8. Freezer capable of holding temperature at -50 to -80°C
9. Polypropylene tubes, 50 ml, with screw caps (Fisher Scientific, Pittsburgh, PA)
10. Micro weighing funnel (Radnoti Glass Technology, Inc., Monrovia, CA), or equivalent
11. Volumetric pipets, 1, 2, and 5 ml; two 10 and 20 ml class A, or equivalent
12. Volumetric flasks, six 100 ml, class A
13. Laboratory refrigerator
14. Eppendorf pipettors, 10-100 µl and 100-1000 µL, or equivalent
15. Eppendorf maxipettor, positive displacement, 1-10 ml, or equivalent

16. HPLC solvent filtering apparatus, 1, 2, or 4 L capacity
17. Nylon-66 HPLC solvent filter, 0.4 m porosity
18. Volumetric flask, 2 L, class A
19. Graduated cylinders, 50 ml, 1 L
20. Intermediate containers, at least 3 glass bottles, 4 L, with Teflon-lined caps. **NOTE**: Plastic storage vessels are not satisfactory for LC solvents.
21. Hewlett-Packard model llC calculator or equivalent
22. Glass repeater pipettors, two 5 ml (Fisher Scientific)
23. Rings and clamps or funnel stand and appropriate laboratory hardware

For each replicate to be analyzed, the following are needed:

24. Separatory funnel, 125 ml, with ground glass stopper and Teflon stopcock
25. Short stem funnel, 75 mm diameter
26. Fluted filter paper, 12.5 cm (Schleicher & Schuell)
27. Pear-shaped flask, 100 ml, with 24/40 standard taper neck with stoppers (Kontes Glass Co., Vineland, NJ)
28. Pasteur pipet
29. Glass autosampler vial or glass test tube

B. Reagents

1. Copy Sulfamethazine standard (Sigma Chemical Co., St. Louis, MO)
2. Potassium dihydrogen phosphate, HPLC grade
3. Methanol, HPLC grade
4. Purified water, distilled, deionized, HPLC grade
5. Chloroform, B&J or equivalent (Burdick & Jackson, Muskegon, MI 49442)
6. Hexane, HPLC grade
7. Solutions (water is considered to be distilled, deionized water)

    1. **Potassium dihydrogen phosphate** (PDP), 0.1 M solution. Dissolve 27.2 g PDP in water, dilute to 2 L, mix, and filter through 0.4 m porosity nylon-66 filter. Store PDP solution at room temperature in intermediate container(s), properly labeled

with expiration date 3 months after date of preparation. In this text, PDP solution means filtered PDP solution.

2. **Mobile phase**. Dilute 600 ml methanol, filtered through nylon-66 filter, to 2 L with PDP solution, and mix. Store mobile phase at room temperature in intermediate container(s), properly labeled with expiration date 3 months after date of preparation of PDP solution.

3. **Flush solution**. Dilute 1200 ml methanol to 2 L with water, mix, and filter through nylon-66 filter. Store flush solution at room temperature in intermediate container(s), properly labeled with expiration date 3 months after date of preparation.

4. **Standard solutions**. All standard solutions have expiration date 3 months after date of preparation of master solution. Store all standard solutions below 10°C. **NOTE**: After washing, rinse all glassware with 1 N HC1 as precaution against sulfamethazine cross-contamination; then thoroughly rinse with water, and finally rinse with methanol. Remove stopcock from separatory funnel for rinses.

    a. **Master solution**. Weigh 100 mg sulfamethazine standard at room temperature in glass weighing boat and transfer to 100 ml volumetric flask. Dissolve in methanol, dilute to volume with methanol, and mix.

    b. **Sulfamethazine solution, 10,000 ng/ml**. Measure 1 ml master solution with 1 ml volumetric pipet into 100 ml volumetric flask, dilute to volume with water, and mix.

    c. **Fortification solution -- sulfamethazine solution 1000 ng/ml**. Transfer 10 ml of the 10,000 ng/ml sulfamethazine solution to 100 ml volumetric flask with 10 ml volumetric pipet, dilute to volume with water, and mix.

Table 1. Recovery of sulfamethazine added to milk at 0, 5, 10, and 20 ppb and incurred in milk[(a)]

5 ppb	5 ppb	10 ppb	10 ppb	20 ppb	20 ppb	Incurred
Rec. ppb	Rec. %	Rec. ppb	Rec. %	Rec. ppb	Rec. %	Found ppb
3.99	79.8	7.51	75.1	14.89	74.5	4.46
4.23	84.6	7.22	72.2	15.07	75.4	4.64
4.40	88.0	7.98	79.8	15.19	76.0	4.29
3.47	69.4	7.51	75.1	14.07	70.4	4.35
4.05	81.0	7.92	79.2	13.84	69.2	4.52
Average	80.6		76.3		73.1	4.45
SD	7.0		3.2		3.1	0.14
CV (%)	8.7		4.2		4.2	3.1

[a] Sulfamethazine was not detected in the 5 control samples. Taken from Weber & Smedley, *J. Assoc. Off. Anal. Chem.* **72**:445-447 (1989).

d. **Solutions for standard curve** (prepare as follows):

20 ppb standard: Dilute 20 ml fortification solution to 100 ml with water, using 20 ml volumetric pipet and 100 ml volumetric flask. (**NOTE**: This is a 200 ng/ml solution, which is equivalent to a 20 ppb standard.)

10 ppb standard: Dilute 10 ml fortification solution to 100 ml with water, using 10 ml volumetric pipet and 100 ml volumetric flask.

NOTE: Residues from 10 ml of milk are extracted into a final 1 ml of PDP, resulting in a tenfold concentration of residues. Therefore, a standard or final extract with a concentration of 100 ng/ml is equivalent to 10 ppb of that analyte in milk. One hundred µl of both sample and standards are injected onto the LC system.

5 ppb standard: Dilute 5 ml fortification solution to 100 ml with water, using 5 ml volumetric pipet and 100 ml volumetric flask.

C. Sample Storage

Store fresh raw milk in refrigerator at <10°c. However, if milk will not be used within 2-3 days, subdivide into polypropylene plastic tubes and store at -80°c. If a freezer of this nature is not available, store samples frozen at as low a temperature as is available. Thaw frozen milk slowly in slightly warm tap water on the day sample is to be analyzed. Mix milk gently before sampling. Some samples have been successfully analyzed 1-2 years after freezing. However, degradation of milk was noted when it was stored at -15°c for only a few months.

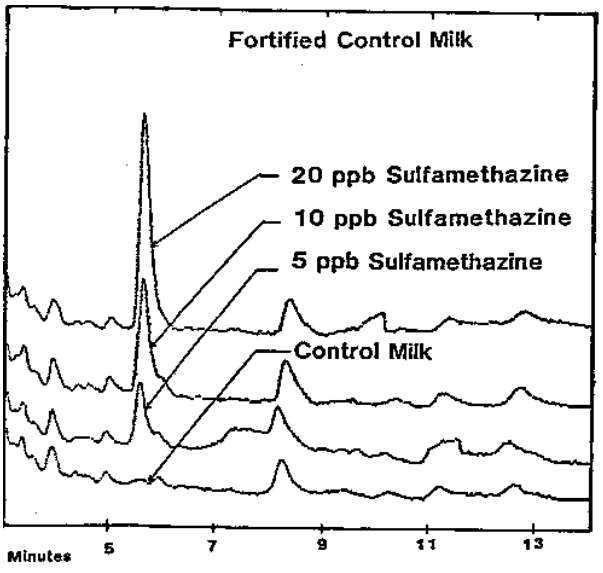

Figure 1. Chromatograms of control milk and fortified control milk. No data were collected during first 3 min after injection to conserve electronic data storage space.

D. Analytical method

1. Copy Extraction of sulfamethazine from milk

   In a hood, set 75 mm short stem funnel with fluted filter paper on rack or stand. Using a 5 ml repeater pipettor, wash filter paper with 5 ml chloroform and discard chloroform wash. Place 100 ml pear-shaped flask under funnel as receiver. Using 10 ml maxipettor, add 10 ml milk to 125 ml separatory funnel. For recovery studies, fortify samples at this point. (**See** Table 1 for expected recoveries.) Add 50, 100, or 200 µl of fortification solution to the 10 ml of milk in the separatory funnel to obtain 5, 10, or 20 ppb fortified samples. Using a graduated cylinder, add 50 ml chloroform to 125 ml separatory funnel, and stopper. Shake mixture of milk and chloroform vigorously for 1 min; excess pressure is then carefully vented through stopper. Shake again for 1 min, vent, and let phases separate for 1 min. Repeat shaking for 1 min, vent, and shake for 1 min more. Vent and let phases separate for at least 5 min.

   VENTING IS A CRITICAL POINT. Venting separatory funnel through stopper is important. **NOTE**: Venting through stopcock often clogs stopcock with milk solids, so that chloroform cannot be conveniently drawn off. Venting through stopper resolves the problem.

   Draw off and filter chloroform through fluted filter paper into 100 ml pear-shaped flask. Using 5-ml repeater pipettor, rinse filter paper twice with 5 ml portions of chloroform, and collect washings in same pear-shaped flask.

2. Prepare sample for injection on HPLC

   Evaporate chloroform solution in pear-shaped flask just to dryness on rotary evaporator at 32 ± 2°C. Using another 5 ml repeater pipettor, add 5 ml hexane to flask, stopper, and dissolve residue by agitating vigorously on vortex mixer for 1 min. Using the 100-1000 µL Eppendorf pipettor, immediately add 1 ml PDP solution to hexane in pear-shaped flask. Agitate vigorously on vortex mixer for about 1 min, 3 or 4 times over a minimum of 15 min.

   CONTACT TIME IS A CRITICAL POINT. Contact time is as important as vigor of agitation. **NOTE**: Recovery improves up to about 15 min. Longer times, up to 1 h, can be safely used, but will not improve recovery.

   Transfer aqueous layer from bottom of flask to autoinjector vial with Pasteur pipet, taking care not to transfer any hexane to autoinjector vial. If autoinjector is not available, transfer PDP solution to glass test tube. The sample is ready for injection.

3. Chromatography (**see** Fig. 1)

   Inject standards at beginning and end of every sample set. Construct standard curve by using peak heights of sulfamethazine standards; calculate sample concentration as described in calculation section.

a. Chromatographic conditions

Heat column to 35 ± 0.2°C. Set mobile phase flow rate to 1.5 ml/min, with UV detector set at 265 nm wavelength. **NOTE**: Use premixed mobile phase and pump isocratically because a small change in the methanol to PDP solution ratio results in a significant change in retention time of sulfamethazine. Let system pump under these conditions for at least 45 min before injecting standards. Set run time at 15 min with equilibrium time of 1 min between runs. **NOTE:** A late eluting peak in some milk samples may appear in the subsequent run, but does not coelute with the sulfamethazine peak. If this causes a problem with the analysis, increase the run by a few minutes as necessary. Inject 100 µl of standard or sample solution. Use flush solution to rinse autoinjector. Set sensitivity and/or recorder ranges to give 75-90% full deflection for 20 ppb standard. Pump flush solution through column for minimum of 45 min as part of system shut-down at end of day.

b. Chromatographic suitability test

Standard curve. Inject duplicate 100 µl aliquots of 5, 10, and 20 ppb sulfamethazine standards. Correlation coefficient of standard curve prepared by using peak heights should be 0.98 or greater. If value is less, repeat injection of standards.

Calculations. Calculate linear regression equation, using least square fit for sulfamethazine standard solutions (concentrations vs peak heights) to obtain values for:

$Y = mX + b;$

then solve for concentrations of unknown, X, from equation of line where Y = peak height at sulfamethazine retention time of samples, and b and m are y-intercept and slope, respectively.

E. Additional comments

- If fresh milk is to be stored more than 2 or 3 days, subdivide sample in 50 ml polypropylene tubes and store between -50 and -80°C.
- We have run more than 1000 injections of samples and standards combined without having to replace the LC-18-DB column.
- Prepared samples, in sealed injection vials, have been stored in the autoinjector (at room temperature) for up to 24 h before being injected. No changes in retention time or peak heights of standard solutions were observed.

This method has been successfully validated by an AOAC collaborative study (4). The original single residue method has been modified to simultaneously determine 10 sulfonamide drugs at 10 ppb and above in raw bovine milk (2). Eight of these drugs were selected to be validated in another AOAC collaborative study (1).

References

1. Smedley, M.D. 1994. Liquid chromatographic determination of multiple sulfonamide residues in bovine milk: collaborative study. *J. AOAC Int.* **77**:1112-1122.

2. Smedley, M.D., and J.D. Weber. 1990. Liquid chromatographic determination of multiple sulfonamide residues in bovine milk. *J. Assoc. Off. Anal. Chem.* **73**:875-879.

3. Weber, J.D., and M.D. Smedley. 1989. Liquid chromatographic determination of sulfamethazine in milk. *J. Assoc. Off. Anal. Chem.* **72**:445-447.

4. Weber, J.D., and M.D. Smedley. 1993. Liquid chromatographic method for determination of sulfamethazine residues in milk: collaborative study. *J. AOAC Int.* **76**:725-729.

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